Effect of Iron Source, Dose and Bioavailability on Zinc and Copper Status of Wistar Rats

By

Henock Mathewos

Advisor. Kaleab Baye (PhD)

A Thesis Research Submitted to the School of Graduate studies of Addis Ababa University Center for Food Science and Nutrition, College of Natural Science

Presented in Partial Fulfillment of the Requirements for the Degree of Master of Science in Food Science and Nutrition Program

24, October 2019

ADDIS ABABA UNIVERSITY

SCHOOL OF GRADUATE STUDIES

COLLEGE OF NATURAL SCIENCES

CENTER FOR FOOD SCIENCE AND NUTRITION

Effect of Iron Source, Dose and Bioavailability on Zinc and Copper Status of Wistar Rats

By

Henock Mathewos

Advisors: Kaleab Baye (PhD)

A Thesis Submitted to the School of Graduate Studies of Addis Ababa University in Partial Fulfillment of the Requirements for the Degree of Master of Science in Food Science and Nutrition.

Approval by Examining Board:	Signature	Date
Dr. Yehune Ayele (External Examiner)		
Dr. Aynadis Tamene (Internal Examiner)		
Dr. Kaleab Baye (Advisor)		
Mrs. Meseret Azene (Chair Person)		

Table of Content

DECLARATION v
Acknowledgments vi
List of Abbreviations/Acronyms vii
List of table x
List of figures xi
Abstract xii
Chapter One 1
1. Introduction 1
1.1 Statement of problem 3
1.2 Significance of the study 6
1.3 Objective 8
1.3.1 General Objective 8
1.3.2 Specific Objective 8
Chapter Two 9
2 Literature Review 9
2.1.1 Iron History 9
2.1.2 Iron Properties 9
2.1.3 Iron Physiological Function 9
2.2 Dietary Sources and Body Regulation of Iron 13
2.2.1 Human Iron Requirement 13
2.2.2 Cause and Consequence of Iron Deficiency 14
2.2.3 Evaluation of Iron Status 15
2.3 Prevalence of Iron Deficiency and Groups at Risk 20
2.3.1. Factors Leads Risk Group to Iron Deficiency Anemia 21
2.4. Anemia Reversing Intervention Strategies 21
2.4.1. Anemia Reversing by Supplementation 22
2.4.2 Anemia Reversing by Dietary Diversification(modification) 22
2.4.3 Anemia Reversing by Fortification 22
2.5 Methods of Assessing Iron Bioavailability and Bioaccessibility 23

2.5.1. In vitro Methods of Estimating Mineral Bioavailability 24
2.5.2. In Vivo Methods of Estimating Iron Bioavailability 28
2.6. Intrinsic and Extrinsic to Food Iron Sources 29
2.6.1. Intrinsic to Food Iron Sources (Dietary Iron Sources) 29
2.6.2. Extrinsic to Food Iron Sources (Non-Food Sources of Iron) 30
2.6.3 Extrinsic Iron Source Study Pattern in Ethiopia 30
2.6.4 Iron Toxicity 31
2.7 Zinc 32
2.7.1 Function of Zinc in Physiology 32
2.7.2. Human Zinc Requirement 33
2.7.3. Sources of Food Rich in Zinc and Factors Affecting Zinc Bioavailability 33
2.7.4. Systemic Zinc Regulation and Dietary Uptake 34
2.7.5. Zinc Deficiency 34
2.7.6. Measuring Human Zinc Status 35
2.8. Copper 35
2.8.1. Function of Copper in Human Physiology 35
2.8.2 Human Copper Requirement 36
2.8.3 Systemic Copper Regulation and Dietary Uptake 37
2.8.4 Copper Deficiency 37
2.8.5 Measuring Human Copper Status 38
2.9. Iron, Zinc and Copper Biological Interaction 39
Chapter Three 41
3. MATERIALS AND METHODS 41
3.1. Study Site, Soil Sample Collection and Preparation 41
3.2. Experimental Design 42
3.3 Iron Sources/Fortificants and Formulated Experimental Diet Iron Analysis 45
3.3.1 Chemical and Reagent 45
3.3.2. Apparatus 45
3.3.3. Procedure 45
3.3.4. Atomic Absorption Spectrophotometer 45
3.3.5 Calculation and Evaluation of Results 46

3.4 Fortificants Supply, Diet Supply and Fortificant Iron Analysis 46
3.4.1. Experimental Diet Dose Setting, Assumption for Assigned Experimental Groups and Preliminary Level for Experimental Diet Formulation 47
3.5. Animal Supply, Base-line Status, End-line Status and Maintenance 51
3.5.1. Study Animal Collection and Screening/Baseline Measurment 51
3.5.2 Study Animal Handling and Ethical Consideration 53
3.5.3. Experimental Diet Feeding 53
3.5.4. Experimental Animal Scarification, Changing Whole Blood to Serum, End-line Iron, Zinc and Copper Analysis 54
3.5.5. Measured Serum Iron, Zinc and Copper for Experimental Animals 54
3.5.5.2 Sample Preparation, Serum Zinc and Serum Copper Analysis 55
3.6. Statistical Analysis 56
Chapter Four 56
4. Results and Discussion 57
4.1. Determining for Fortificants Total Iron Content 57
4.2. Formulated Experimental Diets Total Iron Content 57
4.3 Baseline Characteristics of Experimental Rats 58
4.4. End-line Measurements, Growth Rate and Weight Among Experimental Groups. 59
4.4.1. Growth Rate within the Groups 64
4.5 End-line Measured Liver Weight Characteristics within the Groups 65
4.6. End-line Measured Serum Iron Characteristics within the Experimental Groups 68
4.7. End-line Measured Serum Zinc Characteristics within the Experimental Groups 72
4.8. End-line Measured Copper Characteristics within the Experimental Groups 76
5.Conclusion and Recommendations 80
REFERENCES 82
ANNEX 101
Annex I: Soil Sample Preparation 101
Annex II: Experimental Diet Formulation/Fortification 101
Annex III: Homogenization of Formulated/ Fortified Diet 102
Annex IV: Pelletizing and Drying Experimental Diet 102
Annex V: Iron Content of Fortificants Before Experimental Diet Formulation 103

Annex VI: Formulated Experimental Diet Intended Iron Content Vs Analyzed and Found to be Iron Content (AAS Out Put at Bless Laboratory) 103
Annex VII: Animal Screening and Baseline Measurement 104
Annex VIII: Experimental Animal Feeding and Pre-End-line Protocol (Anesthesia) 104
Annex IX: End-line Measurement and Whole Blood to Serum Changing for Analysis. 105

DECLARATION

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

Signature:  _____ Date:_____

The thesis has been approved for submission by:

Name of Supervisor	Signature	Date
Kaleab Baye(Ph.D)(Advisor)		_____

Acknowledgments

First and foremost, I would like to express my thanks goes to Almighty God, for giving me age, health, wisdom and knowledge to achieve such part of my life.

I would like to express my special thanks to my Advisor Dr. Kaleab Baye who gave me time, material, idea and moral support in the times when I was frustrating, I couldn't achieve it without his unrestricted support like a brother from scratch to reach here.

The author is grateful to Dytes Inc.USA for their donation of AIN-93G rat diet, Hexagon Nutrition INDIA for their donation food grade iron sulfate and electrolyte iron, Ethiopian public health institute staffs specifically Mr.Yergalem Tadesse and Mr. Meseret Woldeyohanes for facilitating experimental rats supply, equipments and working area for experimental animal feeding .

I would like to extend my heartfelt special thanks to Mr. Debebe Hailu, Mr. Bruk Seleshi, Mr. Sisay Ayalew, Mr.Yemane Gebreab, Mrs. Betelhem Taye, Mr. Henock Ashagre, Tigist Tulu and Bless Agrifood staffs for their participation at laboratory, data collection and guiding me throughout the thesis work. I would like to thank all food science and nutrition students whose names are not mentioned here but participated in this study.

Finally, I would like to pass my deepest and heartfelt thanks to my family for their continuous pray and financial support to realize this thesis work.

List of Abbreviations/Acronyms

ACC/SCN	Administrative Committee on Coordination/Subcommittee on Nutrition
AAS	Atomic Absorption Spectrophotometer
AIN-93G	American Institute of Nutritional Formulation for Growing Rats
ANOVA	Analysis of Variance
AOAC	Association of Official Analytical Chemists
ATP	Adenosine Triphosphate
BBM	Brush Border Membrane
BLM	Basolateral Membrane
Caco-2	Human Colon Carcinoma Cell Line
CDA	Copper Deficiency Anemia
CEC	Cation Exchange Capacity
CNS	Central Nervous System
CRP	C-Reactive Protein
CSAE	Central Statistical Agency of Ethiopia
CV	Coefficient of Variation
Cp	Ceruloplasmin
DMT 1	Divalent Metal Transporter
DNA	Deoxyribonuclic Acid
DRI	Dietary Reference Intake
EDHS	Ethiopian Demographic Health Survey
EDTA	Ethylenediamine Tetraacetic Acid
EHNRI	Ethiopian Health and Nutrition Research Institute
ENFCS	Ethiopian National Food Consumption Survey
ENMS	Ethiopian National Micronutrient Survey

ESR	Erythrocyte Sedimentation Rate
FAO	Food and Agricultural Organization of United Nations
FPN	Ferroportin
GI	Gastrointestinal
Hctr 1	High Affinity Human Copper Transporter
HDL	High Density Lipoprotein
HIF	Hypoxia Inducible Factor
HRE	Hemoglobin Regeneration Efficiency
IDA	Iron Deficiency Anemia
IFPRI	International Food Policy Research Institute
IRE	Iron Responsive Element
IRP	Iron Regulatory Proteins
IZiNCG	International Zinc Nutrition Consultative Group
LDL	Low Density Lipoprotein
mRNA	Messenger Ribonucleic Acid
NNS	National Nutrition Survey
NTBI	Non-Transferrin Bound Iron
PCR	Polymerase Chain Reaction
PCV	Packed Cell Volume
RBV	Relative Biological Value
RDA	Recommended Dietary Allowance
RDI	Recommended Daily Dietary Intake
RNA	Ribonucleic Acid
SCF	Scientific Committee of Food
SOD	Superoxide Dismutase

SPSS	Software Statistical Package for Social Sciences
TB	Tuberculosis
TCA	Trichloroacetic Acid
TfR	Serum Transferring Receptor
TIBC	Total Iron Binding Capacity
UL	Tolerable Upper Intake Level

List of table

Page

Table 3.4.1.1 Summarized nutritional composition for experimental groups formulated diets 50

Table 4.2 Base line characteristics of AIN-93G fortified with different fortificants and analyzed iron content of each experimental diets 58

Table 4.3 Baseline rats profiles within the groups 59

Table 4.4 Endline experimental rats profile with in the groups 63

List of figures

Page

Figure 3.2 Design of experiment 44

Figure 4.4 Mean percentage of weight increase within experimental groups(Growth Rate) 64

Abstract

Micronutrient deficiencies constitute important nutritional and public health problems affecting billions of people globally and the prominent one is iron deficiency. To mitigate iron deficiency number of techniques used and fortification is the best interventional strategy that can be used to improve the iron status of population. Recently, there was a concern for extrinsic/geophagy iron for being sole part of Ethiopian Teff cereal, reported for being bioavailable and alleviated iron deficiency *in-vivo* study. In addition, it is presumed that on-top of adequate dietary iron intake the extrinsic iron consumption sum effect will surpass the recommended iron intake. However, its toxicological and potential negative interaction on other micronutrient status effect not yet been discovered. Our study aimed chronic intake of adequate dietary iron combined with excess dose of extrinsic source iron effect on iron, zinc and copper status using *in vivo* model (weaning male Wistar rats). We fed diet formulated with adequate dose of iron sulfate (35 ppm)/Control group was compared with five experimental groups which consumed dietary different dose (adequate/excess) or bioavailability (low/high) index iron without or with excess dose of extrinsic soil source iron formulated diet intake and investigated body weight, liver weight, iron, zinc and copper status for all groups. Excess extrinsic source iron (350 ppm) with adequate dose of electrolyte iron (70 ppm) and excess dose of electrolyte iron (350 ppm) formulated diet fed groups showed statistically insignificant growth rate, liver weight, serum iron and copper level compared to control group($P<0.05$). Similarly, only excess extrinsic source iron (350 ppm) formulated diet consumed group measured parameters were consistent to control group except, serum iron level inconsistency. However, extrinsic iron source (350 ppm) with adequate dose of iron sulfate (35 ppm) formulated diet fed group showed that significantly lower growth rate, higher serum iron and lower serum copper level ($P<0.05$) and presumed to be iron overloaded. While, adequate dose of electrolyte iron formulated diet consumed group showed lower serum iron and higher serum copper status($P<0.05$) and presumed to be iron deficient. All study groups serum zinc findings were not statistically significantly different ($p<0.05$). Since, we investigated mainly serum level we could not reach concrete conclusion for zinc status and presumed findings. Based on our study, in order to reach clear conformation measurements for biomarker, antioxidant agent and organ level study further study in future will fill our gaps.

Therefore, adequate dose of high bioavailable fortificant like iron sulfate combined with excessive dose of extrinsic/soil iron consumption could led to iron-overload and other micronutrient status variation so, while fortification and supplementation programs implementation special attention needed for program and policy developers to consider extrinsic/soil source iron in order to avoid public health issues.

Chapter One

Introduction

1. Introduction

Globally, micronutrients deficiency exists in high prevalence. The most exposed groups are pregnant women and their children under five years reported to be at highest risk. Among these deficiencies iron, iodine, folate, vitamin A and zinc deficiencies which affect many physiological statuses of society (Baily, West & Black, 2015). Of all micronutrient deficiencies, iron deficiency affects more than two billion people worldwide, especially from the population women and children are the victims (WHO, 2015). The prevalence of anemia is particularly high in developing countries, where 293 million of children and 468 million of pregnant women are anemic. Hence, anemia contributes to 20% of all maternal death. Similarly, an estimated 17.3% of world's population is at risk of inadequate zinc intake; the larger figure primarily goes to Sub-Saharan Africa and South Asia accounts the next figure (Wessells & Brown, 2012).

Three main strategies that can be implemented to overcome micronutrient malnutrition are supplementation, dictary diversification and foodfortification (FAO/LLSI,1997). Supplementation through periodic administration of pharmacological preparations in the form of injections, capsules, and tablets are effective, whereby substantial and almost immediate benefits can be brought to the most at-risk groups (Schumann, Elsenhans & Maurer, 1998; Quintaes, Cilla & Barberá, 2015). Policy and program response include food-based strategies, which include dietary diversification and fortification appears the most sustainable approaches to increase the iron and other micronutrients status of a population (Monsen,1998; Quintaes, Cilla & Barberá, 2015). Dietary modifications for reducing iron deficiency anemia involve increased intake of iron rich food such as flesh foods or consumption of ascorbic acid rich fruits and vegetables that enhance non-haeme iron absorption. Among the other dietary methods at globall level which has been widely applied as an interventional approach was fortification (Quintaes, Cilla & Barberá, 2015), due to wider and more sustained impact. However dietary diversity is generally regarded as lesser than fortification since it takes longer time to implement (WHO/FAO, 2006).

Fortification is the process of addition of one or more essential nutrients to food for the purpose of preventing or correcting a demonstrated deficiency of one or more nutrients in the population or specific population groups (Schumann, Elsenhans & Maurer, 1998; Quintaes, Cilla & Barberá, 2015). There are three types of fortifications (mass, target and market-driven) recommended by WHO/FAO, (2006). The public health impact of food fortification depends on a number of

parameters, predominantly the level of fortification, the bioavailability of the fortificants and the amount of fortified food consumed (WHO/FAO, 2006). Hence, the choice of fortificant compound is often a compromised between reasonable cost, bioavailability from diet, and the acceptance of any sensory changes (WHO/FAO, 2006; Quintaes, Cilla & Barberá, 2015). When selecting the most appropriate chemical form of a given micronutrient, the main consideration and concerns are; sensory problems (unacceptable sensory changes like color, flavor, odour or texture), interaction (interaction with food vehicle and with other nutrients and particle size of vehicle and fortificant need to be considered), cost (must not affect the affordability of food) and bioavailability (must be well absorbed from food vehicle)(WHO/FAO,2006; Quintaes, Cilla & Barberá, 2015).

Until recent time, dietary interventions including fortification by many studies focus on intrinsic iron source, yet iron contamination from extrinsic source like cooking utensils (Adish *et al.*, 1999), miller screw wares (Icard-Verniere *et al.*, 2013), and extrinsic/soil contamination was the common one in Ethiopia as discussed by Abebe *et al.*,(2007) and Baye *et al.*,(2014). Most of the extrinsic iron/ contamination in Ethiopia is from soil and is attributed to traditional threshing of grains under the hooves of cattle (Ambaw, 2013; Abebe *et al.*, 2007).

In Ethiopia context, soil iron contamination is not the same for all grains and was found to be higher in Teff because of the small size which means higher suraface area to be contacted with soil (Baye *et al.*, 2014). Also, as disscussed by Baye *et al.*, (2014) and Umeta, West & Fufa (2005) a significant proportion of the iron in the Teff diets of Ethiopia is extrinisic to food, usually referred as contamination iron. Teff is used to be the dominant cereal crop consumed by most society in rual as well as urban part of Ethiopia. Teff is used to make *injera*, a traditional fermented Ethiopian pancake. In-addition, Teff represents 22.6% of cereals grown in Ethiopia(CSAE, 2012).

There were controversies despite, geophagy/extrnisic soil contamination iron source being sole part Teff concerning the bioavailability and bioaccessability of geophagy / extrnisic soil contamination iron source as disscussed by Seima *et al.*,(2013) and Teklu, (2017b). In view of that, dietary extrnisic/soil contamination source iron effect was studied by Teklu, (2017a) and Guja & Baye, (2018) conformed bioavailability and bioaccessability of geophagy/extrnisic soil contamination iron source in hemoglobin regenaration *in vivo* study used to have a positive and potential ability of allevating iron deficiency.

It was reported that extrinsic soil contamination iron source bioavailable and hemoglobin regeneration potential on rat assay; yet, soil contamination source iron existence still not given attention in Ethiopia but extrinsic soil contamination iron intake on top of dietary/fortification could result iron overload/other health problems has been indicated Teklu, (2017a) and Guja & Baye, (2018). In addition, some studies indicated that fortification programs have to consider social and health aspects, including provision against iron over load and excess iron stores may promote cancer and increase the cardiovascular risk (Schumann, Elsenhans & Maurer, 1998). Similarly, dietary based excess dose of iron consumption could result iron overload in both human based survey by Tao & Pelletier, (2009) and EPHI/ENFCS, (2013), animal based experimental study by Arruda *et al.*, (2013).

Apparently, the negative effect as a result of iron overload/oxidative stress due to excess iron consumption would affect zinc and copper (Fairweather-Tait & Southon, 1989; Roughead, Johnson & Hunt, 1999; Akhtar *et al.*, 2010; Ha *et al.*, 2016; Heidari *et al.*, 2016). The researchers have explained the interaction that existed between supportive to the current report of Ethiopian National food consumption survey showing that in Amhara region there was a high prevalence of excessive intakes of iron in the adult population with approximately 64% of women 19-45 years and 89% of urban males that might be at risk of excessive intakes of iron (EPHI/ENFCS, 2013), while Zinc for women (50%) were found to have an inadequate intake. In urban areas the prevalence of inadequate zinc intakes was slightly higher (65%) as per EPHI/ENFCS, (2013). The report made us to presume that soil contamination on top of interventional methods could be the reason. Iron overload state in return affects zinc and copper status used to be current assumed area of our study based on the above (EPHI/ENFCS, 2013) report input.

Therefore, our research tried to see the potential effect of dietary excess dose of extrinsic soil contamination source iron intake on top of adequate dietary iron having either low or high-bioavailabilty index for fortificants based formulated diet chronic consumption effect on iron, zinc and copper status using *in-vivo* assay seen by measuring serum and metabolism status.

1.1 Statement of problem

In January, 2008 Ethiopia had proposed draft to be implemented as national nutritional strategy to be applied until 2015. The national micronutrient guidelines were part of national nutritional

strategy; which focus on supplementation and fortification program for iron, vitamin A, zinc and other micronutrients and for fortification program implementation selecting appropriate local staple foods by both public and private sectors. The strategies were developed by Ministry of Health (FMOH/NNS,2008). Although, food fortification with micronutrients has not yet been developed due to low-levels of food processing industries thus, ministry of health underlined to give attention on the revised strategy by 2013 to expand this sector and encouraging the population to utilize industrially-processed micronutrient fortified foods (FDRE/NNP, 2013). In-addition, in rural area as an optional method local fortification either within the community (such as community grain mills where available), or in the household providing training on basic food preparation, including the use of simple fortification products (for example using *sprinkles* in their cooking) side by side with awareness creation, attitudes changes and practices for improved nutrition and other strategies as per FDRE/NNP, (2013).

Micronutrients are vital for society to be healthy; among the major prevalent micronutrient deficiencies iron and zinc deficiencies exert larger economic and social burden. In Ethiopia, context as per EPHI/ENMS, (2016) highest prevalence of iron deficiency anemia was observed in preschool children 6-59 months of age (34.4%), followed by school age children 5 to 14 year of age (25.6%) and non-pregnant women age 15 to 49 years (17.7%) and nearly similar report was reported by EPHI/ENFCS, (2013). Based on WHO classification, in Ethiopia anemia was moderate public health problem in children 6 to 59 months and 5 to 14 years of age, whereas a mild problem in non-pregnant women.

Likewise, EPHI/ENFCS, (2013) report for anemia and zinc deficiency of targeted group that showed in Amhara region a high prevalence of excessive intakes of iron in the adult population. Statistically approximately 64% of women 19-45 years and 89% of urban males that might be at risk of excessive intakes of iron (EPHI/ENFCS, 2013). While for Zinc for women, 50% were found to have an inadequate intake of zinc (EPHI/ENFCS, 2013). In urban areas the prevalence of zinc inadequate intakes was slightly higher (65%) as per EPHI/ENFCS, (2013) report. Therefore, it was clear that both reports EPHI/ENMS, (2016) and EPHI/ENFCS, (2013) shows that the prevalence of zinc deficiency while higher excessive iron intake, which seems to be correlation and need to be clearly defined.

Simulation of fortification scenarios indicate that the impact of fortification of edible oil and wheat flour varies by the micronutrients (iron, zinc and vitamin A) added for population group of interest

(FMOH/NNS, 2008). Yet, for excessive iron finding could be likely as a result of exposure for excessive dietary iron intake and mostly fortification was done based on recommended daily allowance/WHO set standard. Therefore, the source of excessive iron intake of Ethiopian National Food Consumption Survey report and zinc deficiency prevalence need to be discovered.

Teff is belived to be an excellent source of iron for most of population in our country (Saturni, Ferreti & Bacchetti, 2010; Umeta, West & Fufa, 2005). Most of the iron content for cereals produced in Ethiopia is due to be exposure to iron contamination. The iron source came from soil contamination specifically it is attributed to traditional threshing of grains under the hooves of cattle(Ambaw, 2013; Abebe *et al.*, 2007). This contamination was found to be higher in Teff compared others grains because of higher suraface contact with soil(Baye *et al.*, 2014), and these significant proportion of the iron in the Teff diets of Ethiopia is extrinisic to food, usually referred to as contamination iron.

Extrnisic soil contamination iron source being sole part Teff. However, there was controversies concerning the bioavailability and bioaccessability of extrnisic soil contamination iron source as disscussed by Seim *et al.*, (2013) and Teklu, (2017b). The *in vivo* studies conducted by Teklu, (2017a) and Guja & Baye, (2018) conformed that bioavailability and bioaccessability of extrnisic soil contamination iron source since, it has effect hemoglobin regenaration and potential ability of allevating iron deficiency in similar way to that of non-heme source iron (ferrous sulfate) fortified dietary source.

The possible reason for the above reported by EPHI/ENFCS, (2013) for excess iron and zinc inadequacy scenarios need to be discovered and the assumption goes to extrinsic contamination source of iron intake on top of dietary iron consumption. However, iron, zinc and cooper reported for negative interaction as a result of iron overload/oxidative stress due to excess iron consumption would affect zinc and copper by Fairweather-Tait & Southon, (1989), Roughead, Johnson & Hunt, (1999), Akhtar *et al.*, (2010), Ha *et al.*, (2016) and Heidari *et al.*, (2016). Similarly, due to the reason iron, zinc and copper had common transportation pool, similar chemical nature and negative interaction each other human based reports Angelova *et al.*, (2014), review Sandstrom, (2001) and rat assay based dietary chronic iron consumption experimental studies Fairweather-Tait & Southon, (1989), Roughead, Johnson & Hunt, (1999), Akhtar *et al.*, (2010), Ha *et al.*, (2016) and Heidari *et al.*, (2016). Therefore, our research will try to see the potential effect of excess dose of extrinsic soil contamination source iron on top of adequate dose of dietary iron having low or high-

bioavailability index using *in-vivo* assay on iron, zinc and copper status by measuring serum and metabolism status.

1.2 Significance of the study

Micronutrient deficiencies are global problems. Among the most prevalent micronutrient deficiency iron accounts the major wing followed by zinc. Many interventional methods have been used to reduce those micronutrient deficiencies; supplementation and fortification of local staple food with micronutrients is the major techniques. However, during fortification program application certain consideration needed; that was the existence of micronutrient interaction since one micronutrient will have an effect on another micronutrient status. Based on our case, it is quite obvious that iron has competition effect on zinc and copper status of human being from different studies conducted so far, however the interaction studies were not conducted based on dose dependent and bioavailability level due to ethical issues. Yet, there are number of *in vivo* based studies which can give nearly closed to human based conclusion. In addition, previously conducted studies showed that extrinsic/soil source iron was not bioavailable and bioaccessable. However, iron taken from non-inherent sources like soil, based on researches conducted by Demeke Teklu and Habetamu Guja whether soil contaminated Teff grains has an effect on rat hemoglobin generation showed positive result and we took this as one input to our study for being extrinsic/soil contamination source of excess dose iron on top of adequate dose of dietary source iron consumption effect on iron, zinc and copper status need to be investigated.

In our country fortification and supplementation are the intervention under plan by government and non-governmental organization to overcome micronutrients deficiencies. Staple foods can be fortified by different kinds of fortificants; the fortificant cost, fortificant bioavailability, fortificant easiness to be accessible by near, fortificant nature to food matrix to matchup whether the fortificant has an effect in the shelf-life/off-flavour of food are the criteria used during selection of the right fortificant. After selecting the right fortificant, directly applying the interventional methods is the way, but sometimes the society can get non-inherent sources of iron like soil in addition to the interventional programs (fortification and supplementation), this non-inherent source sum effect has to be identified. This research activity was to check whether non-inherent source (soil in addition) to interventional methods, by combining different possible scenarios like effect of excess dose of soil contamination on top of adequate dose of highly bioavailable iron

fortified food, effect of excess dose of soil contamination on top of adequate dose of less bioavailable iron fortified food, effect of slightly higher dose of soil contamination to food and effect of higher dose of less-bioavailable fortificant in comparison with adequate dose of high bioavailable iron fortified and adequate dose of less bioavailable iron fortified on serum micronutrients(Iron, Zinc and Copper) status, weight(growth) and organ level micronutrients accumulation by employing *in vivo*/rat model experimentation.

The research output will contribute much through the following points.

- It will help to set margin for researchers'/policy makers to consider for human being level different possible combination by dose level and bioavailability type interaction that will exist by taking our study finding as an input.
- Since the government is taking an effort to fortify iron to different local foods as an approach for strategy to control micronutrient deficiency this research will give an insight on what kind and how much percent of fortificant to be added or even not.
- It will be a major input for researchers and policy makers to study for its feasibility at human being level/initiate further research direction at human being level.
- Helps to promote the fortification or supplementation program developers and policy makers to consider the society intended to be part of their interventional program while selecting staple foods/grains and non-inherent iron contamination level in compromised way to set their recommended dietary allowance ultimately to save the population from health problems which are related to iron overload.
- It will benefit the local consumer to be intervened to attain dietary recommendations for iron at the same time can reduce the risk of side effect and toxicity associated with taking high-dose of iron on top of supplement or fortification intake and non-inherent contamination as iron utilizeability.
- It will help policy makers to have an advanced choice whether single or multiple use micronutrient fortification as possible way to avoid micronutrient interaction.

1.3 Objective

1.3.1 General Objective

To investigate the effect of adequate and excessive intake of low and highly bioavailable iron and extrinsic source iron consumption at dietary level on copper and zinc status

1.3.2 Specific Objective

- To investigate whether high dose of diet iron will affect the zinc status
- To investigate whether high dose of diet iron will affect the copper status
- To investigate how the bioavailability of different dietary source iron effect on zinc and copper status when taken in adequate and excessive amounts
- To investigate how the bioavailability of different dietary source iron effect on zinc and copper status when taken in combination with extrinsic source iron at excess amounts
- To investigate iron, zinc and copper interaction

Chapter Two

Literature Review

2 Literature Review

2.1.1 Iron History

A connection between dietary iron supply and disorders of blood was proposed in 16th century, but its physiological basis was not recognized until horse hemoglobin was shown to contain 0.335% iron by Zinoffsky in 1886, this finding was confirmed in other species also (Suttle, 2010). In 1893 Stockman left controversial work showing hemoglobin concentration in anemic women could be rapidly increased by supplementation with ferrous salts (Stockman, 1893). During the 17th century, iron was used to treat chlorosis or green disease (historical names for hypochromic anemia), a condition often resulting from iron deficiency (Guggenheim, 1995). However, it was not until 1932 that the importance of iron was finally settled by the convincing proof that inorganic iron could be used for hemoglobin synthesis (Suttle, 2010).

2.1.2 Iron Properties

Iron exists in one of two oxidation states: the ferrous form (Fe^{2+}) or the ferric form (Fe^{3+}), this chemical property of iron enabled its catalytic role in a multitude of redox reactions necessary to support basic metabolic functions for life. In fact, iron's central role in oxygen and energy metabolism underscores the biologic significance of this element and helps to explain why it is one of the best-studied metal ions in nutrition and health (Wessling-Resnick, 2014).

2.1.3 Iron Physiological Function

Iron plays an important role in the proper structure of the hemoglobin molecule; hemoglobin is the functional unit of the red blood cells and it is a complex protein structure inside the red blood cells that help in delivering oxygen from the lungs to other parts of the body. Iron is a very important component of the hemoglobin molecule; erythropoietin, a molecule secreted by the kidneys, promotes the formation of red blood cells in the bone marrow (Abbaspour, Hurrell & Kelishadi, 2014). Iron sufficiency has a role to play with improved cognitive development (learning ability), body's immune system and work capacity. Iron adequacy during pregnancy is associated with improved outcomes for both mother and infant through preventing morbidity and mortality (Abbaspour, Hurrell & Kelishadi, 2014). Iron is essential for all tissues, present in the

brain from very early life participating in the neural myelination processes, also involved in promoting growth (Iannotti *et al.*, 2006).

Every single tissue in the body needs iron. Iron is required to ensure the body has a functioning metabolism and normal human physiology at the most basic, functional level (Waldvogel-Abramowski *et al.*, 2014). It is a key component in hundreds of proteins and enzymes that provide the foundation for hundreds of required processes of particular significance. Iron is a critical part of hemoglobin, myoglobin, and cytochromes required to supply oxygen throughout the body, regulate cell growth and ensure a healthy cellular environment, and allow for energy metabolism to take place (Waldvogel-Abramowski *et al.*, 2014).

2.1.3.1 Oxygen Transport and Storage

The body contains two heme-containing proteins involved in the transport and storage of oxygen, both of which are found in the blood–hemoglobin and myoglobin, most iron in the body (about 70%) is present in the hemoglobin molecules of the red blood cells (Gupta, 2014). Approximately 300 million molecules of hemoglobin can be found on the surface of every single red blood cell (Gupta, 2014).

Iron gives hemoglobin the ability to carry oxygen from the lungs to the rest of the body and some carbon dioxide back to the lungs to expel, due to nature of hemoglobin's unique ability to acquire oxygen rapidly during the short time it spends in contact with the lungs then is able to release this oxygen as needed as it circulates through the body's tissues (Gupta, 2014). Once the oxygen is delivered the iron (as part of haemoglobin) binds the carbon dioxide which is then transported back to the lung from where it gets exhaled (Gupta, 2014)

2.1.3.2 Cognitive Development

The mechanisms by which iron facilitates cognitive development and neurologic functioning as studies reveal. However, it is likely linked to the iron requirement of enzymes found in the body's neural tissue, their effect on the central nervous system, changes that occur in nerve myelination (an electrically insulating material found around nerves), and the sensitivity of neurotransmitter synthesis to iron status (Mamun & Ghani, 2017). It has also been observed that anemic children interact to a much lesser extent with the environment around them, which may, in and of itself,

affect a child's cognitive development; the influence of iron deficiency in critical periods of fetal development and childhood development has been reviewed on several occasions leading to very similar conclusions (Grantham-McGregor & Ani C, 2001; Iannotti *et al.*, 2006; Mamun & Ghani, 2017).

Research on the effects of iron nutriture on cognition of adolescents and adults has been limited. In college students, serum ferritin concentrations were related directly to characteristics of the electroencephalogram (EEG) and cognitive performance (Sandstead, 2000). A study of nonanemic Fe-deficient high school girls found improved verbal learning and memory after iron repletion (Bruner *et al.*, 1996; Sandstead, 2000). A cross-sectional study in the elderly found that iron status was associated with characteristics of the EEG (Sandstead, 2000). Thus, iron used to among the main cognitive developer micronutrients.

2.1.3.3 Detoxification Neurotransmitters and Hormone Synthesis

Iron is a key component in numerous processes responsible for the detoxification of our bodies, including alcohol metabolism, drug detoxification, and carcinogen excretion in the liver (Grantham-McGregor & Ani C, 2001). The fact that iron forms the core of the protein class called cytochromes. *Cytochrome* P450 enzymes are: to *function* within phase I Liver *Detoxification*. To *detoxify* xenobiotic sources of toxicity, chemicals, alcohols and carcinogens, converting them into water and oxygen (Grantham-McGregor & Ani C, 2001). Iron is also required for the synthesis of cytochromes. Therefore, without cytochromes, which require iron, essential hormones and neurotransmitters could not be produced and key detoxification process would not occur (Grantham-McGregor & Ani C, 2001).

2.1.3.4 Energy Metabolism

The use of energy by our cells also involves cytochromes, cytochromes contain heme at their core and play a key role in cellular energy production (Grantham-McGregor & Ani C, 2001). Cytochromes are part of the electron transport chain within the cell's mitochondria, these cytochromes carry electrons for the synthesis of ATP, the primary energy storage component in cells. Without ATP, and therefore without iron (iron is needed to allow cytochromes to carry electrons to make ATP), energy cannot be stored in our cells and eventually used in the hundreds of processes required by the body (Grantham-McGregor & Ani C, 2001; Wardlaw, 2004).

2.1.3.5 Immune Functioning

Several immune functions require iron; iron must be present to allow for cells to specialize for specific functions throughout the body and for T lymphocytes to rapidly divide, a step essential for the body to mount an immune response. Iron is also needed to create what are called reactive oxygen species used for killing pathogens in systemic controlled manner (Vatansever *et al.*, 2013).

The connection between iron deficiency and susceptibility to infection has been the most discussed topic, especially with respect to malaria; in short, this is because pathogens feed off of iron in the body and the body response during an infection is to decrease serum iron levels (iron in the blood) while increasing levels of ferritin (the iron storage protein) in order to sequester iron from the pathogens (Vatansever *et al.*, 2013).

2.1.3.6 Antioxidant Activity

Heme is an important part of enzymes that help to break down toxic oxygen species in the body, giving it antioxidant properties. However, iron can also catalyze destructive reactions including the formation of free radicals. Iron is unique in that it can switch back and forth between +2 and +3. This is both good and bad (Paes *et al.*, 2011; Heck *et al.*, 2010) while this works to catalyze the movement of electrons (allowing for all the good energy creation and detoxification reactions), it also makes iron very toxic if it roams free outside of the confines of the cellular system and can cause the formation of free radicals (Paes *et al.*, 2011; Heck *et al.*, 2010).

2.1.3.7 DNA Synthesis

Iron is an essential redox element that functions as a cofactor in many metabolic pathways. Critical enzymes in DNA metabolism, including multiple DNA repair enzymes (helicases, nucleases, glycosylases, demethylases) and ribonucleotide reductase, use iron as an indispensable cofactor to function (Paes *et al.*, 2011; Heck *et al.*, 2010). Recent striking results have revealed that the catalytic subunit of DNA polymerases also contains conserved cysteine-rich motifs that bind iron-sulfur (Fe/S) clusters that are essential for the formation of stable and active complexes. In line with this, mitochondrial and cytoplasmic defects in Fe/S cluster biogenesis and insertion into the nuclear iron-requiring enzymes involved in DNA synthesis and repair lead to DNA damage and genome instability (Paes *et al.*, 2011; Heck *et al.*, 2010). In-addition, Iron-dependent enzymes are

required for DNA synthesis making it essential for everything in which DNA is involved from growth, reproduction, and healing to proper immune function (Paes *et al.*, 2011; Heck *et al.*, 2010).

2.2 Dietary Sources and Body Regulation of Iron

Iron is present in foods in two forms, as heme iron, which is derived from flesh foods (meats, poultry, and fish), and as non-heme iron, which is the inorganic form present in plant foods such as cereals, pulses, legumes, grains, nuts, and vegetables. In meat, 30–70% of iron is haem iron, of which 15–35% is absorbed (Hurrell & Egli, 2010); however, non-haem iron, in plant-based diets the absorption is much lower (2-20%) which is strongly influenced by the presence of other food components (Abbaspour, Hurrell & Kelishadi, 2014). The absorption of non-haem iron is increased by meat and ascorbic acid intake, but inhibited by phytates, polyphenols, and calcium (Zimmermann & Hurrell, 2007; Hurrell & Egli, 2010). The quantity of non-heme iron in the diet is many folds greater than that of heme-iron in most plant/cereal based meals; thus, despite its lower bioavailability, nonheme iron generally contributes more to iron nutrition than heme iron (Abbaspour, Hurrell & Kelishadi, 2014).

Iron balance is primarily controlled at the level of intestinal absorption because there is no regulated excretion of iron through the liver or kidneys (Andrews, 2008). Adequate amounts of iron during growth typically results in a 70 kg man accumulating about 4 g of body iron. About 2.5 g of body iron is within hemoglobin and about 1 g is stored as ferritin or haemosiderin, mainly in the liver. Nutritional iron deficiency arises when physiological requirements cannot be met by iron absorption from diet. Loss of blood depletes the iron store since 1 mL blood translated into a 0.5 mg loss of iron (Zimmermann & Hurrell, 2007).

2.2.1 Human Iron Requirement

During gestation, the fetus stores about 250 mg of iron; these stores are drawn on during breastfeeding because breast milk supplies only about 0.15 mg of absorbed iron per day, whereas requirements for absorbed iron are about 0.55 mg per day. Low birth weight infants do not store an adequate amount of iron during fetal life and are at high risk of developing iron deficiency while being breastfed (Zimmermann & Hurrell, 2007). During early infancy iron requirements are met by the little iron contained in the human milk. The need for iron rises markedly 4-6 months after birth and amounts to about 0.7-0.9 mg/day during the remaining part of the first year. Between 1

and 6 years of age, the body iron content is again doubled. Iron requirements are also very high in adolescents, particularly during the period of rapid growth (FAO/WHO, 2004).

Average adult stores nearly 4 grams of iron in his or her body, an exquisite balance between dietary uptake and loss maintains this balance (Abbaspour *et al.*, 2014). About 1 mg of iron is lost each day through sloughing of cells from skin and mucosal surfaces, including the lining of the gastrointestinal tract. Menstruation increases the average daily iron loss to about 2 mg per day in premenopausal female adults and a dietary intake of iron is needed to replace the basal iron loss (Zimmermann & Hurrell, 2007; Abbaspour *et al.*, 2014).

2.2.2 Cause and Consequence of Iron Deficiency

Iron deficiency results from depletion of iron stores and occurs when iron absorption cannot keep pace over an extended period with the metabolic demands for iron to sustain growth and to replenish iron loss (Abbaspour *et al.*, 2014). The primary causes of iron deficiency include low intake of bioavailable iron which is considered to be responsible for around 50% of anemia (Allen *et al.*, 2006a), inflammatory and infectious diseases, especially malaria, blood loss from parasitic infections, and other nutrient deficiencies (vitamin A, riboflavin, folic acid and vitamin B12) are important causes (Brabin, Hakimi, & Pelletier, 2001). Increased iron requirements as a result of rapid growth, pregnancy, menstruation and impaired absorption of iron (Zimmermann & Hurrell, 2007; Abbaspour *et al.*, 2014) are also documented causes. The frequency of iron deficiency rises in female adolescents because menstrual iron losses are superimposed with needs for rapid growth (Harvey *et al.*, 2005). Other risk factors for iron deficiency in young women are high parity, use of an intrauterine device cause increased menstrual blood loss, and vegetarian diets which would be the reasons for iron deficiency anemia (Abbaspour *et al.*, 2014).

Consequences of iron deficiency result in functional changes that may occur with or without anemia. Anemia is described as a condition of low hemoglobin level in blood cells (Abbaspour *et al.*, 2014). Anemia is actually a sign of a disease process rather than a disease itself. It is usually classified as either chronic or acute. This also helps predict how severe the symptoms of anemia may be. In chronic anemia, symptoms typically begin slowly and progress gradually; whereas in acute anemia symptoms can be abrupt and more distressing. Nutritional anemia includes anemia due to deficiency in iron plus deficiencies in folate, vitamins A and B12, and certain trace elements

involved with red blood cell production even though about half of the global burden of anemia is due to iron deficiency (Ouedraogo *et al.*, 2019; Abbaspour *et al.*, 2014). Since iron plays an important role in the proper structure of the hemoglobin molecule, if iron intake is limited or inadequate due to poor dietary intake, anemia may occur as a result and this is called iron deficiency anemia. Iron deficiency anemia can also occur when there are stomach ulcers or other sources of slow, chronic bleeding (like colon cancer, uterine cancer, intestinal polyps, and hemorrhoids) (Abbaspour *et al.*, 2014).

Mild iron deficiency may be compensated by more efficient oxygen delivery from hemoglobin to tissues, redistribution in blood flow to protect brain and heart, and enhanced cardiac output (Wessling-Resnick, 2014). Work performance can become significantly impaired by reduced oxidative energy production as iron deficiency anemia evolves, however, under severe iron deficiency conditions, acidosis can occur (Wessling-Resnick, 2014). Another characteristic of anemia is lack of body temperature regulation under cold conditions that is associated with decreased thyroid hormone synthesis resulting from reduced activity of thyroid peroxidase, which is a heme-dependent enzyme (Zimmermann *et al.*, 2006). Restless legs syndrome arises from impaired dopaminergic function and is also linked to iron deficiency (Salas, Gamaldo & Allen, 2010). Iron deficiency is thought to impair immune function secondary to the important role of iron in generating free radical response to infection (e.g., myeloperoxidase activity), and low iron status is associated with changes in lymphocyte and neutrophil function (Bhaskaram, 2001). Respiratory infections are observed to be more frequent and to last longer in iron-deficient children (De-Silva *et al.*, 2003). During early childhood, low brain levels of iron can also create developmental issues that may not be reversed on iron repletion (WHO, 1992). Iron deficiency increases the incidence of preterm delivery and low birth weight, and patients with very severe cases are at increased risk of maternal and child mortality (Allen *et al.*, 2008).

2.2.3 Evaluation of Iron Status

Iron deficiency and eventually anemia develop in stages and can be assessed by measuring various biochemical indices (Abbaspour *et al.*, 2014). The assessment of iron status can be made by using specific laboratory measurements; this technology is constantly evolving, and no single method or combination of methods is suitable for all purposes (Cook, 1999). The optimal laboratory approach depends on the anticipated severity of the iron lack, the age and sex of the target population, the

potential for associated disease, the feasibility of venous sampling, and the available economic and laboratory resources. Sensitivity and specificity of methods are necessary to characterize changes in iron status (Mei *et al.*, 2005). The preferred methods in approximate order of increasing severity of the iron lack;

I. Hemoglobin
II. Serum ferritin
III. Transferrin Saturation
IV. Zinc-Erythrocyte Protoporphyrin
V. Hepcidin
VI. Serum transferrin receptor (Cook, 1999)

A variety of measurements have been used to identify milder form of iron deficiency, including serum ferritin, transferrin saturation, zinc-erythrocyte protoporphyrin, hepcidin, and more recently the concentration of the soluble fragment of transferrin receptor in serum. Laboratory evidence of milder iron deficiency without anemia will be referred to as iron deficiency (ID). More advanced deficiency is accompanied by a reduction in the packed cell volume or hemoglobin concentration, and thus referred to as iron deficiency anemia (Cook, 1999).

I. Hemoglobin (Hb): is most commonly used because it is inexpensive, easy to perform, and rapid. However, its levels are affected by factors other than iron deficiency because Hb is a function of red blood cells (RBC) production and turnover. Hb levels therefore lack specificity for categorizing iron status. Moreover, mild iron deficiency may not affect Hb levels and these could be drawback of Hb measurement (Mei *et al.*, 2005).

Although the method lacks sensitivity, Hb is a useful screen for iron deficiency in women and children in western societies because iron deficiency is the predominant cause of anemia in these populations and it is less useful in developing countries where anemia is multifactorial (Lynch, 2012). Other micronutrient deficiencies besides iron, such as vitamin B12, folate and vitamin A, can also cause anemia (Ouedraogo *et al.*, 2019). Because of these limitations' hemoglobin measurement cannot be used in isolation to indicate iron status (Cook, 1999).

However, low hemoglobin concentration is a measure of anemia, which is the end stage of iron deficiency (Abbaspour *et al.*, 2014). The Hb determination is in wider use at present because of

the availability of a hand-held battery-operated instrument called Hemocue, which gives immediate and highly-accurate result (Cook, 1999).

II. Serum Ferritin: Mild iron deficiency is better explained by serum ferritin level (Cook, 1999). Importance of the serum ferritin is that a low value in an anemic individual identifies IDA without a doubt; when the concentration is low (<12 μg/L for <5 years of age and <15 μg/L for >5 years of age) iron stores are depleted. Similarly, when the concentration of serum ferritin is ≥15 μg/L indicates the presence of adequate iron stores which is useful for excluding IDA as the cause of anemia (Cook, 1999; Abbaspour *et al.*, 2014). Serum ferritin level 1μg/L approximately as equivalent to 8mg storage of iron in adults (Lynch, 2012), and as an acute-phase reactant, serum ferritin is elevated by acute and chronic infections, inflammatory diseases, malignancies and liver disorders. Thus, the utility of the serum ferritin is enhanced if these disorders are excluded by clinical examination or other laboratory tests such as the erythrocyte sedimentation rate (ESR) or C-reactive protein (Cook, 1999).

Inflammatory disorders also increase circulating hepcidin concentrations, a peptide hormone that blocks iron release from enterocytes and reticuloendothelial system, resulting in iron deficiency (Zimmermann & Hurrel, 2007), because iron balance is primarily controlled at the level of intestinal absorption (Andrews, 2008).

A widely used marker of inflammation is the C-reactive protein (CRP), but the extent of increase of CRP concentration that invalidates the use of serum ferritin to diagnose iron deficiency and CRP values higher than 10–30 mg/L have been used. The problem with CRP is that during the acute-phase response, the increase of CRP concentration is typically of shorter duration than the increase of serum ferritin (Zimmermann & Hurrel, 2007), but still it is preferred over ESR to determine systemic inflammation (Costenbader, Chibnik & Schur, 2007).

III. Transferrin Saturation: calculated serum Fe/total Fe-binding capacity, partly compensates for the limitations of individual measurements, but does not overcome the fundamental problem that plasma iron transport is affected by a wide range of disorders (Cook, 1999). Mean corpuscular volume: a hematological method that has been extensively used to detect Fe-deprived erythrocytes, indicates the average size of circulating erythrocytes (Cook, 1999). Serum transferrin receptor (TfR): transferrin receptor is mostly derived from developing RBCs and therefore reflects the

intensity of erythropoiesis and the demand for iron (Abbaspour *et al.*, 2014). Enhanced synthesis of the transferrin receptors represents the cellular response to a declining iron supply, indicating sever iron insufficiency provided that there are no other causes of abnormal erythropoiesis (Abbaspour *et al.*, 2014). Unlike other methods, it is not affected by chronic inflammation or infections which are often confused with iron deficiency (Cook, 1999; WHO/CDC, 2004).

Finally, the ratio of TfR to ferritin (TfR/ferritin) is designed to evaluate changes in both stored iron and functional iron and thought to be more useful than either TfR or ferritin alone (Cook, Flowers, & Skikne, 2003). TfR/ferritin has been used to estimate body iron stores in both children and adults (Cook *et al.*, 2005). However, the high cost and the lack of standardization of the TfR assay so far have limited the applicability of the method (Yang *et al.*, 2008).

IV. Zinc-Erythrocyte Protoporphyrin: Heme biosynthesis takes place mainly in erythroid precursor cells in the bone marrow (Labbe & Dewanji, 2004). Iron is chelated by protoporphyrin as the final reaction in the heme path-way (Labbe & Dewanji, 2004). This reaction is catalyzed by ferrochelatase on the mitochondrial inner membrane (Labbe & Dewanji, 2004). Iron and zinc compete for the metal binding site of ferrochelatase and when the Fe^{2+}substrate is insufficient, it is substituted by Zn^{2+}, resulting in increased zinc-erythrocyte protoporphyrin formation. Excess ZPP/H formation reflects iron-zinc substrate competition for ferrochelatase in iron-deficient erythropoiesis (Labbe & Dewanji, 2004). Zinc-erythrocyte protoporphyrin is highly responsive to iron status even in borderline deficiency. Conversely, the decrease in zinc-erythrocyte protoporphyrin following iron supple-mentation in pre-anemic states illustrates the ability of zinc-erythrocyte protoporphyrin to respond to marginal changes in iron status (Labbe & Dewanji, 2004). A major advantage of zinc-erythrocyte protoporphyrin measurement is the simplicity with which it can be performed, as it requires only a portable instrument, the direct reading of fluorescence without need for any reagents, and requires minimal professional training (Labbe & Dewanji, 2004).

The zinc-erythrocyte protoporphyrin ratio is highly specific for iron-deficient erythropoiesis. However, it does not distinguish between absolute iron deficiency and iron-deficient erythropoiesis caused by anemia of chronic disease. Thus, a positive test result of zinc-erythrocyte protoporphyrin should be followed by a serum ferritin determination to distinguish iron deficiency

from iron-deficient erythropoiesis associated with inflammation, or the toxic effect of lead exposure (Labbe & Dewanji, 2004). Nevertheless, a zinc-erythrocyte protoporphyrin reading within the reference range is strong evidence of adequate systemic iron supply.

V. Hepcidin: Hepcidin functions by inhibiting the entry to the plasma of iron acquired by intestinal absorption, the recycling of iron derived from catabolism of senescent red blood cells (RBC) in macrophages, and by mobilization of iron stored in the liver. The block of iron flow is achieved by the binding of hepcidin to the iron transporter ferroportin, followed by its internalization and degradation (Kautz *et al.*, 2014).

Hepcidin production is increased by iron excess and by inflammation, and suppressed by both iron deficiency and increased erythropoiesis. Hepcidin production is flexible and changes within hours of introducing stimulatory or inhibitory messages such as iron administration or inflammatory stimulation. Because several opposing messages may present simultaneously, hepcidin output will depend on the relative strength of each. For example, in severe iron deficiency, hepcidin production tends to remain low, even in the presence of inflammation. Similarly, in conditions of ineffective or expanded erythropoiesis, such as in non-transfusion-dependent thalassemias, signals released by bone marrow erythroid precursors tend to override those from replete iron stores (Kautz *et al.*, 2014).

One such erythroid signal, erythroferrone, has been recently identified. erythroferrone is synthesized and secreted by erythroblasts in the marrow and extramedullary sites. The production of erythroferrone is induced by erythropoietin and is also proportional to the total number of responsive erythroblasts (Kautz *et al.*, 2014). Erythroferrone acts on hepatocytes to suppress the production of hepcidin by inhibiting hepatic signaling. By suppressing hepcidin, erythroferrone facilitates iron delivery during stress erythropoiesis, but also contributes to iron overload in anemias with ineffective erythropoiesis. The measurement of hepcidin, unlike other tests used for evaluating iron status, is a direct reflection of the mechanism controlling iron homeostasis. This unique feature of hepcidin represents a major advantage in trying to elucidate the nature of disease and its optimal management also, it can be used as a guide for iron therapy (Kautz *et al.*, 2014).

VI. Serum Transferrin Receptor: is used to measure the level of soluble transferrin receptors, thereby aiding in the evaluation of iron deficiency and in the diagnosis of iron deficiency anemia. Transferrin is an iron binding glycoprotein found in the plasma. Its main function is to control the level of free iron (Cook, 1999; WHO/CDC, 2004).

Serum transferrin receptor levels, combined with measurements of serum ferritin concentrations, provide the most effective assessment of a population's iron status. Unlike serum transferrin receptor levels rise during inflammation, decrease the utility of the indicator in areas where infectious disease is common. Therefore, in areas where inflammation is prevalent, assessing a population's iron status based on serum transferrin receptor levels is most appropriate (Cook, 1999; WHO/CDC, 2004).

2.3 Prevalence of Iron Deficiency and Groups at Risk

Iron deficiency affects more than two billion people worldwide, especially from the population women and children are the victims (WHO, 2015) the prevalence of anemia is particularly high in developing countries, where 293 million of children, 468 million of pregnant women are anemic and anemia contributes to 20% of all maternal death. According to Ethiopian Demographic and Health Survey (EDHS) (2016), the prevalence of anemia in women was 24% and 57% in children under five years 44%; when we look in depth 18% mildly anemic, 5% moderately anemic and 1% severely anemic among women, similarly 25% mildly anemic, 29% moderately anemic and 3% severely anemic among children below age of five.

EDHS data has limitation since it uses only hemoglobin level to determine iron status and no other iron specific tests are done and such surveys have rarely measured iron deficiency or any of the other factors that contribute to the development of anemia and therefore the contributing factors frequently remain unknown (McLean *et al.*, 2008). But country representative good quality study done by Umeta *et al.* (2008), that used a combination of methods employing iron specific test, serum ferritin, along with hemoglobin levels has identified in women of reproductive age in Ethiopia the prevalence of mild to moderate form of anemia was (30.4%), iron deficiency (49.7%) and iron deficiency anemia (17%).

Groups at highest risk of suffering from iron deficiency are found in those parts of a population that have inadequate access to foods rich in absorbable iron during stages of high iron demand

(Umeta *et al.* 2008). These groups correspond to children, adolescents, and women of reproductive age, in particular during pregnancy. In the case of infants and adolescents, the increased iron demand is the result of rapid growth. For women of reproductive age, the principle reason is the excessive blood loss during menstruation. During pregnancy, there is a significant increase in iron requirement due to the rapid growth of the placenta and the fetus and the expansion of the globular mass (FAO/WHO, 2004; Abbaspour *et al.*, 2014).

2.3.1. Factors Leads Risk Group to Iron Deficiency Anemia

The population groups most vulnerable to developing iron deficiency are infants, children, and women of reproductive age. Limited food availability, inadequate or lack of access to health care, poor environmental sanitation and personal hygiene are socioeconomic factors contributing to iron deficiency. Iron deficiency is more prevalent in population groups that have high iron requirements because of rapid growth or blood loss (Munasinghe & Broek, 2006).

Elevated iron needs are also associated with rapid growth, low iron stores at birth caused by the intergenerational link to iron deficiency in pregnancy, hookworm, and malarial infestation. Hookworm infestation leads to gastrointestinal blood loss that contributes further towards such infestation. This increases with age so that older children and adults usually are affected more in areas where hookworm is endemic. The control of intestinal parasites is an integral part of the intervention in such areas. Processes that impair iron absorption and utilization consist of malabsorption syndromes, chronic and/or repeated diarrhea, and rare genetic conditions) (Cade, Moreton & O,Hara, 2005;Munasinghe & Broek , 2006; Gangopadhyay, Karosh & Keith; 2011).

2.4. Anemia Reversing Intervention Strategies

Globally there are three main interventional strategies that can be implemented to overcome micronutrient malnutrition specifically for iron deficiency, supplementation, dietary diversification, and food fortification with iron are the interventions, from the alternatives Food-based strategies, which include dietary diversification and fortification appears in the most sustainable approaches to increase the iron and other micronutrients status of a population(WHO/FAO, 2006; Quintates, Cilla & Barbera, 2015).

2.4.1. Anemia Reversing by Supplementation

Using supplementation as an intervention to fill the gap created through regular/prescribed by physician of pharmacological preparations in the form of injections, capsules, and tablets are effective, whereby substantial and almost immediate benefits can be brought to the most at risk groups; it only improves the micronutrient status of those who receive the appropriately administered medical preparations, leaving behind others, hard to reach or practically unreachable at risk groups (WHO/FAO, 2006; Quintates, Cilla & Barbera, 2015).

2.4.2 Anemia Reversing by Dietary Diversification(modification)

Dietary modifications for reducing IDA involve increased intake of iron rich food such as flesh foods or consumption of ascorbic acid rich fruits and vegetables that enhance non-haeme iron absorption (WHO/FAO, 2006; Quintates, Cilla & Barbera, 2015). Other options include low intake of tea and coffee which inhibit non-haeme iron absorption. Among iron bioavailability suppressors phytate is the main one and techniques such as soaking, germination, and fermentation promote enzymatic hydrolysis of phytic acid in whole grain cereals and legumes by enhancing the activity of endogenous or exogenous phytase enzyme (Hurrell &Egli , 2010). Even use of non-enzymatic methods such as thermal processing, soaking, and milling for reducing phytic acid content in plant-based staples also have been successfully used to degrade the phytate level which inhibit iron absorption taken with the food during dietary diversification as an intervention (Hurrell &Egli , 2010).

2.4.3 Anemia Reversing by Fortification

Fortification is the addition of one or more essential nutrients to food for the purpose of preventing or correcting a demonstrated deficiency of one or more nutrients in the population or specific population groups(WHO/FAO, 2006; Quintates, Cilla & Barbera, 2015); Also, it is used to be the best interventional method for larger population group reaching or national level micronutrients deficiency intervention strategy, the purpose of this strategy is to increase the intake of specific nutrient(s) that have been identified as inadequate in the food. Rapidly changing lifestyle and increasing reliance on refined foods justifies the addition of nutrients to the expanding range of foods in order to ensure nutritional adequacy of a diet (ACC/SCN, 2000).

Food fortification can be mandatory, that is, legislated by law and/or regulated, or it can be voluntary, that is, left to the discretion of the food manufacturer (Nestel & Davidsson, 2002). The first step in a fortification program is the selection of a food that can function as a vehicle for the micronutrient. This food vehicle must be an integral component of the diet of the general population (Lynch, 2005). The main point to be view during fortification of local staple food is used to be the most cost effective, long term approach in reducing the prevalence of iron deficiency anemia (Hurrell, 1997).

2.4.3.1 Cases to be Considered During Fortification Strategies

Minerals are more resistant to manufacturing processes than vitamins but, minerals undergo certain changes when they are exposed to heat, air and light. Minerals such as copper, iron, and zinc are also affected by moisture, and may react with other food components such as proteins and carbohydrates (WHO/FAO, 2006; Quintates, Cilla & Barbera, 2015).

Numerous fortificants are available for iron fortification, the main challenge when selecting is form of iron compound that is adequately absorbed, stable, and does not alter the appearance or taste of the food, even though fortificant bioavailability is crucial, other different aspects must be considered when a source of iron or any mineral has to be selected for fortification. These include particle size, solubility, flavor, color, stability, hygroscopicity, and oxidation (WHO/FAO, 2006; Quintates, Cilla & Barbera, 2015).

For purpose of fortification mostly non-haeme/inorganic sources has been used, the most common fortificants widely used in fortification include iron-EDTA, ferrous sulfate, elemental iron (reduced iron), ferric orthophosphate, ferrous fumarate, sodium ferric pyrophosphate, and other compounds, each fortificant listed above has various advantages and disadvantages (Hurrell, 1997;WHO/FAO, 2008).

2.5 Methods of Assessing Iron Bioavailability and Bioaccessibility

The quantity of a nutrient that is absorbed from a food can be measured at different levels, which can be described by different terms, (Fair-weather-Tait, Collings, & Hurst, 2010) have proposed the following definitions. Bioaccessibility corresponds to the proportion of iron that can be released from food matrix into the lumen of the gastrointestinal tract. This fraction of nutrient potentially available for absorption mostly relies on food composition. Bioaccessibility is

measured by in vitro methods, either by solubility or dialysability. On the other hand,bioavailability of iron corresponds to the proportion of the ingested iron that can be used for normal body functions and thus is measured by in vivo methods. Unlike bioaccessibility, bioavailability measurement is not only depending on food composition but also consider the iron status of the host.

Estimating iron absorption is mainly studied by in vitro methods since it is relatively simple, rapid and inexpensive and carried out through simulating the digestion in gastric and duodenum, followed by dialysis. The proportion of the element diffused through a semi permeable membrane during the process, is the dialysability of element after an equilibration period, being used as an estimate the relative bioavailability of nutrients (Bueno *et al.*, 2013). Studies aimed at the optimization of iron in nutritional formulations should include in vitro methods followed by an assessment of iron absorption in vivo in order to better investigate their metabolic behavior (Bueno *et al.*, 2013). There are different in vivo and in vitro methods of determining iron bioavailability.

2.5.1. In vitro Methods of Estimating Mineral Bioavailability

The in vitro procedures have been very useful for identifying and characterizing factors that affect non-heme iron absorption, but direct comparisons between absorption predicted from the in vitro tests and measurements in human volunteers have only been made in a limited number of published studies (Lynch, 2005). Generally, it is advantageous if human bioavailability testing could be based on preliminary in vitro screening methods to efficiently identify the promising food substance of interest (Beiseigel *et al.*, 2007). There are different methods of determining the in vitro bioavailability of minerals including iron: iron solubility, dialysability, Coca-2 (Frontela, Gracia, & Ros, 2011) and other in vitro methods include phytic acid/mineral molar ratio, and absorption prediction algorithm (Baye *et al.*, 2014).

I. Iron Solubility: This method is employed through exposing foods to a stimulated gastric acid environment and with subsequent measurements of the soluble iron released by the digestion i.e., measuring mineral solubility (Frontela, Gracia, & Ros, 2011).

II. In Vitro Iron Dialysability: This method is used to determine the percentages of dialyzable iron after simulated gastrointestinal digestion of foods; the fraction represents the proportion of iron that can be released from the food matrix into the lumen of the gastrointestinal tract and thus

potentially available for absorption i.e. bioaccessible fraction (Frontela, Gracia, & Ros, 2011; Baye *et al.*, 2014). Hence, dialyzability methods are limited to modeling luminal interactions among the factors as such interactions affect mineral availability (Hotz *et al.*, 2005).

In iron contaminated foods, no difference in iron dialysability was observed irrespective of the processing status; teff flour & its fermented product (injera) as shown in the study by Baye *et al.* (2014), the authors pointed out that mineral absorption inhibitors other than phytate might contributed for that. Earlier findings from human iron absorption studies also showed that dephytinisation in the presence of polyphenols did little to improve iron absorption (Hallberg & Hulthen, 2000).

Improvement in the methodology of dialyzability has been obtained when the human colon carcinoma (Caco-2) cell line has been incorporated (Frontela, Gracia, & Ros, 2011).

III. Caco-2 Cell Model: The human intestinal Caco-2 cell line originally isolated by J. Fogh in Sloan Kettering Institute, New York, from a human colon adenocarcinoma (Fogh *et al.*, 1977), this human colon adenocarcinoma, undergoes in culture a process of spontaneous differentiation that leads to the formation of a monolayer of cells, expressing several morphological and functional characteristics of a mature enterocyte (Sambuy *et al.*, 2005).

To better represent the steric conditions existing in the intestine in vivo, Caco-2 Cells are cultured on permeable filter supports that allow free access of ions and nutrients to the two sides of the cell monolayer (Sambuy *et al.*, 2005). Caco-2 cell line has been extensively used over the last twenty years as a model of intestinal barrier (Sambuy *et al.*, 2005); it is still the best available cell culture model of absorptive small intestinal enterocytes.

However, attempts to establish differentiated enterocytic cell lines in culture have not been fully successful though expressing several brush border enzymes; some of them are expressed at the level higher or lower than human enterocytes do (Sambuy *et al.*, 2005). For instance; oxidative metabolic enzymes are lower than in the human enterocytes, limiting the use of this line as an in vitro model of intestinal oxidative metabolism. On the contrary, the ferric reductase activity associated with brush border that reduces Fe3+ for uptake by the Fe2+ transporter was found to be high in differentiated Caco-2 cells (Ekmekcioglu et al., 1998). The divalent metal transporter (DMT1) is located on the brush border membrane that transports Fe2+; DMT1 expression and

activity is regulated mainly by iron but since it has a broad substrate activity, it can also transport other physiologically relevant divalent cations such as Zn+2, Mn+2, Cu+2 and the toxic metals Cd+2, and Pb+2. Studies by Andrerle *et al.* (2003) and Fleet et al. (2003) were confirmed upregulation of DMT1 during Caco-2 differentiation. Similarly, Caco-2 Cells have an elevated activity of P-glycoproteins (a trans-membrane protein responsible for apical efflux of several substances), higher than the human intestine in vivo, that should be taken in account when these cells are used as a model of intestinal bioavailability (Sambuy *et al.*, 2005). This great variability of Caco-2 cells model arises from cell- and culture related factors and these factors again show huge variation in differentiation of Caco-2 Cells (Sambuy *et al.*, 2005).

According to the study by Beiseigel *et al.* (2007), indirect comparisons with human data indicated that the Caco-2 model can predict the influence of ascorbic acid and polyphenolic compounds on relative iron bioavailability. However, this model has not been directly validated by simultaneously testing identical meals in both humans and the in vitro system. Authors have shown that though providing similar magnitude of total iron from maize varieties to both human and Caco-2 cell model, iron absorption difference was not significant in human and was only marginally significant in Caco-2 cell. The authors then suggested that, the variation might be because in humans the efficiency of iron absorption correlates inversely with body iron status but relative bioavailability measurements (as in Caco-2 cell) appear to be independent of the host's iron status (Beiseigel *et al.*, 2007).

The Caco-2 system is limited to measuring the entrance of iron from a food digestate into the mucosal cell, whereas iron bioavailability for humans also involves serosal transfer from the mucosal cell and subsequent transport and utilization (Beiseigel *et al.*, 2007). Additional testing of in vitro conditions is necessary, because cell passage and differentiation, incubation medium, time, pH, method of cell protection from digestive enzymes, and use of radio-iron variables compared with Caco-2 cell ferritin response variables may all influence in vitro results (Beiseigel *et al.*, 2007). Generally, the method lacks standardized protocol and therefore, reproducibility of results carried out in different settings may vary due to variation in culture, maintenance of the Caco-2 cell line and for specific nutrient/toxicant test to be carried out has no protocol (Sambuy *et al.*, 2005).

IV. Absorption Prediction Algorithms: The amount of iron absorbed from a meal is determined by iron status, the content of heme and non-heme iron, and the bioavailability of the two kinds of iron, which in turn is determined by the balance between dietary factors enhancing and inhibiting the absorption of iron, especially for non-heme iron. It is known that the variation in dietary iron absorption from meals is due to more to differences in the bioavailability of the iron, which can lead to a >10-fold variation in iron absorption, than to a variation in iron content (Bovell-Benjamin *et al.*, 2000; Hallberg & Hulthen, 2000). Algorithm is used to predict the bioavailability of iron in a meal and it is a product of all factors present in the meal that inhibit or enhance iron absorption (Hallberg & Hulthén, 2000).

The algorithm models include all the factors in calculations assuming their effect was independent and additive rather than interactive, which is an important issue in addressing iron bioavailability. The accuracy of the estimations is of concern due to lack of quantitative measurements of bioavailability modifiers, inability to consider interactive effects, and the use of non-iron status measurements (Reddy, 2005). Therefore, algorithm method is more qualitatively than quantitatively useful as it classifies meals or diets as being of high, medium, or low bioavailability and requires further development to accurately predict the influence of polyphenols on human iron absorption (Lynch, 2005; Beiseigel *et al.*, 2007).

Study done by Baye *et al.* (2014), have shown that in foods rich in iron but a high proportion of iron was associated with soil contamination, the knowledge of the exact amount of contaminant iron and its bioavailability may be a prerequisite for proper interpretation of algorithm to predicted absorptions unless the predictions only apply to intrinsic iron.

V. Phytic Acid/Mineral Molar Ratios: Previous attempts to assess or estimate relative mineral bioavailability in Ethiopian staples were based on phytate/mineral molar ratios (Umeta *et al.*, 2005; Abebe et al., 2007). The level of Phytate/ mineral molar ratios have different values to indicate adequacy for different minerals, the desirable level suggested for mineral absorption i.e., phytate:iron < 1, phytate:zinc < 18, phytate:calcium < 0.17 (Ma et al., 2007; Gibson et al., 2010). As phytic acid is strongly inhibitory at low concentrations, complete enzymatic degradation is recommended (>90%). If this is not possible, the phytic acid to iron molar ratio should be decreased to below 1 and preferably below 0.4 (Hurrell, 2004).

Phytate/iron molar ratio >1 is an indication of poor iron bioavailability (Tamanna *et al.*, 2013); however, according to Lestienne *et al.*, (2005), molar ratio of phytate to iron is not a sufficient indicator of iron availability if the level of anti-nutritional factors other than phytate is high. Besides phytates, polyphenols and to some extent calcium and fibers have negative effects on mineral absorption (Hurrell & Egli, 2010).

Moreover, in cereals with large amount of contaminant iron, the validity of using phytate/iron molar ratio to estimate relative bioavailability of the mineral is in question; knowing the exact amount of contaminant iron and its bioavailability may thus be a prerequisite for the use of phytic acid/Fe molar ratios to correctly predict bioavailability in foods containing contaminant iron (Baye *et al.*, 2014).

2.5.2. In Vivo Methods of Estimating Iron Bioavailability

I. Human Study: human trials are the gold standard for evaluating non-heme iron bioavailability (Aragon, Ortiz & Pachon, 2012). However, determining the bioavailability of a mineral in human is complicated by the fact that once a food is consumed, it mixes in the gastrointestinal tract with other foods that are consumed at about the same time or may be because the minerals are present in a mixture of sources available in the diet or meal. The use of extrinsic radio-iron tagging and stable isotopes in humans has partly offers a solution to this problem. Since human iron absorption studies are mainly done in healthy adults using isotopic techniques based on erythrocyte incorporation of either stable or radioactive isotopes (Fidler, 2003), this method adversely expose subjects to radiation and the use of radioisotopes may require monitoring the fate of radioisotopes or stable isotopes of iron in blood (Wienk, Marx, & Beynen, 1999). Moreover, human studies are cumbersome, complicated, costly, and time-consuming to perform (Ismail, 1999; Aragon, Ortiz & Pachon, 2012). These limitations have restricted the use of mineral absorption studies in human for the purpose of identifying specific biochemical components of the diet that explain the inhibitory or enhancing nature of certain foods (Ismail, 1999).

II. Animals Study: in animals, the hemoglobin-repletion bioassay was most often used (Wienk, Marx, & Beynen, 1999). Unlike humans, they are good for efficacy study because their environmental conditions can be controlled properly; however, animal studies have limitations because of different mineral requirements, metabolism, digestive capacity, sensitivity to dietary

factors (promoters and inhibitors of iron absorption) when compared to humans (Aragon, Ortiz & Pachon, 2012), and thus their use is limited because the accuracy of extrapolation to man (Wienk, Marx, & Beynen, 1999). But for evaluating bioavailability or relative biological values of iron from different sources with respect to ferrous sulfate control, hemoglobin-repletion bioassay has been used widely in recent studies (Swain, Newman & Hunt, 2003; Rohner *et al.*, 2007; Weber *et al.*, 2010; Martino *et al.*, 2011; Aragon, Ortiz & Pachon, 2012; Lucia *et al.*, 2013). Moreover, the comparison of in vitro, animal and clinical determinations of iron bioavailability study done by Forbes *et al.* (1989) have showed that the rat model hemoglobin depletion-repletion method serves as the most reliable predictor of iron bioavailability in the human although in vitro methods are promising screening techniques. The biological availability of iron from other diets have been measured by the rat bioassay (Weber *et al.*, 2010; Martino et al., 2011; Lucia *et al.*, 2013) and only single study previously carried out by Urga *et al.* (1998) in Ethiopian diet prepared from teff to show the effect of commonly processed forms of teff (Injera and Kitta) using rat hemoglobin repletion assay method for iron bioavailability.

2.6. Intrinsic and Extrinsic to Food Iron Sources

Iron sources can be broadly classified as intrinsic and extrinsic to food (Harvey *et al.*, 2000), and they are briefly reviewed below.

2.6.1. Intrinsic to Food Iron Sources (Dietary Iron Sources)

Dietary iron presents in food in two forms as heme and non-heme iron. Haem iron comes from animal foods (meat, poultry, seafood and fish) and is absorbed relatively well, its absorption being largely independent of other constituents in the diet (Carpenter & Mahoney, 1992; Hallberg & Hulthen, 2000; Harvey *et al.*, 2000). Animal products contain both heme and non-heme iron (i.e egg, milk and dairy), heme iron is that derived from hemoglobin and myoglobin. Plant materials contain only non-heme iron, the absorption of non-haem iron from the common pool, whether originally intrinsic or extrinsic to food, is subject to the same interactions with other constituents of the diet, as well as to endogenous factors of the host such as the nature of gastric secretions, transit time, rate of erythropoiesis and the size of existing iron stores (Harvey *et al.*, 2000). Heme iron is considered to be nutritionally important as it is higher in bioavailability (>15%) than non-heme iron (Kalpalathika *et al.*, 1991). However, heat treatment/cooking decreases the amount of

heme-iron concentration in food, while non-heme iron increased (Clark *et al.*, 1997; Kongkachuichai *et al.*, 2002).

2.6.2. Extrinsic to Food Iron Sources (Non-Food Sources of Iron)

A significant proportion of the iron in the diets of developing countries is extrinsic to food and usually referred to as contaminant iron (Harvey *et al.*, 2000). Sources of iron extrinsic to food include: contamination of foods from soil, dust and water; metal fragments from milling; that leached from iron or steel pots during processing, storage or cooking; and from the practice of geophagia. These contaminant forms of iron are largely ferric hydroxide and ferric oxide, but they are likely to vary widely in terms of solubility and affinity for reaction with other compounds (Harvey *et al.*, 2000). Thus, iron extrinsic to food can be consumed either through unintentional entrapment during food processing or deliberately via a practice of geophagy.

All iron consumed in the diet is either haem or non-haem and is absorbed from two distinct pools in the lumen of the gastrointestinal tract, largely in the small intestine. The amount of iron absorbed from a meal is determined by the content of heme and nonheme iron among other factors (Hallberg & Hulthen, 2000; Harvey *et al.*, 2000).

It is postulated that geophagy is practiced due to micronutrient deficiencies, cultural influences, and gastrointestinal upsets. Despite their potential to supply micronutrients, soils interfere with bioavailability of micronutrients leading to an increased risk of anemia and can also act as a pathway for ingestion of geohelminths and heavy metals, putting woman and fetus at risk (Kawai *et al.*, 2009; Njiru, Elchalal & Paltiel, 2011).

2.6.3 Extrinsic Iron Source Study Pattern in Ethiopia

An earlier study on the Ethiopian foods cooked in iron, aluminum and clay pots, have shown that there was more crude iron in all foods cooked in iron pots - around twice as much iron in meat and vegetables, and 1.5 times as much iron in legumes- than in food cooked in the other two types of pots (Adish *et al.*, 1999). Moreover, soil contamination of cereals in Ethiopia occurs mainly during traditional threshing under the hooves of cattle for grains like teff (Baye *et al.*, 2014). This traditional threshing method was found to increase iron contents up to 20 times higher in teff than other cereals which were attributed to soil contamination (Abebe *et al.*, 2007).

Teff is generally accepted that the grain is highly nutritious although there is some debate about the precise nutritional value of it (Vinning & McMahon, 2006). Regarding the iron content, some authors have estimated to be high and others reflect this high level is attributed to the dust and dirt that cling to the grain (Vinning & McMahon, 2006). As per the study done by Umeta *et al.* (2005), to evaluate mineral contents of 36 foods consumed in rural Ethiopia has shown Teff enjera as the best source of bioavailable iron of all foods analyzed based on its high iron content and favorable phytate to iron molar ratio. Moreover, the prevalence of IDA has been reported to be lower in some regions of Ethiopia, which has been attributed to Teff forming a staple part of the diet (Bokhari *et al.,* 2012). Whereas, other studies revealed that iron content of Teff vary between geographical regions possibly as a result of soil contamination (Saturni *et al.*, 2010; Bokhari *et al.*, 2012). With respect to soil contamination, teff is extremely exposed and complete removal of the extrinsic iron from Teff is not possible (Baye *et al.*, 2014).

Extrnisic soil contamination iron source being sole part Teff however, there was controversies concerning the bioavailability and bioaccessability of geophagy/extrnisic soil contamination iron source as disscussed by Seim *et al.*, (2013) and Teklu, (2017b). In view of that *in vivo* studies conducted by Teklu, (2017a) and Guja & Baye, (2018) conformed bioavailability and bioaccessability of geophagy/extrnisic soil contamination iron source in hemoglobin regenaration used to have a positive and potential ability of allevating iron deficiency in similar way to that of non-heme source iron(ferrous sulfate)fortified dietary source for showedup bioavailable and bioaccessible characterstics.

Extrnsic soil source iron on top of dietary source of iron(entrinsic) like fortified diets and iron supplement intake, it will create iron over load/toxicity in our society.

2.6.4 Iron Toxicity

Since no mechanism exists for excreting iron, toxicity depends on the amount of iron already in the body and there are ample evidence implicating reactive oxygen species in a number of human degenerative diseases such as atherosclerosis and haemochromatosis (Dabbagh *et al.*, 1994).

Iron over-load in experimental rats has increased in plasma total and high-density lipoprotein cholesterol levels and this effect were correlated with depletion of plasma ascorbic acid; generally, iron overload causes lipid metabolism disturbance and oxidative stress which substantially

depeletion of endogenous antioxidants and lipid peroxidation damage (Dabbagh *et al.*, 1994). Similarly, animal based experimental study conducted by Ha *et al.*, (2016) high dose of iron fed rat's growth retarded, cardiac hypertrophy, anemia, low serum and tissue copper levels and circulating ceruloplasmin activity. Therefore, the assumed iron overload effect at human being level could be comparative. Hence, it needs critical view.

2.7 Zinc

Zinc is found in group IIb of periodic table, and it is among the main micronutrients for human health. Zinc is the second most abundant transition metal in the human body after iron. Studies shows that average adult human body contains 1.4–2.3 g zinc, and nearly 90% is found in muscle and bone (Prasad *et al.*, 2009). Other organs containing estimable concentrations of zinc include prostate, liver, the gastrointestinal tract, kidney, skin, lung, brain, heart, and pancreas. On the cellular level, 30–40% of zinc is localized in the nucleus, 50% in the cytosol and the remaining part is associated with membranes (Plum, 2010).

2.7.1 Function of Zinc in Physiology

Zinc is a common element in human and natural environments and plays an important part in many biological processes (Vasak & Hasler , 2000) which is defined as a micronutrient, is essential for the normal growth and reproduction, also, it plays a key role during physiological of an immune function; in-addition, it is vital for the functionality of more than 300 enzymes, for the stabilization of DNA, and for gene expression.

Zinc is essential and directly involved in catalysis and co-catalysis by the enzymes, which control many cell processes including DNA synthesis, normal growth, brain development, behavioral response, reproduction, fetal development, membrane stability, bone formation, and wound healing (Barceloux, 1999;Mocchegiani *et al.*, 2000).

Zinc ions are hydrophilic and do not cross cell membranes by passive diffusion. Transport has been described having both saturable and non-saturable components, depending on the Zn concentrations present. Zinc ions exist in the expression of genetic information, in storage, synthesis and action of peptide hormones and structure maintenance of chromatin and bio membranes (Tapiero &Tew , 2003).

The biological essentiality of Zn implies the existence of homeostatic mechanisms that regulate its absorption, distribution, cellular uptake, and excretion. Zinc regulates both enzymatic activity and the stability of the proteins as an activator or as an inhibitor ion (Mocchegiani *et al.*, 2000).
In addition to being critical enzymatic cofactors involved in regulation the DNA transcription, other important functions of zinc in humans in broader aspect include; cell proliferation, differentiation and apoptosis, immune response onset and regulation, protein synthesis, DNA metabolism and repair, energy metabolism, vitamin A metabolism, Insulin storage/release, spermatogenesis and steroidogenesis neurogenesis, synaptogenesis and neuronal growth, sequestration of free radicals and protection against lipid peroxidation, cellular division, signal messenger and neuro-transmission and stabilization of macromolecules.

2.7.2. Human Zinc Requirement

Minimum requirement of zinc depends on growth, sex, age, health, and well-being vary with the type of diet consumed, climatic conditions and the existence of stress imposed by trauma, parasitic infestations, infections and zinc bioavailability (Barceloux, 1999;Mocchegiani *et al.*, 2000). Generally, the recommended daily dietary Zinc requirement is estimated at 15 mg/day and then tolerable upper intake level of Zn recommended is 25 mg/day (IZiNCG, 2007).

2.7.3. Sources of Food Rich in Zinc and Factors Affecting Zinc Bioavailability

Lean red meat, whole-grain cereals, pulses, and legumes provide the highest concentrations of zinc 25-50 mg/kg raw weight, processed cereals with low extraction rates, polished rice, and lean meat or meat with high fat content have a moderate zinc content 10-25 mg/kg, fish, roots and tubers, green leafy vegetables, and fruits are only modest sources of zinc <10 mg/kg (FAO/WHO, 2001).

Overall, absorption of zinc depends on the overall composition of the diet. There could be enhancers such as lower-molecular-weight (EDTA and Organic Acids/Citrate) substances has enhancing effect while phytates, micronutrients like iron, calcium and copper has suppression effect due to their competitive nature inside intestine. The risk for competitive interactions seems mainly to be related to high doses in the form of supplements or in aqueous solutions (Sandström, 2001, Hurrell, 2004; Hurrell & Egli, 2010). However, at levels present in food and at realistic fortification levels, zinc absorption appears not to be affected, for example, by iron and copper (Sandström, 2001). People who abstain from eating red meats, vegetarians, vegans, and people

living in developing country who rely mainly on plant-based foods are at higher risk of developing zinc deficiency due to inadequate zinc intake (FAO/WHO, 2001).

2.7.4. Systemic Zinc Regulation and Dietary Uptake

Zinc is lost from the body through the kidneys, skin, and intestine. The endogenous intestinal losses can vary from 7 μmol/day (0.5 mg/day) to more than 45 μmol/day (3 mg/day), depending on zinc intake (Johnson *et al.*, 1993). Urinary and skin losses are of the order of 7-10 μmol/day (0.5–0.7 mg/day) each and depend less on normal variations in zinc intake also, starvation and muscle catabolism increase zinc losses in urine and strenuous exercise and elevated ambient temperatures could lead to losses by perspiration (Johnson *et al.*, 1993).

The body has no zinc stores in the conventional sense. In conditions of bone resorption and tissue catabolism, zinc is released and may be re-utilised to some extent. Human experimental studies with low-zinc diets 2.6–3.6 mg/day (40-55 μmol/day) have shown that circulating zinc levels and activities of zinc-containing enzymes can be maintained within normal range over several months (Johnson *et al.*, 1993; FAO/WHO, 2001) which highlights the efficiency of the zinc homeostasis mechanism. When the dietary Zn content decreases, fecal losses decrease, enabling the efficiency of absorption to increase to almost 100%, there is also decreased urinary Zn excretion and increased cellular retention (Johnson *et.al.*, 1993).

2.7.5. Zinc Deficiency

Both nutritional and inherited zinc deficiency generate similar symptoms and clinical zinc deficiency causes a range from mild effects up to symptoms of severe nature (Prasad, 2015). In-fact, many people particularly in the developing countries consume less than the recommended nutrient intakes (RNIs) for dietary zinc (WHO/FAO, 2004). Epidemiological study has reported that zinc deficiency affects about 1.2 billion people around the world and has been ranked 11th among global risk factors for mortality and 12th for burden of disease (Lopez *et al.*, 2006). Zinc deficiency mostly occurs in developing countries. Similarly, according to (EPHI/ENMS, 2016) in Ethiopia the highest prevalence of zinc deficiency was observed in school children 5 to 14 years of age (35.76%), followed by preschool age children 6 to 59 months of age (35%) and non-pregnant women age 15-49 years (33.8%). According to the IZiNC group recommendation with this high level of zinc deficiency the whole population can be considered as at-risk zinc deficiency (IZiNCG, 2007).

Zn deficiency can mostly occur as a result of inherited or inadequate intake (taking minimal amounts foods rich in zinc), reduced absorption (taking phytate and GI problem), increased losses, or increased demand. The commonest worldwide cause is inadequate intake as a result of a diet low in Zn or rich in phytate (Livingstone, 2015). The population groups most at risk of developing Zn deficiency are those with the greatest physiologic requirements (Livingstone, 2015).

2.7.6. Measuring Human Zinc Status

Plasma zinc concentration was the most frequently used investigation marker of zinc status specifically for zinc deficiency at clinical level. Plasma concentration it is difficult to analyze for zinc and prediction zinc status, there is no set marker for it (Wieringa *et al.*, 2015). Urinary zinc excretion, erythrocyte zinc concentration, mononuclear cell zinc concentration, polymorphonuclear cell zinc concentration, platelet zinc concentration and hair zinc concentration are other types of zinc status measurement (Wieringa *et al.*, 2015).

Plasma zinc status has been the widely used method and the clinical measurement for zinc status. However, plasma zinc concentrations are affected by many other factors, including inflammation, fasting or eating, pregnancy, oral contraceptive use and diurnal rhythm needs to be considered (Wieringa *et al.*, 2015). Another consideration, if deficiency is diagnosed, the possibility of other micronutrient deficiencies should be considered because they tend to coexist and have synergistic effects. For example, deficiencies of vitamin B 12, folate, or ascorbate can all contribute to poor wound healing and should all be considered (Wieringa *et al.*, 2015).

2.8. Copper

2.8.1. Function of Copper in Human Physiology

Copper is a trace metal which acts as an inherent to biological processes, copper group involvement in several key enzymes and is thus essential for the structure and function of the bone marrow and nervous system and essential trace element for humans and animals, in the human copper exists in two forms , the first and second oxidation form, and the mostly occurred in human being is second oxidation state type (Jaiser & Winston, 2010) .The ability of copper to easily attach and accept electrons explains its importance in oxidative reduction processes and in disposing and removing free radicals from the organism (Angelova, Asenova, & Nedkova, 2011).

Copper is a functional component of several essential enzymes, known as copper enzymes for some physiological functions, dependent on the presence these enzymes in the organism (Jaiser & Winston, 2010; Angelova, Asenova, & Nedkova, 2011), are described below; Cytochrome oxidase plays as catalyzing the reduction cytochrome c oxidase is main role player mitochondrial enzyme which catalyzes by reduction oxygen to water and uses this energy during energy prodution for ATP, Lysyl oxidase, the enzyme main role is integrity and elasticity of connective tissues which are essential in heart, blood vessels and bone formation, Ceruloplasmin (feroxidase I) and (feroxidase II) are enzymes which have the ability to oxidize iron (Fe^{2+}) to iron (Fe^{3+}), which are connected to the protein transferrin for transportation to the red blood cells and blood formation in addition facilitates the catalyzation oxidative processes of the disposal of free radicals, many enzymatic reactions needed for proper functioning of brain and nervous system need copper as their central part, enzymes may take part in processes like conversion breakdown and inhibition chemicals needed proper function of brain and CNS, Tyrosinase is required during melanin formation which is needed pigmentation of hair, skin and eyes, Superoxide dismutase (SOD) functions as an antioxidant, which catalyses the conversion of superoxide radicals (free radicals) in hydrogen peroxide, that can subsequently be reduced to water by other antioxidant enzymes , gene expression certain copper containing enzymes are involved in synthesis of proteins in the organism by enhancing or inhibiting the transcription of specific genes and copper is also very important micronutrient studied for essentiality in fighting copper related anemia i.e copper-deficiency anemia.

2.8.2 Human Copper Requirement

The 10th edition of Recommended Dietary Allowances (RDA) did not include an RDA for copper; rather a safe and adequate daily intake was suggested (Livingstone, 2015). Some nutritionists think, committees that establish RDAs should return to the traditions of the first nine editions and make recommendations that meet functional needs, because lack of a recommended dietary allowance for copper may be hazardous to health, (Osredkar *et al.*, 2011) provides the recommended daily dietary intake (RDI) of copper for children and adults and Tolerable upper intake levels for copper.

2.8.3 Systemic Copper Regulation and Dietary Uptake

Copper is absorbed in the duodenum and upper sections of the small intestine then the copper is bound to either serum albumin or histidine and transported through the bloodstream for delivery to tissues or storage in the liver, copper is imported into the hepatocytes via plasma membrane localized high-affinity human copper transporter (hCtr1) (Jaiser & Winston, 2010; Angelova, Asenova, & Nedkova, 2011), hCtr l will guide where the copper has to be provided to tissues, liver and organs, the remaining copper after being utilized will be transported by blood protein ceruloplasmin, almost majority of copper found in serum are attached with ceruloplasmin will be delivered to the liver for storage . The excess copper will be released by bile with stool from certain literatures discussion (Jaiser & Winston, 2010; Angelova, Asenova, & Nedkova, 2011).

Copper is a trace element which can be found in almost every cell of the human organism, the highest concentrations of copper are discovered in the brain and the liver; the central nervous system and the heart have high concentrations of copper as well, about 50% of copper content is stored in bones and muscles (in skeletal muscle it is about 25%), 15% in skin, 15% in bone marrow, 8 to 15% in the liver and 8% in the brain hence, copper mostly at systemic level will be conserved (Angelova, Asenova, & Nedkova, 2011).

2.8.4 Copper Deficiency

Severe dietary deficiency in humans is rare; however, copper deficiency can occur by two means acquired and inherent the acquired is the common type it occurs when the intake and output is unbalanced (Uauy, Olivares, & Gonzalez, 1998). At embryo development level copper deficiency may result in impaired development of the cardiovascular system, bone malformation, and ongoing neurologic and immunologic abnormalities into infancy and beyond (Uauy, Olivares, & Gonzalez, 1998). In adulthood, prolonged marginal copper deficiency has been associated with an increased risk of developing osteoporosis in later life and adverse changes in cholesterol metabolism (Klevay *et al.*, 1984).

Based on studies infants fed cow milk have probability of being victim to copper deficiency than breast fed due to small quantity availability of copper in cow milk, comparatively, infant fed breast in addition to higher content copper availability in mother milk, casein content of human milk will aid as factor for copper absorption (Lönnerdal *et al.*, 1985). Some literatures have elaborated in developing countries infants mostly fed cow milk enriched with refined sugar and fructose and

refined sugar has lowering ability copper absorption, which may result copper deficiency (Uauy, Olivares, & Gonzalez, 1998).

Increased gastrointestinal copper losses usually explain the occurrence of copper deficiency in malabsorption syndromes. Copper deficiency should be suspected in infants with prolonged or recurrent diarrheal episodes, abnormal bile loss, intestinal resections, or loss of intestinal contents (Castillo-Duran *et al.*, 1988), high oral intakes of zinc and iron decrease copper absorption, it will lead to copper deficiency (Ha *et al*, 2016). Among the other causes of copper deficiency, pathological states that cause an inadequate copper supply are becoming more recognized and best example Menkes disease. Menkes disease is genetic related and the best-known disorder of copper deficiency, this cause unrecognized for copper demand and excess state, which result dietary copper not to be absorbed at small intestine and transported via blood brain and central nervous system (Jaiser & Winston , 2010; Angelova, Asenova, &Nedkova, 2011).

2.8.5. Measuring Human Copper Status

The occurrence of mild copper deficiency or excess copper exposure is not easily recognized (Gaetke *et al.*, 2014). Due to lack of sensitive and specific indicators, blood, urine and hair analysis are used to detect copper toxicity. From the blood sample serum copper concentration and ceruloplasmin are the most frequently analyzed copper status measurements used as an indicator, but total ceruloplasmin protein shows copper status but can show us the higher depletion and excess copper status level of individual (Harvey *et al*, 2009). However, review states that there is no updated excelling biomarker method than above stated copper status determining methods despite, the widely held view that there is a lack of sensitive and specific biomarkers of copper status, several putative indexes, including plasma copper, ceruloplasmin, urine, hair analysis, erythrocyte superoxide dismutase, urinary deoxypyridinoline, erythrocyte, platelet glutathione peroxidase, plasma glutathione peroxidase, platelet cytochrome-c oxidase, leukocyte cytochrome-c oxidase, total glutathione, diamine oxidase, and urinary pyridinoline and Cu/Zn superoxide dismutase (Cu/Zn SOD), routinely are test methods in human copper status studies and due to lack of data in her review only serum copper concentration and ceruloplasmin protein used as biomarker (Harvey *et al.*, 2009).

2.9. Iron, Zinc and Copper Biological Interaction

Iron and zinc nutritional deficiencies constitute two of the most important nutritional and public health problems affecting developing countries (Olivares *et al.*, 2012). Supplementation and fortification strategies can help to fight both deficiencies but, both micronutrients due to a competitive binding to DMT1 and Zip14 transporter makes them impossible to apply interventional strategies at the intended dose (Olivares *et al.*, 2012).

Understanding the mechanisms of the interactions between Fe, Zn and Cu are useful for supplementation or fortification programs using both (Iron and Zinc) minerals (Troost *et al.*, 2003). Copper is required for normal erythropoiesis. Copper deficiency causes an iron deficiency-like anemia (Merza *et al.*, 2015; Prasad *et al.*, 2015). The three micronutrients have different interaction with one another. Excess zinc nutritional supplementation is known to stimulate the synthesis of metallothionein in intestinal enterocytes. Copper binds with greater affinity than zinc to metallothionein and the copper will be excreted via intestinal tract and copper deficiency occurs while, zinc level will be elevated (Prasad *et al.*, 2015). When excess Zinc is taken copper will be excreted. Copper is a vital cofactor for various redox enzymes such as ceruloplasmin and cytochrome oxidase, and decreased activity of these enzymes have been postulated to be a potential cause of anemia. Mitochondria isolated from copper-deficient cells failed to synthesize heme from ferric iron and protoporphyrin, that is literally the causes for anemia (Gregg *et al.*, 2002). Moreover, copper homeostasis is closely linked with iron metabolism, since iron and copper have similar physiochemical and toxicological properties (Gaetke *et al.*, 2014; Knez *et al.*, 2015). Study conducted on interaction between anemia and blood levels of iron, zinc and copper in children showed a bit contradictory results, that is for both IDA group and IDA group the analyzed serum copper level is higher than both zinc and iron. In addition, serum zinc level is higher next to copper at both groups (IDA & ID groups). Serum zinc is a bit higher in ID groups than IDA groups and iron is the least level found at both (IDA & ID Group). However, serum iron level at ID group was higher than that of IDA group. When serum iron level is low serum zinc will be higher comparative to iron but, as far as both serum level lower copper will take the advantage of being higher at serum level (Turgut *et al.*, 2007).

Iron-copper interactions in biological systems may be attributed to their positive charges, similar atomic radii, and common metabolic fates. Dietary iron and copper are both absorbed in the

proximal small intestine also, iron and copper must be reduced before uptake into enterocytes and further, both metals are oxidized after export into the interstitial fluids. Enzymatic iron oxidation may occur while copper oxidation is likely spontaneous (Knez *et al.*, 2015). Moreover, both metals are involved in redox reaction in which they function as enzyme cofactors, and both can be toxic when in excess. Some literatures show that a reciprocal relationship between iron and copper has been established, for example, copper accumulates in the liver during iron deficiency, and iron accumulates during copper deficiency (Ha *et al.*, 2016; Klevay, 2001). Copper levels also increase in the intestinal mucosa and blood during iron deprivation. Despite these intriguing observations, the molecular bases of physiologically. Relevant iron-copper interactions are yet to be elucidated in detail.

Chapter Three

Material and Method

3. MATERIALS AND METHODS

3.1. Study Site, Soil Sample Collection and Preparation

The study was conducted in Ethiopian public health institute rodent laboratory and Bless Agrifood Laboratory and partly Addis Ababa University center for food science and nutrition. Our study was initiated with the assumption of excessive dose extrinsic iron/soil contamination intake on top of with or without adequate dose of dietary iron having different bioavailability index effect on iron, zinc and copper status. Hence, soil was used for three group of study as fortificant; excess dose of extrinsic iron/soil contamination only consumed, excess dose of extrinsic iron/soil contamination with adequate dose of electrolyte iron consumed and excess dose of extrinsic iron/soil contamination with adequate dose of iron sulfate consumed were the experimental groups.

We selected vertisol with the reason, recent in vitro study conducted on different soil type to determine iron bioaccessibility using iron fractionation suggested that only cambisol and vertisol soil types had a relatively small portion (0.001% and 0.004% of the total iron) of potentially bioaccessible iron (Smith et al., 2000). The cation exchange capacity of vertisol was significantly higher than cambisol, but both are categorized as a soil having high cation exchange capacity (Hazelton and Murphy, 2007). The highest cation exchange capacity of vertisol was also reported by previous study ranging from 20 to 45 meq/100g (Kebede and Yomoah, 2009). Similarly, contamination of vertisol to white teff flour enhances the exchangeable fraction from10.6% to 11.7% and contamination of cambisol to white teff flour enhances the exchangeable fraction from10.6% to 12.6% (Meseret, 2015). On top of the above-mentioned reasons, for this study we used the most prominent type of soil which was found in Teff growing regions of northern highlands of Ethiopia, that was vertisol.

Vertisol sample was collected from Ginchi Agricultural Research Sub-center. Ginchi Agricultural Research sub-center is located at 75km from Addis Ababa on the way to Ambo at 9^0 02' North latitude and 38^0 12' East longitude with an altitude of 2200 meters above sea level and has an average annual rainfall of 1095 mm, average relative humidity of 58.2%, average maximum and minimum air temperature of 24.6 ^{0}c and 8.4 ^{0} c respectively (Kebede *et al.*, 2016).

During soil sample collection difference in topography, underlying geological material, management history and yield history of the land were observed and composite sampling technique was applied according Carter & Gregorich, (2008).

Soil samples (different vertisol) were collected at depth (15 to 60 cm and 30 to 60 cm combination) using helical auger and ten representative spots were taken from each subsection of cultivated land. The samples were mixed well and a composite sample was taken. The samples were packed in polyethylene bag and transported to Addis Ababa University Food Science and Nutrition Laboratory then after arrival the soil samples were prepared for analysis.

Experimental analysis was conducted at the Center for Food Science and Nutrition research laboratory (experimental diet formulation), Bless Agrifood Laboratory for sample preparation and compositional analysis (mineral analysis for fortificants, formulated experimental diets and serum level minerals) and partly in the Ethiopian Public Health Institute (EPHI) for animal study.

The soil samples were air dried on plastic trays and gently crushed using mortar and pestle and passed through a 0.42mm sieve and poured in flask and closed with aluminum foil then autoclaved at 121°C for 180 minutes at 16psi as per (Environmental and Health Safety, 2018) in-order to sterilize the soil from any microbial contamination then the prepared soil sample was put under polyetelene bag until it was mixed with AIN-93G diet. (See Annex I)

Triplicate samples were taken and analyzed for iron content using AOAC official method 999.10 (Atomic Absorption Spectrometery After Microwave Digestion / see section 3.6), the results were in mean ± standard deviation that was in mg/100 grams before formulating experimental diet.

3.2. Experimental Design

The experimental design was *in vivo* based completely randomized controlled design, thirty-six wining male Wistar Rats were acclimatized and then divided in to six groups. Each group of rats were randomly distributed based on baseline measurements (age, weight, CRP and hemoglobin status). The six groups (one with control group and five experimental groups) were fed AIN-93G with intended iron source at intended dose/bioavailability level fortified (formulated experimental diet) for chronic exposure of two independent variables (dose and bioavailability) effect on zinc and copper status within thirty-five days. Finally, the six groups of animals were sacrificed for

end-line measurements (growth, liver weight, serum iron, serum zinc and serum copper status) across the groups. We tried to see different sources of iron formulated diet consumption effect on iron, zinc and copper status as well as investigated the interaction level that exist and different iron source (bioavailability and dose) independent variables interaction effect on metabolism status. Generally, the experimental study was carried out in four phases as shown in Figure 3.2.

Phase I: Iron content analysis for fortificant, soil sample and formulated experimental diet.

Phase II: Experimental diet formulation based on our assumed area of study.

Phase III: Baseline measurement (CRP, hemoglobin, age and weight) for all experimental rats.

Phase IV: End-line measurement (growth, liver-weight, serum iron, serum zinc and serum copper) based on the end-line measurement evaluating the chronic intake of different source iron effects on copper and zinc status as well as their interaction.

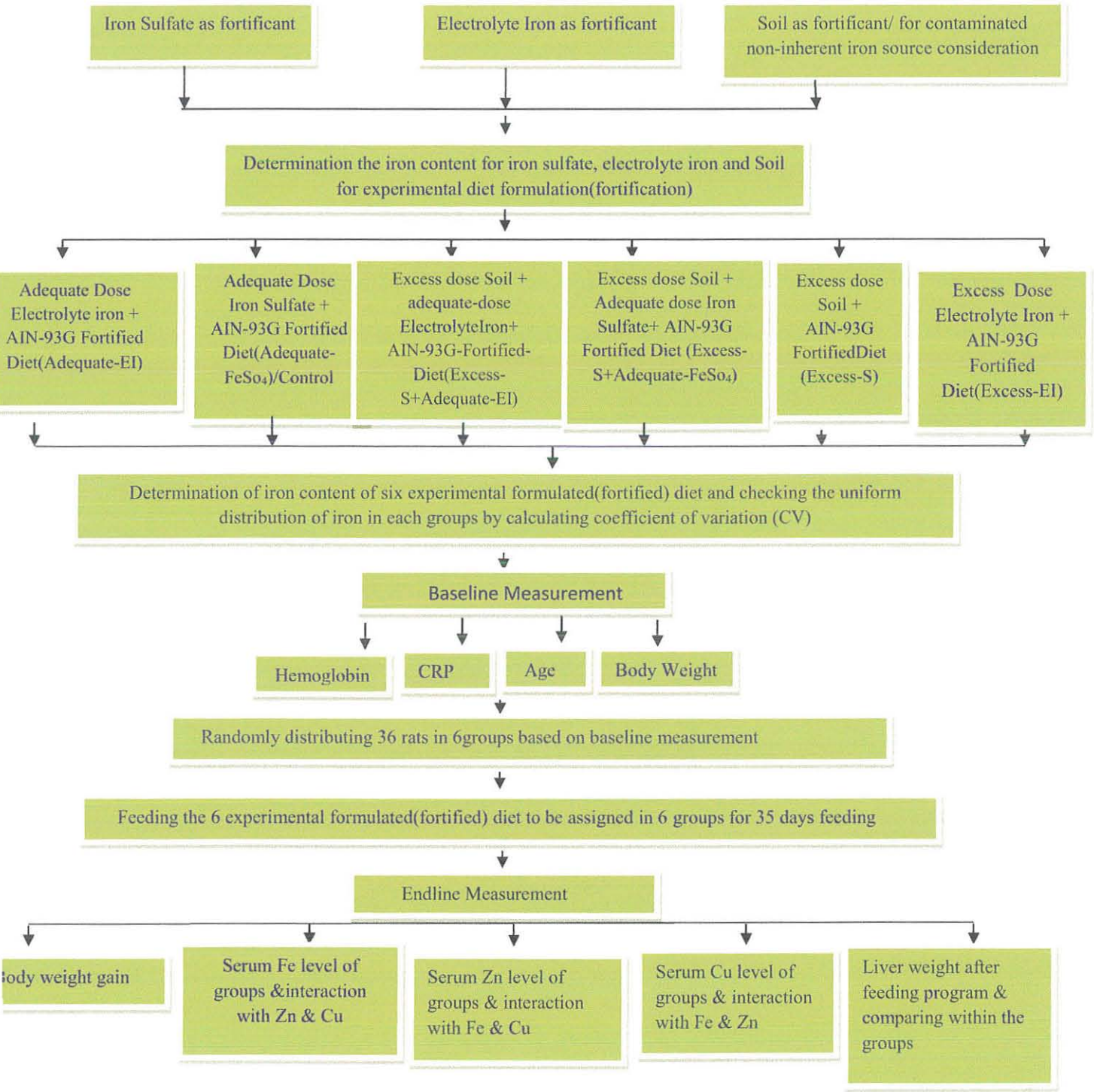


Figure 3.2: Experimental design for chronic exposure of different source iron at variable dose effect on micronutrient status

3.3 Iron Sources/Fortificants and Formulated Experimental Diet Iron Analysis

Iron content was determined by atomic absorption spectrophotometer (AAS) using AOAC official method 999.10 by using Atomic Absorption Spectrophotometry after Microwave Digestion.

3.3.1 Chemical and Reagent

Nitric Acid(65%w/w), Nitric Acid 0.1M,. Nitric acid 3M, Hydrogen peroxide 30%(W/W), Deionized Water, Zinc standard solution, iron standard solution, copper standard solution and working standard solution. (Merck KGaA, St.Louis, Missouri, USA)

3.3.2. Apparatus

Atomic Absorption Spectrophotometer, Hollow Cathode/Electrode discharge lamp, Microwave oven, Teflon digestive vessels, volumetric flasks, funnels(glass/plastic), Plastic bottles and Drying oven.

3.3.3. Procedure: For elemental iron, zinc and copper Analysis

***Cleaning*:** Procedure for glass and plastic ware

***Pre-treatment*:** If product is fresh

Drying: dry the sample in drying oven 105^0c at this stage

$\%\ of\ water = Wf - (Wd \div Wf) * 100$ Where, $H_2O\%$ = water content of the test portion, Wf = weight of the test portion(g) and Wd= weight after drying(g) final result was based on fresh weight, weight test portion before and after drying to obtain water content

Homogenization: Homogenize products using non-contaminating equipment

Digestion: Weighed 2g dry material into digestion vessel. Adding 5 ml HNO_3 and 2 ml 30% H_2O_2. Wait until it was digest based on set program. Transferred a solution into 25 ml volumetric flask and diluted to mark with deionized water.

Dilution: If test solution need to be further diluted (due to high metal concentration) dilute with 3M HNO_3.

3.3.4. Atomic Absorption Spectrophotometer

(Flame technique used for Fe, Zn and Cu concentration determination).

A simplified version of the method of standard addition was to use a matrix -matched curve, which was applicable to products with the same matrix. The test and standard solutions were mixed and used to make a standard curve. This was parallel transferred to origin and was used as the standard curve for the tests that followed and that have been diluted in the same proportion. Measurements were made in the linear range, when the method of addition was used. The concentration of zinc, copper and iron were usually at level suitable for determination by FAAS. When calibration curve was to be used, standard and test solution had the same acid concentration.

3.3.5. Calculation and Evaluation of Results

Concentration of metal in the test samples were calculated according to the formula:

$$C = (a - b) * df * 25 \div m$$

Where, C = concentration in the test sample(mg/kg); a= concentration in the test solution(mg/L); df=dilution factor; b=mean concentration in the blank solutions(mg/L); m=wavelength of the test portion(g). If(a-b) is lower than the detection limit, DL, then (a-b) is replaced by DL technique were used for calculation of the limit of detection in the test sample. The test solution was diluted, the dilution factor(df) was taken into consideration. If the test portion was dried and the result should be based on fresh weight, correct according to the following:

$$CFW = C * 100 - H2O\% \div 100$$

Where, CFW = concentration in the test portion was corrected to fresh weight(mg/kg); $H_2O\%$= The water content of the test portion (%).

When running replicates, the average of the results was given with three significant figures. **Detection limit**- The DL for each metal was calculated as;

$$DL = 3 * Standard\ Deviation\ of\ the\ mean\ of\ the\ blank\ determined (n = \geq 20)$$

3.4. Fortificants Supply, Diet Supply and Fortificant Iron Analysis

The fortificants were supplied from Hexagon Nutrition. Physical appearance black color, moderately fine for electrolyte iron, while iron sulfate was white in color and very fine powder. Both of the fortificants were food grade level with particle size for electrolyte iron(48μm) and iron sulfate(5-10μm). We used the above fortificants which were supplied from Hexagon Nutrition since they were listed as a potential fortificants by World Health Organization. WHO recommends

some iron compounds for cereal/powdery food fortification were ferrous sulfate, ferrous fumarate, ferric pyrophosphate, and electrolytic iron powder (WHO/FAO, 2006; Quintaes, Cilla & Barberá, 2015).

The experimental diet was supplied from American Institute of Nutrition (AIN) named AIN-93G which was intended to be used during the early growth phase and during reproduction of rodent experimental study as per (Reeves *et al.*, 1993), that was without iron incorporated. Though, American Institute of Nutrition (AIN)/Dyets Inc mostly provide the experimental diet containing all nutrients. In our case, we ordered them to supply diet empty/without dietary iron but every components were based on standard of Dyets Inc, since we were to fortify/formulate based on our dose and bioavailability level of fortificant combined with extrinisic/soil source iron as per experimental study design.

3.4.1. Experimental Diet Dose Setting, Assumption for Assigned Experimental Groups and Preliminary Level for Experimental Diet Formulation

Our study was dietary based chronic intake of different sources of iron effects, that was independent variables (bioavailability and dose-level) combination. That was the effect of excess dose of soil contamination/geophagy iron (350 mg/kg or 350 ppm) alone formulated diet consumed(Excess-S group), excess dose of soil contamination/geophagy iron (350 mg/kg or 350 ppm) combined with adequate dose of less bioavailable electrolyte iron dose (70 mg/kg or 70 ppm) formulated diet consumed(Excess-S+Adequate-EI group) and excess dose of soil contamination/geophagy iron(350 mg/kg or 350 ppm) combined with adequate dose of highly bioavailable iron sulfate at dose (35 mg/kg or 35 ppm) formulated diet consumed(Excess-S+Adequate-$FeSo_4$ group) then those experimental groups which consumed excess dose of soil contamination/extrinsic source to be compared with control group.

Similarly, the remaining experimental groups were, excess dose of electrolyte iron less bioavailable fortificant at dose (350 mg/kg or 350 ppm) formulated diet consumed (Excess-EI group) and adequate dose of electrolyte iron/less bioavailable fortificant (70 mg/kg or 70 ppm) formulated diet consumed (Adequate-EI group), then compared against with adequate dose of iron sulfate (at dose 35 mg/kg or 35 ppm) formulated diet consumed (Adequate-$FeSo_4$/control group).

Consequently, the excess dose (350 ppm) was set based on tolerable upper limit intake for human and adjusted the value proportionally to the recommendation for rats (oxidative stress inducing dose) studied by Arruda *et al.*, (2013). In-addition, the adequate dose (35 ppm) set as per Reeves *et al.*, (1993) and WHO/FAO, (2008), recommendation for different fortificants to be added to the foods. However, since electrolyte iron for being less bioavailable type, the adequate dose was adjusted to 70 mg/kg or 70 ppm which was double that of iron sulfate as per recommendation by WHO/FAO, (2006) and Quintaes,Cilla, & Barbera, (2015). Therefore, since we analyzed the fortificants(electrolyte iron, iron sulfate and soil) iron content/fortificant we added adequate dose and bioavailability as per our assumed experimental design or based on the above set dose level to AIN-93G experimental diet while formulating.

3.4.1.1. Experimental Diets Formulation/Fortification and Pelletizing

Before formulation went on the fortificants (electrolyte iron, iron sulfate and soil) iron content was analyzed (as per the method discussed on 3.3 section), then we added the fortificants/soil to the AIN-93G diet based on the intended dose level which was planned for our study design for groups (See Table 3.4.1.1).

To ensure proper mixing of these minerals/fortificants(electrolyte iron, iron sulfate and soil) with the AIN-93G diet, the fortificants total mass to be incorporated were first blended with AIN-93G diet at 1:4 (w/w) that was fortificant to diet with a portion as stated. Following, this blend was added at 1:4 (w/w) ratio first blend/fortified as a fortificant to AIN-93G diet as the second blend. Thirdly we added the second blend/fortified as a fortificant to the remaining diet which need to be fortified/formulated thus, until 4.14kg of diet which was intended for each batches of diet for experimental groups properly mixed as per technique used for wheat flour fortification by Akhtar *et al.*, (2010) and Johnson *et al.*, (2004). Hand operated turbine mixer (3000 rpm/min) was used to mix the fortificants with AIN-93G diet for about 15 minutes at each ratio level formulation steps (Akhtar *et al.*, 2010; Johnson *et al.*, 2004), (See Annex II). Also, we scraped the sides and bottom of the mixing container with straight-edge spatula to make sure there was no unmixed material left in the mixing container fortification principles combination for small scale powdery food based on Akhtar *et al.*, (2010) and Johnson *et al.*, (2004). Finally, the hand operated turbine mixer mixed batches of each experimental diets for six experimental groups were homogenized for 7 minutes

to have uniform level of iron concentration (Akhtar *et al.*, 2010; Johnson *et al.*, 2004). (See Annex III)

As a quality inspection for each batches of experimental diet to check whether the fortified batches of experimental diets for having proper dose of iron we took five samples (minimum number of samples required) as it was recommended by Johnson *et al.*, (2004), Suilburska, Bogdanski & Szulinska, (2013), Dary & Hainsworth, (2008) and WHO/FAO, (2008) then the iron content was analyzed by AOAC official method 999.10 (Atomic Absorption Spectroscopy After Microwave Digestion). The results were in mean ± standard deviation that was in mg/100grams. Ultimately, we used AAS reading for five samples from each batch of formulated diet the iron concentration analyzed samples were further calculated for uniform distribution of iron for six experimental diets by calculating the coefficient of variation (CV) to check the homogeneity of formulated experimental diet (Johnson *et al.*, 2004; Suilburska, Bogdanski & Szulinska, 2013; Dary & Hainsworth, 2008; WHO/FAO, 2008). The six formulated experimental diets were found to be consistent based on Dary & Hainsworth, (2008), WHO/FAO, (2008) and Suilburska, Bogdanski & Szulinska, (2013) with coefficient of variation below 10%.

The formulated batches of diets were agglomerated by adding 1000 to 1050 mL of deionized water for 4.14kg of formulated powdery diet, and made in to paste with the right consistency in stainless steel bowl, then after clumps were forced to pass in to cylindrical shape conical edged plastic test tube which was cut at conical edge with knife as an extruder (with diameter 9mm; length 6cm) in order to have uniform cylindrical shape pelletized diet. Finally, the cylindrical shaped batches of pellets for each experimental group were placed in an oven at 50°C for 24 hours. After 24 hours of drying, the batches of pellets were allowed to cool by storing in the refrigerator (4°C) with zip locked polyethylene plastic bag until feeding program initializes. (See Annex IX)

Table 3.4.1.1. Summarized Nutritional Composition for Formulated Diets Based on Experimental Groups.

Experimental Groups	Adequate-EI + AIN-93G	Excess-EI + AIN-93G	Excess-S+Adequate-$FeSo_4$+ AIN-93G	Excess-S + AIN-93G	Adequate-$FeSo_4$ + AIN-93G	Excess-S+ Adequate-$FeSo_4$ + AIN-93G
Macronutrients(g/kg) of diet for each experimental diet formulated for six groups						
Total CHO	629.48	629.48	629.48	629.48	629.48	629.48
Total Fat	70	70	70	70	70	70
Total Protein	200	200	200	200	200	200
Fiber	50	50	50	50	50	50
Minerals	35	35	35	35	35	35
Micronutrients(mg/kg) of diet for each experimental diet formulated for six groups						
IronFortificant (electrolyte iron/ iron sulfate)	70	350	70	----	35	35
Extrinsic soil source iron	-----	----	350	350	----	350
Zinc	30	30	30	30	30	30
Copper	6	6	6	6	6	6

* Adequate-EI: Adequate electrolyte iron added, Excess-EI: Excess electrolyte iron added, Excess-S+Adequate-EI: Excess dose of Soil with Adequate dose Electrolyte iron added, Excess-S: Excess dose of Soil only added, Adequate-$FeSO_4$: Adequate dose of iron sulphate added and Excess-S+Adequate-$FeSO_4$: Excess dose of Soil with adequate dose of iron sulphate added. All other nutrients were as per source Dyets Inc (Reeves *et al.*, 1993) for all composite except nutrient iron.

3.5. Animal Supply, Base-line Status, End-line Status and Maintenance

The experimental study animal supply, baseline, end-line and maintenance were held according to the standard methods.

3.5.1. Study Animal Collection and Screening/Baseline Measurment

Thirty-six male Wistar Rats (*Rattus novergicus*, albino variety, rodent class) of weaning stage (approximately three weeks to four weeks old or 24.5±1.64 to 26.5±1.51days) with body weight ranging from 70.18±6.76 to 73±5.29 gram was obtained from Ethiopian Public Health Institute Addis Ababa, Animal Department. The acclimatization period was under laboratory condition for five days by keeping them on standard rat ration, deionized water *ad libitum* in order to get them acclimatized with new environment of individual cage (Miyada, Nakajima & Ebihara, 2011).

After acclimatized the animal were housed in an individual cage at light and temperature-controlled room for 12 hours light and 12 hours darkness and 24 ± 3^{0}c after undertaking a test for hemoglobin measurement > 6.4g/dL that was for non-anemic status screening of the base-line status measurement as per Astuti *et al.*, (2017) to differentiate anemic and non-anemic status. We undertook a test for C-reactive protein for exclusion of infected animals from the experiment (Weber *et al.*, 2010) and for both base-line measurement blood from rat was collected at the end of acclimatization period (See Annex VII).

For blood sample collection using tail vein, a rat was placed in restrainer and needle was injected in most visible tail vein and then removed, blood was dripped on glass slide and immediately taken up by microcuvate of HemeCue(IIb 301, Lot No:1408389, Angelholm, Sweeden). The hemoglobin measurements were recorded in duplicate after placing the wipe microcuvate containing blood sample in HemoCue machine as per (Sari *et al.*,2001). If there were a wide variation between two reading of duplicate blood samples, the third measurement were carried out to avoid extreme values arising from operational errors and hemodilution with extracellular fluids (Rohner *et al.* 2007).

All experimental rats were non-anemic and non-infected hence, the thirty-six rats were randomly placed in each six study groups (Adequate-$FeSo_4$, Adequate-EI, Excess-EI, Excess-S+Adequate-EI, Excess-S and Excess-S+Adequate-$FeSo_4$). Base-line weight and age during randomization in to six groups were measured.

3.5.1.1. Blood Sample Collection for Baseline Measurements

Too much blood collected at any one time may cause hypovolemic shock, physiological stress and even death. If smaller volumes were collected too frequently, anemia may result, so we gave care to amount of blood volume we took (Parasuraman, Raveendran, & Kesavan,2010; Kumar *et al.*, 2017).

Lateral tail vein venipuncture*: The* veins located on the lateral aspect of the rat's tail was useful for collecting small volumes (< 0.1 mL) of blood. A slightly larger gauge needle (5cc /14 gauge) can be utilized. Because of the vein's size, larger blood volumes (approximately 1 mL) was obtained from weaning rats. For small blood volume collection, the tail vein was punctured with a small gauge needle, we collected the free-flowing blood for hemoglobin analysis (Parasuraman, Raveendran, & Kesavan,2010; Kumar *et al.*, 2017).

3.5.1.2. Hemoglobin Analysis Using HemoCue Hb 301

Principle of the test*: The* absorbance of whole blood was measured at Hb/HbO_2 isobestic point. It was read by spectrophotometry at 506 and 880 nm to compensate for turbidity.

Specimen*:* Type of sample: capillary (finger stick) or anti-coagulated venous or arterial blood (EDTA or heparin in solid form, no fluoride),

volume of sample: 10 µl of whole blood.

Equipment and reagents

HemoCue Hb 301 analyzer, HemoCue Hb 301 microcuvettes, 4 type AA batteries (1.5 V) and/or mains adapter, Non-sterile, single use gloves, Tissue or gauze, Alcohol swabs, Sterile lancets, Venous blood collection set (needles, syringe, sample tubes), Control solutions (Eurotrol Hb 301control Low/Normal/High), HemoCue cleaner swabs and PipetteParafilm or glass slide

Procedure

- Microcuvettes expiry date and vial opening date were checked.
- Switch on the machine: Press and hold left button and the display was activated.
- Optronic unit was checked.
- When the display showed 3 flashing dashes: the analyzer was ready to use.
- Cuvette holder was pulled out
- The result was displayed

- The first 2-3 drops were wiped
- The microcuvette was filled in one step process. The correct amount of blood (10 µl) was drawn into the microcuvette. The microcuvette were completely filled. Finally, the sample were put in Hemocue to read the hemoglobin.

3.5.2. Study Animal Handling and Ethical Consideration

Animal were handled as per the National Research Council of National Academics Guide for the care and use of laboratory animal (2011). During the whole experimental periods, all rats were receiving *ad libitum* deionized water and experimental diets. All rats were handled in a way that minimizes stress during weight measurement and blood sample collection. Blood sample were taken from lateral vein, tail vein blood sample collection totally avoids the stress resulted from tail incision and convenient for frequent blood sample collection from rats. At the end of the experimental period, all rats were scarified under light diethyl ether anesthesia. The experimental procedures were carried out after the approval /ethical clearance from Ethical clearance review board of college of natural science of Addis Ababa University.

3.5.3. Experimental Diet Feeding

Rats were fed with at animal department of Ethiopian Public Institute. We have six rats in each group which received intended experimental diet. First group of rats received diet containing adequate amount of electrolyte iron and deionized water, second group of rats received diet containing excess amount of electrolyte iron and deionized water , third group of rats received diet containing adequate amount of electrolyte iron and soil contamination with deionized water, fourth group of rats received diet containing excess amount of soil contaminated with deionized water, fifth group of rats received diet containing adequate and highly bioavailable iron sulfate and deionized water and sixth group of rats received diet containing adequate and highly bioavailable iron sulfate and excess soil contaminated with deionized water and the feeding period was for about 35 days (See Annex VIII).

During their feeding period 36 rats were placed in individual cage, we have also put racks in each cages in-order the feces not to be taken again. The cages were cleaned within two days of interval in-order to avoid suffocation.

3.5.4. Experimental Animal Scarification, Changing Whole Blood to Serum, End-line Iron, Zinc and Copper Analysis

The final blood sample was taken and animal scarification were conducted at cultural medicine Study department of Ethiopian Public Health Institute. For final blood sample intake, we used cardiac Puncture for the larger amount of blood collection (Parasuraman, Raveendran, & Kesavan,2010; Kumar *et al.*, 2017).

Cardiac Puncture: Cardiac puncture was the preferred technique for terminal collection of large blood volumes (Parasuraman, Raveendran, & Kesavan,2010; Kumar *et al.*, 2017). The animals for this study had general anesthesia administered, diethyl ether was used as anesthesia and the animals were placed on its back on a solid surface. The xyphoid process was palpated at the caudal aspect of the animal's sternum. A notch was present on both sides of this process. A 1 inch 22 or higher gauge needle attached to a 1 - 3 mL syringe was inserted into either notch and directed toward the heart as determined by palpating for the heartbeat. Once the needle was inserted beneath the skin, gentle negative pressure was applied, by pulling backward on the plunger. The animal was sacrificed at the completion of the procedure prior to awakening from anesthesia. (See Annex IX)

3.5.4.1. End-line Collected Blood Preparation/Changing the Whole Blood to Serum

The blood samples taken with syringe were transferred to yellow top test tube and the test tubes were put inside ice box and transferred to Clinical Chemistry Laboratory of Ethiopian Public Health Institute. The test tubes were put inside centrifuge having speed of 1000rpm for 3minutes, the serum and plasma were separated and then the supernatant(serum) transferred to sample holding tube (yellow top tube) using micropipettes. The prepared serum samples were put inside icebox and taken to Center for Food Science and Nutrition of Addis Ababa University for temporary storage -20 ^{0}c at deep freezer for a month. Finally analyzed at Bless Agri Food Laboratory located Laga-Tafo. (See Annex IX)

3.5.5. Measured Serum Iron, Zinc and Copper for Experimental Animals

Iron, zinc and copper content of thirty-six serum samples were analyzed by using the below methods (See 3.5.5.1 and 3.5.5.2) specifically designated way.

3.5.5.1. Sample Preparation and Serum Iron Analysis

The total serum iron from samples were determined by, dilution of sample 1:2 with a 20% (w/v) trichloroacetic acid (TCA) solution, and heated. This procedure precipitated the plasma protein and removed approximately 95% of hemoglobin iron present. We discarded visibly hemolyzed samples, even though we believed that the TCA removes about 95% of hemoglobin iron.

Equipment and reagents

Trichloroacetic acid (TCA), Polyethylene tube, Heater, Centrifuge, Pyrex-test tube and Atomic Absorption Spectrometer (AAS)

Procedure: we analyzed the supernatant for iron using the conditions listed in the "Standard Conditions". Appropriate iron standards were prepared by diluting the iron stock solution, described in the "Standard Conditions" for iron, with 10% (w/v) TCA. A 10% (w/v) TCA solution was used for the blank.

Since the samples were diluted 1:2 with TCA, we calibrated the instrument to read 2 × the actual concentration of the standards, then concentration was read directly.

3.5.5.2. Sample Preparation, Serum Zinc and Serum Copper Analysis

Copper and zinc content of thirty-six serum samples were analyzed by using the below methods.

Sample Preparation: The serum copper samples were determined; first the samples were diluted with an equal volume of deionized water. In-addition, the serum zinc samples were determined by dilution of the sample 1:5 with deionized water.

Equipment and reagents

10% (v/v) glycerol, 5% (v/v) glycerol, Deionized water, Atomic Absorption Spectrometer (AAS), Copper stock standard solution and Glass test tubes

Procedure: Samples were diluted with deionized water. The analysis was performed against standards prepared in glycerol to approximate the viscosity characteristics of the diluted samples. Determined the concentration of copper and/or zinc using the conditions as per "Standard Conditions". Copper standard was prepared by diluting the copper stock standard solution, described in the "Standard Conditions" for copper, with 10% (v/v) glycerol. A 10% (v/v) glycerol solution was used as a blank solution when determining copper. Zinc standard was prepared by

diluting the stock standard solution, described in the "Standard Conditions" for zinc, with 5% (v/v) glycerol. A 5% (v/v) glycerol solution was used as a blank solution when determining zinc. Finally, the samples were read directly on AAS.

3.6. Statistical Analysis

All analysis was conducted in triplicates and the data were analyzed using the software statistical package for social Science (SPSS) version 21. Descriptive statistics were used and the results were presented as mean and standard deviation. The six experimental diet feeding groups were compared for hemoglobin, initial-weight, CRP and age at baseline level, while at end-line level final-weight, liver weight, growth rate, serum iron, serum zinc and serum copper also, their interaction were run under regression analysis for comparison of independent variables that were for dose and bioavailability power identification within experimental groups. Finally, statistical differences among means were tested by Tucky's *post-hoc* test at 5% significant level ($p<0.05$).

Chapter Four

Results and Discussions

4. Results and Discussion

Fortification (iron sulfate, electrolyte iron and soil) total iron content was analyzed and based on analysis report the experimental diet formulation dose was set as per previously chronic level exposure of adequate or excess dose of iron *in vivo* studies report. The experimental diets were formulated based on bioavailability index(low/high) combined with iron overload impacting dose and adequate dose dietary food. The formulated diets were given to weaning rats, to study it's effect on serum iron, serum zinc, serum copper status of experimental groups were compared against the control group for iron-zinc-copper interaction. In addition, body weight, growth rate, liver weight were measured and compared with experimental control group/Adequate-$FeSO_4$ to strengthen the above measured parameters.

4.1. Determining for Fortificants Total Iron Content

The result for total iron content for fortificants (electrolyte iron, iron sulfate and vertisol iron content) of triplicate samples were analyzed, and the results were in mg/100grams was presented on Annex V. Based on analysis results the mean iron content for iron sulfate, electrolyte iron and soil were 1.9mg100g, 8.96mg/100g and 4532.6mg/100g in respective order.

The triplicate samples of fortificants (soil, electrolyte iron and iron sulfate) were compared each other and showed us significantly different in total iron content ($P<0.05$) . Our study was consistent for Vertisol/soil total iron content analyzed and reported by Azene, (2015) and Teklu, (2017a). However, the total iron content for fortificants were not consistent with that of the label for fortificants attached by supplier Hexagon Nutrition P.L.C, India. Hence, we adjusted the fortificant dose by minor mathematical calculation and the dose was set as per experimental study design.

4.2. Formulated Experimental Diets Total Iron Content

The five formulated experimental diet total iron content results were compared with that of control group for quality assurance purpose. The formulated diets total iron content was statistical significance different ($P<0.05$) as presented in table 4.2.

Though, the fortification/formulation and technical process were handled based on Reeves *et al.*, (1993), WHO/FAO, (2006) and Akhtar *et al.*, (2010), the total iron content for formulated experimental diets were validated by determining the total iron content (for five samples/minimum

number of samples recommended) as quality assurance (Johnson *et al.*,2004; Suilburska, Bogdanski & Szulinska, 2013; Dary & Hainsworth, 2008; Arruda *et al.*, 2013 and WHO/FAO, 2008) reported.

In-addition, the experimental diets CV value was determined by using total iron content. All formulated diets total iron was homogeneous, since CV values were < 5% as presented in table 4.2. The current result for CV values and total iron content were as per Johnson *et al.*, (2004), Suilburska, Bogdanski & Szulinska, (2013), Dary & Hainsworth, (2008) Arruda *et al.*, (2013) and WHO/FAO, (2008).

Table 4.2 Base line characteristics of AIN-93G fortified with different fortificants and iron content of each experimental formulated diet

Diet(4.14kg)	Fortified Diet	Iron Content (mg/100g)
AIN-93G	Adequate Electrolyte Iron (Adequate-EI)	7.57±0.29*[a]
AIN-93G	Excess Electrolyte Iron (Excess-EI)	35.64±0.45*[b]
AIN-93G	Soil with Electrolyte Iron (Excess-S+Adequate-EI)	44.15±1.39*[c]
AIN-93G	Only Soil as Iron Source (Excess-S)	37.55±1.77*[d]
AIN-93G	Adequate Iron Sulfate (Adequate-$FeSo_4$)/Control Diet	3.6±0.04*[e]
AIN-93G	Soil with Iron Sulfate (Excess-S+Adequate-$FeSo_4$)	39.36±0.6*[f]

Values are mean ± standard deviation and superscript (*) with in a row represents statistically significant difference (P<0.05). Different superscript within a row represent % Coefficient of variation value (CV) - a=3.88, b=1.27, c=3.16, d=4.71, e=1.26, f=1.49.

4.3. Baseline Characteristics of Experimental Rats

The baseline characteristics were measured for experimental rats (n=36) before realization of experimental study and the results were presented in Table 4.3 and Annex VII. The baseline

measured (hemoglobin, weight, age and CRP) were not significantly different for all groups ($P<0.05$).

Table 4.3 Baseline Rats Profile within the Groups

Measured Baseline Parameters	Six Experimental Groups					
	AIN-93G+ Adequate-EI	**AIN-93G+ Excess-EI**	**AIN-93G+ Excess-S+ Adequate-EI**	**AIN-93G+ Excess-S**	**AIN-93G+ Adequate-$FeSo_4$**	**AIN-93G+ Excess-S+ Adequate-$FeSo_4$**
Hemoglobin (g/dL)	14.73±3.7[a]	14.75± 3.6[a]	14.16±3.9[a]	14.2±2.6[a]	15.46±2.8[a]	14.06±2.6[a]
Age in days	24.5±1.59[a]	25±1.61[a]	26.2±1.5[a]	25±1.26[a]	24.5±1.67[a]	26±1.26[a]
Initial Weight(g)	70.95 ± 6.05[a]	70.18 ± 6.76[a]	71.38 ± 3.87[a]	71.38 ± 1.54[a]	72.55 ± 7.83[a]	73± 5.29[a]
CRP	Negative	Negative	Negative	Negative	Negative	Negative

Values are mean ± standard deviation and different superscript "a" row represent statistically significance level ($P<0.05$). Where, Adequate-EI- adequate electrolyte iron, Excess-EI- excess electrolyte iron, Excess-S+Adequate-EI - Excess dose of Soil plus adequate dose of electrolyte iron, Excess-S -only excess dose of soil, Adequate-$FeSo_4$-Adequate dose of iron sulfate, Excess-S+Adequate-$FeSo_4$- Excess dose of soil plus adequate dose of iron sulfate to be feed and assigned in to groups based on the above baseline measurement.

Therefore, it is possible to conclude that the randomization process among groups was efficient enough. Rat hemoglobin measurement was adjusted by deducting 1.1 g/dL from each rat measurement for Addis Ababa altitude (2300 meter above sea level) (WHO, 2011).

4.4. End-line Measurements, Growth Rate and Weight Among Experimental Groups.

The end line measured weight within experimental groups were compared using ANOVA for mean ± standard deviation value and there was a statistically significant difference ($P<0.05$) between

groups, end-line weight summarized under Table 4.4. Multiple linear regression result revealed that neither iron dose nor bioavailabilty variable individual effect was not significant in end-line measured weight elevation ($P<0.05$). However, there was significant effect for sum of independent variables (bioavailability and dose of iron) sum effect in elevating end-line weight within groups ($P<0.05$) and ($R^2= 0.08$), that was dietary variables (bioavailability and dose of iron) could influence 8% weight gain variation within the groups.

The end-line weight for experimental groups (Excess-EI, Excess-S and Excess-S+Adequate-EI) compared to the control group no statistically significant difference was observed ($P<0.05$). However, comparative to the end-line weight for control group both experimental groups (Adequate-EI and Excess-S+Adequate-$FeSO_4$) end-line weight was statistically significantly different ($P<0.05$).

The end-line weight for Excess-S+Adequate-$FeSO_4$ group was consistent compared to previous similar chronic study (5 weeks) on weaning rats which consumed dietary high dose of iron with adequate dose copper consumed group reported to be iron overloaded by Ha *et al.*, (2016) and found to have end-line weight(190-200g). Thus, experimental group Excess-S+Adequate-$FeSO_4$ which consumed excess dose of soil iron combined with adequate dose of iron sulfate could have been exposed to iron overload because higher dose of bioavailable form of iron consumption reported in declining growth by Soliman, De-Sanctis & Kalra, (2014).

The end-line weight for control group was significantly higher than Adequate-EI group ($P<0.05$). The first presumed reason for control group and Adequate-EI variation could be, iron sulfate had more relative bioavailability due to particle size difference (Hurrell, 1984; Arredondo *et al.*, 2006 and Degerud *et al.*, 2015) affects greatly dialyzability and solubility at aqueous solution of digestive system (Hurrell *et al.*, 2002). Secondly, compared to iron sulfate the electrolyte iron form precipitate which hinders its absorption in gut due to less bioavailability reported by Swain, Newman & Hunt, (2003). Thirdly, *in-vivo* study-based electrolyte iron formulated diet consumed experimental rats showed significantly higher fecal iron/un-absorbed iron content reported by Hurrell *et al.*, (2002) by resulting low hemoglobin regeneration efficiency.

Similarly, the end-line weight for Adequate-EI group was consistent compared to experimental rat group which consumed dietary low dose of iron with adequate of copper reported by Ha *et al.*,

(2016) to be iron deficient and lower end-line weight (205-210g). Thus, Adequate-EI group might to be mildly/Severely iron deficient and iron deficiency would induce growth retardation.

The end-line weight for excess electrolyte iron consumed experimental group (Excess-EI) was consistent compared to that of control group in our study finding($P<0.05$). Here again the possible reason could be for bioavailability difference reported by Hurrell *et al.*, (2002) and Swain, Newman & Hunt, (2003) and also, the above discussed reasons for Adequate-EI vs control group holds true here too.

The end-line weight for experimental groups (Excess-EI, Excess-S+Adequate-EI and Excess-S) was consistent compared with previous *in vivo* chronic study (5 weeks) on weaning rats which consumed dietary adequate dose of iron combined with adequate copper reported by Ha *et al.*, (2016) for end-line body weight (233-240 g). The end-line weight for experimental groups (Excess-EI, Excess-S+Adequate-EI and Excess-S) agreed compared to another *in vivo* study on weaning male rat which consumed dietary adequate dose of iron reported by Fischer *et al.*, (2002) for end-line weight 263±4 g. Similarly, the end-line weight for experimental groups (Excess-EI, Excess-S+Adequate-EI and Excess-S) were consistent compared to *in vivo* study on weaning male rat which consumed moderately high dose of iron formulated diets without iron overload/lipidperoxidation inducer reported by Fischer *et al.*, (2002) for body weight 261±4 g.

We used excess dose of soil contaminated source iron for experimental groups (Excess-S+Adequate-EI and Excess-S) in addition, they consumed an extra adequate dose of electrolyte iron but, the sum of dietary intake did not affect growth which put us in dilemma. Our finding was contradictory, since excess dose of dietary iron consumption has been reported to induce growth retardation (Ha *et al.*, 2016). In addition, recent studies by Teklu, (2017a; Guja) & Baye, (2018) reported that extrinsic/soil contamination source of iron being bioavailable by inducing hemoglobin regeneration and proper growth. Similarly, on top of adequate dietary iron intake either adequate dose or excess dose of extrinsic soil contamination iron intake would led to iron overload indicated by Teklu, (2017a) and Guja & Baye, (2018). Hence, by taking the above reasons as an input the experimental groups (Excess-S+Adequate-EI and Excess-S) were expected to have lower end-line weight/growth retardation as a result of iron overload.

However, the end-line weight for experimental groups (Excess-EI, Excess-S+Adequate-EI and Excess-S) were normal/unaffected growth status seen and this unexpected finding might be due to three possible reasons. The first reason, soil ingestion may bound to mucosal layer in the intestine for longer period reported by Gonzalez *et al.*, (2004). The second reason, electrolyte iron usually form precipitate in gut reported by Swain, Newman & Hunt, (2003) also, soil mostly contain other extrinsic nutrients which hinders intrinsic iron from being absorbed reported by Hooda *et al.*, (2004) due to interaction effect. The third reason, particle size for both (electrolyte iron/soil) had significant effect in absorption rate reported by many authors Hurrell, (1984), Arredondo *et al.*, (2006) and Eirik *et al.*, (2015); could justify again for finding Excess-S+Adequate-EI and Excess-S proper growth status /without any adverse effect.

In summary, based on our study, excess dose of electrolyte iron formulated diet, excess dose of soil source iron formulated diet, adequate dose electrolyte iron combined with excess dose of soil source of iron formulated diet and adequate dose of iron sulfate formulated diet consumption would not create any difference in end-line weight. However, adequate dose of iron sulfate combined with excess soil source iron consumption presumed to induce iron overload while adequate dose of electrolyte iron presumed to inducing iron deficiency.

Table 4.4. End-line Experimental Rats Profile for Groups

End-line Measured	Experimental Groups					
	AIN-93G+ Adequate-EI	**AIN-93G+ Excess-EI**	**AIN-93G+ Excess-S+ Adequate-EI**	**AIN-93G+ Excess-S**	**AIN-93G+ Adequate-$FeSO_4$**	**AIN-93G+ Excess-S+Adequate-FeSO4**
Final-Weight (g)	151.87± 8.39*[a]	227.55 ± 19.37[a]	225.33 ± 4.53[a]	224.55 ± 13.65[a]	230.21 ± 20.67[a]	134.71 ± 8.61*[a]
Liver Weight(g)	5.97± 1.23*[a]	8.55±0.72[a]	8.19±0.45[a]	8.49± 0.62[a]	8.89±0.89[a]	14.18± 0.84*[a]
Liver/Body Weight (Percentage)	3.93%	3.75%	3.63%	3.77%	3.86%	10.52%
Serum Iron (µg/dL)	88.07± 8.99*[a]	283.63± 8.33[a]	284.35± 8.27[a]	301.43± 5.07*[a]	290.62± 7.26[a]	538.49± 11.28 *[a]
Serum Zinc (µg/dL	222.16± 7.53[a]	226.38± 7.23[a]	221.23± 9.32[a]	224.35± 12.76[a]	222.36± 7.29[a]	219.71± 4.82 [a]
Serum Copper (µg/dL	163.58± 4.39*[a]	152.93± 4.45[a]	148.85± 4.96[a]	152.41± 6.94[a]	154.41± 3.71[a]	115.81± 4.7*[a]

Values are mean ± standard deviation and different superscripts "**a**" row represent statistically tested at ($P<0.05$). Where, Adequate-EI-adequate dose of electrolyte iron, Excess-EI-excess dose of electrolyte iron, Excess-S+Adequate-EI-Soil plus electrolyte iron, SOI- only soil, Adequate-$FeSO_4$-adequate iron sulfate, Excess-S+Adequate-$FeSO_4$ – Excess dose of soil plus adequate dose iron sulfate were the consumed experimental diet type assigned for each group and we found the above end-line measurement.

4.4.1. Growth Rate within the Groups

Growth pattern was calculated and summarized under Figure 4.4. Experimental group (Excess-EI) which consumed diet with excess dose of electrolyte iron grew higher with mean percentage weight gain and followed by control group. The least mean percentage of weight gain was displayed for experimental groups (Adequate-EI and Excess-S+Adequate-$FeSO_4$) showed decreased growth rate (mean percentage of weight gain) compared to all groups.

Experimental groups (Adequate-EI and Excess-S+Adequate-$FeSO_4$) end-line weight and growth rate were significantly lower than the control group($P<0.05$). Supportive to our finding for Adequate-EI either low iron source food or low dose of iron dietary consumption reported to cause lower growth as result of iron deficiency discussed by Dhur,Galan & Hercberg, (1990). However, more recent different experimental studies reported for both (high dose of dietary iron and low dose of dietary iron consumption) were effective in decreasing body weight gain or less growth rate reported by Chen *et al.*, (2000) and Ha *et al.*, (2016). Therefore, our finding for end-line weight and growth rate were consistent.

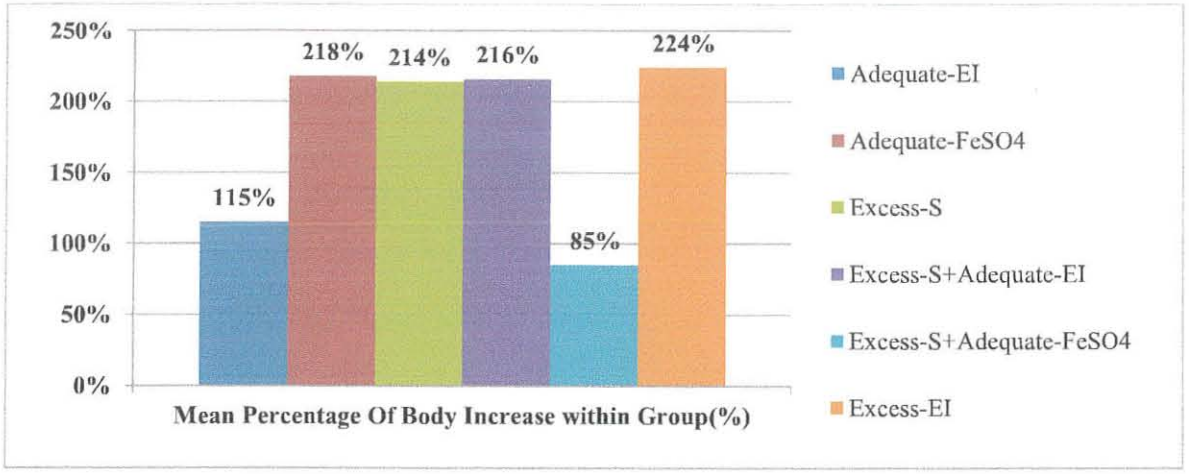


Figure 4.4 Mean Percentage of Body Weight Increase (growth rate).

Adequate- EI-Adequate dose of electrolyte iron, Adequate-$FeSO_4$-Adequate dose of iron sulfate, Excess-S+Adequate-EI-excess dose of soil and adequate dose of electrolyte iron, Excess-S+Adequate-$FeSO_4$- excess dose of Soil and adequate dose of iron sulfate, Excess-S-Excess dose of Soil only and Excess-EI- Excess electrolyte iron formulated experimental diets consumed groups.

4.5. End-line Measured Liver Weight Characteristics within the Groups

The end line measured liver weight within experimental groups were compared using ANOVA for mean ± standard deviation value and there was a statistically significant difference ($p<0.05$) between groups on measured end-line liver weight among experimental groups and presented in Table 4.4. Multiple linear regression result revealed that both iron dose ($R^2 = 0.193$) and bioavailability ($R^2 = 0.342$) variables individual effect was significant in end-line liver weight elevation ($p<0.05$). Based on our experimental study, dose could make 19% variation and bioavailability could make 34% variation in liver weight elevation hence, bioavailability variable was more dominating than dose variable in elevating liver weight with in the groups. However, there was significantly higher for the sum effect of independent variables (bioavailability and dose of iron) in elevating liver weight used to be ($R^2= 0.535$) within groups ($p<0.05$). Therefore, that was dietary variables based on our study could influence 53.5% variation in liver weight gain variation within the groups.

The liver weight for control group was consistent compared to experimental groups (Excess-EI, Excess-S+Adequate-EI and Excess-S) ($P<0.05$). However, the liver weight for experimental groups (Adequate-EI and Excess-S+Adequate-$FeSO_4$) were statistically significant difference compared to control group ($P<0.05$). The liver weight of Excess-S+Adequate-$FeSO_4$ group was the highest, while the liver weight for experimental group AEI group was the least of all groups. In addition, we tried to compare liver to body weight, the three groups (Excess-EI, Excess-S+Adequate-EI and Excess-S) were consistent to the control group, but experimental group (Excess-S+Adequate-$FeSO_4$) showed highly elevated liver to body mass percentage while experimental group Adequate-EI the least value and presented under Table 4.4.

The liver weight for experimental group (Adequate-EI) which consumed adequate dose of electrolyte iron compared to control group was statistically significantly different ($P<0.05$). The liver weight for Adequate-EI group was consistent compared to previous *in vivo* study by Fischer *et al.*, (2002) which consumed low dose of iron (20ppm) without oxidative stress inducing agent resulted liver weight 7 ± 0.2 g. In addition, Fischer *et al.*, (2002) added that low dose of iron declined liver iron concentration approximately by 45% since liver weight used to be correlated with iron amount stored in it.

Similarly, there could number of reasons for Adequate-EI group lower liver weight finding; it has been reported that electrolyte iron (for having larger particle size and correlated to cause less relative bioavailability) reported by Dabbagh *et al.*, (1994). In addition, previously studied electrolyte iron formulated diet consumed rats showed that higher fecal iron content reported by Hurrell *et al.*, (2002) and low hemoglobin regeneration efficiency. Supporting to above mentioned reasons low or adequate dose of electrolyte iron consumption could create insoluble precipitate in gut system and iron deficiency reported by Swain, Newman & Hunt, (2003) justified the liver weight finding for Adequate-EI group.

The liver weight for Excess-S+Adequate-$FeSO_4$ group was significantly higher compared to control group ($p<0.05$). The liver weight for Excess-S+Adequate-$FeSO_4$ group was consistent compared with similar study on rats provided AIN-93G formulated with iron sulfate at high iron doses (1500ppm) with oxidative stress inducing agent consumed liver weight (13.8±0.7g) reported by Fischer *et al.*, (2002). However, our finding for liver weight (Excess-S+Adequate-$FeSO_4$ group) was higher than what they have reported to be higher liver weight with higher liver iron concentration by Fischer *et al.*, (2002) and they discussed that liver weight used to be correlated with liver iron concentration. Similarly, iron mainly stored in liver and during excess iron consumption liver weight used to be elevated since non-heme iron level in the liver increases as reported by Yeung *et al.*, (2004).

Supportively to elevated liver weight finding for (Excess-S+Adequate-$FeSO_4$ group), previous studies by Teklu, (2017a) and Guja &Baye, (2018) explained that extrinsic/soil contaminant source iron was bioavailable in hemoglobin regeneration. In addition, (Teklu, 2017a; Guja &Baye, 2018) indicated that extrinsic/soil contamination iron need to be consider during fortification since, it would cause other serious health problems (iron overload) as a result of adequate dietary dose set to be used as an intervention. Another major point, Excess-S+Adequate-$FeSO_4$ group consumed adequate dose of dietary iron sulfate and the iron sulfate being bioavailable also, it had higher absorption rate reported by Hurrell *et al.*, (2002).

Though, there could be number of reasons for elevated liver weight finding (Excess-S+Adequate-$FeSO_4$ group), having particle size small for iron sulfate would minimize the two iron sources (extrinsic soil source and iron sulfate) interaction for each other or barrier effect with each other

would be minimized if and only if one of them particle size need to be smaller as explained by different authors, Hurrell, (1984), Arredondo *et al.*, (2006) and Degerud *et al.*, (2015). The above raised justifications could have facilitated for increased iron absorption and elevated liver weight for experimental group Excess-S+Adequate-$FeSO_4$. In addition, high dose of iron consumption reported to increase liver weight discussed by Haap *et al.*, (2011) and Alkhatib, *et al.*, (2017) for being liver iron storage organ. This could be correlated directly to iron overload since it had major effect on liver weight increment. Therefore, Excess-S+Adequate-$FeSO_4$ experimental group absorbed higher iron and could to be in iron-overload state since iron used to be store in liver and it showed-up elevated liver weight.

The liver weight for experimental groups (Excess-EI, Excess-S+Adequate-EI and Excess-S) was consistent compared to control group ($P<0.05$). The liver weight for experimental groups (Excess-EI, Excess-S+Adequate-EI and Excess-S) was consistent compared to previous *in vivo* study control group which consumed adequate dose of iron (45ppm) and reported liver weight (8.4±0.4 g) by Fischer *et al.*, (2002). Similarly, since we used excess dose for our experimental groups (Excess-EI, Excess-S+Adequate-EI and Excess-S) hence, we compared with moderately high dose of dietary iron consumed group without oxidative stress inducing agent found to have liver weight 8.7±0.3g reported by Fischer *et al.*, (2002) was consistent to experimental groups (Excess-EI, Excess-S+Adequate-EI and Excess-S) liver weight finding.

However, liver weight finding for experimental groups (Excess-S+Adequate-EI and Excess-S) since they consumed excess dose of iron and our expectation was to get higher liver weight due to the reason liver used to be iron storing organ reported by Haap *et al.*, (2011) and Alkhatib, *et al.*, (2017). In fact, excess iron consumption would result iron overload and it was correlated with higher liver weight reported by Ha *et al.*, (2016) and Klevay, (2001). In addition, previously given explanation (Teklu, 2017a; Guja & Baye, 2018) added that extrinsic/soil contaminant source iron for being bioavailable also, they indicated that interventional methods like fortification to consider the soil contamination source iron since it would affect community health status. Therefore, the above-mentioned reasons contradicted to our finding of proper liver weight for experimental groups specifically for Excess-S+Adequate-$FeSO_4$ and Excess-S group hence, there might be three possible reasons. First, bioaccessibility of soil source iron absorption to be lowered since other material like calcium, magnesium and manganese found to be the integral part of extrinsic source

iron (soil contamination) and soil source iron mostly create intrinsic vs extrinsic mineral interaction reported by Hooda *et al.*, (2004). Secondly, electrolyte iron form precipitate which hinders its absorption in gut to be less bioavailable reported by (Swain, Newman & Hunt, 2003). Thirdly, particle size for electrolyte/soil larger sized fortificants to be less bioavailable discussed by many authors Hurrell, (1984), Swain, Newman & Hunt, (2003), Arredondo *et al.*, (2006) and Degerud *et al.*, (2015).

In spite of the advantages of iron fortification in some foodstuffs, there are few concerns about diverse effects of producing oxidative stress as a result of excessive iron intake on top of normal status (Pouraram *et al.*,2010). Also, high iron consumption reported for production of free radicals that increase oxidative stress which used to be indicator for iron overload reported by Arruda *et al.*, (2013). In addition, high iron absorption would be stored at liver and it was linked with iron overload, iron over load could induce lipid metabolism this may result to fatty liver/lipid peroxidation (hepatic fat accumulation) reported by Ahmed, Latham & Oates, (2012). Even though there were different biomarker for iron overload, lipid peroxidation used to be among the one (Dabbagh *et al.*, 1994). Therefore, based on our study we presumed one group to be iron overloaded but, conformation it is vital to conduct biomarker analysis.

In summary, higher liver weight group (Excess-S+Adequate-$FeSO_4$) was almost identical with studies reported iron overloaded and we presumed our group to be iron overloaded in terms of liver weight finding. In addition, we presumed that adequate electrolyte iron consumption to cause iron deficiency and lower liver weight as compared to control group. High dose of iron consumed groups (Excess-S,Excess-S+Adequate-EI and Excess-EI) did not make any significance effect in liver weight status.

4.6. End-line Measured Serum Iron Characteristics within the Experimental Groups

The end line measured serum iron value for experimental groups were compared using ANOVA for mean ± standard deviation value and there was a statistically significant difference ($p<0.05$) between groups on measured end-line serum iron value presented on Table 4.4. Multiple linear regression result revealed that both iron dose ($R^2 = 0.314$) and bioavailabilty ($R^2 = 0.404$) independent individual variables effect was significant in end-line serum iron level elevation ($p<0.05$). Based on our experimental study dose could make 31% variation and bioavailability

could make 40% variation in serum iron level. Hence, bioavailability variable was more dominating than dose based on our experimental study. However, there was significantly higher effect for sum of independent variables (bioavailability and dose of iron) sum effect in elevating serum iron used to be (R^2= 0.717) within groups ($p<0.05$).

The serum iron level for experimental groups (Excess-EI and Excess-S+Adequate-EI) compared control group was not significantly different ($p<0.05$). However, the serum iron level for experimental groups (Adequate-EI, Excess-S and Excess-S+Adequate-$FeSO_4$) were significantly different compared to control group serum iron level ($P<0.05$).

The serum iron level for experimental group (Excess-S+Adequate-$FeSO_4$) was not consistent compared to control group ($P<0.05$). Similarly, serum iron level for Excess-S+Adequate-$FeSO_4$ group was not consistent compared to previous experimental study on rats which consumed dietary excess dose of iron(1500ppm) combined with adequate dose of copper(6ppm) found to be iron-overloaded and serum iron level (3.82±1.24mg/L ~ 382±12.4μg/dL) reported by Cockella *et al.*, (2005). Hence, the serum iron level for Excess-S+Adequate-$FeSO_4$ was significantly higher than similar study reported to be iron overloaded (Cockella *et al.*, 2005). Iron overload reported for significantly affecting in increasing serum iron indices, expression of liver hepcidin gene and total tissue iron content compared with non-iron loaded reported by Vu'o'ng Lê *et al.*, (2011). Likewise, iron-overloaded rats displayed elevated non-heme iron levels in serum and liver, indicating a condition of systemic iron overload reported by Han & Kim, (2015).

Similarly, the serum iron level for Excess-S+Adequate-$FeSO_4$ was not consistent compared to another previous *in vivo* study on adult male Wistar Rats which consumed enriched bread with ferrous sulfate with doses (210 ppm) group reported by Heidari *et al.*, (2016) serum iron level (434.2±48.854μg/dL) and used to be iron overloaded with antioxidant level changed. The serum iron level for Excess-S+Adequate-$FeSO_4$ group was significantly higher than previous reported serum iron level by Cockella *et al.*, (2005) and Heidari *et al.*, (2016) which were iron overloaded and there could be two possible reasons for variation ; first reason, it has been reported that different level of phytic acid for being sol part of wheat and phytic acid was among the micronutrients inhibitor as discussed by different Authors Baye *et al.*, (2014), Hurrell, (2004) and Hurrell & Egli, (2010); hence, Heidari *et al.*, (2016) reported experimental animals could have

been affected by phytic acid for less systemic level iron. The second possible reason could be age difference, iron is needed at higher level based on age and growth status specially at weaning stage high dose of iron intake has much more Sevier effect as compared adult stage discussed by Hallberg, (2002) and Frazer *et al.*, (2017), so, Excess-S+Adequate-$FeSO_4$ group of rats were at early stage of weaning and possibly could have absorbed excess dose of iron/iron overloaded. Therefore, the serum iron level finding for Excess-S+Adequate-$FeSO_4$ group to be iron overloaded like as presumed in previous sections yet, biomarker analysis demanded.

The serum iron level for Adequate-EI group was not consistent compared to control group ($P<0.05$). In addition, it was significantly lower than serum iron level finding compared to other experimental groups ($P<0.05$). The serum iron level for Adequate-EI was not consistent compared with *in vivo* study on adult male Wistar Rats which consumed iron enriched bread formulated with adequate dose (35ppm) of ferrous sulfate control group reported by Heidari *et al.*, (2016) serum iron level (144.25±23.18 µg/dL) and iron deficient. Similarly, the serum iron level for Adequate-EI was not consistent compared to severely and moderately iron deficient experimental groups of rats which consumed low dose of iron (7ppm) resulted serum iron level (35.5±4.7µmol/L ~198.32±26.25µg/dL) and (19.4± 3.4µmol/L ~108.37±18.99µg/dL) in respective order for the groups reported by Uritski *et al.*, (2004). The serum iron level for Adequate-EI group was significantly lower than previous studies reported to be iron deficient by Heidari *et al.*, (2016) and Uritski *et al.*, (2004). Therefore, based on our study adequate dose of electrolyte iron formulated diet consumed could have been exposed to mild/Sevier iron deficiency but, biomarker analysis demanded.

The serum iron level for experimental groups (Excess-EI and Excess-S+Adequate-EI) were consistent compared to control group ($P<0.05$). The serum iron level for experimental groups (Excess-EI and Excess-S+Adequate-EI) was consistent compared to previous in vivo study on weaning male rats and control group which consumed WWF diet, experimental group which consumed diet formulated with NaFeEDTA(60ppm) and experimental group which consumed diet formulated with NaFeEDTA(60ppm) combined with $ZnSO_4$(30ppm) reported by Akhtar *et al.*, (2010) serum iron level in respective order for the groups (2.82±0.17µg/g ~ 282±17µg/dL), (2.96±0.20 µg/g~296±20 µg/dL) and (3.06±0.12 µg/g~306±12 µg/dL). Similarly, the serum iron level for experimental groups (Excess-EI and Excess-S+Adequate-EI) were consistent compared

to another similar experimental in vivo study on weaning male rats for control groups which consumed dietary adequate dose of iron(35ppm) with adequate dose of copper(6ppm) reported by (Cockella *et al.*, 2005) for serum iron level to be (2.72±1.74mg/L ~272±17.4µg/dL).

Though, we have assigned excess dose for both groups (Excess-EI and Excess-S+Adequate-EI), our expectation was to find higher serum iron level. Indeed, equivalent range of serum iron level finding for Excess-EI and Excess-S+Adequate-EI compared to another similar experimental control group (which consumed adequate dose) finding seems vague however, there could be four possible reasons for our normal range of serum iron level finding. First, the particle size for electrolyte iron was higher and higher particle size for fortificants would result less absorption as discussed by different authors Hurrell, (1984), Arredondo *et al.*, (2006) and Degerud *et al.*, (2015). Secondly, Harvey, Dexter & Darnton-Hill, (2000) discussed that the absorption of exchangeable contaminant iron could be subjected to the same positive interactions with other non-haem iron that was intrinsic/extrinsic to food. Thirdly, electrolyte iron form precipitate after ingested inside intestine and its absorption in gut to be less bioavailable reported by Swain,Newman & Hunt, (2003), Swain,Newman & Hunt, (2007), Hurrell *et al.*, (2002), Sihag *et al.*, (2016) and Ziegler *et al.*, (2010). Fourthly, different studies reported for both (iron fortification/supplementation) of excess iron consumption had a potential of creation inflammation by favoring photogenic anaerobic bacteria growth while suppression normal micro flora bacteria as well as delayed maturation intestinal cells for both rats and human assay due to higher systemic iron as reported by Alexeev *et al.*, (2018), Dostal *et al.*, (2012), Tang *et al.*, (2017) and Zimmermann *et al.*, (2010).

Therefore, by having the above mentioned four reasons in mind and extrapolating it for our finding (Excess-EI and Excess-S+Adequate-EI groups) either excess dose of electrolyte formulated diet or adequate dose of electrolyte combined with excess dose of extrinsic/soil source iron would not create any change in serum iron level yet, serum iron is not the golden standard to confirm iron status.

The serum iron level for experimental group (Excess-S) was not consistent compared with control group ($P<0.05$), that was the most surprising and different thing found in this section. Supportingly to our finding, previously conducted studies on extrinsic/soil contamination source iron was bioavailable also, they indicated that excess dose of soil source iron/extrinsic could cause

community health problems (iron overload) and need to considered when implementation of interventional methods like fortification reported by Guja & Baye, (2018) and Teklu, (2017a). However, the serum iron level finding for Excess-S group was not consistent compared with presumed to be iron overloaded experimental group (Excess-S+Adequate-$FeSO_4$)/ which showed maximum serum iron level. Thus, the elevated serum iron level findings for Excess-S group could possibly have two reasons. Firstly, geophagic soils after ingested may bound to mucosal layer in intestine for longer period which was pretty indicator to be barrier by Gonzalez *et al.*, (2004). Secondly, soil mostly contain other micronutrients as an integral part of it, specifically vertisol had higher cation exchanging capacity as a result, there might be mineral interaction discussed by Gonzalez *et al.*, (2004), Hazelton and Murphy, (2007).

In summary, based on our finding excess dose of extrinsic soil source iron combined with adequate dose of iron sulfate consumed group (Excess-S+Adequate-$FeSO_4$) serum iron level was significantly elevated compared to control group and we presumed to cause iron overloaded. However, excess dose of extrinsic soil source iron formulated (Excess-S) diet consumed group showed significantly higher serum but, it did not reach neither previously reported iron overload studies nor our presumed to be iron overloaded serum iron level. Thus, biomarker tests needed to confirm iron status for Excess-S and Excess-S+Adequate-$FeSO_4$ consumed groups. Adequate-EI consumption induced significantly by lowering serum iron level and we presumed mild/Sevier iron deficiency yet, biomarker and other measurements needed. Finally, from our finding, excess dose of extrinsic soil source iron combined with adequate dose of electrolyte iron formulated diet consumption and excess dose of electrolyte iron formulated diet consumption would not make any significant effect on serum iron level but, other measurements in future needed for confirmation by taking this study research gap as an input.

4.7. End-line Measured Serum Zinc Characteristics within the Experimental Groups

The end line measured serum zinc level for all groups were compared using ANOVA and there was no a statistically significant difference between groups ($P<0.05$), and our serum zinc level findings were presented on Table 4.4. Similarly, multiple linear regression result revealed that neither individual variable (bioavailability or dose) nor, sum (bioavailability and dose of iron

interaction) effect of dietary iron consumption was not significant in affecting serum zinc status within groups ($P<0.05$) and ($R^2=0.019$).

Our serum zinc status findings for all groups which consumed dietary level iron formulated by using two-independent variables (bioavailability and dose combination) were not significant (1.9%) in varying serum zinc status ($P<0.05$). Our serum zinc findings for all group were consistent with the reports by Storey & Greger, (1987), Roughead, Johnson & Hunt, (1999) and Olivares *et al.*, (2012) chronic dietary level excess dose of iron consumption had no any significant effect on serum zinc status.

However, previously acute dietary exposure of iron (12 days) study on weaning male rats which consumed vegetable based infant food formulated by adding extra dose of iron reported by Fairweather-Tait & Southon, (1989) serum zinc level (2.06 µg/ml~206µg/dL) with small reduction in serum zinc status and raised body iron stores. Similarly, cereal based diet with extra dose of iron consumed group reported by Fairweather-Tait & Southon, (1989) significantly higher serum zinc level was reported 2.38µg/ml~238µg/dL.

Similarly, the serum zinc level reported by Fairweather-Tait & Southon, (1989) for rats fed oats with extra dose iron resulted serum zinc level (2.10µg/ml~210µg/dL) was inconsistent compared to control group (which fed on laboratory purified rodent diet) serum zinc level used to be(1.88µg/ml~188µg/dL). The inconsistency for serum zinc level variation reported by Fairweather-Tait & Southon, (1989) could possibly have three reasons. First reason, as reported by Olivares *et al.*, (2012) iron and zinc interaction was mainly depend on food matrix. Second reason, absorption of both inorganic zinc in rat was influenced by host and physiological factors that was mainly take place at lumen of small intestine reported by Kondaiah *et al.*, (2019). Third reason, the higher protein diet would make interaction by forming complex with zinc while, lower protein containing diets would not make interaction could be the profound reason as explained by Fairweather-Tait & Southon, (1989) and Olivares *et al.*, (2012). Therefore, acute dietary adequate dose of iron or acute dietary excess dose of iron intake was effective in increasing body iron store and decreasing zinc serum zinc status.

In contrary to above points, recent chronic exposure of dietary iron study on adult male rats (experimental groups) which consumed whole wheat diets fortified (using NaFeEDTA, Elemental

Iron, $ZnSO_4$ and ZnO), diets were formulated by bioavailability level (low/high) or dose level (adequate/moderately high) combination of iron and zinc then the consumption effect on serum zinc level as reported by Akhtar *et al.*, (2010) within the range (1.60±0.10 µg/g~160±1 µg/dL, to 1.84±0.08 µg/g ~ 184±8 µg/dL). Similarly, the serum zinc level (1.59±0.07µg/g~159±7µg/dL) for control group fed with WFP food adult male rats reported by Akhtar *et al.*, (2010) was statistically consistent compared to serum zinc value of their experimental group ($P<0.05$). Therefore, chronic exposure to different dietary dose with different bioavailability index for iron and zinc consumption was not effective in changing zinc serum zinc status.

The serum zinc status for our experimental groups were consistent compared with serum zinc status reported by Akhtar *et al.*, (2010) since, either adequate dose or excess dose of iron chronic intake did not affect zinc status. However, figuratively Akhtar *et al.*, (2010) reported serum zinc level was smaller than our serum zinc finding and the possible reason could be. First reason, age difference, the older rats consume more than younger and higher micronutrient absorption associated with an improved appetite (Kim & Atallah, 1993; Khan *et al.*, 2014) also, higher dose of micronutrients intake would create micronutrient interactions reported by Ha *et al.*, (2016). Second reason, the experimental diets which has been used by Akhtar *et al.*, (2010) contain phytate, phytate used to be integral part of cereals and it has been reported for being anti-nutritional factor by Windisch & Kirchgessner, (1999). Third reason, laboratory purified diet consumption would favor increment in serum iron/zinc level reported by Fairweather-Tait & Southon, (1989). Therefore, the above-mentioned reasons could have increased serum zinc level for our experimental groups compared to previous chronic dietary study and lower serum zinc level reported by Akhtar *et al.*, (2010).

Another study for chronic exposure of adequate and excess dose of iron consumed male weaning rats provided a profound result reported by Storey & Greger, (1987) revealed that only tissue level zinc was different and the differences were seen in all experimental groups. Moreover, there were limitations to studies on effect of iron on zinc absorption measurement by using circulating concentrations, since circulating concentrations did not necessarily indicate the net zinc uptake discussed by authors Sandstroem, (2001) and Whittaker, (1998). Which could tell us the research gap for our study an our study tried to used serum values to differentiate the interaction level existence for iron, zinc and copper. Therefore, it would make it difficult for our findings to clearly

conclude whether there was interaction level or not for iron and zinc based on measured serum levels.

Nevertheless, our finding on previous sections (4.4 to 4.6) justified that Excess-S+Adequate-$FeSO_4$ (iron overloaded), Adequate-EI (iron deficient) and presumed groups. Likewise, there was other view which support our presumed group for high iron consumed/ iron overloaded compared to different studies by Alexeev *et al.*, (2017), Dostal *et al.*, (2012), Tang *et al.*, (2017) and Zimmermann *et al.*, (2010). It was explained that higher systemic iron could be correlated with iron deficiency since both iron (fortification/supplementation) had a potential for creation inflammation by favoring photogenic anaerobic bacteria growth while suppression normal micro flora bacteria as well as delayed maturation intestinal cells for both rats and human assay (Alexeev *et al.*, 2017; Dostal *et al.*, 2012; Tang *et al.*, 2017 & Zimmermann *et al.*, 2010). Similarly, infection or inflammation level would be high during Sevier iron deficiency and iron overload/oxidative stress status discussed by Uritski *et al.*, (2004). However, it was reported that zinc had a major role in controlling infection or inflammation status occurrence as a result of iron deficiency and iron overload cases (Gammoh & Rink, 2017). Thus, having in mind the above-mentioned points, it was clear that zinc status would decline during both cases (iron deficiency & iron over load) but, difficult to relay on serum status measurement as an indicator.

Similarly, previous study by Vallee & Falchuk, (1993) explained that organisms mostly become susceptible to oxidative stress as a result of iron overload/lipid-peroxidation and zinc deficient status. Likewise, there is important reason which explained that during zinc deficiency the physiological system immediately decrease in protein turnover as well as cell growth to preserve zinc body pools and causes growth retardation reported by Semrad, (1999) which could support indirectly for iron overload presumed group (Excess-S+Adequate-$FeSO_4$) found to be growth retarded in previous sections. Likewise, another important point from past studies reported that simultaneous burden of iron and zinc deficiency occurrence (Ergul *et al.*, 2018) in human (clinical) based study, as well as animal-based study reported by Rodriguez-Matas *et al.*, (1998) and Sandstead, (2000) for showed-up iron deficiency ability in altering metabolism of zinc and copper.

Apart from the above paragraphs raised points, recent review discussed that iron and zinc competition would not occur either excess dose of dietary iron consumption or adequate dose of dietary iron consumption (Olivares *et al.*, 2012) due to two possible reasons. First, it was explained

by Olivares *et al.*, (2012) during excess iron absorption zinc homeostasis capacity will be maintained and zinc level found within the system from running-out. Secondly, during excess dietary iron intake occasion iron and zinc use specifically assigned transportation mechanism for them as discussed by Olivares *et al.*, (2012), and the two reasons seemingly could justify our finding for our experimental groups. Though, our study measurement were fixed to serum and serum value measurement would not clearly show proper micronutrient status by Storey & Greger, (1987) and Ha *et al.*, (2016). Thus, in order to resolve the above contradicting different ideas and reach conformation further study by measuring different organ as well as enzymes related to iron, zinc and copper as a biomarker analysis needed by taking our finding as research gap.

In summary, our study for dietary dose array varied (from adequate to excess intake) combined with bioavailability array varied (from low to high) had no any statistically significant effect on serum zinc status. However, since our study relied on serum level measurement, our study finding could not show clearly micronutrients status so, further biomarker analysis for liver, kidney, spleen with blood micronutrient (Iron, Zinc and Copper) level could be confirmatory. Thus, our study could be an input to initiate future study but, still remains research gap.

4.8. End-line Measured Copper Characteristics within the Experimental Groups

The end line measured serum copper value for experimental groups were compared using ANOVA for mean ± standard deviation value and there was a statistically significant difference between groups($P<0.05$) presented on Table 4.4. Multiple linear regression result revealed that both iron dose ($R^2 = 0.244$) and bioavailability ($R^2 = 0.248$) variables as an individual variable effect was significant in elevation end-line serum copper level ($P<0.05$). Based on our experimental study finding, dose could make 24% variation and bioavailability could make 25% in serum copper level variation within the groups. Thus, bioavailability variable was more dominating than dose. However, the sum effect of independent variables (bioavailability and dose of iron) were significantly higher in elevating serum copper used to be ($R^2= 0.492$) within groups ($p<0.05$). Therefore, our study finding the sum effect for dietary variables (dose combined with bioavailability) were influencing 49% variation in serum copper variation within the groups.

The serum copper level for experimental groups (Excess-EI, Excess-S+Adequate-EI and Excess-S) were not significantly different compared with control group ($P<0.05$). However, the serum

copper level for experimental groups (Adequate-EI and Excess-S+Adequate-$FeSO_4$) were significantly different compared control group ($P<0.05$).

The serum copper level for experimental group (Excess-S+Adequate-$FeSO_4$) was not consistent compared with control group ($P<0.05$). However, the serum copper level for experimental group (Excess-S+Adequate-$FeSO_4$) was consistent compared with previous chronic (42 days) study on weaning male rats which consumed dietary excess dose of iron, marginal non-deficient copper dose and adequate dose of zinc acetate group reported by Klevay, (2001) for serum copper level to be (1.1±0.16µg/ml~110±16µg/dL) and mildly copper deficient.

Similarly, the serum copper level for experimental group (Excess-S+Adequate-$FeSO_4$) was not consistent figuratively compared to chronic study (5 week) on weaning male rats which consumed excess dose of carbonyl iron combined with adequate dose of copper formulated AIN-93G diet group reported by Ha *et al.*, (2016) decreased serum copper level (0.5-0.63µg/ml ~ 50-63µg/dL) and iron overloaded. However, excess dose of iron consumption overall effect in declining serum copper status reported by Ha *et al.*, (2016) was consistent with ours finding for Excess-S+Adequate-$FeSO_4$ group but, the figurative serum copper level variation could have two possible reasons. First reason, excess dose of iron consumed group/iron overloaded reported by Ha *et al.*, (2016) used to consume extremely high dose than previously studied oxidative stress/ hemochromatosis induced dose set by Arruda *et al.*, (2013) and it was reported that extremely excess dose intake critically affects systemic level micronutrients (Sandstroem, 2001; Whittaker, (1998). Second reason, during experimental formulation they have used sucrose, sucrose favors iron metabolism as discussed by Reeves, (1997), which was antagonistic to copper absorption and justifies the figurative level variations.

In support of serum copper level finding for Excess-S+Adequate-$FeSO_4$ group compared to different authors by Klevay, (2001) and Ha *et al.*, (2016) reported that during iron overload state copper level would decline. In addition, copper reported to be an important element in antioxidant catalytic enzyme which can take part in declining susceptibility to oxidative stress also, copper was important element in mobilization and metabolism of iron as discussed by Cockella &Belonje, (2002) and Cockella *et al.*, (2005). Therefore, increment in serum iron status and decreasing serum copper status correlated with iron overload from different studies and it was consistent with

experimental finding for Excess-S+Adequate-$FeSO_4$ group which was presumed to be iron overloaded.

The serum copper level for experimental group (Adequate-EI) was significantly elevated compared to control group ($P<0.05$). The serum copper level for Adequate-EI group in line with earlier discussion by Rodriguez-Matas *et al.*, (1998) which claimed during iron deficiency copper status used to be elevated tremendously while, zinc status will remain unchanged. Despite, Rodriguez-Matas *et al.*, (1998) report was based on measured liver, spleen, sternum and femur. However, other studies supportive to previous justification, Campos *et al.*, (1998) and Garcia *et al.*, (2013) study added that high digestive and metabolic utilization of copper observed in anemic rats compared with their control group. Also, Campos *et al.*, (1998) and Garcia *et al.*, (2013) explained that iron deficiency may increase the absorption of other divalent cations such as copper.

Moreover, Ranganathan *et al.*, (2011) report indicated that an increased in serum and liver copper was correlated with iron deficiency in line with our finding for Adequate-EI group. Correspondingly, human based (on infants) studies revealed that iron deficiency anemia was correlated in increasing serum copper level reported by Turgut *et al.*, (2007) Lonnerdal &Hernell, (1994) and Angelova *et al.*, (2014). Therefore, adequate dose of electrolyte iron consumption could have induced iron deficiency for our experimental group (Adequate-EI). Yet, our measurement sticks on serum so, taking our finding as knowledge gap further biomarker analysis in future will be confirmatory.

The serum copper level for experimental groups (Excess-S, Excess-S+Adequate-EI and Excess-EI) were not significantly different compared to control group ($P<0.05$). Similarly, the serum copper level for experimental groups (Excess-S, Excess-S+Adequate-EI and Excess-EI) were strongly consistent compared to previous similar chronic study (42 days) on weaning male rats which consumed dietary adequate dose of iron with marginal non-deficient dose of copper and adequate dose of zinc acetate group reported by Klevay, (2001) for serum copper level ($1.5\pm0.07\mu g/ml \sim 150\pm7\mu g/dL$).

However, the serum copper level for experimental groups (Excess-S, Excess-S+Adequate-EI and Excess-EI) were not consistent compared with another similarly chronic study (5 weeks) on weaning male rats which consumed dietary adequate dose of carbonyl iron combined with

adequate dose of copper formulated AIN-93G reported by Ha *et al.*, (2016) for serum copper level (1.2-1.3µg/ml ~120-130µg/dL). However, the overall effect was similar but, our experimental groups (Excess-S, Excess-S+Adequate-EI and Excess-EI) serum copper level was significantly higher than Ha *et al.*, (2016) reported figure and the figurative variation could have two possible reasons. First reason, we formulated our experimental diets with excess dose of iron as per previously used to induce iron overload by Arruda *et al.*, (2013), also, we used excess dose for extrinsic soil contamination scenario groups (Excess-S and Excess-S+Adequate-EI) and excess dose of soil contamination bioavailable and bio-accessible as reported by Guja & Baye, (2018) and Teklu, (2017a). Second reason, we used vertisol and vertisol had the highest potential of bioavailable iron compared other soil varieties for having highest cation exchange capacity in the previous invitro study reported by Smith *et al.*, (2000) could justify elevated serum copper level for our experimental groups (Excess-S and Excess-S+Adequate-EI).

Since our measurements were fixed on serum level only but, similar studies done previously tried to check liver, spleen, antioxidant enzymes which solely impart copper, femur, specifically tissue and serum ceruloplasimn activity which used to be marker of copper status reported by Ranganathan *et al.*, (2011) and Pyatskowit & Prohaska, (2008). Therefore, serum iron, zinc and copper level measurements could be our study gap but, by taking our finding as an input further biomarker and other measurements will fill the gap as well as it will lead us to conformation.

In summary, interestingly results were seen on our discussion sections (4.4, 4.5, 4.6, 4.7 and 4.8) with nearly similar fining were observed. That was experimental groups (Excess-S, Excess-S+Adequate-EI and Excess-EI) compared to control group showed up similar finding (weight, growth rate, liver weight, serum iron, zinc and copper); hence, dietary based excess dose of soil contaminated iron intake, dietary based excess electrolyte iron and dietary based adequate dose of electrolyte iron combined with excess dose of soil contaminated iron consumption would not result micronutrients or other physiological status variation. However, our finding and discussions for experimental groups (Adequate-EI and Excess-S+Adequate-$FeSO_4$) in previous sections compared to control group were not similar; hence, dietary based adequate dose of electrolyte iron intake would result iron deficiency and other physiological disorders. Similarly, dietary based adequate dose of iron sulfate combined with excess dose of soil contamination iron intake would result iron overload and other physiological disorders

Chapter Five

Conclusion and Recommendations

5.Conclusion and Recommendations

The current study demonstrated that excess dose of extrinsic soil contamination on top of adequate electrolyte iron fortified diet and excess dose of electrolyte iron fortified diet consumption did not affect iron, zinc and copper status compared to adequate dose of iron sulfate formulated diet consumption. However, excess dose of extrinsic soil contamination source iron based formulated diet consumption did not affect zinc and copper status but, it had significant affected iron status compared to adequate dose of iron sulfate formulated diet consumption. In contrary, our experimental finding for adequate dose of electrolyte iron formulated diet consumption result affected both iron and copper status by resulting iron deficiency. Thus, dietary iron absorption and factors affecting bioavailability (particle-size mineral interaction) in the body would be tightly regulated for both electrolyte iron and extrinsic source iron, since they had higher particle size they could have clogged the lumen as result of physicochemical interaction at individual or both level consumption for the above stated scenarios could justify our findings. Previous studies showed that locally harvested cereals were contaminated with soil and the total iron content was high, also recently *in vivo* based studies showed that soil contamination iron which was extrinsic to food had hemoglobin regeneration as well as bioaccessible; from this paper finding it is possible to conclude that excess dose of soil contaminated iron (extrinsic to food) when combined with routine dietary/fortified food (with iron sulfate at adequate dose) consumption will affect the micronutrients status as well as lead to oxidative stress and the possible reason could be physicochemical (particle-size and mineral interaction) were low. However, extrinsic soil contamination combined with dietary/fortified intake of electrolyte iron compounds will not affect micronutrient status.

From current study excess dose of extrinsic soil contamination on top of adequate iron sulfate based formulated diet consumption affected both iron status and copper status compared to previous studies yet, zinc status was not affected; but, it not enough to reach conformation since serum analysis is golden standard. Likewise, as per most studies indicated, serum value did not indicate the actual micronutrient status but, serum level micronutrients finding were indicatives. Thus, future studies will fill the gap by measuring oxidative status using biomarkers since, both zinc and copper participate in an antioxidant enzyme. Apart from it there is a need for extensive

and exceeding measurements based on *in vivo* study in future needed by taking this study gaps as an input to conclude in general for micronutrients status and oxidative stress level as a result of iron overload based on biomarker indicators.

Extrinsic soil contamination for containing higher total iron which was an integral part of local cereals in our country and for showed up fair bioavailability/utilizability efficiency. Therefore, it is recommended that to reconsider contamination iron effect during iron fortification and supplementation program implementation at national level in-order the society not to be exposed to iron overload, zinc and copper related deficiency.

REFERENCES

Abbaspour, N., Hurrell, R., & Kelishadi, R. (2014). Review on Iron and Its Importance for Human Health. *J Res Med Sci*, 19(2), 164 – 174.

Abebe, Y., Bogale, A., Hambidge, K.M., Stoecker, B.J., Bailey, K., & Gibson, R.S. (2007). Phytate, zinc, iron and calcium content of selected raw and prepared foods consumed in rural Sidama, Southern Ethiopia, and implications for bioavailability. *Journal of Food Composition and Analysis,* 20, 161-168.

Adish, A.A., Esrey, S.A., Gyorkos, T.W., Jean-Baptiste, J., & Rojhani, A. (1999). Effect of consumption of food cooked in iron pots on iron status and growth of young children a randomized trial. *Lancet*, 353(9154), 712-6.https://doi.org/10.1016/S0140-6736(98)04450-X

Ahmed, U., Latham, P.S., & Oates, P.S. (2012). Interactions between hepatic iron and lipid metabolism with possible relevance to steatohepatitis. *World Journal of Gastroenterology*, 18(34), 4651- 4658.doi:10.3748/wjg.v18.i34.4651

Allen, K.J., Buck, N.E., Cheah, D.M.Y., Gazeas, S., Bhathal, P., & Mercer, J.F.B. (2006). Chronological changes in tissue copper, zinc and iron in the toxic milk mouse and effects of copper loading. *BioMetals*, 19(5), 555-564. doi:10.1007/s10534-005-5918-5

Allen, L., Benoist, B.D., Dary, O., & Hurrell, R. (Editors). (2006a). *Guidelines on food fortification with micronutrients. World Health Organization and Food and Agriculture Organization of United Nations*. Retrieved on August 19, 2019 from: https://www.who.int/nutrition/publications/guide_food_fortification_micronutrients.pdf

Allen, K.J., Gurrin, L.C., Constantine, C.C., Osborne, N.J., Delatycki, M.B., Amanda, J., & Nicoll, A.J. (2008). Iron Overload Related Disease in *HFE* Hereditary Hemochromatosis. *N Engl J Med*, 358, 221-230.

Alexeev, E.E., He, X., Slupsky, C.M., & Lonnerdal, B. (2017). Effects of iron supplementation on growth, gut microbiota, metabolomics and cognitive development of rat pups.*PLOS ONE*, 12(6), 1-19. https://doi.org/10.1371/journal.pone.0179713

Alkhatib, B., Al-Domi, H., Shomaf, M., & Abu Irmaileh, B. (2017). Iron Overload Effect on Serum Levels of Glucose, Insulin and HOMA-IR was Influenced by the Type of Fat in Female Sprague-Dawley Rats Fed High-Fat-Diets. *Journal of Clinical Nutrition & Dietetics*,3(3), 1-23. DOI:10.4172/2472-1921.100058

Aragon, I.J., Ortiz, D., & Pachon, H. (2012). Comparison between in vitro and in vivo methods to screen iron bioavailability, *CyTA-Juurnal of Food*, 10(2), 103-111. DOI:10.1080/19476337.2011.596283

Akhtar, S., Rehman, Z.U., Anjum, F.M., Ali, Z., & Nisar, A. (2010). Bioavailability of Iron and Zinc Fortified Whole Wheat Flour in Rats. *Pakistan J.Zool*, 42(6), 771-779.

Ambaw, A. (2013). *Determination of iron fractions of laboratory and field threshed teff flour, fermented past and injera: Implications to iron bioavailability* (Master's Thesis). Addis Ababa University, Center for Food Science and Nutrition.

Andrews, N.C. (2008). Forging a field: the golden age of iron biology. *The American Society of Hematology*, 112(2), 219-230. DOI:10.1182/blood-2007-12-077388

Angelova, M., Asenova, S., & Nedkova, V. (2011). Copper in Human Organism. *Trakia Journal of Sciences*, 9(1), 88-98.

Angelova M.G., Petkova-Marinova, T.V.,Maksym Pogorielov, M.V., Loboda, A.N., Nedkova-Kolarova, V.N., & Bozhinova, A.N.(2014). Trace Element Status (Iron, Zinc, Copper, Chromium, Cobalt, and Nickel) in Iron-Deficiency Anaemia of Children under 3 Years. *Anemia*, 2014, 1-8. http://dx.doi.org/10.1155/2014/718089

AOAC – Association of Official Analytical Chemists. (1997). *Official methods of analysis of the Asscociation of Official Analytical Chemists. Method 973.31 Bioavailability of Iron: Rat Hemoglobin Bioassay.* 16th ed., 3rd Revised, AOAC International, Gaithersgurg, Maryland Chapter 45, page 62-63.

Aragon, I.J., Ortiz, D., & Pachon, H. (2012). Comparison between invitro and invivo methods to screen iron bioavailability. *Journal of Food*, 10(2), 103-111.

Arredondo, M., Salvat, V., Pizarro, F., & Olivares, M. (2006). Smaller iron particle size improves bioavailability of hydrogen-reduced iron–fortified bread. *Nutrition Research*, 26, 235-239.

Arruda, L.F., Arruda, S.F., Campos, N.A., Valencia, F.F.D., & Siqueira, E.M.D.A. (2013). Dietary Iron Concentration May Influence Aging Process by Altering Oxidative Stress in Tissues of Adult Rats. *PLOS ONE*, 8(4), 1-12. doi:10.1371/journal.pone.0061058

Astuti, R., Subagyo, H.W., Muis, S.F., & Widianarko, B. (2017). Influence of Fortified Tempe with Iron and Vitamin A to Increase Hemoglobin Level of Rats with Iron Deficiency Anemia. *Pakistan Journal of Nutrition*, 16, 90-95. DOI: 10.3923/pjn.2017.90.95

Azene, M. (2015). *Iron fractionation of cereals contaminated with different types of Ethiopian soils and its consequence* (Master's Thesis). Addis Ababa University, Center for Food Science and Nutrition.

Bailey, E.L., West Jr, K.P., & Black, R.E. (2015). The epidemiology of Global micronutrient deficiencies. *Annals of Nutrition and Metabolism,* 66(Suppl 2), 22-33. doi: 10.1159/000371618.

Barceloux, D.G. (1999). Zinc. *Zinc Clin Toxicol,* 37, 279-292.

Baye, K., Mouquet-Rivier, C., Icard-Vernie`re, C., Picq, C., & Guyot, J.P. (2014). Changes in mineral absorption inhibitors consequent to fermentation of Ethiopian injera: implications for predicted iron bioavailability and bioaccessibility. *International Journal of Food Science and Technology*, 49, 174-180.doi:10.1111/ijfs.12295

Beiseigel, J.M., Hunt, J.R., Glahn, R.P., Welch, R.M., Menkir, A., & Maziya-Dixon, B.B. (2007). Iron bioavailability from maize and beans: a comparison of humanmeasurements with Caco-2 cell and algorithm predictions. *American Journal of Clinical Nutrition*, 91(Suppl), 1484-1491

Bhaskaram, P. (2001). Immunobiology of mild micronutrient deficiencies. *British Journal of Nutrition, 85(S2), S75.* doi:10.1079/bjn2000297

Brabin, B.J., Hakimi, M., & Pelletier, D. (2001). An Analysis of Anemia and Pregnancy Related Maternal Mortality. *The Journal of Nutrition*, 131(2), 604-615. https://doi.org/10.1093/jn/131.2.604S

Brouillard, M.Y., & Rateau, J.G. (1989). Adsorption potency of 2 clays, smectite and kaolin on bacterial enterotoxins, In vitro study in cell culture and in the intestine of newborn mice. *Gastroenterol Clin Biol*,13(1), 18-24.

Buchowski, M.S., Mahoney, A.W., & Kalpalathika, M.P.V. (1989). Non-heme iron absorption, apparent iron absorption and hemoglobin regeneration efficiency in anemic and normal rats fed dietary heme and non- heme iron at various levels. *Nutritional Research*, 9, 773-783.

Bueno, N. B., de Melo, I. S. V., de Oliveira, S. L., & da Rocha Ataide, T. (2013). Very-low-carbohydrate ketogenic diet v. low-fat diet for long-term weight loss: a meta-analysis of randomised controlled trials. *British Journal of Nutrition, 110(07), 1178–1187.* doi:10.1017/s0007114513000548

Cade, J.E., Moreton, J.A., OHara, B. (2005). Diet and genetic factors associated with iron status in middle aged women. *American Journal of Clinical Nutrition*, 82, 813-820.

Campos, M.S., Pallares, I., Moratalla, A., Lopez-Aliaga, I., Gomez-Ayala, A.E., Hartiti, S., Alferez, M.J.M., Barrionuevo, M., & Lisbona, F. (1996). Bioavailability of Fe, Ca, P and Mg in Fe-Deficient Rats Treated with Different Sources of Dietary Iron. *Nutrition Research*, 16(4), 683-693. doi:10.1016/027-5317(96)00046-2

Campos, M.S., Barrionevo, M., Alferez, M.J., Gomez-Ayala, A.E., Rodriguez-Matas, M.C., Lopez Aliaga, I., & Lisbona, F. (1998). Interactions among iron, calcium, phosphorus and magnesium in nutritionally iron-deficient rats. *Experimental Physiology*, 83, 771-781.https://doi.org/10.1113/expphysiol.1998.sp004158

Carter, M.R., & Gregorich, E.G. (2008). Soil Sampling and Methods of Analysis. *European Journal of Soil Science*, 36, 100-151. https://doi.org/10.1111/j.1365-2389.2008.01052_5.x

Central Statistical Agency of Ethiopia (CSAE). (2012). *Agricultural Sample Survey 2011/2012*: Report on Area and Production of Crops. Addis Ababa. Retrieved on August 18, 2019, from http://213.55.92.105/nada3/index.php/ddibrowser/309/download/2119

Chen, K., Suh, J., Carr, A.C., Morrow, J.D., Zeind, J., & Frei, B. (2000). Vitamin C suppresses oxidative lipid damage in vivo, even in the presence of iron overload. *American Journal of Physiology Endocrinology and Metabolism*, 279, 1406-1412. http://doi.org/10.1152/ajpendo.2000.279.6.E1406

Cockell, K.A., & Belonje, B. (2002). The carbonyl content of specific plasma by dietary copper deficiency in rats. *Journal of Nutrition*, 132, 2514-2518.

Cockella, K.A., Wotherspoon, A.T.L., Belonjea, B., Fritz, E.M., Maderea, R., Hidiroglou, N., Plouffea, L.J., Ratnayake, W.M.N., & Kubowb, S. (2005). Limited effects of combined dietary copper deficiency/iron overload on oxidative stress parameters in rat liver and plasma. *Journal of Nutritional Biochemistry*, 16, 750-756. doi:10.1016/j.jnutbio.2005.04.003

Cohen, N.L., Keen, C.L., Hurley, LS., &Lonnerdal, B. (1985). Determinants of Copper-Deficiency Anemia in Rats. *Journal of Nutrition*, 115(6), 710-725. https://doi.org/10.1093/jn/115.6.710

Cook, J.D. (1999). Defining Optimal Body Iron. *Proceeding of the Nutrition Society*, 58, 489-495.

Cook, J.D., Flowers, C.H., & Skikne, B.S. (2003). The quantitative assessment of body iron. *Blood*, 101,3359-3364.

Cook, J.D., Boy, E., Flower, C., & Daroca, M.C. (2005). The influence of high-altitude living on body iron. *Blood*, 106, 1441-1446.

Costenbader, K.H., Chibnik, L.B., & Schur, P.H. (2007). Discordance between erythrocyte sedimentation rate and C-reactive protein measurements: clinical significance. *Clin Exp Reheumatol,* 25(5), 746-749.

Crowe, A., & Morgan, E.H. (1996). Iron and Copper Interact during Their uptake and Deposition in the Brain and Other Organs of Developing Rats Exposed to Dietary Excess of the Two Metals. *Journal of Nutrition*, 126(1), 183-194. https://doi.org/10.1093/jn/126.1.183

Dabbagh, A.J., Mannion, T., Lynch, S.M., & Frei, B.(1994). The effect of iron overload on rat plasma and liver oxidant status in vivo. *Biochem. J.*, 300, 799-803.

Dary, O., & Hainsworth, M. (2008). Technical Determination of Fortification Levels and Standards for Mass Fortification. *The Food Fortification Formulator Hand-Book of USAID*, 1-29.

Degerud, E.M., Manger, M.S., Tor, Strand, T.A., & Dierkes, J. (2015). Bioavailability of iron, vitamin A, zinc, and folic acid when added to condiments and seasonings. *Annals of New York Academy of Sciences*,1357(1), 29-42. doi: 10.1111/nyas.12947

De-Silva, A., Atukorala, S., Weerasinghe, I., & Ahluwalia, N. (2003). Iron Supplementation improves iron status and reduces morbidity in children with or without upper respiratory tract infections: randomized controlled study in Colombo, Sri Lanka. *American Journal of Clinical Nutrition,* 77, 234-241.

Dhur, A., Galan, P., & Hercberg, S. (1990). Effect of Decreased Food Consumption During Iron Deficiency Upon Growth Rate and Iron Satus Indicators in The Rat. *Ann Nutr Metab*, 34, 280-287.

Dostal, A., Chassard, C., Hilty, M. F., Zimmermann, M.B., Jaeggi, T., Rossi, S., & Lacroix, C. (2012). Iron Depletion and Repletion with Ferrous Sulfate or Electrolytic Iron Modifies the Composition and Metabolic Activity of the Gut Microbiota in Rats. *The Journal of Nutrition*, 142(2),271-277. doi:10.3945/jn.111.148643.

Dufner-Beattie, J., Wang, F., Kuo, Y.M., Gitschier, J., Eides, D., & Andrews, G.k. (2003). The acrodermatitis enteropathica gene ZIP4 encodes atissue-specific, zinc-regulated zinc transporter in mice. *The Journal of Biological Chemistry*, 278(35), 33474-33481.DOI 10.1074/jbc.M305000200

Elseweidy, M.M., & AbdEl-Baky, A.E. (2008). Effect of dietary iron overload in rat brain:oxidative stress, neurotransmitter level and Serum metal ion in relation to neurodegenerative disorder. *Indian Journal of Experimental Biology,* 46, 855-858.

Ergul, A.B., Turanoglu, C., Karakukcu, C., Karaman, S., & Torun, Y.A. (2018). Increased Iron Deficiency and Iron Deficiency Anemia in Children with Zinc Deficiency. *The Euroasian Journal of Medicine,* 50,34-37. doi: 10.5152/eurasianjmed.2017.17237

Esamai, F., Liechty, E., Ikemeri, J., Westcott, J., Kemp, J., Culbertson, D., Miller, L.V., Hambidge, K. M., & Krebs, N.F. (2014). Zinc Absorption from Micronutrient Powder Is Low but Is not Affected by Iron in Kenyan Infants. *Nutrients*,6, 5636-5651. doi:10.3390/nu6125636

Ethiopian Centeral Statstical Agency and ICF International. (2016). *Ethiopian Demographic and Health Survey* (EDHS):key Findings. Calverton, Maryland, USA: CSA and ICF International.

Ethiopian Public Health Institute (EPHI/ENFCS). (2013). *Ethiopian National Food Consumption Survey (ENFCS).* Addis Ababa: Retrieved on August 19, 2019, from https://www.ephi.gov.et/images/pictures/National%20Food%20Consumption%20Survey%20Report_Ethiopia.pdf

Ethiopian Public Health Institute (EPHI/ENMS). (2016). *Ethiopian National Micronutrient Survey Report* (ENMS). Addis Ababa: Retrieved on August 19, 2019, from https://www.ephi.gov.et/images/pictures/download2009/National_MNS_report.pdf

Fairweather-Tait, S.J., & Southon, S. (1989). Studies of Iron:Zinc Interactions in Adult Rats and the Effect of Iron Fortification of Two Commercial Infant Weaning Products on Iron and Zinc Status of Weanling Rats. *Journal of Nutrition*, 119, 599-606.

Fairweather-Tait, S.J., Collings, R., & Hurst, R. (2010). Selenium bioavailability current knowledge and future research requirements. *The American Journal of Clinical Nutrition*, 91(Supp), 1484-1491.

Federal Ministry of Health (FMOH). (2008). *National Nutrition Strategy*. Addis Ababa: Federal Ministry of Health. Retrieved on August 20, 2019 from, https://extranet.who.int/nutrition/gina/sites/default/files/ETH%202008%20National%20Nutrition%20Strategy_1.pdf

Fields, M., Lewis, C.G., Lure, M., & Antholine, W.E. (1992). The Influence of Gender on Developing Copper Deficiency and on Free Radical Generation of Rats Fed a Fructose Diet. *Metabolism,* 41(9), 989-994.

Fischer, J.G., Glauert, H.P., Yin, T., Sweeney-Reeves, M.L., Larmonier, N., Black, M.C.(2002). Moderate Iron Overload Enhances Lipid Peroxidation in Livers of Rats,but Does Not Affect NF-kB Activation Induced by the PeroxisomeProliferator, Wy-14,6431,2. *Journal of Nutrition*, 25, 2525-2531.

Fogh, J., Fogh, J.M., Orfeo, T, (1977). One hundred and twenty-seven cultured human tumor cell lines producing tumer in nude mice. *J Natl Cancer Inst*, 59, 221-226.

Food and Agricultural Organization of the United Nations (FAO/LLSI). (1997). *Preventing micronutrient malnutrition: A guide to food-based approaches.* Washington D.C: Food and Agricultural Organization of the United Nations. Retrieved on August 15, 2019 from, http://fao.org/3/x0245e/x0245e01.pdf.

FAO/WHO. (2004). *Expert Consultation on Human Vitamin and Mineral Requirements, Vitamin and mineral requirements in human nutrition: Report of joint FAO/WHO expert consolation.* Retrieved on August 21, 2019 from https://apps.who.int/iris/bitstream/handle/10665/42716/9241546123.pdf?sequence=1

Frazer, D.M., Wilkins, S.J., Darshan, D., Cornel, Mirciov, C.S.G., Dunn, L.A., & Anderson, G.J. (2017). Ferroportin Is Essential for Iron Absorption During Suckling, But Is Hyporesponsive to the Regulatory Hormone Hepcidin. *Cellular and Molecular Gastroenterology and Hepatology*, 3, 410-421. https://linkinghub.elsevier.com/retrieve/pii/S2352345X16301485

Frontela, C., Gracia, C.M., & Ros, G. (2011). Phytic acid content and "in vitro" iron, calcium and zinc bioavailability in bakery products: The effect of processing. *Journal of Cereal Science* 54(1),173-179.

Gaither, L.A.& Eide, D. J. (2001). The human ZIP1 transporter med mediates zinc uptake in human K562 erythroleukemia cells. *The Journal of Biological Chemistry*, 275, 5560-5564. doi: 10.1074/jbc.275.8.5560

Gammoh, N.Z., & Rink, L. (2017). Zinc in infection and inflammation. *Nutrients*, 9, 599-624.

Gangopadhyay, R., Karosh, M., & Keith, L. (2011). Anemia and Pregnancy: A link to Maternal Chronic Diseases. *American Journal of Clinical Nutrition*, 115(Suppl 1), 11-15.

Gonzalez R., Medina S., Martinez-Augustin O., Nieto A., Galvez J., Risco S., and ZarzueloA., (2004). Anti-inflammatory effect of diosmeetite in hepten-induced colitis in the rat. *Br J Pharmacol*, 141, 951-960.

Garcia-Casal, M.N., Ramirez, J., & Leets, I. (2009). Bioavailability from electrolytic and reduced iron in humans is enhanced by NaFe-EDTA and vitamin A in corn and wheat flours. Effect of serum retinol status. *African Journal of Food Science*,3(5),131-138. http://www.academicjournals.org/ajfs

Garcia, Y., Diaz-Castro, J., Lopez-Aliaga, I., Alferez, M.J.M., Ramos, A., & Campos, M.S. (2013). Bioavailability of Fe, Cu, Zn and antioxidant defence in anemic rat supplemented with a mixture of heme/non-heme Fe. *Journal of Food and Nutrition Research,* 52(2), 128-138.

Government of Federal Democratic Republic of Ethiopia (FDRE/NNP). (2013). *National Nutrition Program*. Addis Ababa: Federal Ministry of Health and Federal Ministry of trade. Retrieved on August 20, 2019 from https://extranet.who.int/nutrition/gina/sites/default/files/ETH%202013%20National%20Nutrition%20Programme.pdf

Grantham-McGregor, S. & Ani, C. (2001). A Review of Studies on the Effect of Iron Deficiency on Cognitive Development in Children. *Journal of Nutrition*, 131, 614-668.

Green, R.S., & Turner, J.C. (1974). The Manufacturer of Small Quantities of Rats Food Pellets in the Laboratory. *Laboratory Animals*, 8, 131-132.

Gulec, S., & Collins, J.F. (2014). Molecular Mediators Governing Iron-Copper Interactions. *Annu Rev Nutr*, 34, 95-116. Doi:10.1146/annurev-nutr-071812-161215

Guggenheims, S. (1995). Definition of Clay and Clay Mineral; Joint Report of the AIPEA Nomenclature and CMS Nomenclature Committees. *Clays and Clay Minerals*, 43(2), 255-256.

Guja, H., & Baye, K. (2018). Extrinsic iron from soil contributes to Hb regeneration of anemic rats: implications for foods contaminated with soil iron. *British Journal of Nutrition*, 119, 880-886. doi:10.1017/S0007114518000338

Gupta, C.P. (2014). Role of Iron (Fe) in Body. *Journal of Applied Chemistry*, 7(11), 38-46.

Ha, J.H., Doguer, C., Wang, X., Flores, S.R., & Collins, J.F. (2016). High-Iron Consumption Impairs Growth and Causes Copper-Deficiency Anemia in Weanling Sprague-Dawley Rats. *PLOS ONE*, 11(8), 1-19. https://doi.org/10.1371/journal.pone.0161033

Haap, M., Machann, J., Von-Friedeburg, C., Schick, F., Stefan, N., Schwenzer, N.F., Fritsche, A., Haring, H.U., & Thamer, C. (2011). Insulin sensitivity liver fat: role of iron load. *J Clin EndoMeta*,96(6), 958-961. doi:10.1210/jc.2010-2682

Hallberg, L., & Hulthen, L. (2000). Prediction of dietary iron absorption: an algorithm for calculating absorption and bioavailability of dietary iron. *American Journal of Clinical Nutrition*, 4(3), 749-756.

Hallberg, L. (2002). Advantages and disadvantages of an iron-rich diet. *European Journal of Clinical Nutrition*, 56(Suppl), 12-18.

Han, M., & Kim, J. (2015). Effect of Dietary Iron Loading on Recognition Memory in Growing Rats. *PLOS ONE*,10(3), 1-20. doi:10.1371/journal.pone.0120609

Harvey, P.W., Dexter, P.B., & Darnton-Hill, I. (2000). The impact of consuming iron from non-food sources on iron status in developing countries. *Public Health Nutrition*, 3(4), 375-383.

Harvey, L.J., Armah, C.N., Dainty, J.R., Foxall, R.J., Lewis, D.J., Langford, N.J., & Fairweather-Tait, S.J. (2005). Impact of menstrual blood loss and diet on iron deficiency among women in the UK. *British Journal of Nutrition*, 94, 557-564. DOI:10.1079/BJN20051493

Harvey, L.J., Ashton, K., Hooper, L., Casgrain, A.L., & Fairweather-Tait, S.J. (2009). Methods of assessment of copper status in humans: a systematic review. *American Journal of Clinical Nutrition*, 89(Suppl), 2009-2024.

Heck, D.E., Shakarjian, M., Kim, H.D., Laskin, J.D., & Vetrano, A.M. (2010). Mechanisms of oxidant generation by catalase. *Ann NY Acad Sci*, 1203, 120-125. doi:10.1111/j.1749-6632.2010.05603. x.

Heidari, S., Omidi, A., Gheisari, H.R., & Nazifi, S. (2016). Effect of Iron Enriched Bread Intake on the Oxidative Stress Indices in Male Wistar Rats. *Zahedan J Res Med Sci*, 18(9), 5020-5027. doi: 10.17795/zjrms-5027.

Hooda, P.S., Henry, C.J.K., Seyoum, T.A., Armstrong, L.D.M., & Fowler, M.B. (2004). The potential impact of soil ingestion on human mineral nutrition. *Science of the Total Environment*, 333, 75-87. doi:10.1016/j.scitotenv.2004.04.023

Howard, L., Buchowski, M., Wang, B.J., & Miller, D.D., (1993). Bioavailability of Electrolytic Iron in Fortified Infant Cereal Determined by Hemoglobin Repletion in Piglets. *Nutritional Research*, 13, 287-295.

Hotz, C., Haene, J.D., Woodhouse, L.R., Villalpando, S., Rivera, J.A.,& King, J.C. (2005). Zinc Absorption from Zinc Oxide, Zinc Sulfate, Zinc Oxide + EDTA, or Sodium-Zinc EDTA Does Not Differ When Added as Fortificants to Maize Tortillas. *The Journal of Nutrition*, 135(5), 1102-1105.

Hurrell, R.F. (1984). Bioavailability of Different Iron Compounds Used to Fortify Formulas and Cereals:Technological Problems. *Iron Nutrition in Infancy and Childhood*, 8, 147-179.

Hurrell, R., Bothwell, T., Cook, J.D., Dary, O., Davidsson, L., Fairweather-Tait, S., Hallberg, L., Lynch, S., Rossado, J., Walter, T., & Whittaker, P. (2002). The Usefulness of Elemental Iron for Cereal Flour Fortifcation:A SUSTAIN Task Force Report. *Nutrition Reviews*, 60(12), 391-406.

Hurrell, R.F. (2004). Phytic acid degradation as a means of improving iron absorption. *International Journal for Vitamin and Nutrition Research*, 74(6), 445-452. DOI: 10.1024/0300-9831.74.6.445

Hurrell, R. & Egli, I. (2010). Iron bioavailability and dietary reference values. *American Journal of Clinical Nutrition*, 91(5), 1461-1467. http://doi.org/10.3945/ajcn.2010.28674F

Iannotti, L.L., Tielsch, M.J., Black, M.M., & Black, R.E. (2006). Iron supplementation in early childhood: Health benefits and risks. *American Journal of Clinical Nutrition*, 84, 1261-1276.

Icard-Verniere, C., Hama, F., Guyot, J-P, Picq, C., Diawara, B., & Mouquet-Rivier, C. (2013). Iron contamination during in-field milling of millet and sorghum. *Journal of Agriculture and Food Chemistry,* 61(43), 10377-83. doi:10. 1021/jf402612k.

International Food Policy Research Institute (IFPRI). (2016). Crop Production in Ethiopia: Regional Patterns and Trends. Washington D.C., International Food Policy Research Institute (IFPRI). Retrieved on August 17, 2019 from, https://www.researchgate.net/publication/290263344_Crop_production_in_Ethiopia_Regional_patterns_and_trends

International Zinc Nutrition Consultative Group (IZiNCG). (2007). *Conclusions of the Joint WHO/UNICEF/IAEA/IZiNCG Interagency Meeting on Zinc Status Indicators* (Food and Nutrition Bulletin).28(3), 1-7. https://www.who.int/nutrition/publications/micronutrients/FNBvol28N3supsep07.pdf

Ismail, M. (1999). The use of CaCo-2 cells as an in vitro method to study bioavailability of iron. *Mal J Nutr*, 5, 31-45.

Jaiser, S.R., & Winston, G.P. (2010). Copper deficiency myelopathy. *J. Neurol*, 257, 869-881.

Johnson, M.A. (1989). Influence of ascorbic acid, zinc, iron, sucrose and fructose on copper status. *Advances in Experimental Medicine and Biology*, 258, 29-43.doi:10.1007/978-1-4613-0537-8

Johnson, P.E., Hunt, C.D., Milne, D.B., & Mullen, L.K. (1993). Homeostatic control of zinc metabolism in men: zinc excretion and balance in men fed diets low in zinc. *American Journal of Clinical Nutrition*, 57, 557-565.

Johnson, Q., Mannar, V., Ranum, P., Wesley, A., & Ranum, P. (2004). Vitamin and Mineral Fortofication of Wheat Flour and Maize Meal. *Fortification Handbook for Micronutrient Initiative.*

Kaganda, J., Matsuo, T., & Suzuki, H. (2002). The effect of dietary iron levels on changes in iron status and zinc dependent enzyme activities in rats fed two levels of dietary zinc. *Journal of Nutr-SciVitaminol*, 48, 461-466. DOI:10.3177/jnsv.48.461

Kalantar-Zedeh, K., Rodriguez, R.A., & Humphrey, M.H. (2004). Association between serum ferritin and measures of inflammation, nutrition and iron in haemodialysis patients. Nephrol Dial Transplant. *Nephrology Dialysis Transplantation*, 19, 141-149. DOI: 10.1093/ndt/gfg49

Kautz, L., Jung, G., Valore, E.V., Rivella, S., Nemeth, E., Ganz, T. (2014). Identification of erythroferrone as an erythroid regulator of iron metabolism. *NatGenet*, 46(7), 678-684.

Kebede, G., Assefa, G., Feyissa, F., & Mengistu, A. (2016). Seed yield and yield components of vetch species and their accessions under Nitosol and Vertisol conditions in the central highlands of Ethiopia. *International Journal of Development Research*, 06(07), 8692-8701.

Kim M., & Atallah, T. (1993). Intestinal solubility and absorption of ferrous iron in growing rats are affected by different dietary pectins. *Journal of Nutrition*, 123, 117–124.

Kim, B.E., Wang, F., Dufner-Beattie, J., Andrews, G. K., Eide, D. J., & Petris, M. J. (2004). Zn^{2+} stimulated endocytosis of the mZIP4 zinc transporter regulates its location at the plasma membrane. *The Journal of Biological Chemistry*,279(6), 4523-4550. DOI 0.1074/jbc.M310799200

Klevay, L.M. (2001). Iron overload can induce mild copper deficiency. *Journal of Trace Elements Medicine and Biology*,14(4), 237-240.

Klevay, L.M. (2016). IHD from Copper Deficiency: A Unified Theory. *Nutrition Research Reviews*, 29(2),1-8. http://doi:10.1017/S0954422416000093

Kondaiah P., Yaduvanshi, P.S., Sharp, P.A., and Pullakhandam, R. (2019). Iron and Zinc Homeostasis and Interactions: Does Enteric Zinc Excretion Cross-Talk with Intestinal Iron Absorption? *Nutrients,* 11, 1871-1885.

Kordas, k., & Stoltzfus, R.J. (2004). New Evidence of Iron and Zinc Interplay at the Enterocyte and Neural Tissues. *Journal of Nutrition*,134, 1295-1298.

Kumar, M., Dandapat, S., Sinha, M.P., Kumar, A., & Raipat, B.S., (2017). Different blood collection methods from rats: A review. *Balneo Research Journal*, 8(1), 46-50. DOI: http://dx.doi.org/10.12680/balneo.2017.141

Labbe, R.F., & Dewanji, A. (2004). Iron assessment tests: transferrin receptor vis-à-vis zinc protoporphyrin. *Clin Biochem*, 37(3), 165-174.

Liu, D., He, H., Yin, D., Que, A., Tang, L., Liao, Z., Huang, Q., & He, M. (2013). Mechanism of chronic dietary iron overload-induced liver damage in mice. *Molecular Medicine Reports*,7(4), 1173-1179. https://doi.org/10.3892/mmr.2013.1316

Livingstone, C. (2015). Zinc: Physiology, Deficiency, and Parenteral Nutrition:Review. *American Society for Parenteral and Enteral Nutrition*, 3, 1-12.

Lonnerdal, B., & Hernell, O. (1994). Iron, zinc, copper and selenium status of breast-fed infants and infants fed trace element fortified milk-based infant formula. Acta Paediatrica, 83(4), 367-373. http://doi.org/10.1111/j.1651-2227.1994.tb18121.x

Lopez, A.D., Mathers, C.D., Ezzati, M., Jamison, D.T., & Murray, C.J. (2006). Global and regional burden of disease and risk factors, 2001: systematic analysis of population health data. *Lancet*, 367(9524), 1747-1757.

Lucia, C.M.D., Toste, M.G.V., Silveira, C.M.M., Bordalo, L.A., Roddrigues, F.C., Ana, H.M.P. *et al.* (2013). Iron bioavailability in Wistar rats fed with fortified rice by Ultra Rice® technology with or without addition of yacon flour (Smallanthus sonchifolius). *Archivos Latinoamericanos De Nutrition*, 13(1), 64-73.

Lynch, S. (2005). The precision of in vitro methods and algorithms for predicting the bioavailability of dietary iron. *Int J Vitam Nutr Res*, 75(6), 436-445.

Lynch, S. (2012). The rationale for selecting and standardizing iron status indicators. In world Health Organization. Report*: Priorities in the assessment of vitamin A and iron status in populations*. Panama City, Panama, Geneva, World Health Organization. Page 55-62. Retrieved August, 24, 2019, from http://www.who.int/nutrition/publications/micronutrients/background_Paper3_report_assessment_vitAandIron_status.pdf

Mamun, A.A., & Ghani, R.B.A. (2017). The role of iron and zinc in cogenitive development of children. *Asian Journal Medical and Biological Research*, 3(2), 145-151. doi: 10.3329/ajmbr.v3i2.33561

Mei, Z., Cogswell, M.E., Parvanta, I., Lynch, S., Beard, J.L., Stoltzfus, R.J., & Grummer-Strawn, L.M. (2005). Hemoglobin and Ferritin are Currently the Most Efficient Indicators of Population

Response to Iron Interventions: An Analysis of Nine Randomized Controlled Trials. *Journal of Nutrition*, 135, 1974-1980.

Merza, H., Sood, N., & Sood, R. (2015). Idiopathic hyperzincemia with associated copper deficiency anemia: a diagnostic dilemma. *Clinical Case Reports*, 3(10), 819-822. doi:10.1002/ccr3.344

Miyada, T., Nakajima, A., & Ebihara, K. (2011). Iron bound to pectin is utilized by rats. *British Journal of Nutrition*, 106, 73-78. doi:10.1017/S007114510005842

Mocchegiani, R., Muzzioli, M., & Giacconi, R. (2000). Zinc and immuno resistance to infections in ageing: new biological tools. *Trends Pharmacol Sci*, 21, 205-208.

Monsen, E.R. (1998). Iron nutrition and absorption: dietary factors which impact iron bioavailability. *J Am Diet Assoc*, 88(7), 786-790.

Munasinghe, S., & Broek, N.V.D. (2006). Anemia in pregnancy in Malawi a review. *Malawi Medical Journal*, 18(4), 160-175.

O'Dell, B.L. (1985). Bioavailability of and Interactions Among Trace Elements. *Trace Elements in Nutrition of Children*, 3,41-63.

Oladiji, T.A. (2003). Tissue levels of iron, copper, zinc and magnesium in iron deficient rats. *Nigerian Society for Experimental Biology*,14,75-81.

Olivares, M., Fernando, Pizarro., Manuel, Ruz., Daniel, Lo´pez de Roman˜a. (2012). Acute inhibition of iron bioavailability by zinc: studies in humans. *Biometals*, 25, 657-664. DOI 10.1007/s10534-012-9524-z

Oppenheimer, S.J. (2001). Iron and Its Relation to Immunity and Infectious Disease. *Journal of Nutrition*, 131, 616-635. http://doi.org/10.1093/jn/131.2.616S

Oteiza, P.I., Olin, K.L., Fraga, C.G & Keen, C.L. (1995). Zinc Deficiency Causes Oxidative Damage to Proteins, Lipids and DNA in Rat Testes. *The Journal of Nutrition*,125(4), 823-829.http://doi.org/10.1093/jn/125.4.823

Ouedraogo, I., Zamane, H., Sawadogo, A.Y., Kiemtore, S., Kain, D.P., Ouattara, A., Swadogo, T.P., Ouedraogo, A., & Tleba/Bonane, B. (2019). Anemia in pregnancy in an African setting after preventive measures. *Open Journal of Obstetrics and Gynecology*, 9, 10-20. https://doi.org/10.4236/ojog.2019.91002

Paes, M.C., Cosentino-Gomes, D., De-Souza, C.F., Nogueira, N.P.D.A., & Meyer-Fernandes, J.R. (2011). The Role of Heme and Reactive Oxygen Species in Proliferation and Survival of Trypanosoma cruzi. *Journal of Parasitology Research*, 2011, 1-8. doi:10.1155/2011/174614

Parasuraman, S., Raveendran, R., & Kesavan R. (2010). Blood sample collection in small laboratory animals. *Journal of Pharmacol Pharmacotheraputics*, 1(2), 87-93. doi: 10.4103/0976-500X.72350

Pouraram, H., Elmadfa, I., Siassi, F., Motlang, A.D., Heshmat, R., & Abtahi, M. (2010). Oxidative Stress among Non-Anemic Adults following Flour Fortification with Iron: Baseline Data. *Annals of Nutritional Metabolism*, 56, 283-287. DOI: 10.1159/000290421

Prasad, R., Hawthorne, B., Durai, D. & McDowell I. (2015). Zinc in denture adhesive: a rare cause of copper deficiency in a patient on home parenteral nutrition. *BMJ Case Report*, 1-3. doi:10.1136/bcr-2015-211390.

Prohaska, J.R., & Heller, L.J. (1982). Mechanical Properties of the Copper-Deficient Rat Heart. *Journal of Nutrition*, 112(11), 2142-2150. https://doi.org/10.1093/jn/112.11.2142

Pyatskowit, S.W., &Prohaska, J.R. (2008). Copper deficient rats and mice both develop anemia but only rats have lower plasma and brain iron levels. *Comp Biochem Physiol C Toxicol Pharmacol*, 147(3), 316-323.

Quintaes, K.D., Cilla, A., & Barberá, R. (2015). Iron bioavailability from cereal foods fortified with iron. *Austin Journal of Nutrition and Metabolism*, 2(3), 1012-1021. doi: 10.1080/10408398.2013.866543

Ranganathan, P.N., Lu, Y., Jiang, L., Kim, C., & Collins, J.F. (2011). Serum ceruloplasmin (CP) protein expression and activity increases in iron deficient rats and is further enhanced by higher dietary copper intake. *Blood Journal*, 118(11), 3146-3156. doi:10:1182/blood-2011-05-352112

Reeves, P.G., Nielsen, F.H., George, C., & Fahey, J. (1993). AIN-93 Purified Diets for Laboratory Rodents: Final Report of the American Institute of Nutrition AdHoc Writing Committee on the Reformulation of the AIN-76A Rodent Diet. *The Journal of Nutrition*, 123(11), 1939-1951. http://doi.org/10.1093/jn/123.11.1939

Reeves, P.G. (1997). Components of the AIN-93 Diets as Improvements in the AIN-76A Diet and Toxicological Research. *Journal of Nutrition*, 127(5 suppl), 838-841.doi:10.1093/jn/127.5.838S.

Rodriguez-Matas, M.C., Lisbona, F., Gomez-Ayala, A.E., Lopez-Aliaga, I., & Campos, M.S. (1998). Influence of nutritional iron deficiency development on some aspects of iron, copper and zinc metabolism. *Laboratory Animals*,32, 298-306.

Rohner, F., Ernst, F.O., Arnold, M., Hilbe, M., Biebinger, R., Ehrensperger, F., Pratsinis, S.E., Langhans, W., Hurrell, R.F., Zimmermann, M.B. (2007). Synthesis, characterization and bioavailability in rats of ferric phosphate nanoparticles. *Journal of Nutrition*, 137, 614-619.

Rossander-Hultn, L., Brune, M., Sandstrm, B., Lnnerdal, B., & Hallberg, L. (1991). Competitive inhibition of iron absorption by manganese and zinc in humans. *American Journal of Nutrition*, 54, 151-156. DOI:10.1093/ajcn/54.1.152

Roughead, Z.k., Johnson, L.K., & Hunt, J.R. (1999). Dietary Copper Primarily Affects Antioxidant Capacity and Dietary Iron Mainly Affects Iron Status in a Surface Response Study of Female Rats Fed Varying Concentrations of Iron, Zinc and Copper. *The Journal of Nutrition*, 129(7), 1368-1376. https://doi.org/10.1093/jn/129.7.1368

Rowley, D.A, &Halliwell, B. (1982). Superoxide-dependent formation of hydroxyl radicals from NADH and NADPH in the presence of iron salts. *Federation of European Biochemical Societies*, 142(1), 4-7. DOI:10.1016/0014-5793(82)80214-7

Salas, R.E., Gamaldo, C.E., & Allen, R.P. (2010). Updated in restless legs syndrome. *Curr Opin Neurol,* 23(4), 401-406.

Sambuy, Y., De Angelis, I., Ranaldi, G., Scario, M.L., Stammati, A., & Zucco, F. (2005). The CaCo-2 cell lines as model of the intestinal barrier: influence of cell and culture related factors of CaCo-2 cell functional characteristics. *Cell Biology and Toxicology*, 21, 1-26.

Sandstead, H.H. (2000). Causes of Iron and Zinc Deficiencies and Their Effect on Brain. *Journal of Nutrition*, 130, 347-349.

Sandstrom, B. (2001). Micronutrient interactions: effects on absorption and bioavailability. *British Journal of Nutrition*, 85(Suppl2), S181-S185. https://www.ncbi.nlm.nih.gov/pubmed/11509108

Sari, M., De-Pee, S., Martini, E., Herman, S., Sugiatmi, Bloem, M.W., & Yip, R.(2001). Estimating the prevalence of anemia: a comparison of three methods. *Bulletin of Word Health Organization*, 79, 506-511.

Saturni, L., Ferretti, G., & Bacchetti, T. (2010). The gluten-free diet: safety and nutritional quality. *Nutrients*,2(1), 16-34. doi: 10.3390/nu2010016

Schumann, K., Elsenhans, B., & Maurer, A. (1998). Iron supplementation. *Journal of Trace elements in medicine and biology,* 12(3), 129-140. doi.org/10.1016/S0946-672X (98)80001-1.

Seim, G.L., Ahn, C.I., Bodis, M.S., Luwedde, F., Miller, D.D., Hillier, S., Takoc, E., Glahn, R.P., & Young, S.L. (2013). Bioavailability of iron in geophagic earths and clay minerals, and their effect on dietary iron absorption using an in vitro digestion/Caco-2 cell model. *Food Funct*, 4(8), 1263-1270. doi:10.1039/c3fo30380b

Semrad, C.E. (1999). Zinc and Intestinal Function. *Current Gastroenterol Reports,* 1(5),398-403. doi:10.1007/s11894-999-0021-7

Sihag, M.K., Sharma, V., Goyal, A., Aroraa, S., & Kapila R. (2016). In vivo assessment of iron bioavailability from fortified pearlmillet based weaning food. *Journal of The Science of Food and Agriculture,* 1,1-6. https://doi.org/10.1002/jsfa.7651

Sandstead, H.H. (2000). Causes of Iron and Zinc Deficiencies and Their Effects on Brain. *Journal of Nutrition,* 130, 347-349.

Smith B., Rawlins B., Cordiero M., and Hutchins M., (2000). The bioaccessibility of essential and potentially toxic trace elements in tropical soils from geophagic earths. *J. Geol. Soc.*, 157, 884–892

Stockman, R. (1893). The Treatment of Chlorosis by Iron and Some Other Drugs. *The British Medical Journal* 23, 881-885.

Storey, M.L., & Greger, J.L. (1987). Iron, Zinc and Copper Interactions: Chronic versus Acute Responses of Rats. *The Journal of Nutrition*, 117(8), 1434-1442. https://doi.org/10.1093/jn/117.8.1434

Soliman, A.T., De-Sanctis, V., & Kalra, S. (2014). Anemia and growth. *Indian Journal of Endocrinology and Metabolism*, 18(Suppl 1): S1-S5. doi: 10.4103/2230-8210.145038

Sukalski, K.A., LaBerge, T.P., & Johnson, W.T. (1997). In vivo oxidative modification of erythrocyte membrane proteins in copper deficiency. *Free Radic Biol Med*, 22(5), 835-842. https://doi.org/10.1016/S0891-5849(96)00430-3

Suliburska, J., Bogdanski, P., & Szulinska, M. (2013). Iron Excess Disturbs Metabolic Status and Relative Gonad Mass in Rats on High Fat, Fructose, and Salt Diets. *Bio Trace Elem Res*, 151, 263-268. Doi10.1007/s12011-012-9548-9

Suttle, N.F. (2010). *Mineral Nutrition of Livestock.* (4th ed.). Wallingford: CAB International, Oxfordshire OX10 8DE, UK. Retrieved August 19, 2019, from http://www.ucv.ve/fileadmin/user_upload/faculated_agronomia/Production_Animal/Minerals_in_Animal_Nutrition.pdf

Swain, J.H., Newman, S.M., & Hunt, J.R. (2003). Bioavailability of Elemental Iron Powders to Rats Is Less than Bakery-Grade Ferrous Sulfate and Predicted by Iron Solubility and Particle Surface Area. *Journal of Nutrition*, 133, 3546-3552.

Swain, J.H., Johnson, L.K., & Hunt, J.R. (2007). Electrolytic Iron or Ferrous Sulfate Increase Body Iron in Women with Moderate to Low Iron Stores. *The Journal of Nutrition*, 137, 620-627.

Tang, M., Frank, D.N., Hendricks, A.E., Ir, D., Esamai, F., Liechty, E., Hambidge, K.M., & Krebs, N.F. (2017). Iron in Micronutrient Powder Promotes an Unfavorable Gut Microbiota in Kenyan Infants. *Nutrients*, 9,764-766. doi:10.3390/nu9070776

Tao M., & Pelletier, D.L. (2009). The effect of dietary iron intake on the development of iron overload among homozygotes for haemochromatosis. *Public Health Nutrition*, 12(10), 1823-1829.

Teklu, D.(2017a). *Rat Hemoglobin Regeneration Efficiency of Teff Contaminated with Vertisoil and Cambisol Varieties* (Master's Thesis). Addis Ababa University, Center for Food Science and Nutrition.

Teklu, D.(2017b). The Unresolved Wonder on Contaminant Iron Bioavailability: The Way Forward (Review Article). *Journal of Nutritional Health and Food Science*, 5(2), 1-5. http://dx.doi.org/10.15226/jnhfs.2017.00195

Troost, F.J., Brummer, R.J.M., Dainty, J.R., Hoogewerff, J.A., Bull, V.J., & Saris, W.HM.(2003). Iron supplements inhibit zinc but not copper absorption in vivo in ileostomy subjects. *American Journal of Clinical Nutrition*, 78, 1018-1023. http://doi.org/10.1093/ajcn/78.5.1018

Turgut, S., Polat, A., Inan, M., Turgut, G., Emmungil, G., Bican M., Karakus T.Y., & Genc O. (2007). Interaction Between Anemia and Blood Levels of Iron, Zinc, Copper, Cadmium and Lead in Children. *The Indian Journal of Pediatrics*, 74(9), 827-830.

Umeta, M., West, C.E., & Fufa, H. (2005). Content of Zinc, Iron, Calcium and their absorption inhibitors in foods commonly consumed in Ethiopia. *Journal of Food Composition and Analysis*, 18, 803-817.

Umeta, M., Haidar, J., Demissie, T., Akalu, G. & Ayana, G. (2008). Iron Deficiency Anemia Among Women of Reproductive Age in Nine Adminstrative Regions of Ethiopia. *Ethiopian J Health Dev*, 22(3), 252-258.

Urga, K., Narasimha, H.V., Sasikala, B.V., & Vishwanatha, S. (1998). Bioavailability of Iron and Zinc from Teff in Rats. *Bull Chem Soc Ethiop*, 12(2), 95-103.

Uritski, R., Barshack, I., Bilkis, I., Ghebremeskel, K., & Reifen, R. (2004). Dietary Iron Affects Inflammatory Status in a Rat Model of Colitis. *Journal of Nutrition*, 134, 2251-2255.

Uauy, R., Olivares, M., Gonzalez, M. (1998). Essentiality of copper in human. *Journal of Clinical Nutrition*, 67(5), 952-959.

Vallee, B., & Falchuk, K. (1993). The Biochemical Basis of Zinc Physiology. *Physiological Reviews*, 73(1),79-118. DOI:10.1152/physrev.1993.73.1.79

Vasak, M., & Hasler, D.W. (2000). Metallothioneins: New Functional and Structural Insights. *Curr Opin Chem Biol*, 4, 177-183.

Vatansever, F., De-Melo, W.C.M.A., Avci, P., Vecchio, D., Sadasivam, M., Gupta, A., Chandran, R., Karimi, M., Parizotto, N.A., Yin, R., Tegos, G.P., & Hamblin, M.R. (2013). Antimicrobial strategies centered around reactive oxygen species - bactericidal antibiotics, photodynamic therapy and beyond. *FEMS Microbiol Rev*, 37(6), 955-989. doi:10.1111/1574-6976.12026.

Vu'o'ng Lê, B., Khorsi-Cauet, H., Villegier, AS., Bach, V., & Gay-Quéheillard, J.(2011). New rat models of iron sucrose-induced iron overload. *Experimental Biology and Medicine*, 236, 790-799. doi: 10.1258/ebm.2011.010298

Waldvogel-Abramowski, S., Waeber, G., Gassner, C., Buser, A., Frey, M.B., Favrat, B., & Tissot, J-D. (2014). Physiology of Iron Metabolism. *Transfusion Medicine and Hemotherapy*, 41, 213-221. DOI:10.1159/000362888

Weber, T.K., Freitas, C.K., Amanico, O.M.S., & Morais, M.B. (2010). Effect of dietary fibre mixture on growth and intestinal iron absorption in rats recovering from iron-deficiency anaemia. *British Journal of Nutrition*, 104, 1471-1476. doi:10.1017/S0007114510002497

Wienk, K.J., Marx, J.J., & Beynen, A.C. (1999). The concept of iron bioavailability and its assessment. *Eur J Nutr*, 38(2), 51-75.

Wessling-Resnick, M. (2010). Iron Homeostasis and the Inflammatory Response. *Annu Rev Nutr*,30, 105-122. doi: 10.1146/annurev.nutr.012809.104804

Wessling-Resnick, M. (2014). Iron. In Ross, A.C., Caballero, B., Cousins, R., Tucker, K.L., Ziegler, T.R.(Eds.). *Modern Nutrition in Health and Disease*. (11th Ed). (Pp.176-186).

Wessells, K.R., & Brown, K.H. (2012). Estimating the Global Prevalence of Zinc Deficiency: Results Based on Zinc Availability in National Food Supplies and the Prevalence of Stunting. *PLoS ONE*, 7, 1-11. doi: 10.1371/journal.pone.0050568.

Wilson, M.J. (2003). Clay mineralogical and related Characteristics of geophagic materials. *Journal of Chemical Ecology*, 29(7), 1525-1545.DOI:10.1023/A:1024262411676

Wieringa, F.T., Dijkhuizen, M.A., Fiorentino, M., Laillou, A., & Berger, J. (2015). Determination of Zinc Status in Humans: Which Indicator Should We Use? *Nutrients*, 7, 3252-3263.

Windisch, W., & Kirchgessner, W. (1999). Tissue zinc distribution and exchange in adult rats at zinc deficiency induced by dietary phytate additions: II. Quantitative zinc metabolism of 65Zn-labelled adult rats at zinc deficiency. *Journal of Animal Physiology and Animal Nutrition*, 82,116-124. doi:10.1111/j.1439-0396.1999. 00220.x

World Health Organization (WHO). (1992). *The Prevalence of Anemia in women: A Tabulation of Available Information*. Geneva: World Health Organization. Retrived August 23, 2019, from http://whqlibdoc.who.int/hq/1992/WHO_MCH_MSM_92.2.pdf

World Health Organization (WHO). (2005). *Nutrients in Drinking Water*: *Water, Sanitation and Health Protection and the Human Environment.* Geneva, World Health Organization. Retrieved on August 15, 2019 from https://www.who.int/water_sanitation_health/dwq/nutrientsindw.pdf

World Health Organization/ Food and Agricultural Organization of the United Nations (WHO/FAO). (2006). *Guidelines on food fortification with micronutrients*. Geneva, World Health Organization. Retrieved on August 17, 2019 from http://apps.who.int/iris/handle/10665/43412

World Health Organization/ Food and Agricultural Organization of the United Nations (WHO/FAO). (2008). *Recommendations on Wheat and Maize Flour Fortification Meeting Report: Interim Consensus Statement*. Geneva, World Health Organization.

World Health Organization (WHO). (2011). Haemoglobin concentrations for the diagnosis of anaemia and assessment of severity. Vitamin and Mineral Nutrition Information System. Geneva, World Health Organization, (WHO/NMH/NHD/MNM/11.1). Retrieved on December 11, 2018 from, http://www.who.int/vmnis/indicators/haemoglobin.pdf.

World Health Organization (WHO). (2015). *Micronutrient deficiency, iron deficiency*. Geneva, World Health Organization. Retrieved on August 15, 2019 from, http://www.who.int/nutrition/topics/ida/en/

Whittaker, P. (1998). Iron and zinc interactions in humans. *American Journal of Clinical Nutrition*, 68, 442-446.

Yang, Z., Dewey, K.G., Lonnerdal, B., Hernell, O., Chaparro, C., Adu-Afarwuah, S., *et al*. (2008). Comparison of Plasma ferritin concentration with the ratio of plasma transferrin receptor to ferritin in estimating body iron stores: Results of 4 intervention trial. *American Journal of Clinical Nutrition*, 87, 1892-1898.

Yeung, C.K., Zhu, L., Glahn, R.P., & Miller, D.D. (2004). Iron Absorption from NaFeEDTA Is Downregulated in Iron-Loaded Rats. *Journal of Nutrition*,134(9), 2270-2274. doi:10.1093/jn/134.9.2270.

Yi-chen, J., Yong-ye, S.,Wei-lei, G.,Yuan-yuan, L., & Ma, Ai-guo, M.(2017). Effects of Iron Overload on Growth and Intestinal Mucosa in Rats. *International Journal of Sciences*, 6(3), 1-5. DOI: 10.18483/ijSci.1212

Young, Sera.L., Wilson, M.Jeffrey., Miller, Dennisl., & Hillier, Stephen.(2008). Toward a Comprehensive Approach to the Collection and Analysis of Pica Substances, with Emphasis on Geophagic Materials. *PLOS ONE*,3(9), 3147-3150. doi:10.1371/ journal.pone.0003147.

Yu, S. (1993). *Copper Metabolism and Its Interactions with Dietary Iron, Zinc, Tin and Selenium in Rats.* Thesis Wageningen. - With ref. - With summary in Dutch and Chinese. *Pp1-171.* Retrieved on August 20, 2019 from http://doi.edepot.wur.nl/201153

Ziegler, E.E., Fomon, S.J., Nelson, S.E., Jeter, J.M., & Theuer, R.C. (2010). Dry Cereals Fortified with Electrolytic Iron or Ferrous Fumarate Are Equally Effective in Breast-fed Infants. *Journal of Nutrition,*141, 243-248. doi:10.3945/jn.110.127266

Zimmermann, M.B., Biebinger, R., Rohner, F., Dib, A., Zeder, C., Hurrell, R.F., Chaouki, N. (2006). Vitamin A Supplementation in Children with Poor Vitamin A and Iron Status Increases Erythropoietin and Hemoglobin Concentrations without Changing Total Body Iron. The American Journal of Clinical Nutrition, 84(3), 580-586. http://doi.org/10.1093/ajcn/84.3.580

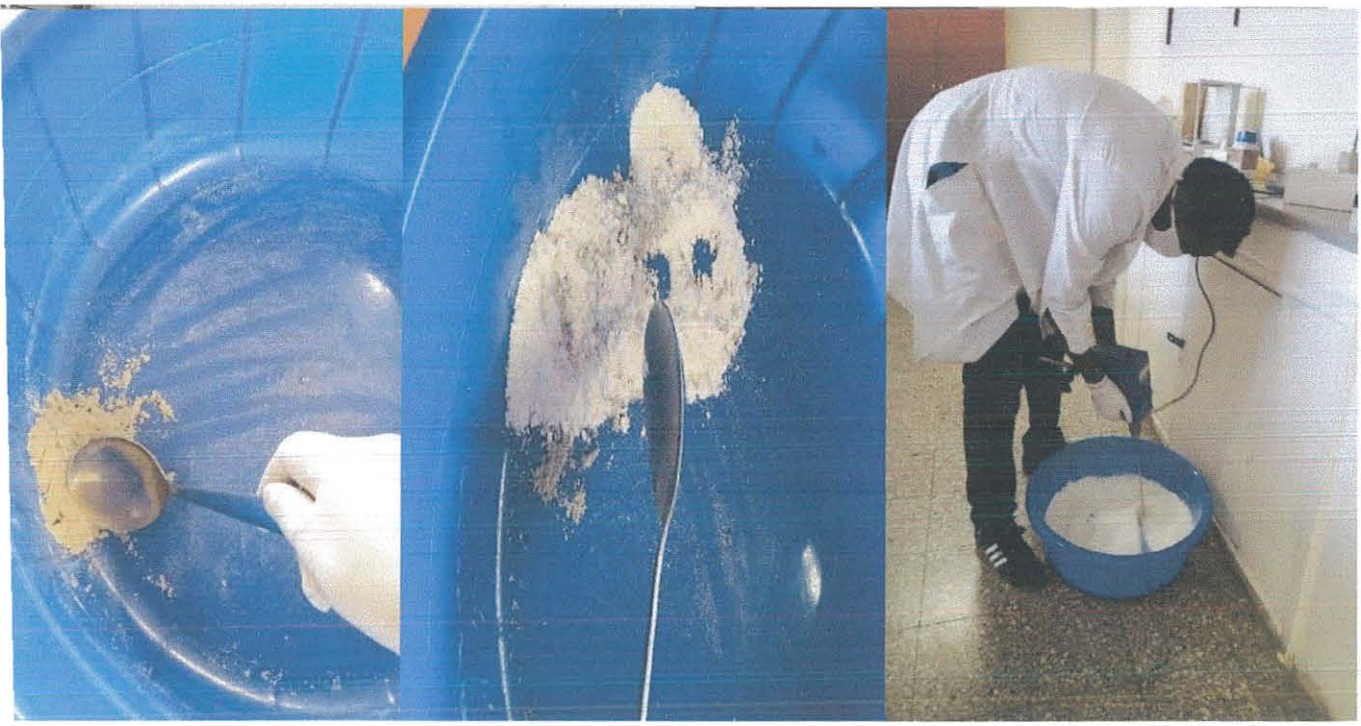
Zimmermann, M.B., & Hurrell, R.F. (2007). Nutritional iron deficiency. *Lancet*, 370(9586), 511-520.

Zimmermann, M.B., Chassard, C., Rohner, F., Goran, E.K.N., Nindjin, C., Dostal, A., Utzinger, J., Ghattas, H., Lacroix, C., & Hurrell, R.F. (2010). The effects of iron fortification on the gut microbiota in African children: a randomized controlled trial in Coˆ te d'Ivoire. *Journal of Clinical Nutrition*, 92(6), 1406-1415. https://doi.org/10.3945/ajcn.110.004564

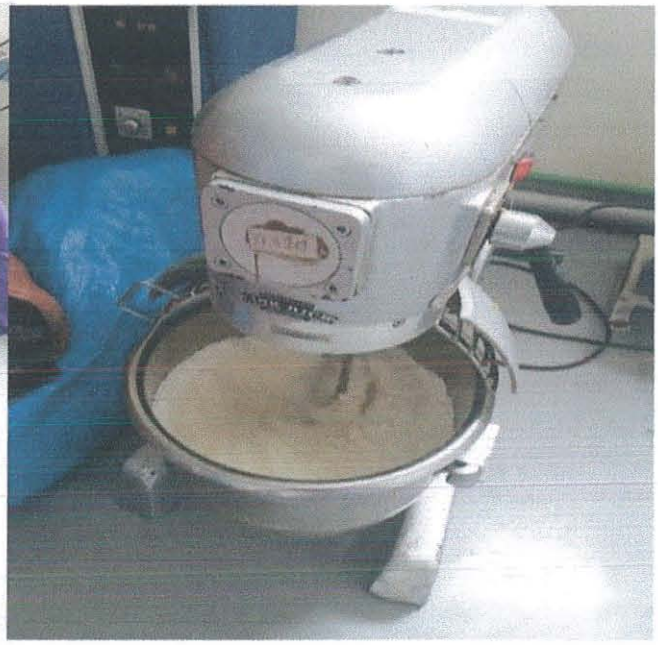
ANNEX

Annex I: Soil Sample Preparation

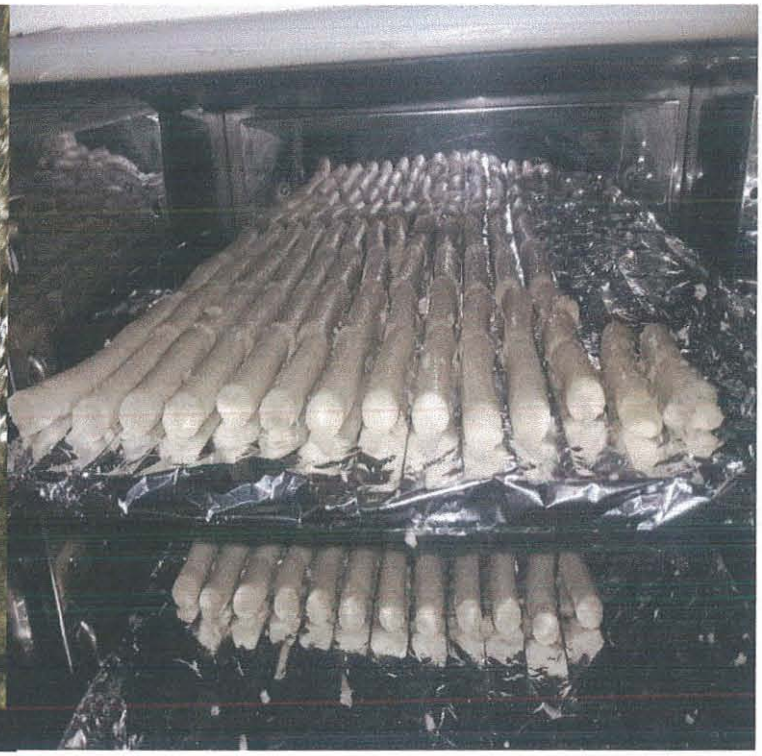
Annex II: Experimental Diet Formulation/Fortification



Annex III: Homogenization of Formulated/Fortified Powder Diet



Annex IV: Pelletizing and Drying Experimental Diet

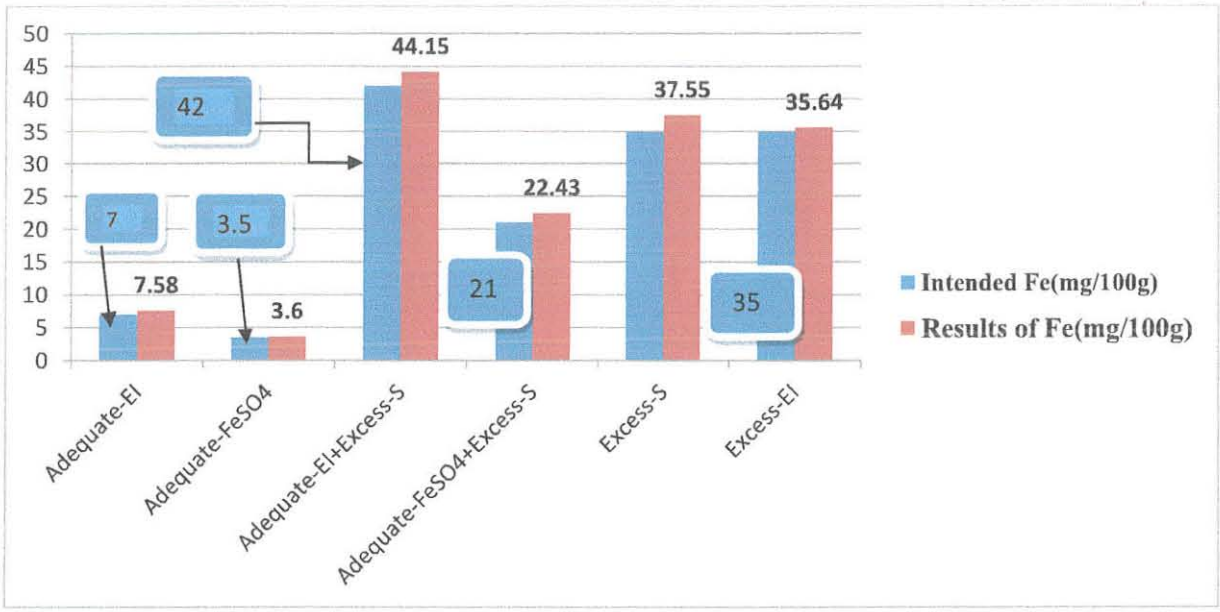


Annex V: Iron Content of Fortificants Before Experimental Diet Formulation

Fortificant Type	Result in mg/100g
Iron Sulfate	1.9±0.035*[a]
Electrolyte Iron	8.96±0.45*[b]
Soil	4532.6±0.23*[c]

* All results were expressed in mean ± standard deviation and superscripts (a, b and c) within a row represent significance level at ($p<0.05$) and (*) - superscript represent mean ± standard deviation significantly different each other in iron content.

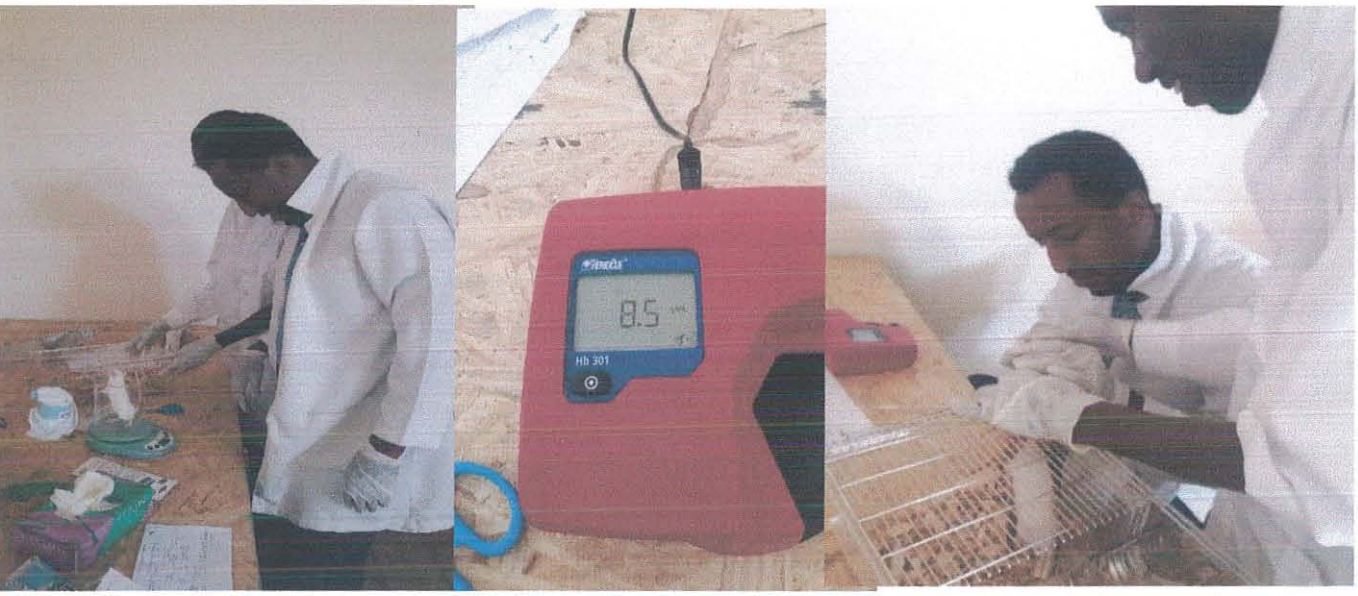
Annex VI: Formulated Experimental Diet Intended Iron Content Vs Analyzed and Found to be Iron Content (AAS Out Put at Bless Laboratory)



*Adequate-EI- Adequate electrolyte iron, Adequate-$FeSO_4$-Adequate iron sulfate, Excess-S+Adequate-EI - Excess dose of soil combined with adequate dose of electrolyte iron, Excess-S+Adequate $FeSO_4$ – Excess dose of soil combined with adequate dose of iron sulfate, Excess-S - Only excess dose of Soil and Excess-EI- Excess dose of Electrolyte Iron were the fortificants type added to experimental diet. Intended Iron- Mean iron content expected to be incorporated in

mg/100g and Iron Result- After fortification of experimental diet analyzed for mean iron content in mg/100g.

Annex VII: Animal Screening and Baseline Measurement



Annex VIII: Experimental Animal Feeding and Pre-End-line Protocol (Anesthesia)

Annex IX: End-line Measurement and Whole Blood to Serum Changing for Analysis.