

**The potential of fermentation and
blanching in improving bioaccessibility and
bioavailability of iron and zinc in African
leafy vegetables**

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DEDICATION

This thesis is dedicated to God. He strengthened me to accomplish this work. All the glory and honour belong to Him.

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I commit to my Lord Jesus Christ - Proverbs 16:3.

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ABSTRACT

Introduction

Iron and zinc deficiencies remain a significant problem in sub-Saharan Africa. Consumption of African leafy vegetables (ALVs) have shown potential in alleviating these deficiencies. However, ALVs also contain high levels of antinutrients which have some inhibitory effects on the absorption of iron and zinc. Processing methods such as blanching and spontaneous fermentation are effective in reducing antinutrients. The reduction of antinutrients in ALVs could improve iron and zinc bioaccessibility and bioavailability. The study aimed to compare mineral and antinutrients content of cooked ALVs to blanched-dried and spontaneously fermented-dried ALVs and with the overall aim to evaluate the bioaccessibility of iron and zinc from these processed ALVs and to predict their iron and zinc bioavailability using the Caco-2 cell assay model.

Methods

Five ALVs, viz. spider plant (*Cleome gynandra*), amaranth (*Amaranthus hybridus*), wild jute (*Corchorus olitorius*), cowpea leaves (*Vigna unguiculata*) and Ethiopian kale (*Brassica carinata* A. Baun) were procured in Botswana. The ALVs were separated into three processing techniques: cooked (as typically prepared and consumed in households when fresh), blanched-dried, and spontaneously fermented-dried (consumed in households when out of season). Mineral (iron, zinc, calcium, phosphorus, magnesium and aluminium) contents were analysed by inductively coupled plasma optical emission spectroscopy (ICP-OES). Total phenolics, condensed tannins, total oxalates and phytate content were analysed. The bioaccessibility of iron and zinc from differently processed ALVs were also analysed using *in vitro* dialysability assay, and results were used to calculate contributions these ALV could make towards iron and zinc absolute requirements for women aged 19 to 50 years. The two ALVs with the best iron and zinc bioaccessibility were chosen to predict iron and zinc bioavailability using the *in vitro* digestion combined with the use of Caco-2 cells assay to simulate mineral uptake. Iron uptake by Caco-2 cells from processed ALVs was measured by determining ferritin formation in the cells using the Human ferritin ELISA kit. In contrast, zinc uptakes by the Caco-2 cells were analysed using inductively coupled plasma mass spectrometry (ICP-MS).

Results

Compared to their cooked counterparts the mineral content of spontaneously fermented-dried ALVs was significantly ($p \leq 0.05$) lower for iron in wild jute (44%) and spider plant (14%); for magnesium in spider plant (14%), Ethiopian kale (7%), and amaranth (8%) and calcium in amaranth (13%). In contrast, compared to the cooked ALVs, the mineral content of spontaneously fermented-dried ALVs was significantly ($p \leq 0.05$) higher for iron in amaranth (11%) and Ethiopian kale (83%); for zinc in cowpea leaves (17%), wild jute (22%) and Ethiopian kale (21%), for magnesium in wild jute (12%) and cowpea (14%) and calcium in cowpea (21%), spider plant (8%) and wild jute (12%). Compared to their cooked counterparts the mineral content of blanched-dried ALVs was significantly ($p \leq 0.05$) lower for iron in cowpea (39%), spider plant (35%), wild jute (65%) and amaranth (12%), for magnesium in spider plant (14%) and Ethiopian kale (21%), for calcium in Ethiopian kale (23%), and for phosphorus in amaranth (30%) and Ethiopian kale (8%). In contrast, compared to cooked counterparts, mineral contents of blanched-dried ALVs were significantly ($p \leq 0.05$) higher for iron in Ethiopian kale (49%), for zinc in Ethiopian kale (24%) and amaranth (6%), for calcium in wild jute (72%), phosphorus in amaranth (19%). Aluminium levels of cooked ALVs varied from 9.71 to 75.8 mg/100 g, db. However, the aluminium content of blanched-dried and spontaneously fermented-dried ALVs was significantly ($p \leq 0.05$) lower for all ALVs compared to their cooked counterparts except in Ethiopian kale which had significantly ($p \leq 0.05$) higher contents in the blanched-dried and spontaneously fermented-dried samples. Cooked spider plant had the highest aluminium content (75.8 mg/100 g, db) compared to other cooked ALVs.

Condensed tannins were significantly ($p \leq 0.05$) lower in spontaneously fermented-dried spider plant (14%), wild jute (40%), cowpea (58%) and amaranth (46%) than in their cooked counterparts. Significantly ($p \leq 0.05$) higher levels of condensed tannins were found in spontaneously fermented-dried Ethiopian kale (52%) than its cooked counterpart. Significantly ($p \leq 0.05$) lower contents of condensed tannin contents were found in blanched-dried spider plant (19%) and wild jute (47%) compared to their cooked counterparts. In comparison, significantly ($p \leq 0.05$) higher contents were found in blanched-dried cowpea (27%) than in its cooked counterpart. Significantly higher ($p \leq 0.05$) total phenolic content (TPC) was found in spontaneously fermented-dried cowpea (29%) and spider plant (48%), than in their cooked counterparts while no significant differences ($p > 0.05$) were found in TPC in all blanched-dried ALVs compared to their cooked counterparts except for wild jute with significantly ($p \leq 0.05$) lower contents. Significantly lower ($p \leq 0.05$) total oxalate contents were found in all spontaneously fermented-dried and blanched-dried ALVs compared to their cooked counterparts. All blanched-dried ALVs also had significantly ($p \leq 0.05$) lower soluble oxalates contents compared to their

cooked counterparts. Phytate contents were significantly ($p \leq 0.05$) lower in spontaneously fermented-dried amaranth (22%) and spider plant (25%) compared to their cooked counterparts. There were no significant ($p > 0.05$) differences found between phytate contents of all blanched-dried ALVs and their cooked counterparts except for spider plant which had 24% lower phytate content than its cooked counterpart.

The Principal Component Analysis (PCA) graph showed that total oxalates, soluble salts, and magnesium were positively correlated. Moreover, there was a strong positive correlation between phytate and magnesium contents. Iron, zinc, and aluminium were also closely associated.

The percentage of iron bioaccessibility from cooked, blanched-dried and spontaneously fermented-dried ALVs ranged from 1.6 to 15.3%, 1.8 to 7.8% and 1.5 to 11.5%, respectively. The amount of iron bioaccessible from processed ALVs ranged from 0.47 to 3.20 mg/100 g, db, 0.38 to 2.06 mg/100 g, db and 0.37 to 3.81 mg/100 g, db respectively. Significantly ($p \leq 0.05$) higher percentages of iron bioaccessibility were found in spontaneously fermented-dried Ethiopian kale compared to its cooked counterpart. Compared to cooked counterparts, percentage iron bioaccessibility and amount of iron bioaccessible in all the blanched-dried ALVs showed no significant ($p > 0.05$) differences except for Ethiopian kale. Significantly ($p \leq 0.05$) lower amounts of bioaccessible iron were found in blanched-dried amaranth compared to its cooked counterpart. The percentage zinc bioaccessibility from cooked, blanched-dried and spontaneously fermented-dried ALVs ranged from 8.4 to 40.9%, 16.6 to 25.1% and 12.2 to 30.8%, respectively. The amount of zinc bioaccessible from cooked, blanched-dried and spontaneously fermented-dried ALVs ranged from 0.47 to 3.20 mg/100 g, db, 0.38 to 2.06 mg/100 g, db and 0.37 to 3.81 mg/100 g, db. This study found no significant ($p > 0.05$) differences in percentage zinc bioaccessibility between the same type of cooked and spontaneously fermented-dried ALVs while significantly ($p \leq 0.05$) higher amount of bioaccessible zinc was observed in blanched-dried amaranth (14.5%) than in its cooked counterpart.

The percentage iron contribution estimation that could be made by 300 g (as consumed) of ALV cooked, blanched-dried and spontaneously fermented-dried towards iron absolute requirements for women aged 19 to 50 years were 14.5% to 50.3%, 6.1% to 40.6% and 3.9% to 48.1%, respectively. The estimated zinc contributions cooked, blanched, and spontaneously fermented-dried ALVs could make towards zinc absolute requirements for women aged 19 to 50 years were 2.9% to 34.9%, 8.5% to 51.9% and 6.0% to 57.2%, respectively. Amaranth has the potential to contribute over 40% (0.58 mg/day) towards AR for iron irrespective of processing. About zinc, the spider plant is estimated to contribute between 34.9 to 57.1% (0.61 to 1.0 mg/day) towards zinc absolute requirements for women aged 19 to 50 years. Still, these contributions are just relative

not actual values as physiological situations *in vivo* are not similar to *in vitro* situations; hence the need for human intervention studies ascertain iron and zinc bioavailability from these ALVs.

Ferritin formation in Caco-2 cells exposed to simulated gastrointestinal digestates of differently processed ALVs ranged from 0.31 ng ferritin/mg cell protein to 2.35 ng ferritin/mg cell protein. Compared to cooked ALVs, the amount of ferritin formation in cells exposed to spontaneously fermented-dried and blanched-dried ALVs were not significantly ($p > 0.05$) different. Concerning zinc, cooked amaranth (31.2%) had significantly ($p \leq 0.05$) higher percentages of zinc retained by Caco-2 cells compared to blanched-dried amaranth (3.1%). At the same time, no significant differences were found between differently processed spider plants. Zinc transport percentages in Caco-2 cells varied from 1.8 to 15.8%. Zinc uptake by the Caco-2 cells (calculated as the sum of cell retention and transport) was significantly ($p \leq 0.05$) lower from blanched-dried amaranth (approximately five times less) compared to cooked counterpart. The amount of zinc uptake in both spontaneously fermented-dried amaranth and spider plant compared to their cooked counterparts was not significant ($p > 0.05$).

Conclusions

ALVs had high levels of iron and zinc, so their consumption could be beneficial to rural communities with limited access to food and that are at risk of iron and zinc deficiencies. Spontaneously fermented-dried ALVs had similar levels of iron, zinc, and phosphorus as cooked vegetables and suggest that processing ALVs by fermentation could be as good as freshly cooked and therefore, consumption of spontaneously fermented-dried ALVs can be encouraged. Blanching and spontaneous fermentation further removed some of the soil contamination from the ALVs which remained after regular cleaning as shown by low levels of aluminium contents. Though blanched-dried and spontaneously fermented-dried ALVs had lower contents of antinutrients it did not translate to enhanced iron and zinc bioaccessibility compared to their cooked counterparts; hence other ALV components probably influenced mineral bioaccessibility. The uptake of iron and zinc by Caco-2 cells from ALVs is seriously impaired by the presence of high levels of antinutrients. Most antinutrients are dose-dependent, hence levels which were present after processing still had some inhibitory effects on iron and zinc uptake. Consumption of ALVs could be encouraged concurrently with other locally available foods which are rich sources of enhancers of iron and zinc (tomatoes, oranges and Mopani worms) to influence iron and zinc absorption. There is a need to deepen knowledge on the bioavailability of iron and zinc from ALVs and the magnitude of effects of antinutrients on iron and zinc bioavailability mostly when ALVs are consumed concurrently with cereal staples. The findings of the current *in vitro* studies can only be used to predict the direction of iron and zinc bioavailability from processed ALVs but not

the actual amount of mineral available for absorption, as *in vitro* models cannot simulate all *in vivo* conditions.

Keywords: African leafy vegetables (ALVs), iron, zinc, antinutrients, bioaccessibility, bioavailability, spontaneous fermentation, blanching

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LIST OF ABBREVIATIONS

AA	Ascorbic acid
AAS	Atomic spectrometry
ALVs	African Leafy Vegetables
AR	Absolute Requirements
ATCC	American Type Culture Collection
BCA	Bicinchoninic acid assay
CA	Citric Acid
Caco-2	Colorectal adenocarcinoma
CAF	Central Analytical Facilities
CAS	Central Analytical Facilities
DALYS	Disability adjusted life years
DCYTB	Duodenal cytochrome B
DMT1	Divalent metal cation transporter 1
DNA	Deoxyribonucleic acid
EBSS	Earle's Balanced Salts Solution
ELISA	Enzyme-linked immunoassay
FAO	Food Agriculture Organisation
FBS	Foetal Bovine serum
FE	Ferritin
Fe	Iron
Fe ²⁺	Ferrous

Fe ³⁺	Ferric
FPN	Ferroportin
GI	Gastrointestinal Tract
GBD	Global burden disease study
HCP1	Haem carrier protein 1
HERPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	High performance liquid chromatography
HRE	Haemoglobin repletion efficiency
HREC	Human Research Ethics Committee
HRG-1	Haem responsive gene-1
ICP-MS	Inductively Coupled Plasma Mass Spectrometry
ICP-OES	Inductively Coupled Plasma Optical Emission Spectroscopy
IDA	Iron Deficiency Anaemia
IP3	Inositol tetrphosphate
IP4	Inositol tetrphosphate
IP5	Inositol pentaphosphate
IP6	Inositol hexaphosphate
IQR	Interquartile Range
LAB	Lactic acid bacteria
MEM	Minimum Essential medium
MMP	Multiple Micronutrient Powder
MNDs	Micronutrient deficiencies
MS	Mass spectrometry

MT	Metallothionein
MWCO	Molecular weight cut off
NCDs	Non-Communicable Diseases
PCA	Principal Component Analysis
PCBP2	Poly C binding protein 2
Phy*Ca : Zn	Phytate * Calcium : Zinc
Phy : Fe	Phytate : Iron
Phy : Zn	Phytate : Zinc
PPO	Polyphenol oxidase
RDA	Recommended Dietary Allowances
SANAS	South African National Accreditation System
SDS	Sodium dodecyl sulphate
TPC	Total phenolic content
UNICEF	United Nations Children's Fund
WBA	Women of Childbearing Age
WHO	World Health Organisation
ZIPs	Zinc related proteins
Zn	Zinc
ZnO	Zinc oxide
ZnSO ₄	Zinc sulphate
ZNT	Zinc Transporter

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CHAPTER 1: INTRODUCTION

1.1 Background

Iron and zinc are the two most critical minerals deficient globally, with about 2 billion of the world population being iron deficient and 17.3% of the global population estimated to have inadequate zinc intake with the highest estimates found in Asia (19.4%) and Africa (23.9%) (Bailey *et al.*, 2015). Pregnant women and children under five are at risk of zinc deficiency, and these primarily causes morbidities in developing countries (De Benoist *et al.*, 2007). The highest prevalence of iron deficiency found in Africa is in children (67.6%) and pregnant women (57.1%) (Lopez *et al.*, 2016). Iron and zinc deficiencies in sub-Saharan Africa result from inadequate dietary intake of animal sources of iron and zinc, especially for households relying mainly on cereal staples (Fanzo, 2012). Plants-based diets contain non-haem iron which has low bioavailability (López *et al.*, 2004). The bioavailability of zinc and iron from plant-based diets are additionally inhibited by dietary components (Gibson *et al.*, 2010; Gibson *et al.*, 2018). Food insecurity and shifting of dietary patterns contribute to malnutrition in sub-Saharan Africa (Damman *et al.*, 2008; Frison *et al.*, 2006). The global nutrition trends contribute not only to micronutrient deficiencies but also to risk factors for diet-related diseases such as diabetes, obesity and cardiovascular diseases (Damman *et al.*, 2008). Food-based strategies have been deemed a priority in meeting mineral requirements and reducing the incidence of chronic diseases (Lartey, 2008). Dietary diversification has been reported to be crucial in increasing mineral intakes and reducing deficiencies (Boy *et al.*, 2009). Diet diversification could be encouraged by the use of locally available foods that could be rich sources of iron and zinc. However, knowledge regarding foods that are part of the traditional food system is crucial (Frison *et al.*, 2006).

African leafy vegetables (ALVs), for example, cowpea (*Vigna unguiculata*), amaranth (*Amaranthus hybridus*), spider plant (*Cleome gynandra*) and wild jute (*Corchorus olitorius*), have been a part of food systems in many African countries for many generations. ALVs play a significant role in food security for rural people in sub-Saharan Africa who have limited food sources (Uusiku *et al.*, 2010). In most cases, cooked ALVs are eaten as a relish, accompanying staples such as maize, millet or sorghum porridges (Bvenura & Afolayan, 2015; Kruger *et al.*, 2015). Various studies found that ALVs contains protein, fibre, ascorbic acid (AA), magnesium, iron, zinc and calcium (Ndlovu & Afolayan, 2008; Schönfeldt & Pretorius, 2011; van Jaarsveld *et al.*, 2014). Previous studies reported higher levels of calcium, iron and zinc in ALVs than in exotic vegetables (Ndlovu & Afolayan, 2008; Orech *et al.*, 2007). In these studies, ALVs were compared

to cabbage (*Brassica oleracea* var. *capitata*), kale (*Brassica oleracea* var. *acephala*) and spinach (*Spinacia oleracea*).

ALVs have the potential to remedy food insecurity issues in sub-Saharan Africa; however, these green leafy vegetables are seasonal and not available throughout the year (Dweba & Mearns, 2011; Hart, 2011). Surveys by Matenge *et al.* (2012) and Keller *et al.* (2005) reported that the consumption of ALVs in urban areas was low due to consumers' lack of knowledge and ALVs not readily accessible in the local markets. To encourage the consumption of ALVs when out of season, traditionally ALVs are blanched and fermented in combination with drying- and stored and later reconstituted and used when out of season (Wafula *et al.*, 2016; Hart, 2011; Vorster *et al.*, 2009). However, these methods have some limitations, such as that drying in direct sunlight has been associated with contamination and vitamins and mineral losses (Dweba & Mearns, 2011; Muchoki *et al.*, 2007; Thandeka *et al.*, 2011). Nevertheless, freshly spontaneously fermented and blanched ALVs are also commonly consumed (Masarirambi *et al.*, 2010; van der Hoeven *et al.*, 2013). Although ALVs have high levels of essential minerals, they also contain high amounts of antinutrients (Yang & Keding, 2009), which inhibit their iron and zinc bioavailability. Several studies conducted in sub-Saharan Africa ascertained that ALVs contain a substantial amount of essential minerals, especially iron and zinc (Faber *et al.*, 2010; Kruger *et al.*, 2015; Orech *et al.*, 2007; Uusiku *et al.*, 2010; van Jaarsveld *et al.*, 2014). However, a challenge that undermines the potential of ALVs is the presence of high levels of antinutritional factors (Kruger *et al.*, 2015). Amaranth, cowpea and spider plant contain high levels of phytate, condensed tannins and total phenolic contents (Kruger *et al.*, 2018; Kruger *et al.*, 2015). These antinutrients reduce protein digestibility and hinder essential minerals (iron and zinc) absorption through the formation of insoluble complexes significantly when present in high levels (Gibson *et al.*, 2010), reducing the bioavailability of these essential minerals. Phytate binds with minerals to form stable insoluble complexes with them, especially iron and zinc, making them unabsorbable (Kumar *et al.*, 2010b). Zinc bioavailability is also affected by the combined the inhibitory effect of phytate and calcium (Lönnerdal, 2000). Bioavailability of iron and zinc from plant based diets can be predicted using phytate-mineral ratios (Gibson *et al.*, 2010). Phytate : iron molar ratios (Phy : Fe) values below the critical value of 1 predict iron that is favourably bioavailable, and molar ratios above the critical value of 1 predict severely impaired iron bioavailability (Hurrell, 2004). Phytate : zinc (Phy : Zn) molar ratios above the critical value of 15 predicts zinc bioavailability which is severely impaired (FAO & WHO, 2005). Therefore, plant-based diets with high levels of phytates

have low iron and zinc bioavailability, which could result in essential mineral deficiencies if no other sources of iron and zinc consumed.

Evidence suggests that processing techniques such as fermentation and blanching reduce antinutrients in ALVs (Akello, 2014; Elisha *et al.*, 2016; Fadupin *et al.*, 2014; Wafula *et al.*, 2016). Akello (2014) reported that phytates in cowpea, spider plant and pumpkin (*Cucurbita moschata*) were reduced by 19%, 31% and 41% respectively after spontaneous fermentation. Other authors also reported a significant decrease in oxalates, polyphenol and tannin content after fermentation of cowpea leaves (Ifesan *et al.*, 2014; Muchoki *et al.*, 2010; Oulai *et al.*, 2015; Waluchio, 2016). During blanching, cell membranes rupture and some soluble compounds leach out into the blanching liquid (Natesh *et al.*, 2017), some antinutritional components leaches out into the blanching medium, resulting in reductions in levels. Previous studies also reported that blanching was effective in reducing levels of phytates, polyphenols, tannins, and oxalates in ALVs (Essack *et al.*, 2017; Fadupin *et al.*, 2014; Oboh, 2005). It should be noted that both blanching and fermentation were found to cause mineral losses during ALVs processing (Mepba *et al.*, 2007; Ortiz *et al.*, 2017), however, these processes could influence the bioavailability of essential minerals (iron and zinc) due to reduction of antinutrients that have some inhibitory effects on their absorption. In this regard, there is a need to evaluate mineral and antinutrient contents of differently processed ALVs (cooked, blanched-dried and spontaneously fermented-dried) and also the bioaccessibility and bioavailability of iron and zinc from these processed ALVs.

1.2 Problem statement / Rationale of the study

There is limited research data available on the mineral and antinutrient contents of variously processed ALVs; information available deals with mostly raw ALVs (Uusiku *et al.*, 2010; van Jaarsveld *et al.*, 2014). This research will evaluate minerals, antinutrients content and iron and zinc bioaccessibility of cooked, spontaneously fermented-dried and blanched-dried ALVs. ALVs are seasonal, and it makes their use throughout the season/year problematic, which results in post-harvest losses and a decline in consumption when out of season (Matenge *et al.*, 2012; Wafula *et al.*, 2016). In this study, ALVs were cooked as they are typically cooked and consumed, blanched-dried, and spontaneously fermented-dried as commonly preserved to be used when out of season. However, this study did not assess the effect of preservation on ALVs but rather on how ALVs are commonly processed to be used later when out of season. The ALVs were cooked

simulating how they are usually prepared in households when in season (freshly cooked) and out of season (preservation type of method) as depicted by the experimental design (**Figure 1-1**). As stated, blanching and fermentation have the potential to reduce antinutrients levels in ALVs. However, still these methods also result in the reduction of mineral content hence the need to assess the mineral content of variously processed ALVs. Previous studies reported that cooked ALVs are good sources of iron and zinc but there is limited information on the mineral value of spontaneously fermented-dried and blanched-dried ALVs. (Kruger *et al.*, 2018; Kruger *et al.*, 2015; Schönfeldt & Pretorius, 2011; van Jaarsveld *et al.*, 2014). There is a need for sustainable production of ALVs for commercialization to augment the use of exotic green leafy vegetables. Compositional data from this study can be used to guide researchers and farmers when advocating for commercialisation of non-commercialized to penetrate the markets to improve nutrition security. As stated above, inadequate dietary intake of animal sources of causes iron and zinc deficiencies in sub-Saharan Africa especially for poor households as diets are mainly cereal staples (Fanzo, 2012). Variously processed ALVs with reduced antinutrients possibly could have enhanced iron and zinc bioavailability. To the best of my knowledge, no research has been published before on the bioaccessibility of iron and zinc from variously processed ALVs and predicted iron and zinc bioavailability from variously traditionally processed ALVs using Caco-2 cell assay. Evidence suggests that ALVs are commonly consumed in sub-Saharan Africa especially by rural communities (Uusiku *et al.*, 2010), so data generated by this study will benefit a broad audience when policymakers are advocating for the use of ALVs in nutritional strategies, especially in planning food security programmes for vulnerable groups. Data generated by this study on ALVs could be used in sub-Saharan Africa to encourage nutritional diversification to increase mineral intake and food security

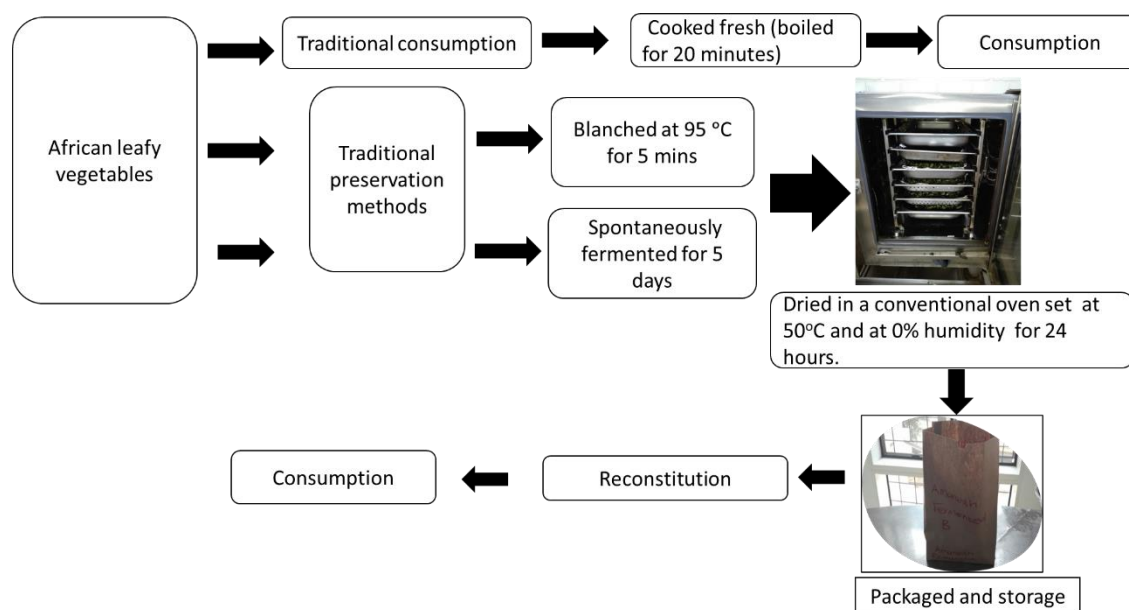


Figure 1-1: Experimental design illustrating traditional processing methods of African leafy vegetables

1.3 Aim

The study aimed to compare mineral and antinutrients content of cooked ALVs to blanched-dried, and spontaneously fermented-dried ALVs and with the overall aim to evaluate the bioaccessibility of iron and zinc from these processed ALVs and to predict their iron and zinc bioavailability using Caco-2 cell assay. This study also compared the mineral contents of four uncommercialised ALVs to Ethiopian kale which is commercialised. These processed ALVs could be used to address essential mineral deficiencies for consumers with limited access to animal food sources of iron (haem) and zinc. Though this study dried the vegetables after blanching and spontaneous fermentation as they would be for safe storage and use when out of season, this study did not evaluate ALVs preservation.

1.4 Specific objectives

The objectives of this study are:

- To compare the mineral and antinutrient content of cooked, blanched-dried, and spontaneously fermented-dried ALVs.
- To compare the bioaccessibility of iron and zinc in cooked, blanched-dried, and spontaneously fermented-dried ALVs, using an *in vitro* dialysability assay.
- To compare the estimated bioavailability of iron and zinc from cooked, blanched-dried, and spontaneously fermented-dried ALVs using an *in vitro* Caco-2 cell culture assay.

1.5 Study hypotheses

Hypothesis 1

ALVs blanched at the maximum blanching time of five minutes will have similar mineral contents as their cooked counterparts.

Blanching is conducted to inactivate quality changing enzymes that cause deterioration reactions that result in undesirable colour, texture, odour and off-flavours in vegetables (Huang *et al.*, 2015). Blanching is associated with leaching out of minerals from the plant material into the blanching medium which is discarded after processing (Reis, 2017). Still, the shortest adequate blanching time of maximum time of 5 minutes is associated with negligible mineral losses (Acho *et al.*, 2015). Cooked ALVs are consumed together with the cooking liquid and minerals that leach out into the cooking liquid is consumed, henceforth their mineral contents will be like that of ALVs blanched at the maximum blanching time of 5 minutes.

Hypothesis 2

Spontaneously fermented-dried ALVs will have substantially lower mineral contents compared to their cooked counterparts.

During fermentation, the lactic acid bacteria (LAB) hydrolyse fibre and loosen the food matrix resulting in increased amounts of minerals leaching into the sugar-salt solution used for fermentation (Liang *et al.*, 2008), and the fermentable mediums are discarded before the drying

process contributing to minerals losses through leaching (Swain *et al.*, 2014). Due to leaching out of minerals into the discarded fermentable mediums, fermented ALVs will have lower mineral contents compared to their cooked counterparts in which the cooking liquid is not discarded.

Hypothesis 3

Blanched-dried ALVs will have similar levels of condensed tannins, phytates, total phenolic content and total oxalates as their cooked counterparts.

As stated, ALVs will be subjected to blanching at the shortest adequate blanching time of 5 minutes associated with minor mineral losses. The degree of reduction of antinutrients in ALVs during blanching depends on the duration of exposure to blanching medium, temperature and the type of the vegetable (Essack *et al.*, 2017). Blanching at the duration between 10 to 15 minutes has been reported to be effective in reducing levels of oxalates in vegetables (Obboh, 2005; Yadav & Sehgal, 2003). During blanching, the decrease in levels of soluble oxalates, phenolic compounds and phytates is associated with cell wall rupturing causing antinutrients to leach out into the blanching medium (Natesh *et al.*, 2017; Reis, 2017; Obboh & Rocha, 2007). However, it is assumed that blanching ALVs at the maximum blanching time of 5 minutes will result in insignificant amounts of antinutrient losses which will be similar to that in cooked ALVs because the cooking liquid is not discarded hence leached out minerals into the cooking liquid is not lost.

Hypothesis 4

Spontaneously fermented-dried ALVs will have substantially lower levels of condensed tannins, total phenolic content, total oxalates and phytates than their cooked counterparts.

Spontaneous fermentation effectively reduces levels of phytates, oxalates, condensed tannins and total phenolic contents from ALVs (Essack *et al.*, 2017; Muchoki *et al.*, 2010; Wafula *et al.*, 2016). During fermentation, LAB releases phytase, which degrades phytate, thus reducing its levels in the food (Gupta *et al.*, 2015). At pH lower than 6, LAB degrades oxalates for carbon and energy (Simpson *et al.*, 2009), attributing to its reduction. During fermentation, the reduction of total phenolic contents is attributed to the activation of polyphenol oxidase (PPO) which is responsible for polyphenol hydrolysis (Duodu, 2011). Fermentation also decreases condensed tannins due to the production of enzymes which degrade complex tannins to release free tannins which are lost through leaching out into the discarded fermentable medium (Taylor & Duodu,

2015), therefore, fermented ALVs will have lower antinutrients compared to their cooked counterparts.

Hypothesis 5

Blanched-dried ALVs will have similar estimated amounts of iron and zinc availability than their cooked counterparts.

As stated in hypothesis 3, a negligible amount of antinutrients will be lost from blanched-dried ALVs due to shortened blanching time. Consequently, it is assumed that both cooked and blanched-dried ALVs will have similar levels of antinutrients. Oxalates bind iron and zinc to form insoluble salts (Penniston, 2014), which makes these essential minerals unavailable for absorption. Besides, condensed tannins and total phenolic contents (especially flavonoids) form insoluble complexes with iron and zinc rendering them unabsorbable (Friman *et al.*, 2004; Lesjak & Srai, 2019; Towo *et al.*, 2006). Moreover, condensed tannins are associated with the formation of insoluble complexes with protein inhibiting digestive enzymes activation which are responsible for breaking down of food matrix to release minerals in the lumen (Shahidi & Ambigaipalan, 2015), and these undesirably affect iron and zinc bioavailability. Therefore, similar levels of antinutrients in both blanched-dried and cooked ALVs will have similar effects on iron and zinc availabilities.

Hypothesis 6

Spontaneously fermented-dried ALVs will have substantially have higher estimated iron and zinc bioavailability compared to their cooked counterparts.

Household processing methods such as fermentation are effective in reducing phytates, oxalates, condensed tannins and total phenolic contents from ALVs (Essack *et al.*, 2017; Muchoki *et al.*, 2010; Wafula *et al.*, 2016). As stated, these antinutrients have some inhibitory effects on iron and zinc bioavailability. Phytates binds to iron and zinc and form insoluble phytate-mineral complexes which decrease their absorption (Kumar *et al.*, 2010b; Lopez *et al.*, 2002), but during spontaneous fermentation, phytate is degraded by activation of the phytase enzyme which occurs in acidic pH conditions ranging from 4.5 to 5.5 (Sanz-Penella *et al.*, 2012). The reduction of phytate is concomitant to the improvement of mineral solubility and uptake. Also, polyphenols and condensed tannins are reduced during fermentation (Taylor & Duodu, 2015; Duodu, 2011), these consequently reduce the formation of insoluble complexes formed between these antinutrients

and essential minerals rendering them unabsorbable. Therefore, reduction of antinutrients during fermentation reduces their inhibitory effects on iron and zinc bioavailability.

1.6 Research design

The study is in three phases, which are elaborated further on in the methodology section. Four ALVs were selected based on their current consumption as reported by previous studies conducted in sub-Saharan Africa (Faber *et al.*, 2010; Flyman & Afolayan, 2006b; Maroyi, 2011; Masarirambi *et al.*, 2010; Smith & Eyzaguirre, 2007; Steyn *et al.*, 2001; Uusiku *et al.*, 2010; Vorster *et al.*, 2009). These include cowpea leaves (*Vigna unguiculata*), wild jute (*Corchorus olitorius*), spider plant (*Cleome gynandra*) and amaranth (*Amaranthus hybridus*). Ethiopian mustard (*Brassica carinata* A. Baun), which is already commercialised in various countries such as Botswana, Kenya and Ethiopia (Bosekeng, 2019; Kebede & Bokelmann, 2017; Odongo *et al.*, 2017), was chosen as a control (commercialised ALV). The commercialised vegetables reported to have originated from Ethiopia, and its other common names are Abyssinian cabbage, Ethiopian kale or African cabbage (Bosekeng, 2019; Odongo *et al.*, 2017). ALVs were bought from a local farmer in Kgatleng district in Botswana, and the commercialised ALV was purchased from the local market in Kanye, Botswana. All ALVs were processed at the National Food Technology Research Centre in Botswana, via three traditional processes, namely cooked, blanched-dried, and spontaneously fermented-dried. The first phase involved the analysis of minerals and antinutrients content of processed ALVs. The second phase conducted the *in vitro* dialysability assay on iron and zinc from variously processed ALVs. The third phase which is the last stage, predicted the bioavailability of iron and zinc from variously processed ALVs using an *in vitro* / Caco-2 cell culture model assay, choosing ALVs which had better iron and zinc bioaccessibility, as evaluated in the second phase.

1.7 The research team and their contribution to this PhD thesis

Table 1-1 shows the persons involved in this research and the roles they played in this study.

Table 1-1: Research team and their contribution

Team member	Role and responsibility
Prof Marius Smuts	The promoter of the PhD student. Designed and conceptualized the study. Supervised the overall running of the study and provided technical direction on the interpretation of the results and overall write-up of this thesis.
Dr Johanita Kruger	Co-promoter of the student. Provided guidance and technical direction on all laboratory work concerning nutrient and anti-nutrient content of ALVs and <i>in vitro</i> dialysis analysis.
Dr Angelique Lewies	Co-promoter of the student. Provided guidance and technical direction on all laboratory work concerning cell work, guided the student in data analysis and result interpretation. Provided technical help in the write-up of the thesis
Prof. Jeannine Baumgartner	The assistant promoter of the student. Technically guided the student in the writing of the thesis.
Tiyapo Mongwaketse	PhD student, participated in the design and conceptualizing of the study, processed samples, laboratory analysed samples, conducted <i>in vitro</i> dialysis method and Caco-2 cell models. Analysed and interpreted data. Wrote and edited the thesis.

1.8 Thesis outline

The thesis has five chapters, and the references used in each chapter are provided at the end of the thesis and referenced using the North-West University Harvard style of referencing style. The first chapter outlines the problem statement and the motivation of the study. The chapter also looked at the gaps in research concerning ALVs, their nutrient and antinutrient contents and how these influences the bioaccessibility and bioavailability of iron and zinc. The chapter also outlines the aim and the objectives of the research including the hypotheses.

Chapter two reviews the literature on the nutritional situation at a globally, regionally and country-specific levels. In the case of country level, the iron and zinc nutrition situation were based on Botswana situation as one of the countries in sub-Saharan Africa utilising the ALVs studied by

the current study. The chapter further reviews iron and zinc deficiencies, associations between iron and zinc deficiencies, factors that affects iron and zinc status in the body, approaches at country level, that could be adopted to prevent and alleviate iron and zinc deficiencies. Besides, the chapter reviewed the literature on ALVs, their mineral and antinutrients content, and evaluated processing techniques to reduce iron and zinc inhibitors from ALVs. Also, the chapter evaluated dietary components that inhibit iron and zinc bioavailability and also enhancers of iron and zinc absorption. Lastly, *in vitro* assays of assessing iron and zinc bioaccessibility and bioavailability including their limitations are described in this chapter.

Chapter three describes the study desi and gives information on methods used for sample collection, processing methods of ALVs, the laboratory methods used for the analysis of the nutrient and antinutrient contents of ALVs, as well as methods used in the assessment of the bioaccessibility and predicted bioavailability of iron and zinc from ALVs. The chapter further describes the statistical methods performed to analyse the data. Chapter four describes and summarises data obtained from the study, discusses, and compare the results of the current finding to previous studies. Chapter five summarises the findings of the study and explains how these findings may contribute to iron and zinc nutrition research and its implications in policy formulation. The chapter first evaluates ALV processing techniques and some methodological considerations for future studies. Both the study limitations and strengths are reviewed and discussed. The chapter also deals with the significant findings of the study. In addition, recommendations for future research are made in this chapter. This thesis also has some additional information attached after the reference list as addendums. A manuscript under review with the Journal of Food Science and Technology is included as an addendum 1, a poster presented at the 2018 Nutrition Congress based on this research is also included as addendum 2, ethics exemption letter as addendum 3 and language editing certificate as addendum 4 respectively.

CHAPTER 2: LITERATURE REVIEW

2.1 Overview

This chapter reviews the literature on the nutrition situation globally, regionally and in Botswana as one country in sub-Saharan Africa in which African Leafy Vegetables (ALVs) evaluated are commonly consumed. Also, the ALVs used in this study were harvested in Botswana. The chapter further reviewed iron and zinc deficiencies, associations between iron and zinc deficiencies, factors that affect iron and zinc status in the body as well as approaches at country level, that could be adopted to prevent and control iron and zinc deficiencies. In addition, the chapter reviewed the literature on ALVs, their mineral and antinutrients content, and evaluated processing techniques to reduce iron and zinc inhibitors from ALVs. Further, the chapter evaluated and discussed the dietary components of ALVs that inhibit and enhance iron and zinc absorption. Lastly, *in vitro* assays of assessing iron and zinc bioaccessibility and bioavailability including their limitations are described in this chapter.

2.2 The global nutrition situation

According to the 2017 global burden of diseases study (GBD), 11 million deaths were linked to poor diets and half of all the diet related diseases were associated with diets high in sodium, low in fruits and whole grains (Afshin et al., 2019). The 2019 GBD reported that high fasting plasma glucose, high blood pressure and high body mass index were among the fifteen leading causes of global attributable disability adjusted life years (DALYS) (Vos et al., 2020). There is inadequate intake of micronutrients such as zinc, iron, calcium and vitamin A globally (Beal et al., 2017). In addition, 140 million children globally are still stunted though the prevalence of stunting among children aged five and below is decreasing, on the other hand, 7.3% of children are wasted (FAO/IUWA, 2019). There has been a rise in overweight and obesity globally contributing to 4 million deaths per annum (FAO/IUWA, 2019). However, the WHO Global Action Plan for control and prevention of non-communicable diseases (NCDs) 2013-2020 outlines a policy that promotes healthy diets and physical activity (CEPAL, 2020). However, a double burden of malnutrition (both under-nutrition and over-nutrition) exists globally (FAO/IUWA, 2019). The prevalence of both under-nutrition and overweight could exist in the same individual, household and community (WHO, 2016). The primary causes of malnutrition in developing countries have been associated with food insecurity (Manap & Ismail, 2013). Household food insecurity results in monotonous diets based mainly on grains, roots, and tubers which are inadequate in minerals (Kennedy,

2009). In addition, there have been trends of diets shifting towards an increased reliance on processed foods, high in salt, fat, sugar, low in complex carbohydrates in developing countries (Popkin *et al.*, 2012; Walls *et al.*, 2018), and most of these diets are less nutritious and linked to a high prevalence of mineral and vitamin deficiencies. The global nutrition trends depict a high prevalence of risk factors for diet-related diseases such as diabetes, obesity and cardiovascular diseases (Damman *et al.*, 2008), so control measures should be adopted to alleviate the situation. Some authors recommended that food-based strategies such as dietary diversification should be prioritised in meeting micronutrient requirements and reducing the incidences of chronic disease (Allen, 2008; Boy *et al.*, 2009), as this will encourage the consumption of local foods. As stated above, iron and zinc deficiencies are major problems globally. About 2 billion of the world population are iron deficient, and it is also estimated that 17.3% of the global population have inadequate intake of zinc with the highest estimates found in Asia (19.4%) and Africa (23.9%) (Bailey *et al.*, 2015). Pregnant women and children under five are at risk of zinc deficiency, and these primarily cause morbidity in developing countries (De Benoist *et al.*, 2007).

2.2.1 Nutrition situation in sub-Saharan Africa

Most of the nutritional problems reported above found globally are more prevalent in sub-Saharan Africa. In the 21st century, sub-Saharan Africa has faced two fundamental development challenges; poverty and rapid development (Frayne *et al.*, 2014), with almost half of the children living in extreme poverty (UNICEF, 2015). It is projected that by 2030, nine out of ten of the world's children living in extreme poverty would be living in sub-Saharan Africa (UNICEF, 2016). The region needs some attention in improving the nutritional situation. The global hunger index report state that the region has the highest level of food insecurity (von Grebmer *et al.*, 2016). One-third of children are stunted in sub-Saharan Africa in 2016, representing the world's highest rate of stunting among children (World Bank, 2016). About 17.8% of children in sub-Saharan Africa are underweight, whereas 35.5% are facing stunting, and 8.2% are wasted (UNICEF, 2016).

The main contributors of malnutrition in sub-Saharan Africa are micronutrient deficiencies, food insecurity, and the shifting of dietary patterns. Sub-Saharan Africa has the highest rates of anaemia (46%-71%) and Vitamin A deficiency (48%) prevalence in children less than five years of age (Stevens *et al.*, 2016; Stevens *et al.*, 2013). The highest prevalence of iron deficiency found in sub-Saharan Africa is in children (67.6%) and pregnant women (57.1%)(Lopez *et al.*, 2016). In addition, the prevalence of iodine deficiency is at 42%, and estimated inadequate zinc nutrition (25%) is also high in sub-Saharan Africa (Andersson *et al.*, 2005; Muthayya *et al.*, 2013). Due to

poverty, iron and zinc deficiencies in most communities in sub-Saharan Africa are caused by inadequate dietary intake of animal sources of iron and zinc as diets are mainly cereal staples (Fanzo, 2012). Predictably, NCDs will be the leading cause of death in sub-Saharan Africa by 2030 (Naik & Kaneda, 2015), because there is enough evidence that most of the countries in the region are undergoing through a nutrition transition where obesity is emerging as a critical problem, while under-nutrition remains a significant burden (Bosu, 2015; Kandala & Emina, 2016; Steyn & Mchiza, 2014). The transition is also evident through the increase in the prevalence of obesity in school-going children and pregnant women in the region (Muthuri *et al.*, 2014; Onubi *et al.*, 2016). The situation is worsened by urban migration which led to a decline in subsistence farming, which previously influenced the production of staple foods and use of indigenous foods to diversify diets (Damman *et al.*, 2008; Frison *et al.*, 2006).

2.2.1.1 Botswana nutritional situation

Botswana, like any other developing country in sub-Saharan Africa, is currently facing changes in eating patterns and food preferences. This changes in eating patterns result in the high demand for foods high in carbohydrates, such as white rice and pasta and other processed foods, instead of indigenous foods (Clausen *et al.*, 2005; Maruapula & Chapman-Novakofski, 2007). There is a lack of data on the nutritional composition of Botswana's indigenous foods. Lack of knowledge on the composition of indigenous food resources has resulted in less use and appreciation of available indigenous foods (Uusiku *et al.*, 2010).

Botswana is facing a double burden of malnutrition, i.e., the occurrence of both obesity and under-nutrition due to overconsumption of foods high in simple carbohydrates, fat, and salt but low in vital micronutrients and fibre, and micronutrient deficiencies. In a study conducted by Mahgoub *et al.* (2006) the levels of wasting, stunting, and underweight in children under the age of three years were reported to be 5.5%, 38.7% and 15.6% respectively. Also, the Botswana Family Health Survey of 2007 reported that 13% of children under five years of age were underweight, 26% stunted, and 7.2% wasted (Central Statistics Organisation, 2009). In a study conducted in Botswana to estimate the prevalence and determinants of under-nutrition in adults, about 20% of males and 10% of the sample population are underweight (Letamo & Navaneetham, 2014). However, obesity is also prevalent in the population. In a study conducted in 2007, more women (23%) were overweight compared with men (13%) and that 15% of women were obese compared with 3% of men (Letamo, 2011). The prevalence of overweight and obesity in adults is on the rise; a high prevalence of risk factors for diet-related diseases, i.e. overweight and obesity (36.8%),

particularly in women (50%), was reported in Botswana (Kwape, 2012). Micronutrient deficiencies are also a challenge in Botswana; however, there is no recent research conducted on the micronutrient status of both adults and children in Botswana. Nevertheless, the 1994 micronutrient survey reported that about 38% of children surveyed had anaemia, and 35% had marginal vitamin A status (Aplogan *et al.*, 1996). There is a need for up-to-date data on the zinc status in Botswana.

About 30% of Botswana are living below the poverty datum line (Botswana Statistics, 2011). The incidence of poverty is increasing in rural areas (Sekwati *et al.*, 2013). A study reported that about 38% of Botswana were food insecure, and issues of food insecurity were also in the rise in urban areas (MuGuire, 2015). In a study to estimate the dimensions of urban food insecurity in a city, 62% of households were severely food insecure (Acquah *et al.*, 2014). Food security alleviation in both rural and urban areas can improve by diversifying diets (Legwaila *et al.*, 2011). In a study conducted in adults, there was a low variety of fruits, vegetables, and dairy consumed, respectively (Clausen *et al.*, 2005). There is enough evidence on the utilisation of ALVs in diets in Botswana and their contribution to mineral intakes (Flyman & Afolayan, 2006b; Kasimba *et al.*, 2019). Hence, there is a need to encourage communities to consume African leafy vegetables (ALVs) and fruits to diversify their diets. Green leafy vegetables are the most common traditional food plants capable of providing a crucial source of food for people (Legwaila *et al.*, 2011). Consumption of traditional African diets curb incidences of chronic diseases (Walker, 1995), and promotes the use of local foods, knowledge of the foods that are part of the traditional food system is vital (Frison *et al.*, 2006). However, in Botswana, there is minimal documentation on the nutritional composition of local food material available. Data that is available is at best equivocal; therefore, there is a need for more evidence on the composition of indigenous foods consumed in Botswana. More information on indigenous foods that are usually consumed by the population of Botswana will make it easier to include indigenous foods in nutrition programmes aimed at addressing essential mineral deficiencies.

2.3 Iron deficiency and its effects on functions in the human body

There is enough evidence that iron deficiency is a worldwide crisis, with over 2 billion of the world population iron deficient (Bailey *et al.*, 2015), and with a quarter of the world population anaemic (Pasricha *et al.*, 2013). The World Health Organization (WHO) stated that about 42% of pregnant women, 30% of non-pregnant women (aged 15 to 50 years) and 47% of preschool children are anaemic (McLean *et al.*, 2009), this shows that vulnerable groups are at risk of iron deficiency. However, iron deficiency is widespread, with 1.2 billion suffering from iron-deficiency anaemia

(IDA) (World Health Organization, 2008). Inadequate iron (Fe) intake and absorption, increase in requirements during growth, and excessive losses of iron may result in iron deficiency (Pasricha *et al.*, 2013). Women of childbearing age (WBA) and preschool children are at risk of IDA (Black *et al.*, 2013; Pasricha *et al.*, 2013). WBA have higher iron requirements due to loss of iron during menstruation and require more iron during pregnancy and childbirth (Bothwell, 2000) while preschool children need more iron for a rapid expansion of red cell mass during child growth (Zimmermann & Hurrell, 2007). Iron deficiencies in children affect the cognitive function and result in reduced growth and development, leading to poor memory, reduced intelligence, and poor school performance in children (Carter *et al.*, 2010; Ignacio, 2014). The severity and duration of iron deficiency determine whether the cognitive effects will be reversible (Zimmermann & Hurrell, 2007). Previous studies associated iron deficiency to impaired immunity (Ekiz *et al.*, 2005; Jonker & van Hensbroek, 2014), and often resulted in increased childhood mortality and morbidity. During pregnancy, iron deficiency increases the risk of premature delivery and low birth weight (McArdle *et al.*, 2014). In a study conducted in rats, IDA during pregnancy resulted in a higher mortality rate and underweight pups (Gambling *et al.*, 2003). The same study found that IDA during pregnancy increased the risk of perinatal mortality and morbidity and may increase the risk of disease later in life. Considering the adverse effects of IDA, it is crucial to prevent and control iron deficiencies by increasing dietary iron intake.

2.3.1 Zinc deficiency and functions in the human body

Based on the analysis of the National Food Balance Sheets, approximately 17% of the world population is zinc (Zn) deficient (Wessells & Brown, 2012). However, there is less information available on the prevalence of zinc deficiency because of non-specific features of zinc deficiency and lack of sensitive biomarkers to detect zinc deficiency (Krebs, 2013). Previous surveys evaluated zinc deficiency in children using zinc protoporphyrin concentration of $>70 \mu\text{mol/mol}$ haem in washed erythrocytes (Baumgartner *et al.*, 2012; van der Hoeven *et al.*, 2016). As with iron deficiency, Southeast Asia and sub-Saharan Africa are also at higher risk of zinc deficiency. About a third of the population has inadequate zinc intake, and stunting rate for schoolchildren is at 40% (Brown *et al.*, 2004; Wessells & Brown, 2012). Diets in developing countries are predominantly plant-based, and the intake of animal source food is low (Gibson *et al.*, 2010). Plant-based diets contain low amounts of zinc and high amounts of inhibitors of zinc that affects its availability (Gibson *et al.*, 2018). Complementary cereal-based foods are mostly zinc deficient (Dewey & Adu-Afaruwah, 2008), and few global policies for zinc interventions available are advocating for the use of zinc supplements and strongly recommending fortification of

complementary foods (Shrimpton *et al.*, 2005). However, zinc bioavailable from food and supplements cannot be stored in the body for future use, except in infants, hence there is a need for adequate dietary zinc supply (King *et al.*, 2016). Breastfed infants are at highest risk of zinc deficiency after six months of breastfeeding when the zinc intake from breast milk becomes inadequate to meet their requirements (Brown *et al.*, 2009). Animal based sources of zinc should be advocated for as complementary foods to increase zinc intake. Consumption of meat has been associated with improved zinc intake in infants (Krebs *et al.*, 2006).

In addition, zinc plays a significant role in DNA transcription and gene expression (King *et al.*, 2016), which is essential for child growth and development. Krebs (2013) associated zinc-responsive growth faltering in infants and toddlers with low dietary intake of zinc. Zinc deficiency negatively affects the immune system and results in thymic atrophy, lymphopenia and compromised cell and antibody-mediated responses that lead to increased rates and periods of infections (Fraker & King, 2004). An increase in infections may be an early sign of zinc deficiency (King *et al.*, 2016). Over 50% of child mortality and morbidity rates due to diarrhoea are associated with poor zinc nutrition (Raiten *et al.*, 2015). Positive evidence exists that zinc supplementation reversed impaired immune functions and reduced diarrhoea and pneumonia mortality by 13% and 15%, respectively (Ibs & Rink, 2003; Yakoob *et al.*, 2011). Besides, a review concluded that therapeutic use of zinc for the treatment of zinc deficiency in children reduced diarrhoea, the number of stools passed, and diarrhoea duration as well as respiratory infection (Liberato *et al.*, 2014). To back up the evidence of the positive effect of zinc supplementation in reducing the duration of diarrhoea, WHO and UNICEF released a joint statement advocating for oral zinc supplementation to reduce deaths caused by diarrhoea (UNICEF & World Health Organization, 2004). Similarly to iron deficiency, zinc deficiency also affects cognitive function a study conducted in pregnant women where cognition was affected by zinc deficiency (Stoecker *et al.*, 2009).

2.3.2 Association between iron and zinc deficiencies

There are limited studies available on the association between iron and zinc deficiencies. Zinc plays a role in iron metabolism as it is the co-factor of several enzymes in the body, and there is a link between zinc deficiency and IDA (Kelkitli *et al.*, 2016). Kelkitli *et al.* (2016) reported low zinc serum levels in anaemic patients than non-anaemic patients in a study that evaluated the relationship between iron and zinc deficiencies. Another study conducted on pregnant women reported that providing combined iron and zinc supplementation improved the haematological

status of women who were iron deficient during early pregnancy (Saaka, 2012). These two studies show that there is a causal relationship between iron and zinc deficiencies. It has been advised that IDA patients should have their zinc levels tested and supplemented with zinc (Kelkitli *et al.*, 2016).

2.4 African leafy vegetables

ALVs, like other green leafy commercialised vegetables, could be used in nutrition strategies to alleviate iron and zinc deficiencies in sub-Saharan Africa. There are different species of ALVs in sub-Saharan Africa. ALVs are defined as any leafy vegetables, which have been a part of the food systems in Africa for generations (Smith & Eyzaguirre, 2007). Although the term ALV usually refers to the leafy parts of the vegetables, they may also include young succulent stems and flowers (Vorster *et al.*, 2007). Usually ALVs are natural and gathered from the wild, though it can also be cultivated (Van Rensburg *et al.*, 2007). Most of the ALVs that are semi-cultivated are seasonal and highly perishable, and a couple of authors recommend preservation methods development to extend shelf the life of these vegetables (Bvenura & Afolayan, 2015; Dweba & Mearns, 2011; Masarirambi *et al.*, 2010; Matenge *et al.*, 2012; van der Hoeven *et al.*, 2013). Previous studies reported that the most common traditional methods of preserving ALVs were sun-drying of fresh leaves and sun drying of blanched or cooked leaves (Faber *et al.*, 2010; Matenge *et al.*, 2012; van der Hoeven *et al.*, 2013). Other studies reported spontaneous fermentation (Muchoki *et al.*, 2010; Wafula *et al.*, 2016). The preservation of ALVs increases their consumption when out of season. Each region in Africa has specific common ALVs (Smith & Eyzaguirre, 2007). Some of the common ALVs found in Southern Africa are displaced (

Table 2-1) There is a huge information gap on the nutrient content of ALVs, mostly when cooked and preserved (Dweba & Mearns, 2011). The availability of compositional content of ALVs positively promotes consumer's confidence concerning consumption and use of ALVs (Dweba & Mearns, 2011; Uusiku *et al.*, 2010).

Table 2-1: ALVs mostly found in Southern Africa

Scientific name	Common name
Amaranthus retroflexus	pigweed
Amaranthus hybridus	amaranth
Cleome gynandra	spider plant
Corchorus olitorius	wild jute
Vigna unguiculata	cowpea leaves
Cucurbita maxima	pumpkin leaves
Citrallus lanatus	bitter pumpkin
Brassica rapa l. Subs.chinensis	chinese cabbage
Aloe saponaria	common soap aloe
Chenopodium album	lambs quarters
Bidens pilosa	blackjack
Mormodica balsamina	bitter gourd
Lagenaria siceraria	wild melon
Abelmoschus esculentus	okra
Colocassia esculenta	taro

Adapted from Flyman & Afolayan (2006b); Legwaila *et al.* (2011); Masarirambi *et al.* (2010); Ndoro *et al.* (2007); Nguni and Mwila (2007); Van Rensburg *et al.* (2007)

2.4.1 Mineral content of African leafy vegetables

ALVs contain high levels of protein, fibre, ascorbic acid (AA), magnesium, iron, zinc and calcium (Bvenura & Afolayan, 2015). This review will focus on ALVs commonly consumed in Southern Africa such as amaranth, spider plant, wild jute, and cowpea leaves (Mabala, 2018). For comparable purposes results reported per wet weight basis were converted to dry weight basis using moisture percentage conversion factor and calculated as shown below.

$$Cd = \frac{Cw}{(Ps)} \times 100$$

Where;

Cd = concentration in dry weight

Cw = concentration in wet weight

Ps = Percentage solids (100 – percentage moisture)

The mineral content of raw wild jute was compared to that of spinach (*Spinacia oleracea*) and cabbage (*Brassica oleracea*) (**Table 2-2**). The results show that raw wild jute contained higher levels of iron, zinc and magnesium compared to the raw cabbage (Ndlovu & Afolayan, 2008). It should be noted that ALVs are mostly consumed cooked, therefore, it is vital to have the mineral content of the cooked vegetable. Previously the nutritional superiority of ALVs over exotic vegetables compared to raw sample data was reported (Orech *et al.*, 2007), however, the nutrient content does change during cooking; hence data from raw vegetables could give inaccurate contents when directly extrapolated for cooked vegetables. There are some studies which compared cooked ALVs and exotic commercialised vegetables. High contents of iron in cooked wild jute (17.6 mg/100 g, db) (Schönfeldt & Pretorius, 2011) compared to cooked cabbage (0.1 mg/100 g, db) (Kawashima & Soares, 2003) were previously reported. However, zinc contents were substantially lower in raw wild jute (2.9 to 5.1 mg/100, db) compared to raw spinach (10.6 mg/100 g, db) (Ndlovu & Afolayan, 2008; van Jaarsveld *et al.*, 2014). The same species of wild jute harvested in different locations and similarly processed had different levels of minerals. Environmental factors such as climate and soil fertility may contribute to these variations (Uusiku *et al.*, 2010), such as amount of rainfall the plant was exposed to and the amount of sunlight.

Table 2-2: Mineral content of wild jute compared with cabbage and spinach

species	Fe	Zn	Ca	Mg	P	Raw/cooked
mg/100 g, db						
Wild Jute ^{1*}	22.8	5.1	35	56	26	Raw
Cabbage ¹	5.6	3.1	ND	28	12	
Spinach ¹	41.1	10.6	61	43	44	
Wild jute ^{3*}	17.6	2.9	1519	426	578	Cooked
Wild jute ^{2*}	38.4	7.3	3310	419	780	
Cabbage ⁴	0.1	0.2	47	14	-	

db = Dry basis, ND (not detected), - not reported, * same ALV species from different locations, Fe = iron, Zn = zinc, Ca = calcium, Mg = magnesium, P = phosphorus ¹Ndlovu & Afolayan, 2008 ² Schönfeldt & Pretorius, 2011 ³ van Jaarsveld *et al.*, 2014, ⁴ Kawashima & Soares, 2003.

Table 2-3: Mineral content of raw and cooked amaranth

Study	Fe	Zn	Ca	Mg	P	Raw/Cooked
mg/100 g, db						
Van der Walt <i>et al.</i> , (2009)*	94.9	4.2	1772	871	-	Raw
Van der Walt <i>et al.</i> , (2009)*	14.8	0.6	2700	1400		
Odhav <i>et al.</i> , (2007)	21	18	2363	1317	427	
van Jaarsveld <i>et al.</i> , (2014)	28.3	3.9	2461	1344	450	
Schönfeldt & Pretorius, (2011)	85	7	2720	1050	649	Cooked
Kruger <i>et al.</i> , (2015)	16.4	2.8	947	-	-	

db = Dry basis , – (not reported), * same ALV species from different locations, Fe = iron, Zn = zinc, Ca = calcium, Mg = magnesium, P = phosphorus.

Different authors studied amaranth mineral contents (**Table 2-3**). Both raw and cooked amaranth had higher levels of iron than zinc (Kruger *et al.*, 2015; Nesamvuni *et al.*, 2001; Odhav *et al.*,

2007; Schönfeldt & Pretorius, 2011; van Jaarsveld *et al.*, 2014). In general, plant-based diets have lower zinc contents (Kruger *et al.*, 2015), and zinc bioavailability from plant-based diets is lower. In a study that compared the zinc status of vegetarians to non-vegetarians, low dietary zinc intake and serum levels were found in vegetarians than in non-vegetarians (Foster *et al.*, 2013).

Table 2-4: Mineral content of raw and cooked cowpea

Study	Fe	Zn	Ca	Mg	P	Raw/Cooked
mg/100 g, db						
van Jaarsveld <i>et al.</i> , (2014)	26.7	2.4	2461	352	290	Raw
Van der Walt <i>et al.</i> , (2009)	97.9	6.1	1722	292	—	
Schönfeldt & Pretorius, (2011)	31.4	4	2429	441	645	
Schönfeldt & Pretorius, (2011)	33	5.5	1659	379	623	Cooked
Kruger <i>et al.</i> , (2018)	14.6	6.9	—	—	—	

db = dry basis, - not reported, Fe = iron, Zn = zinc, Ca = calcium, Mg = magnesium, P = phosphorus.

Table 2-5: Mineral content of raw and cooked spider plant

Study	Fe	Zn	Ca	Mg	P	Raw/cooked
mg/100 g, db						
Van der Walt <i>et al.</i> , (2009)*	89.7	8.4	1944	848	-	Raw
Van der Walt <i>et al.</i> , (2009)*	38.1	43.7	3100	1311	-	
Schönfeldt & Pretorius, (2011)	90.5	6.3	393	924	930	
van Jaarsveld <i>et al.</i> , (2014)	16.8	8.3	1856	608	1104	
Schönfeldt & Pretorius, (2011)	116	8	2120	732	880	Cooked

db = Dry basis, - not reported, * same ALV species from different locations, Fe = iron, Zn = zinc, Ca = calcium, Mg = magnesium, P = phosphorus, ¹; ²; ³.

The mineral contents of both raw and cooked cowpea leaves and spider plant were assessed by different authors (**Table 2-4,**

Table 2-5). During cooking ALVs minerals may leach out into the cooking water and may be lost if the cooking water is discarded before laboratory analysis (Mziray, 1999), and this may account for the variation found between different vegetable species. ALVs contains high levels of iron, zinc, calcium and magnesium (**Table 2-2, Table 2-3, Table 2-4,**

Table 2-5). For households that have limited access to exotic vegetables due to demographic and economic status, ALVs could possibly be considered as an essential source of these minerals (van der Hoeven *et al.*, 2016). A study conducted in children in a rural area found that consumption of ALVs contributed significantly to iron and calcium intakes (Faber *et al.*, 2007). However, the high mineral levels in ALVs do not always translate to high mineral bioavailability because of other vegetable components that can influence bioavailability. There is enough evidence that ALVs contains high levels of antinutrients that hinders the absorption of iron, zinc and calcium (Kruger *et al.*, 2015; Uusiku *et al.*, 2010). There is a need to assess the bioavailability of iron and zinc from ALVs.

2.5 Role of African leafy vegetables in food security and meeting dietary requirements

A review concluded that ALVs could play a significant role in food security among the rural people in sub-Saharan Africa (Uusiku *et al.*, 2010). Traditionally these vegetables are cooked and eaten as a relish, together with a starchy staple such as maize, millet or sorghum porridge (Bvenura & Afolayan, 2015; Kruger *et al.*, 2015; Uusiku *et al.*, 2010). These vegetables can contribute positively to the world's food production, as they can withstand harsh conditions and are more tolerant to pathogens than improved varieties, thus requiring fewer chemicals and pesticides compared with exotic vegetables (Dweba & Mearns, 2011; Hart, 2011). In addition, ALVs have a better shelf-life than non-indigenous vegetables (Hart, 2011). Poor households, rather than their wealthier counterparts, are found to be the ones using these vegetables (Matenge *et al.*, 2012; Van Rensburg *et al.*, 2007).

A study conducted in Molati, a rural village in the Limpopo province of South Africa reported that about 72% of the households surveyed eat ALVs at least twice a day (Hart, 2011). The same observation of frequent consumption of ALVs was in Nomathiya, a rural village in the KwaZulu-Natal province of South Africa (Thandeka *et al.*, 2014). ALVs are often dried and stored for consumption when they are out of season (Bvenura & Afolayan, 2015; Faber *et al.*, 2010; Matenge *et al.*, 2012; Odhav *et al.*, 2007). Almost 94% of the households surveyed in a study in South Africa relied on ALVs as their primary source of vegetables in winter, when they are out of season (Hart, 2011). A study in Eswatini also reported that the diet of people was dependent on a variety of seasonally available ALVs (Masarirambi *et al.*, 2010). In rural settings, ALVs are used mostly as a relish accompanying staples in most households (Matenge *et al.*, 2012). The consumption of ALVs as relish accompanying staple foods could play a significant role in the management of hunger and micronutrient deficiencies (Mavengahama *et al.*, 2013). ALVs consumption could reduce food insecurity and hunger (Legwaila *et al.*, 2011). Also, a study conducted in Zambia reported that ALVs are a source of income for many households hence could alleviate poverty in rural households (Nguni & Mwila, 2007).

Numerous studies have indicated that ALVs have the potential to contribute to food security. Therefore, to advocate their use, there is a need for data on their nutritional contribution towards dietary requirements. There are only a few studies which have evaluated the contribution of ALVs to dietary requirements. The lack of compositional data of ALVs makes it challenging to determine their nutritional contribution (Faber *et al.*, 2007). A study conducted in South Africa evaluated the potential contribution of ALVs towards dietary reference intakes. It reported that pigweed (Amaranth), and cowpea leaves could contribute more than 75% of the Recommended Dietary Allowance (RDA) of iron for children, and that pumpkin leaves can contribute 50 to 75% RDA of iron for children (van Jaarsveld *et al.*, 2014). A study conducted on the potential contribution of ALVs and maize porridge composite meals to iron and zinc nutrition reported that 100 g of ALVs could contribute approximately 34% of the iron RDA for children and 14% of the iron RDA for women. At the same time, the vegetable can contribute 12% of the zinc RDA for children and 5% of the zinc RDA for women (Kruger *et al.*, 2015). Both studies concluded that the ALVs contribution to RDA for zinc is low (Kruger *et al.*, 2015; van Jaarsveld *et al.*, 2014). Limited studies had considered evaluating the bioavailability of minerals from ALVs (Kruger *et al.*, 2015; Muchoki *et al.*, 2010). A study conducted by Kruger *et al.* (2015) observed that iron bioaccessibility from amaranth was 9.2%. From the two studies conducted in South Africa, ALVs can contribute to

dietary intake of minerals. But, their antinutrients content negatively effect on mineral absorption(Essack *et al.*, 2017).

2.6 Antinutrient content of African leafy vegetables

ALVs contain antinutritional factors that hinder the absorption of minerals such as iron, zinc and calcium. Several studies have investigated the antinutrients content of ALVs (**Table 2-6**). Some of the antinutrients found in ALVs include phytates, oxalates, phenolic compounds especially total phenolic content and condensed tannins (Kruger *et al.*, 2015; Uusiku *et al.*, 2010). These antinutrients form insoluble complexes with essential minerals rendering the mineral mainly unavailable for absorption by the human body. Hence, there is a need to evaluate levels of antinutritional factors in ALVs to establish their effects on mineral absorption (Hailu & Addis, 2016).

Table 2-6: Antinutrients content of ALVs

Study	Species	Phytate	Total phenolic content	Condensed tannins	Total oxalates	Raw/cooked
mg/100 g, db						
Kruger et al., 2015	Amaranth	2089	1234	203	-	Cooked
Kruger et al., 2018	Cowpea	721	5295	1021	-	
Ndlovu & Afolayan, 2008	Wild jute	117	-	-	-	Raw
Mwanri et al., 2018	Spider plant	0.3	-	-	48.3	

db = Dry basis, - (not reported).

A study that evaluated antinutrients in amaranth reported high levels of phytates(2089 mg/100 g), condensed tannins (203 mg/100 g, db) and total phenolic content (1234 mg/100 g, db)(Kruger *et al.*, 2015). Though ALVs contain high levels of iron and zinc (**Table 2-2, Table 2-3, Table 2-4,**

Table 2-5), the levels of antinutritional factors pose a challenge for iron and zinc absorption. Several studies have investigated the phytate content of ALVs, and it has been reported to be the primary inhibitor of iron and zinc. Phytic acid (the unstable free form) with its negatively charged phosphates sites strongly bind with cations of K, Fe, Zn, Mg and Mn at physiological pH (Bohn *et al.*, 2008). The phytic acid-mineral complex formed is usually referred to as phytate (Coulibaly *et al.*, 2011), and it is known for its negative effect on mineral bioavailability. A study that predicted mineral bioavailability in wild jute using phytate-mineral molar ratios reported high molar ratios of phytate to calcium, phytate to zinc, and phytate to iron, which predicted low bioavailability of these essential minerals (Ndlovu & Afolayan, 2008). However, the study was conducted in a raw sample, and there is evidence that phytate content reduces during cooking (Oboh *et al.*, 2005). It has been previously reported that reduction of antinutritional factors depended upon the type of processing (cooking and sun drying) of the vegetables (Hailu & Addis, 2016). In his thesis, Lee (2010) reported that cooking wild vegetables for a long time and at high temperatures significantly reduced their antinutrient composition. Consequently, this prolonged cooking reduced the mineral content in the vegetables. As reviewed, it is apparent that ALVs contain antinutritional factors which bind essential minerals and inhibit their absorption. Therefore, mineral concentration of ALVs could not be directly used to predict their bioavailability. Several studies recommended that appropriate processing methods should be investigated to reduce antinutrients content in ALVs (Bvenura & Afolayan, 2015; Hailu & Addis, 2016), these processing methods should be shared with communities to reduce inhibitors of essential minerals and enhance mineral availability.

2.7 Challenges of the use of African leafy vegetables

Several challenges influence the unsustainable incorporation of ALVs into diets. Urbanisation and shifting to western diets have been identified as a significant challenge that causes a decline in the consumption of ALVs (Dweba & Mearns, 2011; Matenge *et al.*, 2012; Smith & Eyzaguirre, 2007; van der Hoeven *et al.*, 2013; van Jaarsveld *et al.*, 2014; Vorster *et al.*, 2009). Due to deforestation and the clearing of land near homesteads owing to industrialisation, ALVs are not readily available in urban areas (Keller *et al.*, 2005). Hence studies insisted that the unavailability of ALVs resulted in low consumption, especially in urban areas (Matenge *et al.*, 2012; van der Hoeven *et al.*, 2013). Low consumption of ALVs is associated with food insecurity and micronutrient deficiencies in rural communities (Flyman & Afolayan, 2006a).

Other authors alluded to the fact that communities' knowledge of ALVs is diminishing (Flyman & Afolayan, 2006a; Van Rensburg *et al.*, 2007), which also influence low consumption of ALVs. Lack of awareness on the importance of ALVs especially among the younger generation, as they do not know which plants are considered ALVs is linked to diminishing use of ALVs (Flyman & Afolayan, 2006a; Matenge *et al.*, 2012; van der Hoeven *et al.*, 2013; Van Rensburg *et al.*, 2007). This lack of awareness of the importance of ALVs has resulted in low intakes of ALVs and a lack of dietary diversity (Flyman & Afolayan, 2006a). It has also resulted in false and negative perceptions on the use of ALVs, such as the fear of poisoning due to harvesting the wrong plants (Bvenura & Afolayan, 2015). Studies recommended that elders should be encouraged to influence the younger generation to use ALVs (Matenge *et al.*, 2012; van der Hoeven *et al.*, 2013). There is enough evidence that, compared with young women, older women are more knowledgeable about ALVs available in their locations (Dweba & Mearns, 2011; Matenge *et al.*, 2012; Modi *et al.*, 2006; Odhav *et al.*, 2007; van der Hoeven *et al.*, 2013). Young women associate ALVs with rural communities and older generations (Dweba & Mearns, 2011; van der Hoeven *et al.*, 2013). ALVs are viewed as food that cannot be served to guests as they are not highly valued (Dweba & Mearns, 2011). Due to insufficient supply of ALVs especial in urban areas, younger generations are not keen to walk long distances to gather ALVs as mostly are semi-cultivated (Keller *et al.*, 2005), hence, this impact their consumption negatively.

Even though ALVs contain high levels of essential minerals, their consumption is minimal compared to modern commercialised vegetable types. Previous surveys conducted in South Africa revealed that individuals preferred commercially grown vegetables on the market over ALVs (Faber *et al.*, 2010; van der Hoeven *et al.*, 2013). It has been hypothesised that commercially grown vegetables are widely advocated and promoted by Agricultural and Extension workers as evidenced by health and agricultural policies available promoting commercially grown vegetables, both for marketing and consumption, at the expense of ALVs (Van Rensburg *et al.*, 2007; Vorster *et al.*, 2007). Previous surveys have revealed that consumers perceive ALVs as food for the poor (Faber *et al.*, 2010; van der Hoeven *et al.*, 2013), and consumed in times of drought (Hart, 2011). ALVs are not accessible to market to more substantial populations; therefore, farmers are more enthusiastic about cultivating commercially grown vegetables, which are accessible and secure to market (Keller *et al.*, 2005). Exotic vegetables provide a monetary value; hereafter, they are popular and preferred compared to ALVs (Dweba & Mearns, 2011).

Most countries have laws which prohibit people from entering grazing lands where most of the ALVs grow, which has a direct impact on the accessibility of the wild vegetables (Keller *et al.*,

2005). Laws should be re-evaluated and made more lenient so that people in rural areas have access to ALVs (Dweba & Mearns, 2011).

Under-utilisation of ALVs has been linked to their seasonality, making them available only fresh in one season in other sub-Saharan countries. Several studies have reported that ALVs are available fresh only in summer, while during winter, dried ALVs are commonly used (Dweba & Mearns, 2011; Hart, 2011; Masarirambi *et al.*, 2010). Preservation has been identified as a solution to ensure the availability of ALVs when out of season (Vorster *et al.*, 2009). Traditionally ALVs are dried in direct sunlight without blanching, and the method has been reported to result in vitamin losses and contamination by dust (Flyman & Afolayan, 2006a). Several studies are calling for the evaluation of proper drying methods of ALVs to minimise vitamins losses and contamination (Bvenura & Afolayan, 2015; Masarirambi *et al.*, 2010; Thandeka *et al.*, 2011). Only a few researchers have looked at how different processing methods influence the nutritional composition of ALVs (Ndlovu & Afolayan, 2008; Odhav *et al.*, 2007; Orech *et al.*, 2007). New knowledge in processing ALVs could improve ALVs accessibility even out of season. Limited information available on the mineral composition of ALVs is linked to negligence and underutilisation of ALVs, resulting in various authors questioning their suitability in supplying nutrients towards meeting daily nutrient requirements (Bvenura & Afolayan, 2015). As previously stated, high levels of antinutrient presence in ALVs negatively affect the absorption of essential minerals making the bioavailability of minerals from these ALVs questionable.

The use of ALVs needs to be encouraged both for commercial cultivation and for household consumption, as well as to supplement household income (Hart, 2011; Odhav *et al.*, 2007). Education can change people's attitudes towards ALVs (Bvenura & Afolayan, 2015). Younger generations need education on the use of ALVs. Research institutions and universities should be encouraged to research these vegetables and come up with solutions for appropriate processing methods to ensure their availability throughout the season. Several studies have recommended marketing and commercialisation of ALVs (Hart, 2011; Matenge *et al.*, 2012; Vorster *et al.*, 2007), this will ensure accessibility of vegetables to all communities to improve iron and zinc status.

2.8 Factors that affect iron and zinc status of the body

Both iron and zinc deficiencies occur when physiological requirements are not met due to malabsorption of these essential minerals from the diet. Zinc and iron are mostly present in the

same food, and their absorption is affected by the same antinutritional factors (Gibson *et al.*, 2000; Gibson *et al.*, 2010). Therefore, their deficiencies may coincide. Plant-based diets natural have low iron and zinc bioavailability (Gibson *et al.*, 1997), due to the presence of inhibitors of iron and zinc.

2.8.1 Iron (Fe)

About 30-70% of iron is present in animal sources (haem iron), of which 15-35% is bioavailable (Hurrell, 2002). The other form of iron which is present in plant-based sources is non-haem iron, absorption of which ranges from 2% to 20% (Zimmermann *et al.*, 2005b). The low bioavailability of non-haem iron is due to phytates, polyphenols and calcium, which inhibit iron absorption, but meat and ascorbic acid increase its absorption (Zimmermann *et al.*, 2005b). Except for calcium, dietary inhibitors that impair non-haem iron do not impair haem iron absorption (Sandstead, 2000). Calcium inhibits both non-haem and haem iron absorption. It has been suggested that early consumption of cow's milk in infants could contribute to iron deficiency (Moy, 2006). IDA has been associated with intestinal parasitic infections such as hookworm (*Necator americanus*) which causes intestinal inflammation and blood losses (Hesham *et al.*, 2004; Stephenson *et al.*, 2000). In rural Malaysia, the presence of intestinal parasitic infections, was significantly associated with an increase in the prevalence of IDA (Ngui *et al.*, 2012). Programmes that control parasites or periodic deworming should be encouraged periodically to control hookworms which cause IDA. In cases where the prevalence of IDA is higher than 50%, it was recommended that deworming should be conducted twice yearly for preschool and school-aged children and WBA (Pasricha *et al.*, 2013).

Impaired iron absorption also results in IDA (Zimmermann & Hurrell, 2007). During digestion, sufficient gastric acid is needed in the stomach to maintain ferric iron forms in solution (Dickey, 2002). Gastric acid is crucial in the availability of non-haem iron (Annibale *et al.*, 2003). Therefore, patients with gastric conditions such as atrophic body gastritis and those that have undergone gastric surgery are at risk of IDA (Annibale *et al.*, 2003). Gastric conditions are more common in the elderly (Andr s *et al.*, 2008), and might be a substantial cause of IDA in this group.

2.8.1.1 Intestinal absorption of Iron

The absorption of iron occurs predominantly in the duodenum and upper jejunum (Abbaspour *et al.*, 2014). Both haem and non-haem iron absorption occur on the apical brush border membrane of the small intestine by independent pathways (**Figure 2-1**). Iron rapidly oxidises into the insoluble ferric (Fe^{+3}) form at physiological pH (Ems & Huecker, 2019). For iron to be absorbed, it should be in the ferrous state (Fe^{+2}) or haem form (Abbaspour *et al.*, 2014). In the proximal duodenum, gastric acid lowers the pH activating ferric reductase enzyme, duodenal cytochrome B (DCYTB) which catalyses the reduction of insoluble Fe^{+3} to absorbable Fe^{+2} (Abbaspour *et al.*, 2014; Ems & Huecker, 2019). Therefore, gastric acid production plays a significant role in the solubility and uptake of ferric iron. In the intestinal lumen, the reduced ferric iron is taken up by divalent metal cation transporter 1 (DMT1) across the apical membrane and into the enterocytes (Ems & Huecker, 2019). DMT1 only transports iron in the Fe^{+2} state (Nishito & Kambe, 2018). Transported Fe^{+2} iron in enterocytes is stored as ferritin and transported into circulation through Fe^{+2} transporter ferroportin (FPN) (Abbaspour *et al.*, 2014). The mechanism of intracellular iron transport and metabolism involves chaperone proteins such as poly C binding protein 2 (PCBP2) (Shi *et al.*, 2008). PCBP2 binds to DMT1 and FPN, and functions to deliver iron from the apical side to the basolateral side of the intestinal epithelial cells (Nishito & Kambe, 2018). The only efflux route of cellular iron is FPN and regulated by hepcidin levels (Ems & Huecker, 2019). Hepcidin binds FPN inducing its internalization and degradation, which prevents iron absorption into enterocytes (Ems & Huecker, 2019). If hepcidin levels are low and FPN is not down-regulated Fe^{+2} is released from the cells and oxidised back into the non-absorbable Fe^{+3} , subsequently binding to transferrin for it to be soluble and transported into cells (Ems & Huecker, 2019).

Considerable studies had been conducted but the pathway for haem iron absorption is still unclear. Haem iron uptake occurs through two haem transport proteins, i.e. haem carrier protein 1 (HCP1) and haem responsive gene-1 (HRG-1) (Przybyszewska & Żekanowska, 2014). Haem iron is transported across the apical membrane by HRG-1 and HCP1 importers into the enterocytes and catabolised by haem oxygenase to generate iron in Fe^{+2} state (Knutson, 2017; Nishito & Kambe, 2018). The generated Fe^{+2} subsequently metabolised in the same pathway as for absorption of non-haem iron (Przybyszewska & Żekanowska, 2014). HRG-1 influences the transport of haem iron into the cytosol via the endocytosis pathway due to its high affinity for haem iron (White *et al.*, 2013). Though HCP1 is involved in haem uptake, it has a high affinity to folate and functions as folate transporter (Qiu *et al.*, 2006). Further investigations are needed to understand the absorption of haem iron fully.

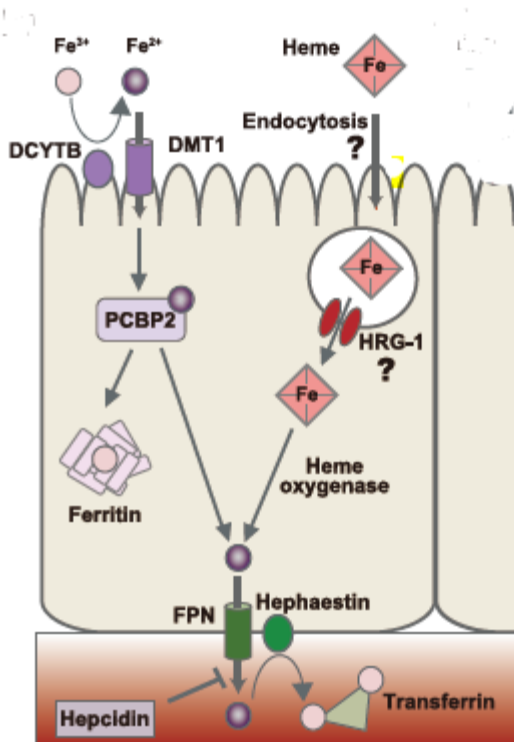


Figure 2-1: Absorption mechanism of iron.

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2.8.2 Zinc (Zn)

To maintain zinc status, the regulation of zinc levels in the body is crucial. Its excretion maintains zinc homeostasis through the gastrointestinal tract (King *et al.*, 2016). Excretion declines when zinc intake is low, and when zinc intake increases, more excretion occurs to maintain zinc balance (King *et al.*, 2016). Increases in zinc absorption occur in late pregnancy and lactation (Fung *et al.*, 1997), and also among children with diarrhoea (Bitarakwate *et al.*, 2003). Pregnant women and children are at high risk of zinc deficiencies if they consume diets low in zinc (Wessells & Brown, 2012).

As previously stated, ALVs contains high levels of zinc (Table 2-2, Table 2-3, Table 2-4,

Table 2-5) but plant-based diets are associated with low zinc bioavailability (Platel & Srinivasan, 2016). Dietary components such as phytates and insoluble dietary fibres in plant-based diets negatively affect the absorption of zinc (Gibson *et al.*, 2010). There is enough evidence that zinc from animal sources is more readily absorbed than that from plant-based diets (King *et al.*, 2016; Gibson *et al.*, 2010; Hunt *et al.*, 2008), therefore, sources of zinc will determine the extent of zinc absorption.

Zinc absorption is influenced by the presence of other minerals during absorption. The mechanism of interaction between iron and zinc is not fully understood (King *et al.*, 2016). Due to their similar ionic properties iron and zinc influence the transport and absorption of one another across the hepatocytes and enterocytes (Knez *et al.*, 2017). However, there have been conflicting findings reported on iron and zinc interaction. Walker and co-workers previously summarised that there was a non-competitive absorption between zinc and iron (Walker *et al.*, 2005) while other authors concluded that there was a positive interaction between iron and zinc absorption (Chang *et al.*, 2010; Harvey *et al.*, 2007; Hininger-Favier *et al.*, 2007). A positive correlation has been found between zinc deficiency and anaemia in pregnant women and children (Gebremedhin *et al.*, 2014; Ma *et al.*, 2004). However, high doses of soluble iron and zinc are capable of producing a negative interaction between these two essential minerals, but the effect was not observed when iron and zinc were given in the form of meals (de Romana *et al.*, 2005; Esamai *et al.*, 2014; Lönnerdal, 2000).

2.8.2.1 Intestinal absorption of zinc

Zinc uptake occurs in the small intestine through a carrier-mediated mechanism (**Figure 2-2**). Zinc transporter (ZNT) and Zrt-/Irt- related proteins (ZIPs) are the two zinc transporter families that control the influx and efflux of zinc (Nishito & Kambe, 2018). There are about 14 ZIP and nine ZNT transporters identified (Hara *et al.*, 2017). The transporter that is essential for the uptake of zinc on the apical membrane in the small intestine is the ZIP4 transporters (Hashimoto *et al.*, 2016). Then the ZNT1 exports the absorbed zinc (by the intestinal epithelial cells) into the portal blood (Hennigar & McClung, 2016). The process in which zinc is transported from the apical membrane by ZNT1 to the basolateral membrane by the ZNT1 in the enterocytes is unclear (Nishito & Kambe, 2018). It is also unclear how metallothionein (MT) influences the zinc transporters (ZIP4 and ZNT1) in the regulation of zinc uptake (Krebs, 2000). MT has a high affinity in binding cellular zinc (King *et al.*, 2016).

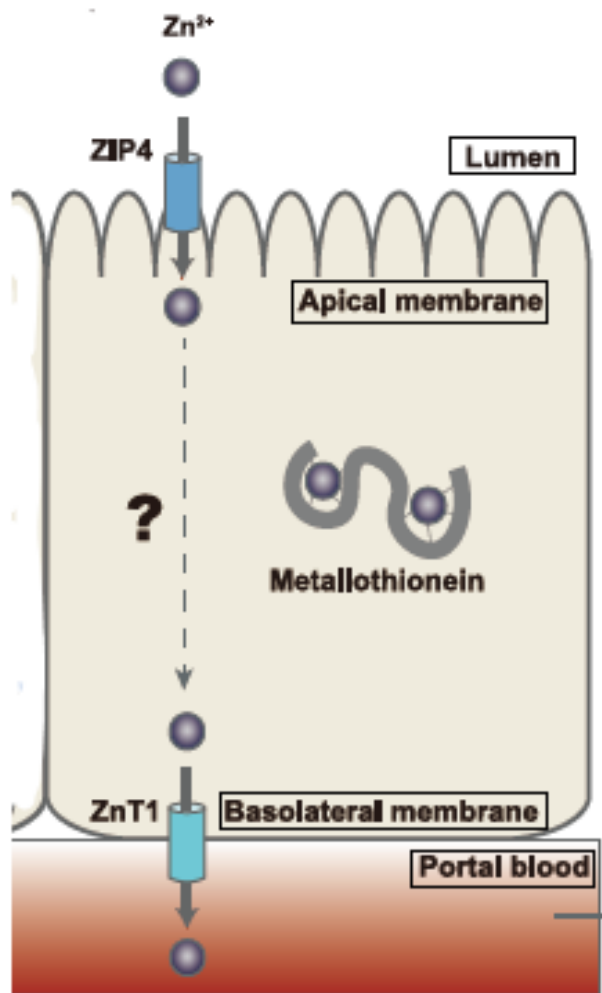


Figure 2-2: Absorption mechanism of zinc.

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2.9 Approaches to prevent and alleviate iron and zinc deficiencies

Due to the complex absorption mechanism of iron and zinc, not all dietary minerals consumed are absorbable (FAO & WHO, 2005). Therefore, approaches such as fortification, supplementation, biofortification and dietary diversification have been crucial in addressing iron and zinc deficiencies (Kumar *et al.*, 2019). There is enough evidence on the effectiveness of these approaches towards alleviating zinc and iron deficiencies as summarised below (**Table 2-7**).

2.9.1 Supplementation

Supplementation with iron and zinc has been extensively studied. There is strong evidence available that indicates that supplementation with zinc and iron is effective (Black *et al.*, 2004; Wieringa *et al.*, 2007). Several studies conducted in children with diarrhoea supplemented with oral zinc observed a decrease in stool output, duration of diarrhoea and the severity of acute diarrhoea (Bhatnagar *et al.*, 2004; Brooks *et al.*, 2005; Lukacik *et al.*, 2008). Although there is enough evidence to support the use of zinc during diarrhoeal episodes, it is not yet clear what the most effective method is to deliver the supplements. However, it has been suggested that it may be beneficial to add zinc to oral rehydration therapy (Shrimpton *et al.*, 2005). Supplementation with zinc has been beneficial in children with low birth weight and growth failure (Ohiokpehai, 2003). Despite these promising results, currently, there is no routine large-scale programme implemented targeting zinc supplementation.

Iron supplementation has long been promoted (Pasricha *et al.*, 2013). Different oral supplements exist. Also, different iron-containing compounds have varying costs and bioavailability (Pasricha *et al.*, 2013). Several studies have investigated iron supplementation and found positive effects. A study that supplemented anaemic patients with elemental iron with daily doses of 15 mg or 50 mg as liquid ferrous gluconate or 150 mg of ferrous calcium citrate tablets for sixty days reported a significant increase in haemoglobin and ferritin levels (Rimon *et al.*, 2005). Other authors evaluated iron supplementation during pregnancy and found that supplementation with iron during pregnancy improved the mother's iron stores, significantly lowered incidences of low birth weight infants and incidences of low preterm birth weight infants (Cogswell *et al.*, 2003; Scholl, 2005). Due to cost, sometimes it is not possible for countries to have periodic supplementation of these essential minerals to vulnerable groups, hence balanced diversified diets are encouraged to meet daily mineral needs.

2.9.2 Fortification

Fortification is usually used at the national level to prevent deficiencies of iron, zinc and other nutrients. It usually targets existing staple foods (Harrison, 2010; Pasricha *et al.*, 2013). However, it is often difficult to target foods eaten by infants and young children (Detzel & Wieser, 2015). Infants are at risk of iron and zinc deficiencies, so suitable food vehicles for fortification with iron and zinc need exploration to make appropriate food choices.

Zinc oxide (ZnO) and zinc sulphate (ZnSO₄) are the most commonly used and accepted forms of zinc fortification (Romaña *et al.*, 2002). Nations such as Mexico, Indonesia and South Africa, fortify maize meal and wheat flour with zinc (Gibson, 2006). A desktop study in South Africa showed that the national fortification programme could improve the intake of mineral and vitamins such as iron, folate, niacin, riboflavin and vitamin B₆ (Steyn *et al.*, 2008). Other studies have also found positive outcomes when fortifying with zinc. In a study in Thailand, the use of a multi-micronutrient fortified seasoning powder led to improved zinc status in primary school children (Winichagoon *et al.*, 2006). In agreement, another study observed similar results of improved zinc absorption in South African children fed highly inhibitory maize porridge. This maize porridge was supplemented with a micronutrient powder, containing low doses of highly absorbable iron and zinc and an exogenous phytase, which breaks down phytates and is active at gut pH (Troesch *et al.*, 2011).

The most common iron fortificants are ferrous sulphate and ferrous fumarate (Pasricha *et al.*, 2013). Both fortificants are highly bioavailable (Pasricha *et al.*, 2013). Bioavailable iron reacts with other food components, resulting in rancidity, undesired sensory and colour changes (Hurrell, 2002). In a study that examined the effect of maize meal porridge fortified with ferrous fumarate, there was an observation of a decrease from 45% to 17% in the proportion of infants with anaemia in the group given fortified maize meal porridge (Faber *et al.*, 2005). However, in another study, the fortification of brown bread with ferrous fumarate at levels which did not result in colour change did not lead to an improvement of the iron status of children (Van Stuijvenberg *et al.*, 2008).

Home fortification using multiple micronutrient powders (MMP) in infant feeding is usual because iron-rich complementary foods are not easily accessible (Pasricha *et al.*, 2013). Clinical trials have confirmed that MMP are more effective than liquid ferrous in controlling anaemia (Zlotkin *et al.*, 2003). The use of MMP among infants and young children, 6-23 months old, living in locations with different prevalences of anaemia has been reported to be effective (De-Regil *et al.*, 2013). In

a study done in South Africa, the use of MMP increased serum ferritin and body iron stores and decreased both iron and zinc deficiencies in children by 30.8% and 11.8% respectively (Troesch *et al.*, 2011). The challenges of using MMP include cost, limited availability and accessibility to vulnerable groups (Pasricha *et al.*, 2013).

2.9.3 Dietary diversification

Fortification and supplementation require stable infrastructure and finances. Dietary diversification is more sustainable, economical, and culturally acceptable (Gibson, 2006). Dietary diversification results in an increase in the variety of food consumed, which promotes proper nutrition (Frison *et al.*, 2006). A review concluded that the consumption of ALVs improved dietary diversity and quality (Cogill, 2014). Though ALVs have high levels of antinutrients (**Table 2-6**), fermentation can reduce antinutrients (Gibson *et al.*, 2010). There is a need to promote the consumption of zinc and iron from animal sources simultaneously with plant-based diets to diversify diets (Gibson, 2006; Pasricha *et al.*, 2013). Though dietary diversity encourages the consumption of a variety of food and access to nutritious food (Nair *et al.*, 2016), it needs behavioural change which can be difficult.

2.9.4 Biofortification

Biofortification is the addition of minerals and vitamins in a crop during plant breeding and agronomic practices (Bouis, 2018). Micronutrient deficiencies especial essential minerals could be reduced by increasing the production of mineral-rich food and increasing dietary diversity (Bouis & Saltzman, 2017). Increasing the content of iron and zinc during crop production could benefit vulnerable groups and economically disadvantaged populations in rural areas who generally will have no access to commercially fortified foods and mineral supplements (Saini *et al.*, 2016). In most of the cases, biofortification has been used to complement other existing interventions such as mineral fortification, supplementation and dietary diversity to prevent and control essential mineral deficiencies (Bouis & Saltzman, 2017).

Several studies demonstrated the effectiveness of biofortified crops in improving dietary iron and zinc intakes. A study in iron-depleted university women in Rwanda reported more significant increases in total body iron (0.5 mg/kg) and haemoglobin levels (3.8 g/L) in the group that consumed iron biofortified beans for 18 weeks compared to the control group (Haas *et al.*, 2017).

Similar results of increases in total iron and serum ferritin were reported in adolescents after consuming biofortified pearl millet flatbread for four months twice daily (Finkelstein *et al.*, 2015). The biofortification of maize resulted in the reduction in the prevalence of inadequate zinc intake among rural residents, where the prevalence reduced by 56% and 47% for adults and children respectively (Denova-Gutiérrez *et al.*, 2008). In an absorption study, improved iron and zinc uptake were observed in Caco-2 cell after exposed to biofortified indica rice (Trijatmiko *et al.*, 2016). In contrast, a study that used biofortified pellet millet reported low cell ferritin values (Tako *et al.*, 2015) and the presence of high levels of polyphenols in pellet millet were associated with low ferritin values. Polyphenols probably seriously impaired iron absorption by the Caco-2 cells. A review concluded that iron biofortified crops were an efficacious intervention to improve iron status, and the effects were highest among subjects who were iron deficient at baseline (Finkelstein *et al.*, 2017), this may suggest the greatest potential to benefit. Biofortification of leafy vegetables has been previously studied. A study that biofortified rape (*B. napus*) and redroot pigweed (*A. retroflexus*) reported an increased iodine concentration in the biofortified vegetables (Ligowe *et al.*, 2020). Similar results of increased mineral content of biofortified leafy vegetables were also reported when red and green pigmented lettuce (*Lactuca sativa* L.) was biofortified with iron (Giordano *et al.*, 2019). Therefore, there is a need to explore the biofortification of ALVs to improve their iron and zinc contents. Though ALVs are mostly wild growing some agronomist explored their cultivation and published production guidelines (Jansen van Rensburg *et al.*, 2012). In addition, previous studies suggested that plant breeding approaches should produce new varieties of crops with reduced levels of antinutrients and improved iron and zinc bioavailability (La Frano *et al.*, 2014; Shrimpton *et al.*, 2005).

Table 2-7: Summary of the effectiveness of the strategies to prevent iron and zinc deficiencies

Strategy	Study details	Outcomes	References
Supplementation	Children with diarrhoea supplemented with oral zinc	decrease of stool output and duration of diarrhoea	Bhatnagar <i>et al.</i> , (2004); Brooks <i>et al.</i> , (2005)
	Anaemic patients supplemented with elemental iron with daily doses of 15 mg or 50 mg liquid ferrous gluconate or 150 mg ferrous calcium citrate for sixty days	Significant increase in haemoglobin and ferritin levels	Rimon <i>et al.</i> , (2005)
	Supplementation with iron during pregnancy	Improved mother's stores and lowered incidences of low birth weight	Cogswell <i>et al.</i> , (2003); Scholl, (2005)
Dietary Diversification	Dietary score evaluated among adult women	Serum retinol concentration increased	Fujita <i>et al.</i> , (2012)
	Increased in number of food groups consumed including fruits and vegetables	Serum ferritin and serum retinol increased among children in Uganda	Acham <i>et al.</i> , (2013)
	Diversity of vegetable consumption by young children from four to thirteen types	Anaemia prevalence decreased	Talukder <i>et al.</i> , (2010)
	Home gardens focusing on the production of yellow and dark green leafy vegetables	Increased serum retinol concentration of 2 to 5 year olds	Faber <i>et al.</i> , (2002)

Strategy	Study details	Outcomes	References
	Impact of home gardens on nutrient intake and access to food and dietary diversity	Increased dietary diversity, iron and vitamin A intakes improved among pre-school children	Selepe & Hendriks, (2014)
	Maize porridge fortified with ferrous sulphate and fed infants	Decrease from 45% to 17% of infants with anaemia	Faber <i>et al.</i> , (2005)
Fortification	High phytate maize porridge home fortified with multiple micronutrient powder with low doses of highly absorbable iron and zinc	Improved zinc absorption Reduction of iron and zinc deficiencies by 30.8% and 11.8% respectively	Troesch <i>et al.</i> , (2011)
	Iron biofortified beans supplemented to iron-depleted university women in the Philippines	Increased body iron stores and haemoglobin levels	Haas <i>et al.</i> , (2005)
Biofortification	Biofortified pearl millet fed to adolescents for 4 months	Increased iron stores and serum ferritin	Finkelstein <i>et al.</i> , (2015)
	Biofortification of leafy vegetables with iodine (rape and redroot pigweed)	Increased iodine concentration in the biofortified vegetables	Ligowe <i>et al.</i> , (2020)
	Biofortification of green and red pigmented lettuce with iron	Increased iron content in the biofortified samples	Giordano <i>et al.</i> , (2019)

2.10 Current strategies to alleviate micronutrient deficiencies in Botswana

Deficiencies of iron and zinc are still a significant challenge in Botswana (**Section 2.2.1.1**). Different approaches had been in place to alleviate the problem, and this review will discuss some of those strategies currently in place. After the 1994 micronutrient status study, vitamin A supplementation was launched (Botswana Government, 2009). This programme targets children aged 6-59 months and the distribution of the vitamin A capsules is biannual. The vitamin A supplementation coverage rate in Botswana is reported by the World Bank to be 91.1% (World Bank, 2011).

Botswana also developed the vulnerable-group feeding scheme programme, which was initially intended for drought seasons but was made a blanket programme in 1999 (Ministry of Health, 2005). This programme targets all children under five, aged from 6-59 months, and vulnerable groups such as orphans, mothers of multiple births, and selected pregnant and lactating women and patients with tuberculosis. The under-five food rationing aims to prevent protein-energy malnutrition (Ministry of Health, 2005). These provisions consist of food products called Tsabana and Malutu, which are pre-cooked sorghum-soya cereals nutritionally fortified with some micronutrients. To date, there are no studies that have evaluated the effectiveness of this feeding programme.

Moreover, fortification is not mandatory in Botswana; therefore, other groups not targeted by the scheme may still not receive adequate micronutrients. In Botswana, 95 % of salt is fortified with iodine to prevent iodine deficiency (Ministry of Health, 2005); however, no information is available on the prevalence of iodine deficiency in Botswana. Fortification is not mandatory in the country, although most food products in Botswana are imported from South Africa where the fortification of flour and maize meal is mandatory.

2.11 Dietary components that affect iron and zinc absorption

The antinutrients levels and mineral enhancers present in the diet influences mineral bioavailability of minerals (Gibson *et al.*, 2018). Plant-based diets contain high levels of antinutrients (Popova & Mihaylova, 2019). Major antinutrients found in plant-based diets include phytates, oxalates, condensed tannins, total phenolic content and oxalates (Natesh *et al.*, 2017). Antinutrients affect the absorption and utilisation of iron and zinc as they bind to the mineral and rendering it unabsorbable (Gibson *et al.*, 2018; Popova & Mihaylova, 2019). ALVs have high levels of antinutrients, hence bioavailability of iron and zinc is probably severely impaired. Therefore, it is critical to discuss how each of these components inhibits zinc and iron bioavailability in this section.

2.11.1 Phytate

The primary inhibitor of zinc and iron in plant-based diets including ALVs is phytate (**Figure 2-3**), and it forms insoluble complexes with iron and zinc making them unavailable for absorption (Gibson, 2006; Gibson *et al.*, 2010). Phytate binds mineral cations (**Figure 2-4**) and forms insoluble phytate-mineral complexes rendering minerals unabsorbable (Lopez *et al.*, 2002). Many plant tissues stores phosphorus primarily as phytic acid known as inositol hexaphosphate (IP6) or phytate (when in salt form) (Kumar *et al.*, 2010b). It accounts for 85% of the stored phosphorus (Oatway *et al.*, 2001). Other forms of phosphorylated inositol phosphate found in plant-based diets are inositol pentaphosphate (IP5), inositol tetrphosphate (IP4) and inositol triphosphate (IP3) (Dahdouh *et al.*, 2019). The presence of phytate in plant-based diets negatively affects the absorption of non-haem iron and zinc (Hurrell, 2002; Lönnerdal, 2000). Iron and zinc deficiencies result from consumption of high phytate diets over time especially when relying on plant-based staple foods as a source of these minerals (Al Hasan *et al.*, 2016; Platel & Srinivasan, 2016). People living in developing countries who are dependent on plant-based diets are at risk of these mineral deficiencies (Gibson *et al.*, 2010). However, the effect does not depend only on the phytate levels in food but on the molar ratios of phytate:mineral, which serves as an indicator to predict if minerals will be less bioavailable or not (Hotz & Brown, 2004; Hotz *et al.*, 2003). These molar ratios estimate mineral absorption. Phytate to zinc (Phy : Zn) molar ratios less than 5 predict relatively high zinc bioavailability, while Phy : Zn molar ratios between 5 to 15 predict moderate zinc bioavailability and Phy : Zn molar ratios more than 15 predict low zinc bioavailability (Hotz *et al.*, 2003). Phytate also forms complexes with zinc and calcium, and these complexes are even less soluble, decreasing zinc absorption and serving as a reasonable estimate for measuring zinc

bioavailability (Bindra *et al.*, 1986). The molar ratio of phytate and calcium to that of zinc ($\text{Phy} \cdot \text{Ca} : \text{Zn}$) measures the combined inhibitory effect of phytate and calcium on zinc bioavailability and molar ratios higher than 200 predict greatly impaired zinc bioavailability (Bindra *et al.*, 1986). Regarding iron, the desired molar ratios of phytate to iron ($\text{Phy} : \text{Fe}$) to predict good iron bioavailability should be less than 1, ratios above 1 indicate that iron bioavailability that is seriously impaired (Hurrell, 2004).

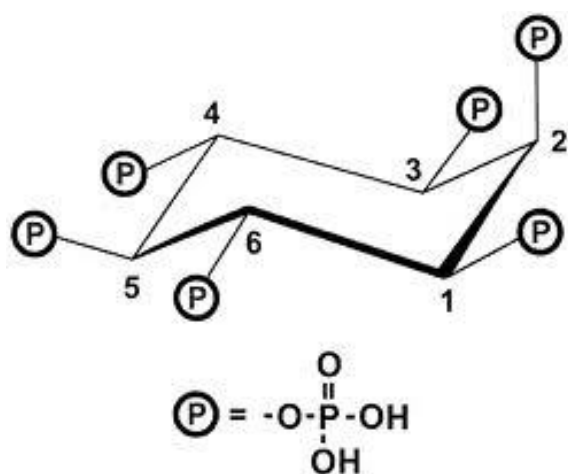


Figure 2-3: Chemical structure of phytic acid.

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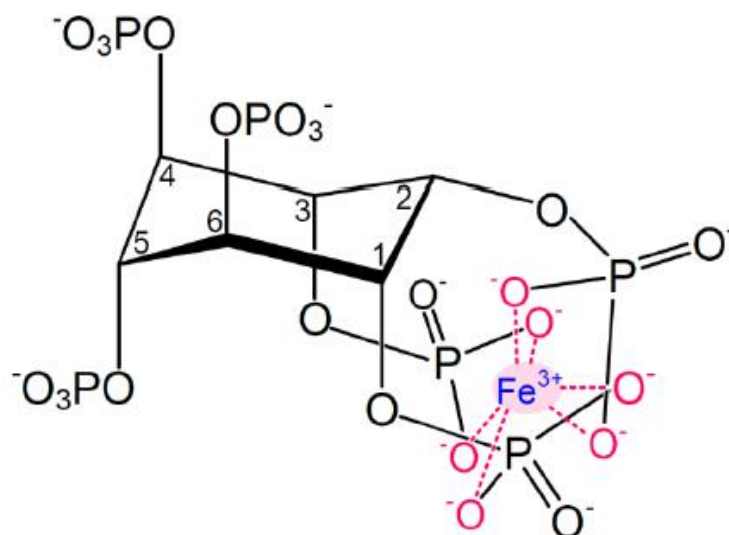


Figure 2-4: Structure of monoferric phytate demonstrating how Fe^{3+} is chelated.
 Reproduced with permission from a paper by Nielsen *et al.* (2013).

During non-haem iron absorption (**Figure 2-1**), the chemical form of phytate present in the gut determines the chelating affinity of phytate to iron (Nielsen *et al.*, 2013). Inositol rings which are more phosphorylated account for a robust chelating effect of phytate on iron and low solubility of the mineral (Nielsen *et al.*, 2013). Also, phytate demonstrates a dose-dependent effect on the absorption of iron (Ems & Huecker, 2019). For non-haem iron to be absorbed, it has to be in the ionic state (Mahan & Raymond, 2016). In the ionic state, the mineral is unstable and prone to sequestering by phytate (Goff, 2018). During the transportation of Fe^{+2} into the enterocytes, DMT1 receptors compete for the available iron with phytate (Lynch, 2002).

Phytate content in plant-based diets also affects zinc absorption. During digestion, zinc is released as free ions and binds to phytate to form insoluble complexes (Roohani *et al.*, 2013). Phytate is the principal dietary factor known to limit the bioavailability of zinc by binding to zinc firmly in the gut (Hambidge, 2010). Therefore, the consumption of phytate-rich diets is the leading cause of zinc deficiency in humans (Adams *et al.*, 2018). Previous studies using animal models reported that phytate rich diets impaired zinc absorption (Lönnerdal, 2000; Shockravi *et al.*, 2012). Same as iron, the effect of phytate on zinc absorption is dose-dependent (Lazarte *et al.*, 2015a), hence phytate-mineral ratios are used to predict zinc bioavailability. Despite the adverse effects of phytate on zinc absorption, the human intestinal lumen has a limited ability to hydrolyse phytate because of inability to produce the hormone phytase (Greiner & Konietzny, 2006). The inositol

phosphates actively impairing zinc absorption are IP6 and IP5 (Lönnerdal, 2000). In a study that investigated zinc absorption in humans from white bread containing 400 μmol of IP4, IP5 and IP6, about 41.5, 18.1 and 14.3% of zinc were absorbed respectively (Sandström & Sandberg, 1992). Another study gave confirmatory results using the Caco-2 model assay, reported that IP5 and IP6 strongly affected the uptake and rate of uptake of zinc by Caco-2 cells from plant derived foods than other inositol phosphates (Han *et al.*, 1994). The reductions in zinc transport were 44, 71, 89 and 96% by adding 100 μmol of IP3, IP4, IP5 and IP6 respectively.

A study which evaluated the inhibitory effects of phytate on both iron and zinc reported low bioavailability of these essential minerals (Pushpanjali & Santosh, 1996). The authors attributed the low bioavailability to the composition of the diets which consisted mainly of cereals, which have high amounts of phytate. Similar results were also reported in another study which looked at common Bolivian foods which consisted of cereals, legumes and tubers (Lazarte *et al.*, 2015a). Both studies showed that high concentrations of phytate from plant-based diets negatively affect the bioavailability of iron, zinc and calcium. Therefore, nutritional processing methods that reduce phytate and enhance mineral absorption are advised (Hemalatha *et al.*, 2007b; Hotz & Gibson, 2007). Phytate-mineral molar ratios can only estimate mineral absorption and hence *in vitro* and *in vivo* studies are needed to validate the outcome.

Despite the negative role of phytate in iron and zinc absorption, some studies have shown that it may have beneficial roles as an anti-oxidant and anti-carcinogen (Kumar *et al.*, 2010b; Singh *et al.*, 2003; Vucenik & Shamsuddin, 2003). However, this is outside the scope of this thesis.

2.11.2 Phenolic compounds

Phenolic compounds are abundantly present in ALVs (Moyo *et al.*, 2018). These are products of secondary metabolism of plants (Shahidi & Ambigaipalan, 2015). Phenolic compounds contribute to astringency, colour, bitterness and odour of the food product (Shahidi & Ambigaipalan, 2015). The carboxylic and hydroxylic groups in polyphenols form complexes with mineral cations and thereby inhibiting their absorption (Shahidi *et al.*, 2005).

Phenolic compounds are classified based on the number of the hydroxyl groups attached to the benzene ring (Pereira *et al.*, 2009). The main dietary phenolic compounds are phenolic acids (**Figure 2-5**), flavonoids (**Figure 2-6**) and tannins (**Figure 2-8**) (Hurrell *et al.*, 1999). Currently,

there are more than 8000 known phenolic structures, and within them, 4000 flavonoids have been identified (Tsao, 2010). The flavonoids consist of two benzene rings which are separated by a propane unit and abundantly present in plants (Kumar & Pandey, 2013). There is an association of flavonoids to decreased non-haem absorption (Lesjak & Srai, 2019). Quercetin strongly chelates non-haem iron in the duodenum reducing its basal transport (Lesjak *et al.*, 2014). The flavonoids prefer to chelate with iron at its 3-hydroxyl and 4-carbonyl group (Leopoldini *et al.*, 2006). The other mechanism in which flavonoids can reduce iron absorption is through complexing with Fe^{+3} with more stability than Fe^{+2} (**Figure 2-7**) making non-haem iron unabsorbable (Perron & Brumaghim, 2009). In a review of traditional Southern African leafy vegetables, leaves of amaranth and cowpea predominantly contained flavonoids (quercetin and rutin) (Sivakumar *et al.*, 2018). Therefore, iron availability from ALVs could be limited due to inhibitory effects from flavonoids.

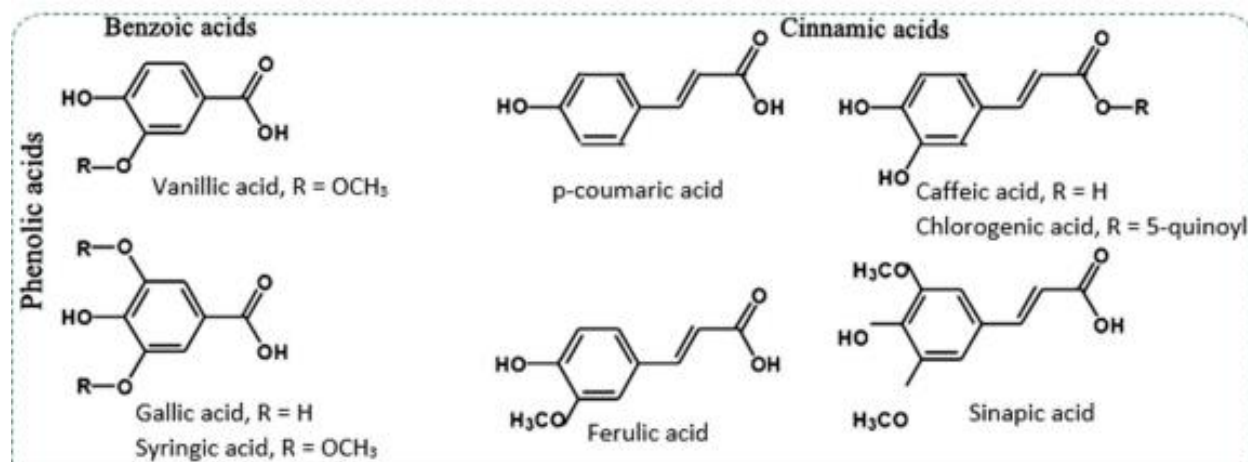


Figure 2-5: Basic structure of phenolic acids.

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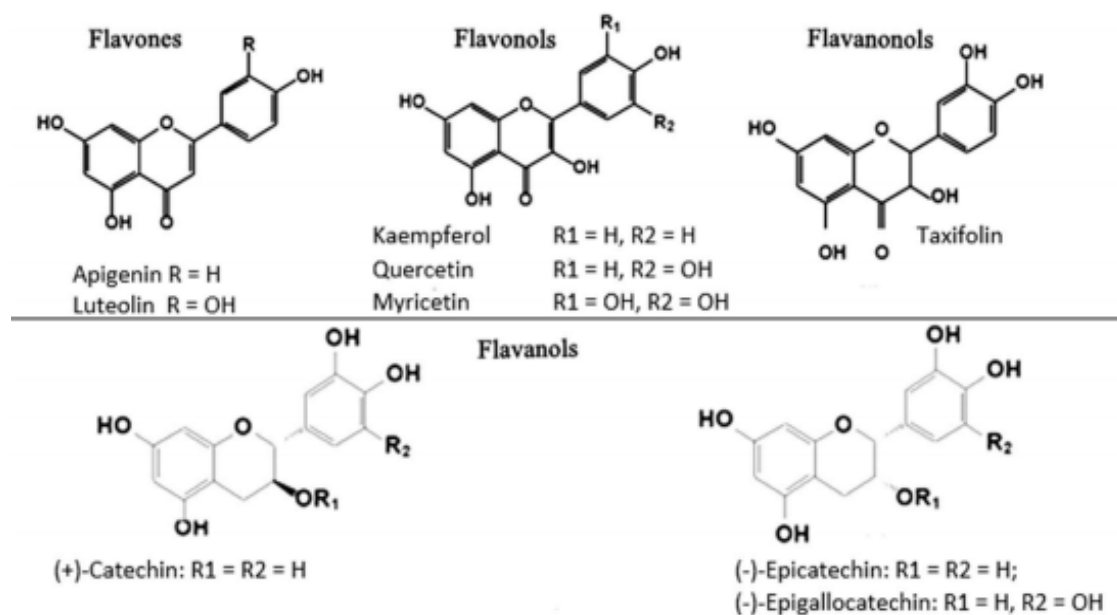


Figure 2-6: **Basic structure of flavonoids.**

Reproduced with permission from a paper by Tanase *et al.* (2019)

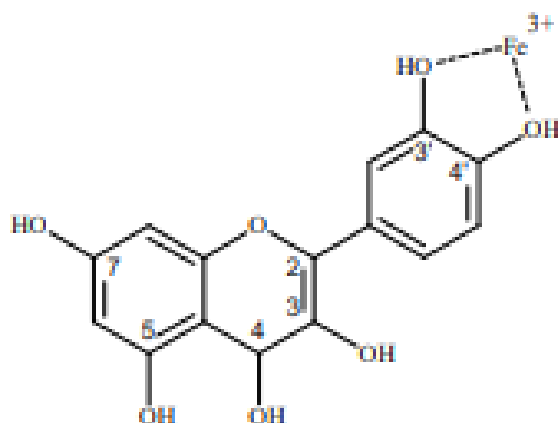


Figure 2-7: **Flavonoids (Quercetin) chelating Fe^{+3} .**

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Phenolic acids are mainly in elevated concentrations in leaves (Tanase *et al.*, 2019). Examples of phenolic acids include the hydroxybenzoic and hydroxycinnamic acids. Phenolic acids are insoluble bound compounds that support the formation of rigid cell wall structures (Shahidi & Yeo, 2016). Phenolic acids are found esterified with tartaric, quinic acids or carbohydrates (Lafay & Gil-Izquierdo, 2008). Phenolic acids containing a galloyl and catechol groups form insoluble complexes with iron rendering the mineral unabsorbable (Towo *et al.*, 2006). In a study that evaluated iron-chelating properties of phenolic acids bearing catechol and galloyl groups, reported that chlorogenic acid is the most potent chelator of iron (Andjelković *et al.*, 2006). In contrast, the same study reported that protocatechuic acid is the weakest chelator of iron (Andjelković *et al.*, 2006). The strength of the chelating effect of the galloyl group on iron depends on the galloy groups available. A single galloyl (3,4,5 trihydroxybenzene) group showed a low iron-binding capacity with a binding efficiency of 25% (Khokhar & Apenten, 2003).

Tannins have high molecular weight and are responsible for the bitter, astringent test in vegetables (Shahidi & Yeo, 2016). Gallic acid, catechin, tannic and chlorogenic acids belong to tannin compounds (Seigler, 1998). The two classes of Tannins are hydrolysable and condensed tannins (Shahidi *et al.*, 2005). Condensed tannins (**Figure 2-8**) are formed by carbon-carbon bond polymerization of flavane-3-ol (Chang *et al.*, 2019). They have a digestibility-reducing effect on proteins and carbohydrates (Koenig & Beauchemin, 2018). The ability of condensed tannins to form insoluble complexes with proteins inhibit digestive enzymes; hence these could affect the whole digestive process functionality (Shahidi & Ambigaipalan, 2015). The number of OH-groups in tannins determines its strength in iron-binding (Khokhar & Apenten, 2003). Hydrolysable tannins (**Figure 2-9**) hydrolyse efficiently in acid, alkaline or hot water conditions (Shahidi *et al.*, 2005). These tannins are classified as gallotannins and ellagitannins (Das *et al.*, 2019). Tannins complexes with iron making it unavailable for absorption (**Figure 2-10**). At pH ranges between 1-7 tannins forms complexes with iron which may be mono and bis-type complexes (Friman *et al.*, 2004).

A study that investigated the effect of phenol-containing beverages on the inhibition of non-haem iron reported an inhibitory effect on iron absorption (Hurrell *et al.*, 1999). Tea, coffee and cocoa drinks lowered the bioavailability of iron (Hurrell *et al.*, 1999). Similarly, a study that evaluated the effect of phenolic compounds in red wine on the iron absorption from vegetables and composite meals reported an inhibitory effect on iron absorption (Lucarini *et al.*, 2000)

Regarding zinc, a study performed in rats indicated that a diet that contains tannic acid does not affect the absorption of zinc, copper and manganese but reduces iron absorption (Afsana *et al.*, 2004). However, results from an *in vitro* dialysability assay indicated that the presence of tannins hinders zinc absorption (Lestienne *et al.*, 2005). Another study also reported that proanthocyanin binds zinc with high affinity, hindering its transport across the apical membrane of the enterocytes (Kim *et al.*, 2011). The difference may be due to the type of food and structure (Shahidi *et al.*, 2005) and the method used to assess the absorption (Carbonell-Capella *et al.*, 2014). Conflicting results reported by *in vitro* dialysability assay and animal studies may need to be confirmed further in human studies.

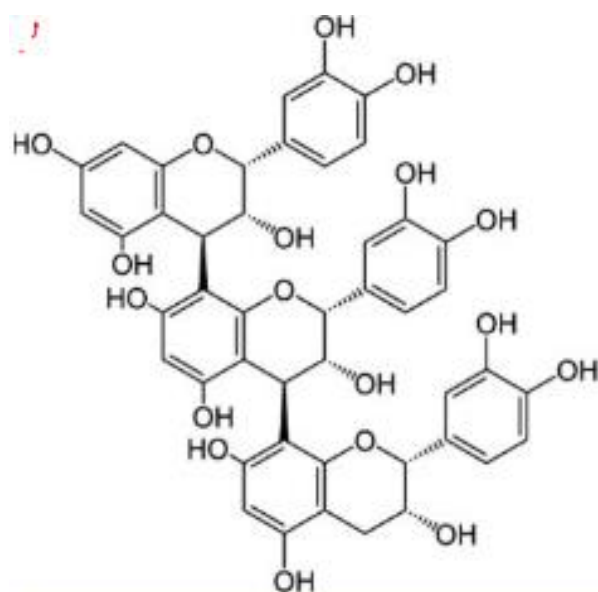


Figure 2-8: Condensed tannins structure.

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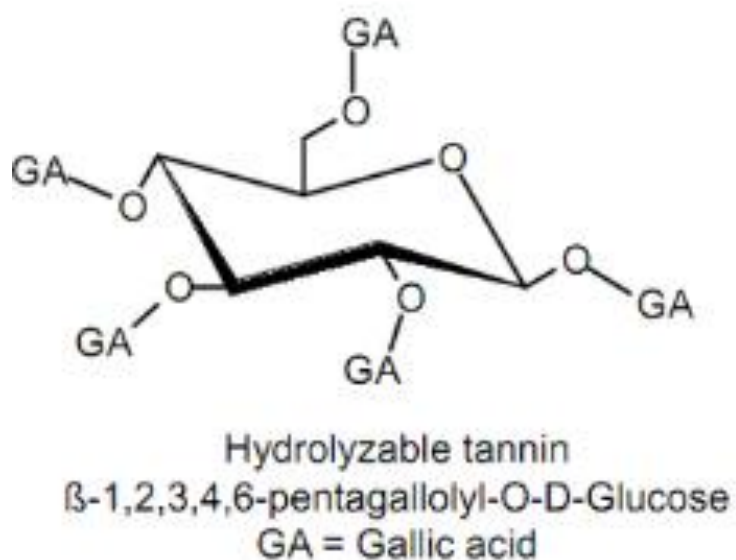


Figure 2-9: Hydrolysable tannins structure.

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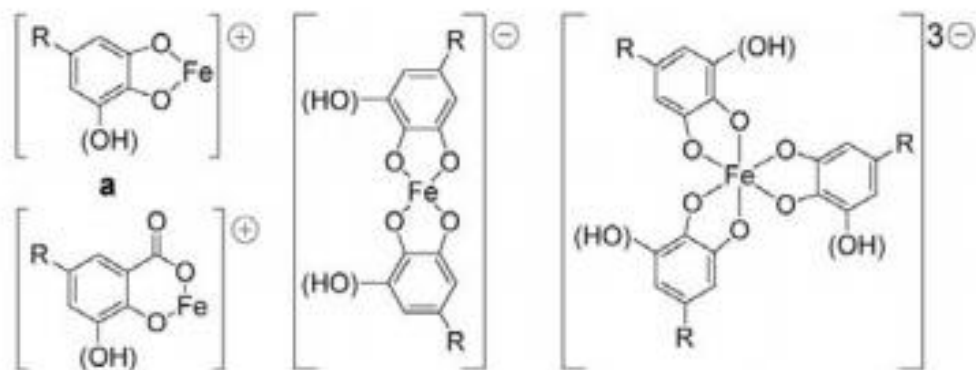


Figure 2-10: Tannin-iron complexes.

Reproduced with permission from a paper by Friman *et al.* (2004). R=residual part of tannin

2.11.3 Oxalates

Oxalates are naturally occurring molecules in plants and have an inhibitory effect on mineral absorption (Penniston, 2014). Oxalates increase as the plant ages and over-ripens (Słupski *et al.*, 2011). Oxalate has been extensively studied. Oxalates, also known as oxalic acid, and its salts are end products of metabolism in plant tissues (Noonan & Savage, 1999). Oxalic acids bind with iron, zinc, calcium and magnesium to form insoluble salts and also bind with sodium, potassium and sulphate to form soluble salts (Noonan & Savage, 1999; Penniston, 2014). These insoluble salts are unabsorbable rendering essential minerals unavailable for absorption in the gastrointestinal tract (GI). Oxalate is removed from the body by excretion through urine in the form of calcium oxalate (Alelign & Petros, 2018). Oxalate precipitates with calcium to form calcium oxalate, which results in the formation of kidney stones (Noonan & Savage, 1999; Słupski *et al.*, 2011). When the molar ratios of oxalates to calcium exceed 9 : 4, then the adverse effect of oxalate on calcium absorption is more pronounced (Noonan & Savage, 1999). Magnesium has a higher affinity to oxalates, thus when dietary magnesium levels are higher than calcium levels, it reduces the ability of oxalate to crystallise into stones (Kmieciak *et al.*, 1997). Oxalates form stable salts with Fe^{3+} and Fe^{2+} (Genannt Bonsmann *et al.*, 2008), and the Fe^{2+} oxalate is insoluble (Savage *et al.*, 2000). Oxalate complexes with Fe^{2+} blocking its absorption by the epithelial cells (Salovaara *et al.*, 2002), making iron unavailable for absorption. The mechanism in which oxalates inhibit iron and zinc bioavailability is unclear (Gibson, 2007).

An early study reported that 1 g of calcium oxalate added into a cabbage diet reduced iron absorption significantly (Gillooly *et al.*, 1983). In contradictory, iron absorption from wheat bread rolls with kale (10.7%) did not differ significantly from wheat rolls with kale plus 1.26 g potassium oxalate (11.5%) (Genannt Bonsmann *et al.*, 2008). The variation between the two studies could be associated with calcium, as it has direct dose-related inhibiting consequences on iron absorption (Hallberg *et al.*, 1991). The study by Gillooly *et al.* used calcium oxalate, and the calcium probably had some natural inhibitory effects on iron absorption hence results found.

From this review, high oxalate contents may pose a health risk. Compared to tubers, bulb and shoot vegetables, ALVs contained the highest amount of oxalates (Champ, 2002; Santamaria *et al.*, 1999). Previously amaranth has been found to contain high levels of total oxalate (9.1 g/100 g db) (Larsen *et al.*, 2003). So, oxalate content in amaranth has the potential to form insoluble complexes with calcium rendering calcium unavailable for absorption. Also, daily consumption of amaranth may not be advisable as it may expose humans to kidney stones.

2.11.4 Dietary fibre

Part of the plant material which is resistant to enzymatic digestion is called dietary fibre (Dhingra *et al.*, 2012). Dietary fibre binds to essential minerals and makes them unavailable for absorption. Green leafy vegetables, including ALVs, are rich in dietary fibre (Natesh *et al.*, 2017). Dietary fibre is classified based on gastrointestinal solubility, site of digestion and the type of polysaccharide present in the food product (Tunland & Meyer, 2002). Dietary fibre can be water-soluble or insoluble. Water-soluble dietary fibre includes pectin, gum and mucilage and water-insoluble fibre includes lignin, hemicellulose and cellulose (Dhingra *et al.*, 2012). Foods containing dietary fibre negatively affect zinc and iron absorption, possibly because fibre-containing foods also contain phytate (Lönnerdal, 2000; Thebaudin *et al.*, 1997). Some studies reported that it is difficult to separate the effects of fibre and phytate on the absorption of the essential minerals (Lönnerdal, 2000; Torre *et al.*, 1991). An early study reported that the removal of phytates improved zinc and iron bioavailability in the presence of dietary fibre (Morris & Ellis, 1989).

There is contradictory information on the negative effect of dietary fibre on iron and zinc absorption. In a study conducted in rats, which were fed a marginally zinc-deficient diet with the addition of insoluble dietary fibre (cellulose) reported a significant increase in iron and zinc absorption (Hayashi *et al.*, 2001). Contradictorily, a study which evaluated the effects of addition of soluble dietary fibre in infant formula reported a reduction in iron and zinc absorption (Bosscher *et al.*, 2003). The addition of high esterified pectin, low esterified pectin and oligofructose into the formula reduced iron bioavailability by 2.3%, 2.1% and 2.2% respectively. About zinc, the addition of locust bean gum and maltodextrin into formula reduced zinc bioavailability by 6.8% and 5.4% respectively (Bosscher *et al.*, 2003). Similar results of decrease in iron absorption were observed in a multiple nutrient formulation with the addition of soluble dietary fibre (Luciana *et al.*, 2012). However, in contrast, a study reported no significant effects on iron absorption after adding soluble fibre to a diet for iron-deficient anaemic rats (Feltrin *et al.*, 2009). The differences in the results may be due to the type of fibres used. The binding capacity of dietary fibre to minerals depends on the type of dietary fibre component available in the food product (Baye *et al.*, 2017). An earlier study which investigated the binding capacity of five insoluble components of dietary fibre reported that cellulose has a stronger binding capacity to zinc than hemicellulose and lignin (Claye *et al.*, 1996). Also, the methodological issues such as duration of the study and also not matching mineral contents of the diet may result in conflicting results (Shah *et al.*, 2009) and not

controlling for other inhibitors of iron and zinc absorption may result in conflicting results (Baye *et al.*, 2017). There are currently no studies which solely support the notion that dietary fibre on its own inhibits the utilisation of minerals. So the impairment effect of dietary fibre on iron and zinc absorption is associated with phytate contents (Baye *et al.*, 2017; Kumar *et al.*, 2010b). The level of dietary fibre and phytate content greatly varies in ALVs (Uusiku *et al.*, 2010); therefore, their impact on iron and zinc absorption will also vary.

2.11.5 Calcium

It has been widely documented that calcium inhibits iron absorption. Several studies conducted in humans, found the negative inhibitory effect of calcium on iron regardless of whether was given as calcium salts or dairy products (Lönnerdal, 2010). ALVs also contain appreciable amounts of calcium (**Table 2-2, Table 2-3, Table 2-4,**

Table 2-5). Calcium has been found to inhibit iron absorption in a single test meal setting (Minihane & Fairweather-Tait, 1998). Calcium inhibits the absorption of both non-haem and haem iron (Milman, 2020). As previously stated, the bioavailability of non-haem iron is naturally low because of the presence of antinutrients. The inhibitory effect of calcium on iron probably will further reduce its absorption. Several single test studies (Cook *et al.*, 1991; Hallberg *et al.*, 1991; Lynch, 2000), found that iron absorption was impaired by dairy products and calcium supplements due to the presence of iron and calcium simultaneously in the intestine and also when iron and calcium are administered combined in a fasting state.

A reduction by 50-60% of iron absorption was found in human subjects when doses of 300-600 mg of calcium were added to wheat rolls for short term dietary interventions (Hallberg *et al.*, 1991). However, long-term supplementation with calcium appeared not to have adverse inhibitory effects on iron absorption. In a study that supplemented healthy women with calcium for one month found that calcium did not affect iron bioavailability (Ríos-Castillo *et al.*, 2014). A study that assessed the association between calcium intake and iron status using a three-day food record also found no effect in serum ferritin and body iron stores of women and children (Van de Vijver *et al.*, 1999). A review concluded that the inhibitory effects of calcium on iron uptake shown in single test meals studies become complex in daily life dietary settings (Milman, 2020). Calcium interferes with the

transportation of iron through the enterocytes, possibly at absorption process which is typical for both haem and non-haem (Hallberg *et al.*, 1993). In an *in vitro* study using Caco-2 cells there was an increase in ferroportin expression at the basolateral membrane observed after addition of calcium resulting in a decrease in iron efflux and increase retention of iron in the cells (Lönnerdal, 2010).

Calcium alone does not seem to have a direct effect on zinc absorption in humans (Lönnerdal, 2000). A review concluded that the effect of calcium on zinc availability is less pronounced (Roohani *et al.*, 2013). However, calcium has the tendency to form complexes with phytate and zinc that are insoluble and consequently have an inhibitory effect on the absorption of zinc (Lönnerdal, 2000), hence making zinc unavailable for absorption.

2.12 Processing techniques to reduce inhibitors of iron and zinc in African leafy vegetables

ALVs contains high levels of phytates, oxalates, tannin and total phenolic contents (**Table 2-6**); therefore, the reduction of these antinutritional factors could improve their iron and zinc absorption. Antinutritional factors reduce the absorption of iron and zinc, as previously discussed (**Section 2.6**). Ways of reducing antinutrients in ALVs need exploration to improve essential mineral bioavailability (Essack *et al.*, 2017; Popova & Mihaylova, 2019). Home processing methods should reduce nutrient inhibitors (Hotz & Gibson, 2007). Previous studies reported that household processing methods reduced the contents of phytate, oxalates, tannin and total phenolic contents in plant-based foods (Gibson *et al.*, 2000; Gibson *et al.*, 1997; Hurrell, 2002; Noonan & Savage, 1999; Shahidi & Ambigaipalan, 2015). Household processing methods such as spontaneous fermentation and blanching are effective in reducing levels of antinutritional factors in ALVs (Essack *et al.*, 2017; Muchoki *et al.*, 2010; Wafula *et al.*, 2016). Although these processing methods are effective in the reduction of antinutritional factors, they also negatively result in mineral losses. A study that evaluated the effect of spontaneous fermentation on cowpea leaves reported a reduction in the content of iron (1.50 µg / g to 0.89 µg / g), calcium (16.37 µg / g to 14.52 µg / g), zinc (0.08 µg / g to 0.04 µg / g) and magnesium (0.67 µg / g to 0.38 µg / g) after fermentation (Kasangi *et al.*, 2010). The loss of minerals was due to discarding the liquid used for fermentation; hence minerals leached into the discarded liquid.

2.12.1 Spontaneous fermentation

Spontaneous fermentation is a method commonly used in sub-Saharan Africa to maintain quality, reduce post-harvest losses and ensure availability and consumption of ALVs when out of season (Masarirambi *et al.*, 2010; Natesh *et al.*, 2017; Wafula *et al.*, 2016). ALVs are used widely in the preparation of fermented products (Kasangi *et al.*, 2010). This method of preservation allows extended storage, which allows the consumption of ALVs throughout the year (Habwe *et al.*, 2008). Fermented ALVs are valuable, cheap and nutritionally beneficial to households in the developing world (Elisha *et al.*, 2016). Fermentation is beneficial in the reduction of antinutritional factors such as phytates, tannins and oxalates, which has inhibitory effects on iron and zinc absorption (Ifesan *et al.*, 2014). There is currently no literature available on the potential of spontaneous fermentation in improving iron and zinc absorption from ALVs.

During spontaneous fermentation, no starter cultures are added to the raw food to aid its fermentation and usually described as natural fermentation (Groenenboom *et al.*, 2019). Spontaneous fermentation occurs through micro-organisms naturally occurring in the raw food material supported by the conducive environment supporting their growth (Demarigny, 2012). Spontaneous fermentation occurs due to the presence of a mixed colony of micro-organisms such as yeast, bacteria and fungi (Chaudhary *et al.*, 2018). Lactic acid bacteria (LAB) and yeast are responsible for lactic acid fermentation and ethanol fermentation, respectively (Faria-Oliveira *et al.*, 2015). During lactic fermentation, LAB synthesizes lactic acid as the main by-product hence results in the acidification of the food product (Mokoena *et al.*, 2016). LAB includes species of the genus *Lactococcus*, *Lactobacillus*, *Streptococcus*, *Enterococcus* and *Weissella* (Todar, 2004). LAB contributes to the texture and taste of fermented food products and prevents food spoilage through the production of lactic acid, which inhibits the growth of food spoilage bacteria (Groenenboom *et al.*, 2019). During fermentation LAB breakdown sugars (such as glucose) in the food to lactic acid, carbon dioxide and ethanol (Todar, 2004). LAB obtains energy during carbohydrate metabolism, therefore, they can only function in environments with sugars (Saeed A & Salam A, 2013).

Spontaneous fermentation of ALVs in sub-Saharan Africa is home-based and conducted at small scale production (Oguntoyinbo *et al.*, 2016). ALVs do not contain enough sugar to support the growth of LAB, and therefore, for active fermentation to occur, the addition of fermentable carbohydrate is needed (Wafula *et al.*, 2016). In studies that examine the effect of fermentation on ALVs, a fermentable carbohydrate was routinely added (Kasangi *et al.*, 2010; Muchoki *et al.*,

2007). A study reported that, by adding 3% glucose to cowpea leaves, a fermentation with the highest concentration of lactic acid of 0.06% and with a pH of 4.7 could be obtained (Kasangi *et al.*, 2010). Except for Ethiopian kale (*Brassica carinata* A. Braun), which contains enough fermentable sugar, ALVs need about 2-3% of fermentable sugar for active fermentation to occur (Wafula *et al.*, 2016). The schematic fermentation process (**Figure 2-11**) summaries the spontaneous fermentation of ALVs.



Figure 2-11: Schematic diagram of the fermentation process of vegetables.
It is adapted from Swain *et al.* (2014) with some modifications.

Several researchers evaluated the potential of fermentation in reducing antinutrients in plant-based diets. Spontaneous fermentation degrades phytate and enhances mineral utilisation (Humer & Schedle, 2016). The mechanism of phytate reduction in fermentation occurs when phytate is degraded by activation of the phytase enzyme, which is naturally present in plants and cereal-based foods (Kumar *et al.*, 2010b). Phytase becomes activated at acidic pH conditions ranging from 4.5 to 5.5 (Sanz-Penella *et al.*, 2012). Phytase hydrolyses very stable phytate to free myo-inositol phosphates freeing divalent minerals, making them available for absorption (**Figure 2-12**). Sufficient degrading of phytate avert complexing and subsequent precipitation of minerals into insoluble complexes, in that way improving mineral absorption (Troesch *et al.*, 2013). The effectiveness of fermentation in reducing phytate content positively reported in cereals and grains. These studies reported the positive effect of fermentation on phytate content in sorghum and flour (Hemalatha *et al.*, 2007a; Kruger *et al.*, 2012; Makokha *et al.*, 2002; Oke & Bolarinwa, 2011). Furthermore, a study from India reported a reduction in phytate content in traditional Indian foods spontaneously fermented (22.2%) (Chandorkar & Kafila, 2012). However, there are limited studies on the effect of fermentation on phytate content in ALVs. In a study that evaluated the effect of fermentation on ALVs, the phytate content of fermented ALVs ranged from 27.5 – 70.67 mg / 100 g compared with unfermented ALVs, where the phytate content ranged from 40.0 -83.44 mg / 100 g (Akello, 2014). A decrease in the phytate content of cowpea leaves (19%), spider plant (31%) and pumpkin leaves (41%) after fermentation was reported (Akello, 2014).

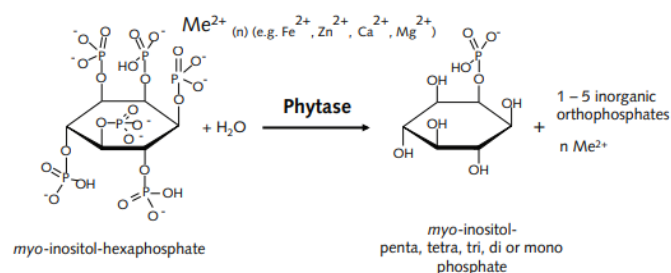


Figure 2-12: The activation of phytase on the phytate molecule.
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As stated, oxalates bind with iron, zinc, calcium and magnesium to form insoluble salts which hinder the bioavailability of these nutrients. Fermentation decreases the content of oxalate in food (Noonan & Savage, 1999). The mechanism by which fermentation reduces oxalate content in a

food sample is through the pH of the medium (Wadamori *et al.*, 2014). At pH lower than 6, the proportion of fully deprotonated divalent oxalate ions ($C_2O_4^{2-}$) is inversely proportional to its binding effect with mineral cations to form insoluble oxalates (Simpson *et al.*, 2009). Therefore, a reduction in pH reduces the proportion of insoluble oxalates formed and hence limits its inhibitory effects on minerals. Also, during fermentation, LAB degrades oxalates for carbon and energy, reducing its inhibitory effects (Goldfarb *et al.*, 2007). In a study that evaluated the levels of antinutrients in ALVs, a decrease of 72% of total oxalate content in the spontaneously fermented spider plant was observed (Akello, 2014). However, the study by Akello reported no effect on cowpea leaves. Similar results of no effect on oxalate content in fermented cowpea leaves were also reported by Muchoki and others (2010). Longer duration of fermentation results in more reduction of oxalates as the population of microbial species is produced (Oke & Bolarinwa, 2011). However, although cowpea leaves were fermented for 48hrs (Akello, 2014) and 16 days (Muchoki *et al.*, 2010) respectively, different durations did not result in any differences in oxalate content in these studies. Differences in results from these studies may be due to other factors, such as vegetable genetic appearances, levels of oxalates in the vegetable and method of oxalate analysis used in each study.

Phenolic acids, flavonoids and condensed tannins negatively affect the bioavailability of iron and zinc (Shahidi & Ambigaipalan, 2015). Fermentation could mitigate the adverse effects of these phenolic compounds on essential minerals bioavailability. During fermentation condensed tannins such as proanthocyanin are hydrolysed to phenols through the action of polyphenol oxidase (PPO) (Emmambux & Taylor, 2003). Acidic conditions cause the cleavage of proanthocyanins into flavan-3-ols, resulting in oxidation and transformation to quinones (Chen *et al.*, 2001). In a study conducted on fermented cassava leaves, there was a reduction of 86% in total tannin content in fermented leaves compared to unfermented leaves (Waluchio, 2016). A review concluded that the decrease in tannins after fermentation is due to enzymes activation during fermentation which causes the degradation of complexes of tannins and releases free tannins which then leach through the discarded fermentable medium attributing to a decrease in tannins content (Taylor & Duodu, 2015). Also, a reduction of tannins may be due to their ability to bind with proteins and other components, and these reduce their extractability and degradation by microbial enzymes (Beta *et al.*, 2000). A study which investigated the effect of fermentation on tannins in pearl millet reported an increase in tannin contents and the decrease was qualified to be due to the hydrolysis of condensed tannins such as proanthocyanins (Osman, 2011).

After spontaneous fermentation of cowpea leaves, a decrease of 28% of total phenols was observed (Muchoki *et al.*, 2010). The decrease of total phenolic content is due to the enzymatic activity of polyphenol oxidase (PPO) and peroxidase, which uses phenolic compounds as substrates (Adebo & Gabriela Medina-Meza, 2020). There are limited studies on the effect of fermentation on total phenols. However, studies conducted in fermented sorghum reported contradictory results. A decrease in total phenols was reported in sorghum (Adebo *et al.*, 2018; Dlamini *et al.*, 2007) while other studies reported an increase (Đorđević *et al.*, 2010; Mohapatra *et al.*, 2019). The increase in phenolic compounds during fermentation is due to the release of bound compounds which are mainly in the cell walls (Gan *et al.*, 2016). The transformation of condensed tannins during fermentation results in an increase in total phenolic content (Taylor & Duodu, 2015). This change has adverse effects on mineral bioavailability (Nkhata *et al.*, 2018). Also, during fermentation, the action of β -glucosidase hydrolyses phenolic glucosides to release free phenols which could increase (Adebo & Gabriela Medina-Meza, 2020). The acidic conditions causes the production of hydrolytic enzymes which degrade the cell walls resulting in free phenolics (Wang *et al.*, 2019)

Fermentation could be beneficial in the reduction of phytates, oxalates, total phenolic and condensed tannin contents in ALVs. Reduction in these antinutrients in ALVs will subsequently improve mineral absorption.

2.12.2 Blanching

Blanching reduces antinutrients in plant-based foods through leaching out into the discarded blanching mediums (Hotz & Gibson, 2007; Nwosu, 2010). Blanching involves thermal processing, where food is immersed in water to improve its quality by inactivating enzymes that result in food spoilage (Huang *et al.*, 2015). The appropriate blanching duration is vital, as blanching vegetables for longer results in loss of texture, flavour and colour while shorter duration stimulates enzymatic action and spoilage (Huang *et al.*, 2015). Blanching reduces antinutrients through the rupturing of plant cell walls, and this results in soluble compounds leaching into the medium used for blanching (Natesh *et al.*, 2017).

A blanching duration of between 10 to 15 minutes, in a study conducted in amaranth, fenugreek (*Trigonella foenum grecum*) and spinach resulted in a significant reduction of phytates and oxalates (Obboh, 2005; Yadav & Sehgal, 2003). Another study also reported that blanching

decreased phytates (58%) and oxalates (33%) in pumpkin leaves (*Cucurbita maxima*) (Fadupin *et al.*, 2014). However, the same study reported a significant loss of iron (41%), β -carotene and AA (31% each), potassium (64%) and sodium (50%). Blanching has also caused mineral and vitamin losses through leaching in the blanching medium (Mepba *et al.*, 2007). However, the hydrolysis of phytate is influenced by pH and temperature (Greiner & Konietzny, 2006). Phytate is heat stable and not easily destroyed by blanching; however, prolonged exposure to heat may lead to activation of phytase which helps in the breaking down of phytate (Kumar *et al.*, 2010b). Prolonged duration of blanching has a negative effect on minerals due to losses through leaching (Fadupin *et al.*, 2014; Kruger *et al.*, 2014). There is a need for exploring other processing methods which can reduce oxalates and phytates in ALVs and at the same time preserving mineral losses.

During blanching, cell membranes rupture, and this results in the release of phenolic compounds by leaching out into the blanching water (Oboh & Rocha, 2007). Several authors reported the reduction of phenolic compounds after blanching of ALVs (Bamidele *et al.*; Moyo, 2016; Zoro *et al.*, 2014). Blanching results in heat degrading of tannin molecules which changes their chemical reactivity, resulting in their reductions (Alonso *et al.*, 1998). The concentration and structure of tannin determine the extend of processing (Gazzani *et al.*, 1998). Mosha *et al.* (1995) reported that blanching significantly reduced tannin content in vegetables. In support, another study reported a decrease of 62.3% of tannins in blanched bitter yam (Egbunu & Nzewi, 2016a). The reduction in both studies was attributed to leaching out into the blanching medium which is always discarded after the process. Reduction of blanching time to less than 5 minutes is crucial to minimise mineral loss (Oulai *et al.*, 2015). The duration of the blanching is vital to avoid affecting the quality of the food.

2.12.3 Addition of exogenous phytase

During food processing, isolated phytase is added into food to optimise phytate dephosphorylation by naturally occurring enzymes (Kumar *et al.*, 2010b). Free inorganic phosphorus, along with a chain of intermediate myo-inositol phosphates, is released when phytase sequesters orthophosphate groups from the inositol ring of phytic acid (Debnath *et al.*, 2005). Phytase catalyses the sequential release of phosphate from phytate (**Figure 2-12**). Humans cannot produce phytase in their mono gastric stomach (Vashishth *et al.*, 2017); thus necessity a need to supplement. The microbial phytases are commercially available, making their use in food

processing feasible (Kumar *et al.*, 2010b). However, their efficacies depend on pH and temperature (Vashishth *et al.*, 2017). So, the added phytase should be active during food preparation and processing. The isolated exogenous phytase should have a strong phytate degrading power even at room temperatures, heat resistant and active over a broad range of pH (Vashishth *et al.*, 2017).

Addition of phytase during food processing has been effective (Bedford, 2000). A study that investigated phytate hydrolysis by phytase in cereals reported that iron solubility increased from 3% to 53% in wheat and from 5% to 21% in the rye (Sandberg & Svanberg, 1991). Similar results of an increase in iron absorption after dephytinization of phytate by the addition of exogenous phytase in rice porridge (1.73 to 5.34%), oats (0.33 to 2.79%) and maize (1.80 to 2.79%) were obtained (Hurrell *et al.*, 2003). When phytase was added to soybean/maize/sorghum ready-to-use food, a significant reduction of phytic acid (2.09 – 0.84 g / 100 g) was reported (Akomo *et al.*, 2016). There is enough promising evidence on the efficacy of phytase to degrade phytate in foods, but there is no information available on their use in ALVs. There is a need of research to explore the use of isolated exogenous phytase in reducing phytate content in ALVs.

2.13 Dietary components that enhance iron and zinc absorption

The balance between antinutritional factors and enhancers of iron and zinc, along with the essential mineral status of an individual, determines the bioavailability of that mineral (Gibson *et al.*, 2010). Despite all the antinutrients that inhibit the absorption of iron and zinc as discussed above by this review (**Section 2.11**), there are also some dietary components which enhance the absorption of iron and zinc.

2.13.1 Ascorbic Acid

Ascorbic acid (AA) enhances the absorption of non-haem iron from plant-based diets (Dasa & Abera, 2018). AA acts as a reducing agent by chelating Fe^{3+} , preventing it from forming insoluble bonded iron complexes (Thankachan *et al.*, 2008). It also reduces Fe^{3+} to a more absorbable Fe^{2+} iron (Teucher *et al.*, 2004). Therefore, AA enhances iron solubility. For AA iron absorption enhancing ability to be more pronounced, the meal should be subsequently consumed with AA (Teucher *et al.*, 2004). An earlier study validated that taking AA hours before the iron-containing meal did not improve iron absorption (Cook & Monsen, 1977). Consuming nutrients together as

far as iron absorption is concerned, counteracts technological difficulties that may result due to the instability of AA during food processing and storage (Teucher *et al.*, 2004). AA is sensitive to light, oxygen and heat (Singh *et al.*, 2016a). Heat destroys AA through the oxidation process (Naidu, 2003). A review reported that there was a lack of reduction in AA in iron absorption studies due to the addition of AA before cooking in test meals (Teucher *et al.*, 2004).

AA has been shown to enhance iron absorption by counteracting the inhibitory effects of phytate. In a study that served phytate-containing bread rolls with 50 g AA, iron absorption increased from 7.1% to 14.2%, and when doubling AA, iron absorption increased from 6.5% to 19.5% (Hallberg *et al.*, 1989). In meals containing antinutritional factors that reduce iron absorption, the action of AA in promoting iron absorption is more pronounced (Teucher *et al.*, 2004). For meals to effectively promote iron absorption in meals containing low to medium levels of antinutrients, the molar ratios of AA to iron should be 2:1 (Dasa & Abera, 2018). A study validated an increase in iron absorption by 291% in the IDA group when AA was added to the meal at a molar ratio of 2:1 (Thankachan *et al.*, 2008). Higher molar ratios of AA to iron are a requirement to promote iron absorption in meals with high levels of antinutrients (Dasa & Abera, 2018). The addition of AA at a molar ratio of 4:1 increased iron absorption by 350% and 343%, respectively, for the IDA and control group, were observed (Thankachan *et al.*, 2008).

The long-term effects of AA in improving iron absorption may be less than previously predicted in single meal studies (Dasa & Abera, 2018). In a study that evaluated the effect of AA intake on non-haem iron absorption reported no significant effect on the average iron absorption in the three days of the dietary period (Cook & Reddy, 2001).

AA has been reported not to influence zinc absorption because zinc does not need transformation to an oxidation state for intestinal absorption (Nair *et al.*, 2013). Earlier studies which evaluated the effect of AA on zinc absorption reported negative results of no improvement in iron absorption (Sandström & Cederblad, 1987; Solomons *et al.*, 1979). Consumption of AA rich foods with other meals containing iron antinutritional factors could be a vital strategy in improving iron status in people but not zinc.

2.13.2 Meat and meat products

Plant-based proteins differ with animal-based proteins in terms of amino acids composition and presence of antinutrients which affects their digestibility (Mariotti & Gardner, 2019). Most plant-based diets are low in one or more essential amino acids such as leucine, lysine, methionine and

tryptophan (Lonnie *et al.*, 2018). ALVs has been found to contain essential amino acids but at low levels (Ntuli, 2019). Previously the positive influence of animal-based proteins on non-haem iron has been reported, animal flesh such as poultry, meat, fish and seafood increases the absorption of non-haem iron and zinc from plant-based diets if consumed concurrently (Gibson, 2007). The absorption of non-haem iron in diets containing meat, chicken or fish were four times more than in diets containing eggs, milk and cheese (Bowman & Russell, 2001). Different animal muscle proteins such as beef, lamb, liver, chicken and pork were reported to enhance non-haem absorption differently (Reddy *et al.*, 2006). The mechanism by which meat and its products increase non-haem iron absorption is unclear (Dasa & Abera, 2018).

Nevertheless, Hurrell *et al.* (2006) hypothesized that meat stimulates the production of gastric acid in the stomach, promoting iron solubilisation. The solubilized iron is chelated by the amino acids under acidic conditions and maintained soluble for intestinal digestion and absorption hence enhancing non-haem absorption (Hurrell *et al.*, 2006). Incorporating animal tissues into diets counteracts the inhibitory effect of phytate (Bæch *et al.*, 2003). A study that compared the bioavailability of non-haem iron from bean meal cooked with salmon and one cooked without, showed consuming 196 g of kidney bean with 227 mg of phytate simultaneously with 100 g of salmon improved iron absorption by 48% in young iron-deficient women (Navas-Carretero *et al.*, 2008). Another study that investigated the levels of meat factor needed to promote iron absorption from iron complexed with phytic or tannic acid by Caco-2 cells, the presence of 1 g of fish doubled iron uptake (Engle-Stone *et al.*, 2005). The addition of protein overcomes the inhibitory effect of a meal rich in phytate-tannin (Navas-Carretero *et al.*, 2008). Also, similar results of enhanced non-haem bioavailability were reported when 50 g and 75 g of pork were added to basic meals in a phytate-rich meal. However, the quantity of protein used influenced the results as a lesser amount of pork (25 g) did not have any effect on iron bioavailability (Bæch *et al.*, 2003). The exact amount of animal protein expected to improve iron absorption is unknown (Lim *et al.*, 2013). Although animal protein enhances non-haem absorption, minerals from the animal source may also hinder its absorption. The addition of milk and yoghurt does not enhance iron bioavailability (Rosado *et al.*, 2005), due to an increase in levels of calcium from dairy products which inhibit iron absorption (Hallberg, 1998). Calcium phosphate forms an insoluble aggregate macromolecule with non-haem iron (Baney IV, 1991). The inhibitory effect of calcium on iron absorption is dose dependent. No adverse effects were observed at less than 40 mg of calcium per meal, while the maximum inhibitory effect is reached at more than 300 mg of calcium per meal (Grinder-Pedersen *et al.*, 2004).

Zinc absorption is considerably higher in the presence of animal sources of protein than plant-based sources (Maares & Haase, 2020). Previous studies reported that zinc absorption from plant-based diets tends to increase with protein intake (Engle-Stone *et al.*, 2005; Lönnerdal, 2000). During protein digestion amino acids such as cysteine and methionine bind to zinc from plant-based diets keeping it in a solution resulting in improved zinc absorption (Lönnerdal, 2000). As stated, plant-based proteins are relatively low in these essential amino acids hence a need to consume animal-based proteins to enhance zinc absorption. The addition of milk and yoghurt to a plant-based diet increased zinc bioavailability in women by 50% to 68%, respectively (Rosado *et al.*, 2005). Similar results of enhancing zinc bioavailability in a study which studied the effect of beef and soya proteins on absorption were found (Etcheverry *et al.*, 2006). A beef meal enhanced zinc bioavailability by 13.7% compared with a soy meal (10.1%). Therefore, protein sources quality and quantities used in enhancing iron and zinc absorption are crucial. Plant-based diets, including ALVs, contain high levels of condensed tannins (**Table 2-6**). As previously discussed, condensed tannins form tannin-protein complexes, which prevents protein digestion in the stomach (Feitosa *et al.*, 2018). Lack of protein degradation could affect the release of amino acids which are crucial in enhancing iron and zinc absorption.

2.13.3 Other organic acids

There are various organic acids other than ascorbic acids, such as citric, malic and lactic acid, present in foods. The most common group of organic acids consists of carboxylic acids, which contain one or more carboxyl groups (-COOH) (Quitmann *et al.*, 2013). They also contain the functional sulphonc acid (-SO₂OH) group, which is found mainly in onions and garlic (Quitmann *et al.*, 2013; Vaidya *et al.*, 2009). These acids are effective in enhancing the bioavailability of iron and zinc from plant-based diets (Hemalatha *et al.*, 2005; Scheers *et al.*, 2016). However, these acids are less effective than AA in stimulating iron absorption owing to their weak chelation, which may keep the metal ion in solution and aid its uptake by mucosal cells (Lönnerdal, 2000).

Several studies have evaluated the effects of different organic acids on the absorption of iron and zinc in plant-based diets. The use of sulfonic acid increased zinc and iron absorption. Sulfonic acid resulted in an increase in iron bioaccessibility in cereal (9.4 – 65.9%) and pulses (9.9 – 73.3%) (Gautam *et al.*, 2010a). In the same study, zinc bioaccessibility also increased in cereal (10.4 – 159.4%) and pulses (9.8 -49.8%) (Gautam *et al.*, 2010a). Garlic and onions are good sources of sulphur-containing amino acids such as cysteine (Corzo-Martínez *et al.*, 2007). An increase in the concentration of sulphur-containing amino acids, such as methionine and cysteine, increases the

bioavailability of iron and zinc (Ruel, 2001). However, there are limited studies that evaluate the effect of garlic and onion on the mineral accessibility of other vegetables. The use of garlic and onion as a means of improving iron and zinc bioavailability should be explored.

A study investigated the effect of citric acid (CA) on zinc bioaccessibility in rice and chickpeas, using *in vitro* dialysability assay (Hemalatha *et al.*, 2005). The bioaccessibility increased by 40% and 31% in rice and chickpeas, respectively, with the addition of CA in the meals (Hemalatha *et al.*, 2005). CA has been effective in enhancing iron absorption. Iron dialysability was 12-fold higher with CA added to the dough (Porres *et al.*, 2001). A study on ALVs reported that lime juice-acidulated sauces improved iron and zinc solubility from 42.2% to 66.8% and 54.0% to 82.0% respectively (Mawouma *et al.*, 2017).

2.14 Bioaccessibility and bioavailability of iron and zinc

The quantity of nutrients released from food samples after digestion to be absorbed in the gastrointestinal lumen is called bioaccessibility (Etcheverry *et al.*, 2012). The proportion of ingested minerals in the food, absorbed and utilised for normal metabolic and physiological functions and stored in the body, is described as bioavailability (Gibson, 2007). Bioaccessibility does limit the extent of nutrient bioavailability; that is, lower bioaccessibility significantly lowers bioavailability (Etcheverry *et al.*, 2012). Antinutritional factors in food (**Table 2-6**) have adverse effects on mineral absorption. The high mineral content in the food matrix does not always translate into higher mineral bioaccessibility and bioavailability (Carbonell-Capella *et al.*, 2014). Bearing in mind the high antinutrient levels in ALVs, it is crucial to assess the bioaccessibility and bioavailability of these minerals from ALVs. Different *in vitro* (simulated laboratory experiments) and *in vivo* (studies in animals and humans) studies have been conducted to assess the absorption of iron and zinc (Etcheverry *et al.*, 2012). *In vivo* models evaluate how the mineral components are absorbed through the intestinal wall into the blood for circulation while *in vitro* models simulate conditions of GI under controlled conditions and estimate bioavailability (La Frano *et al.*, 2014).

2.14.1 Methods to assess iron and zinc bioaccessibility

The bioaccessibility method of assessing iron and zinc accessibility involves enzymatic digestion under simulated conditions of the stomach and intestines (Etcheverry *et al.*, 2012). Bioaccessibility studies provide useful knowledge on possible interactions between food components and nutrients, food processing practices and food preparation, type of food matrix

and the likelihood for a nutrient to be absorbed (Galanakis, 2017). These *in vitro* studies are used as screening, categorizing and ranking tools and cannot substitute *in vivo* studies (Dias *et al.*, 2018). Bioaccessibility methods include solubility and dialysability assays (Etcheverry *et al.*, 2012).

2.14.1.1 Solubility

During *in vitro* solubility, the pH of the food sample is adjusted to two using hydrochloric acid (HCl) to simulate the gastric conditions of the GI after which there is an addition of pepsin. The food sample digested after pepsin addition (

Figure 2-13) then pH is adjusted to six and pancreatin is added with the continuation of digestion of the food sample (Au & Reddy, 2000). The supernatant is collected to measure the soluble mineral (Dias *et al.*, 2018). In the case of iron, the approach only deals with non-haem iron solubility since haem is directly absorbed (**Figure 2-1**) and enters enterocyte as haem (Nishito & Kambe, 2018). The supernatant mineral contents are analysed by one of the following; mass spectrometry (MS), atomic absorption spectrometry (AAS), inductively coupled plasma atomic emission spectrometry (ICP-AES) and high performance liquid chromatography (HPLC) (Etcheverry *et al.*, 2012). The percentage of solubility is calculated based on the amount of soluble mineral relative to the total amount of mineral in the food sample (Bryszewska, 2019).

2.14.1.2 *In vitro* dialysability assay

The *in vitro* dialysability assay involves the factionalism of the sample using a dialysis bag (Kapsokefalou *et al.*, 2005). The dialysis bag usually contains a base (sodium bicarbonate) to neutralise the pH in the intestines (Cardoso *et al.*, 2015). The model is a modification of the solubility model. The method simulates the digestive conditions in the stomach and the intestine.

In most cases, agitation simulates the peristaltic movements with optimum temperature, and pH maintained (Kabak & Ozbey, 2012). To account for the physical and rheological reality in human digestion, the model divides the digestive process into two phases (Knockaert *et al.*, 2012). The two phases are gastric and intestinal (**Figure 2-13**) and the pH of each phase differs. The two phases represent the stomach and the small intestine (Cardoso *et al.*, 2015). The methodology considers that conditions in the digestive tract change over time and simulates them. The *in vitro* dialysability assay measures soluble minerals of lower molecular weight based on equilibrium

dialysis (Etcheverry *et al.*, 2012). Following the gastric phase, which is similar to the one in the solubility model, there is an inclusion of a dialysis tubing of a specific molecular weight cut off (MWCO) (Dias *et al.*, 2018). As previously stated, that the dialysis tubing contains a buffer, the buffer usually diffuses out of the bag into the peptic digest to neutralise it (Etcheverry *et al.*, 2012). After incubation, a pancreatin/bile mixture is added, followed by incubation. The amount of mineral in the dialysate (dialysis bag) is analysed to determine the total dialysable mineral (Bryszewska, 2019). The mineral contents in the dialysate are analysed, similar as in the solubility model. According to Etcheverry *et al.* (2012), the notion of dialysability is that dialysable compounds are available for absorption into the small intestine. The dialysability assay for iron was earlier introduced by Miller *et al.* (1981), consequently other studies modified the method to assess the bioaccessibility of other minerals such as zinc, calcium and magnesium (Kruger *et al.*, 2018; Kruger *et al.*, 2015; Silva *et al.*, 2017).

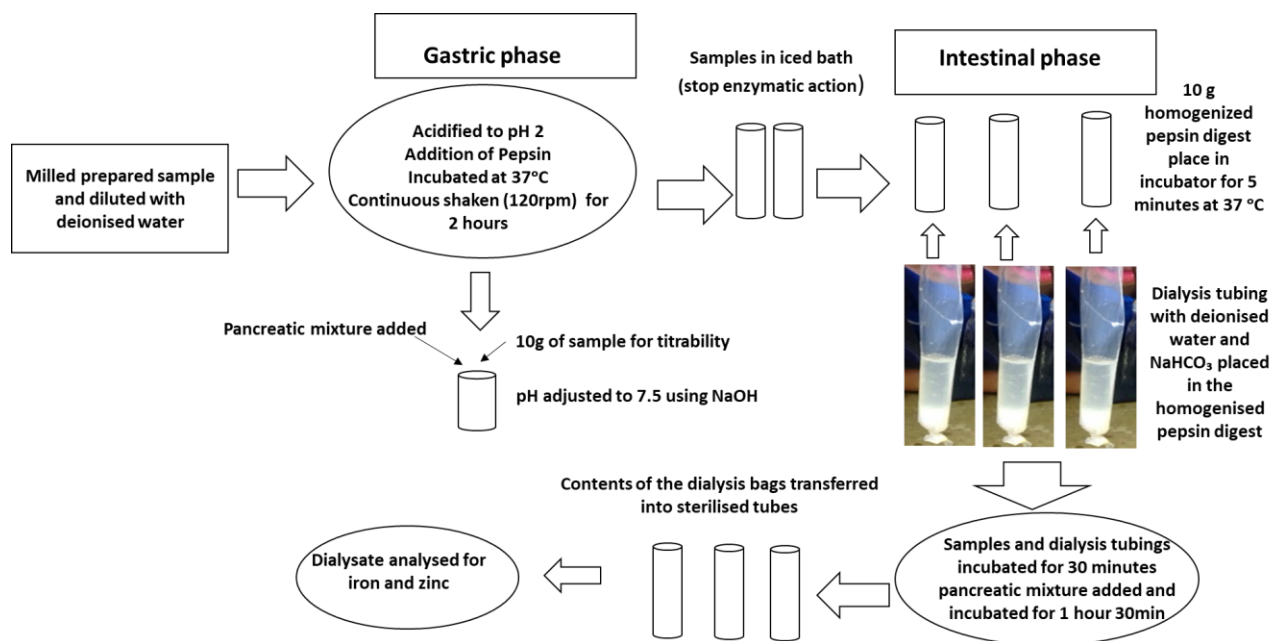


Figure 2-13: Schematic diagram of in vitro dialysability.
Adapted from the method used by Kruger *et al.* (2012).

A comparison of data from *in vitro* dialysability assays and human studies in an inter-laboratory study that involved nine laboratories found an agreement between *in vitro* dialysability results and human study results (Luten *et al.*, 1996). Chiplonkar *et al.* (1999) found that *in vitro* iron and zinc dialysability studies correlated with human studies data strongly. On the contrary, a study that assessed the bioaccessibility of carotenoids and vitamin E from the primary dietary sources validated *in vitro* simulated models against human studies (Reboul *et al.*, 2006). The study found that spinach lutein was more bioaccessible *in vitro* than in humans. They point out that their disagreements in results may be due to other factors such as incubation duration, pH and centrifugation specifications which differ from one study to another. These pose a challenge in comparing simulated *in vitro* results (Reboul *et al.*, 2006). The reliability of *in vitro* dialysability assay data is questionable; for instance, small polyphenol iron complexes dialyzed while high molecular weight protein ferritin that is bioavailable is excluded (La Frano *et al.*, 2014). Also, the model fails to evaluate the effect of high dose consumption of minerals on absorption because of a lack of biological feedback mechanism (Fairweather-Tait *et al.*, 2005). Therefore, simulated *in vitro* models may underestimate or overestimate the direction of mineral bioavailability due to challenges cited above.

The bioaccessibility of iron and zinc from ALVs has been previously studied. In a study that evaluated the contribution of ALVs and maize composite porridge meals towards iron and zinc nutrition reported that iron bioaccessibility ranged from 6.7% (0.9 mg/100g) to 45.2% (5.1 mg/100g). In comparison, zinc bioaccessibility ranged from 6.4% (0.63 mg/100g) to 12.7% (1.63 mg/100g) (Kruger *et al.*, 2015). The bioaccessibility of zinc from ALVs in this study was observed to be lower compared to iron.

2.14.2 The use of cell cultures to predict mineral bioavailability

The use of both cell culture and *in vitro* digestion models present an alternative model to predict iron and zinc bioavailability especial for studies not easy to conduct in animals and humans due to ethical issues (Dias *et al.*, 2018). The bioavailability of minerals from food is predicted by the human colorectal adenocarcinoma cells (Caco-2) through the determination of mineral uptake, transport or both (Etcheverry *et al.*, 2012). Caco-2 cells are epithelium cells derived from the small intestine (Verhoeckx *et al.*, 2015). Caco-2 cell line simulates the absorption in the human intestinal epithelium (Etcheverry *et al.*, 2012). Caco-2 cells cultured appropriately on Transwell permeable

inserts differentiate to contain microvilli-like structures with features typical of absorptive enterocytes with brush borders as found in the small intestine (Verhoeckx *et al.*, 2015). Transwell permeable inserts are permeable support that fits into the conventional multi-well cell culture plates and has a tiny, porous membrane (Miller *et al.*, 2017). Transwell permeable inserts offer a simplified technique of culturing polarized cells as their membrane allows uptake and secretion of molecules by the Caco-2 cells on both the basal and apical surfaces promoting metabolic activities that simulate what occurs in the human body (Miller *et al.*, 2017). Caco-2 cells are seeded on the Transwell permeable inserts which facilitates the formation on a monolayer which simulate the intestinal epithelium, the apical chamber simulating the intestinal lumen and the basolateral side representing the serosal blood side (**Figure 2-14**) (Langerholc *et al.*, 2011). The duration needed for Caco-2 cells to fully differentiate and form confluent and tight monolayers in Transwell inserts is three weeks (Hubatsch *et al.*, 2007). The Transwell permeable inserts allow collection and measurement of minerals absorbed through the apical membrane and released through the basolateral membrane (Etcheverry *et al.*, 2012). Caco-2 cells are deemed the gold standard of the intestinal model when cultured on Transwell permeable inserts (Tan *et al.*, 2018). Cells cultured on Transwell inserts develop tight junctions, have brush-border microvilli, and the intestinal epithelium layer of these cells contains metabolic enzymes (Verhoeckx *et al.*, 2015). The formation of a tight monolayer with the Caco-2 cell model exhibits a feature that allows physical and biochemical barriers for the passage of ions and small molecules through the cells (Tan *et al.*, 2018). As these cells simulate human epithelial cells, they have been widely used in mineral absorption studies (Tan *et al.*, 2018). For instance, essential minerals can be traced from the apical compartment, transported into the cell through the intestinal epithelium. The method is combined with an *in vitro* digestion model to predict bioavailability and absorption of minerals from digested food samples (Lichtenstein *et al.*, 2015). However, the Caco-2 cell model lacks the mucus layer on of the essential component of the intestinal epithelium (Etcheverry *et al.*, 2012). The human intestinal epithelium is composed of enterocytes that include goblet cells (**Figure 2-14**) for producing and secreting mucin (Johansson *et al.*, 2013; Leal *et al.*, 2017). Mucus provides an imperative physiological luminal factor for intestinal zinc uptake and absorption (Maares *et al.*, 2018). However, a study by Kim *et al.* (2012) reported that the combination of peristaltic motion and fluid flow in a microfluidic device resulted in Caco-2 cells showing intestinal villi-like structures with physiological growth up to seven hundred microns in height. An increase in intestine-specific functions, including mucus function, was also observed. To offset the effects of lack of mucus layer which results in detrimental effects on mineral absorption, it is recommended that the mucus layer be simulated by adding isolated (porcine) mucins on top of

the three-dimensional Caco-2 monocultures (Jin *et al.*, 2006; Maares *et al.*, 2018). However, the isolated and purified mucin *in vitro* does not display the same viscoelastic and gel-forming properties of the GI mucous layer found *in vivo* (Schömig *et al.*, 2016). Also, isolated mucins are not like transmembrane mucins which form a fraction of the mucus *in vivo* (Johansson *et al.*, 2013). Therefore, it is advisable to co-culture Caco-2 cells together with goblet cell line HT-29MTX (Maares & Haase, 2020) to facilitate mucus production. The strength of this type of co-culture is its ability to optimize the cellular permeability of conventional Caco-2 monocultures (Maares & Haase, 2020). The cell model also does not have intestinal blood fluid flow and peristaltic motions (Maares & Haase, 2020). Intestinal peristaltic movements support the transport of chyme along the intestine, and it enhances mechanical food component degradation (Welcome, 2019). This is an essential factor that affects digestion and availability of mineral for absorption.

A dialysis membrane secured with a silicone o-ring is placed on top of the cell monolayer to prevent enzymes from reaching the cells to avoid cell enzymatic degradation by enzymes from the intestinal digest applied (Glahn, 2009). In some methods, the intestinal digest is heated at 100 °C to inactivate the enzymes used in digestion (Frontela *et al.*, 2009). However, this method can have an impact on protein bioavailability because protein is denatured at 100 °C (Frontela *et al.*, 2009). Alternatively, enzymes can be inactivated by acidifying the digest to pH 2 (Frontela-Saseta *et al.*, 2011). These steps are representative of the physiologically processes of the *in vivo* conditions (Etcheverry *et al.*, 2012).

Caco-2 cell models are efficient and straightforward to conduct (Kumar *et al.*, 2010a). Animal models are time-consuming, labor-intensive, costly and also present concerns on ethical issues (Tan *et al.*, 2018). Also, animal models are generally not able to predict results in humans accurately (Shanks *et al.*, 2009). Therefore, this makes the *in vitro* model using the Caco-2 cells the appropriate choice for prediction of nutrient absorption in humans. The *in vitro* digestion method combined with the Caco-2 cell model is usually used to screen mineral bioavailability before human trials are conducted (Hur *et al.*, 2011). Also, this model has been validated and correlated with data from humans (Cilla *et al.*, 2018).

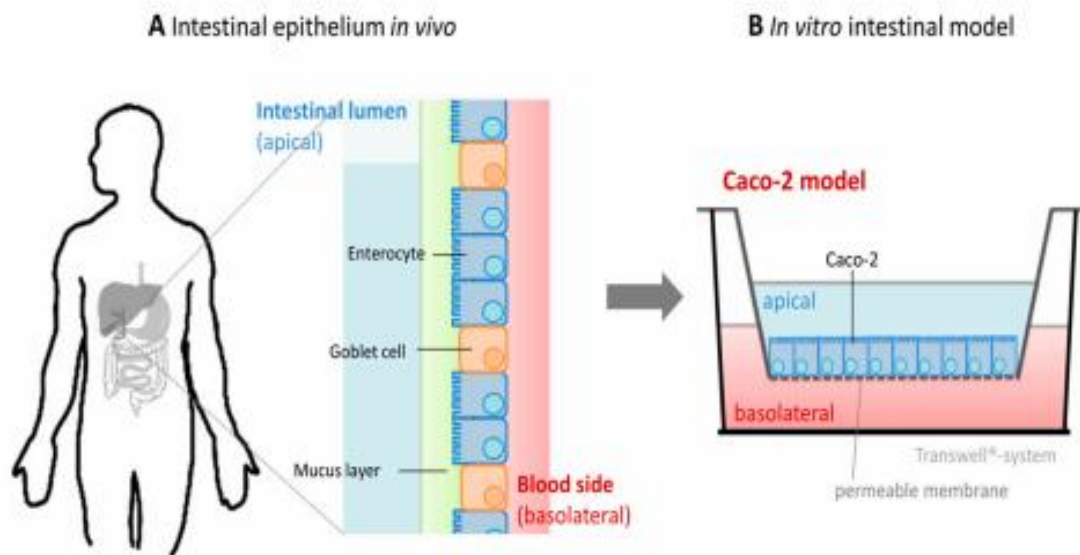


Figure 2-14: The three-dimensional in vitro cellular intestinal model schematic representation.

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2.14.3 Prediction of Iron and zinc bioavailability using the Caco-2 model assay

Mineral bioavailability is estimated based on the *in vitro* digestion, measurement of the solubility of the mineral and the measurement of mineral absorption by the Caco-2 cell line (Cilla *et al.*, 2018). Therefore, the *in vitro* digestion method combined with the Caco-2 cell model is a suitable method for estimating bioavailability in food by measuring nutrient uptake and transport by the cells (Eagling *et al.*, 2014). The method is suitable for measuring the bioavailability of iron and zinc from food products (Etcheverry *et al.*, 2012). The Caco-2 cell line stores iron in the form of protein, *i.e.* ferritin (Glahn, 2009; Sandberg, 2010). Iron uptake from the cells is measured as ferritin formation by the Caco-2 cells (Scheers *et al.*, 2014; Viadel *et al.*, 2007). Ferritin formation by the Caco-2 cell line effectively predicts iron bioavailability *in vitro* (Glahn, 2009). Ferritin formation positively correlates with cell iron content (Viadel *et al.*, 2007). Caco-2 cell ferritin formation is assessed through the enzyme-linked immunosorbent assay (ELISA) from kits commercially available, and these are the same kits that are used to measure human ferritin in clinical settings (Tako *et al.*, 2015).

Zinc bioavailability is also estimated by evaluating the nutrient uptake and transport by Caco-2 cells (García-Nebot *et al.*, 2013). Unlike iron uptake, which can be estimated by cell ferritin formation, biomarkers for zinc uptake are not available (Dias *et al.*, 2018). Metallothionein has been proposed as a biomarker for zinc uptake; however, it stores and binds other minerals such as copper and selenium (Bell & Vallee, 2009). Therefore, this protein cannot be used as a biomarker for zinc as it is not zinc specific (Etcheverry *et al.*, 2012). However, zinc uptake and transport from the Caco-2 cell line are measured through atomic spectrometry methods (García-Nebot *et al.*, 2013).

Also, the bioavailability of iron and zinc using Caco-2 cell assay can be predicted by extrinsic or intrinsic labelling of test food samples with radioactive isotopes of Fe and Zn (Dias *et al.*, 2018). The test samples are labelled with radioactive tracers added either extrinsically or intrinsically (Van Campen & Glahn, 1999). Intrinsic labelling the isotope tag is incorporated during plant growth (King *et al.*, 2000). Extrinsic labelling, which is the most used with Caco-2 cells, involves adding the isotope directly into the test meal (House, 1999). Previous bioavailability studies estimated uptake by extrinsic labelling of test meals with radiolabelled tracers of ^{59}Fe for iron and ^{65}Zn for zinc (Jou *et al.*, 2012). Then radioactivity is counted in the gamma counter (Van Campen & Glahn, 1999). The procedure assumes that the isotopes added extrinsically into the test meals exchanges with all forms of the element that naturally occur in the meal, so both extrinsic and unlabelled native element uptake is from the shared pool (King *et al.*, 2000). Different studies compared the effect of intrinsic and extrinsic labelling and mineral uptake (Boza *et al.*, 1995; Fairweather-Tait *et al.*, 1991; Meyer *et al.*, 1983). These studies concluded that uptake of minerals depends on the type of food used.

The use of Caco-2 cell assay to measure bioavailability of iron and zinc is validated against human studies. In a study by Beiseigel *et al.* (2007) which compared Caco-2 cell uptake data to human absorption measuring iron uptake from a different type of beans similarities between the *in vitro* and *in vivo* assay were reported for one bean type. They stated that different polyphenol composition of the beans might have attributed to the difference in mineral uptake in both beans. In agreement, a similar trend of similarities between *in vitro* and *in vivo* studies was observed in a study that evaluated the effect of ascorbic acid on iron absorption in a synthetic meal (Aragón *et al.*, 2012). The *in vivo* and *in vitro* data were strongly correlated ($r = 0.9765$; $P = 0.0008$). Also, the same study validated *in vitro* and *in vivo* methods by assessing the effect of iron absorption promoter Na_2EDTA and three iron fortificants (ferrous fumarate, NaFeEDTA and Ferrous sulphate) on non-haem iron absorption in Guatemalan girls aged 12-13 years consuming a meal

of maize tortillas with refried black bean paste. The *in vitro* results displayed a similar trend to the *in vivo* results for NaFeEDTA and ferrous sulphate but not for Na₂EDTA and ferrous sulphate. Variability between *in vitro* and *in vivo* studies were linked to large intersubject variability in iron uptake based on different iron need in human subjects. In agreement, a study by (Luten *et al.*, 1996) reported a statistically significant correlation between *in vitro* and *in vivo* methods. In contrast, a study by (Sarriá & Vaquero, 2001) that compared *in vivo* and *in vitro* methods in evaluating iron and zinc bioavailability reported no correlation between *in vitro* and *in vivo* data.

The use of the *in vitro* digestive method in combination with Caco-2 cell culture models is useful in estimating the bioavailability of iron and zinc; however, the assay has some limitations as discussed above, one must be mindful of their limitations when inferring data from *in vitro* to *in vivo* situations.

2.14.4 The use of animal and human studies for the measurement of mineral absorption

Animal and human studies look at the actual absorption of minerals by the body (Cardoso *et al.*, 2015). Iron bioavailability may be assessed in animals through haemoglobin repletion assay to obtain the relative measure of iron uptake (Dias *et al.*, 2018). Usually, at the beginning of the study, a baseline blood sample is collected to measure haemoglobin concentration, then the animals are fed test diets for two to three weeks and the final blood samples are collected to evaluate haemoglobin concentrations (La Frano *et al.*, 2014). To calculate haemoglobin repletion efficiency (HRE), the blood volume is estimated from body weight (Tako *et al.*, 2009). Although many animal studies have been previously conducted and identified as appropriate models to predict mineral bioavailability in humans, no animal model can accurately simulate human physiological responses (La Frano *et al.*, 2014). Although rat model studies are widely used to evaluate the bioavailability of iron in food their mechanism of iron absorption, haemoglobin repletion and depletion differ from that of humans (Tako *et al.*, 2009). The use of rodents to assess iron may not be accurate as they endogenously synthesise AA which promotes iron absorption (Sharp, 2005). Therefore, rats may overestimate non-haem iron bioavailability in plant-based diets in humans. The level of phytase activity is high in rats compared to humans which could overestimate non-haem iron bioavailability in humans (Sharp, 2005). A study by Iqbal *et al.* (1994) reported a phytase activity level of 30 times less in human intestines than those in rat tissue. The use of poultry may be suitable and sensitive to evaluate iron bioavailability because of their quick

response to iron deficiency (Tako *et al.*, 2011). Modern broiler chicken grows faster and responds to mineral deficiencies such as iron and have limited ability to synthesize AA (Tako *et al.*, 2016; Tako *et al.*, 2011). The use of broilers in previous studies showed a good agreement with results obtained through the Caco-2 assay (Tako *et al.*, 2009). Piglets have also been used to determine iron bioavailability due to similar GI anatomy and physiology between humans and pigs (Dias *et al.*, 2018).

The use of stable and radioactive isotopes in human studies allows for discrimination between the dose of mineral provided and the natural forms of the mineral, allowing for a more accurate evaluation of bioavailability (La Frano *et al.*, 2014). Radioactive and stable isotope techniques are well established and developed to measure iron and zinc bioavailability in humans (Carbonell-Capella *et al.*, 2014; Fernández-García *et al.*, 2009). The use of stable isotopes involves body counting to determine retention levels and the distribution patterns of the mineral isotope in the human body (Butler *et al.*, 2017). Radio-isotopes are forms of an element that emits radiation, while stable isotopes are naturally occurring in nature and food products in fixed amounts (IAEA, 2012). The disadvantage of using radioactive isotopes in human studies is their infinite half-lives (IAEA, 2012). This introduces a time limitation factor for storage of the isotope before study inception, collection, and analysing of samples after dosing (IAEA, 2012). Also, there is a difficulty in disposing of radioactive waste, and a licence is needed to administer radioactive isotopes to human subjects (IAEA, 2012). Stable isotopes are used for both children and adults as they are considered safe (Abrams, 1999; Hurrell *et al.*, 2003; Manary *et al.*, 2000). Stable isotopes are used to determine differences in bioavailability that can result due to dietary factors using test meals that have been biofortified, fortified, processed to reduce inhibitors and have enhancers of mineral absorption (Sheftel *et al.*, 2018). Food samples are evaluated for iron and zinc bioavailability by extrinsically labelling with the mineral isotope (**Figure 2-15**, **Figure 2-16**). For iron bioavailability, two test meals are created with different iron isotopes, and their bioavailability is evaluated by comparing ratios of the staple isotopes before and after dosing to determine the amount of iron in red blood cells (Walczyk *et al.*, 2005). Previous studies used stable iron isotopes such as $^{58}\text{FeSO}_4$ and $^{57}\text{FeSO}_4$ to determine iron bioavailability by measuring the isotopic dilution through the blood (Cercamondi *et al.*, 2014; IAEA, 2012; Petry, 2014).

Zinc absorption is measured by administering baseline zinc intravenously and on day one and two zinc isotopes are administered orally with test meals then urine samples are collected at different days for analysis (Rosado *et al.*, 2009). Zinc bioavailability is commonly measured

through the dual tracer ratio method, where one isotope is orally administered, and the other isotope is intravenously administered (Butler *et al.*, 2017). For zinc stable isotopes ^{67}Zn , ^{68}Zn and ^{70}Zn were utilized to determine zinc bioavailability through measurements of the isotopic labels in urine (Davidsson & Haskell, 2011; Rosado *et al.*, 2009).

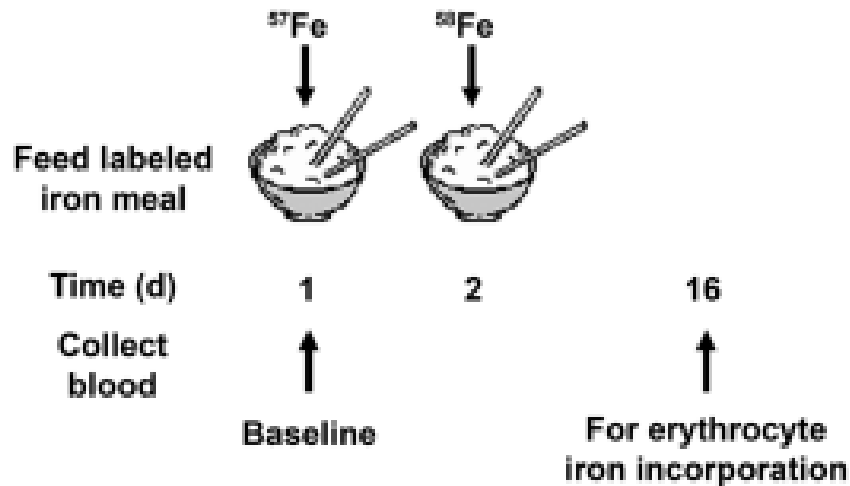


Figure 2-15: Extrinsically labelling of test meals with stable iron isotopes.
Reproduced with permission from a paper by Sheftel *et al.* (2018).

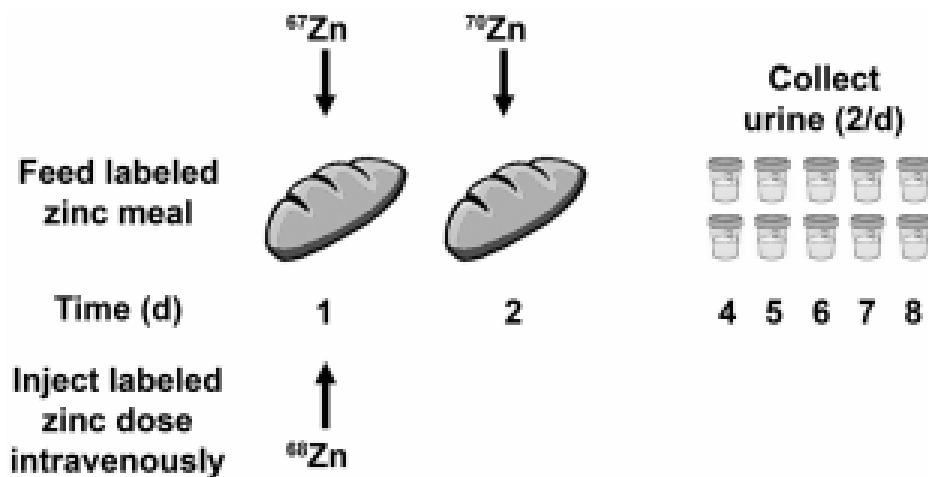


Figure 2-16: Extrinsically labelling of test meals with stable zinc isotopes.
 Reproduced with permission from a paper by Sheftel *et al.* (2018)

Human studies on iron and zinc bioavailability assessment are more accurate and allow selection of the target population (Etcheverry *et al.*, 2012). But they require ethical considerations, expensive, labour intensive and also absorption can be influenced by other factors (age, sex and illness) (Carbonell-Capella *et al.*, 2014; Fernández-García *et al.*, 2009; Van Campen & Glahn, 1999). Hence, there is a need to predict iron and zinc bioavailability *in vitro* before conducting human studies.

2.15 The influence of different processing methods on the bioaccessibility and bioavailability of iron and zinc

As previously reported, processing methods such as fermentation and blanching reduce inhibitors that affect iron and zinc absorption in ALVs (**section 2.12**). In a review conducted by Krishnan and Meera (2018), they reported that household processing methods (soaking, fermentation, blanching and use of mineral absorption enhancers) positively influenced iron and zinc bioaccessibility in pearl millet either by preventing inhibitory factors of these minerals or by reducing the antinutritional factors. In agreement, another review by Raes *et al.* (2014) also reported that different household processing methods were associated with a reduction of

antinutritional factors in plant-based foods which could be a positive impact on mineral bioaccessibility. There are limited data on the influence of processing on the bioaccessibility of iron and zinc from ALVs using the *in vitro* dialysability assay. However, previous studies evaluated the bioaccessibility of iron and zinc from other plant-based foods such as cereals and legumes (**Table 2-8**). The studies conducted by different authors showed that processes such as fermentation, soaking and addition of organic acids could have a positive impact on iron and zinc bioaccessibility. An increase of zinc (71 and 50% respectively) and iron (227 and 127% respectively) bioaccessibility was reported in a study that investigated the effect of fermentation on cereals and pulses commonly eaten in India (Hemalatha *et al.*, 2007a). In agreement, a study conducted in fermented maize, sorghum, and millet also reported the positive effect of fermentation on iron and zinc bioaccessibility (Gabaza, 2018). A 30% increase in iron bioaccessibility was also reported in genetically modified sorghum porridge made from the non-tannin line (Kruger *et al.*, 2012). The reduction of phytic acid in fermented products (sorghum) reported by previous studies has been linked to phytate degradation (Kruger, 2012; Towo *et al.*, 2006). Also, the drop in pH conditions during fermentation may produce organic acids, enhancing iron and zinc bioavailability (Hotz & Gibson, 2007). A review summarised that fermentation poses great potential in reducing antinutritional factors that bind iron and zinc in African fermented cereal porridges (Gabaza *et al.*, 2017).

The positive influence of blanching ALVs to reduce antinutritional factors that prevent iron and zinc absorption has been previously studied (Essack *et al.*, 2017; Mosha *et al.*, 1995), but no studies assessed the bioaccessibility of these essential minerals after blanching.

Lactic fermentation enhanced iron bioavailability in a vegetable mixture of carrots (40%), white cabbage (15%), turnips (25%) and equal parts of parsnip, celery and onion (20%) predicted by Caco-2 cell assay (Scheers *et al.*, 2016). The study report that Fe^{3+} was more hydrated, resulting in improved absorption as hydration assists in preventing the iron from forming insoluble complexes (Ems & Huecker, 2019). The same study also evaluated the effect of fermentation on the bioavailability of iron and zinc from lactic fermented vegetable mixture added on meals in humans (Scheers *et al.*, 2014). Enhanced iron absorption was observed when fermented vegetable mixtures replaced fresh vegetables in high phytate (5.2 to 10.4%) and low phytate meals (13.6 to 23.6%). Contradictory results of no effect on iron bioavailability were observed in rats when the influence of fermentation and germination on iron and zinc bioavailability from intrinsically labelled infant weaning foods was evaluated. The study observed no differences ($p < 0.05$) in iron bioavailability between rats fed fermented and non-fermented weaning products.

The differences in the results may be due to the levels of antinutritional factors present after fermentation of the products. Phytate is the primary antinutritional inhibitor of iron in cereals (Gibson *et al.*, 2010).

Previous studies obtained conflicting results on zinc bioavailability after fermentation. Scheers *et al.* (2014) reported an insignificant effect on zinc absorption after addition of fermented mixed vegetables into meals in a human study. The authors attributed the lack of increase in zinc bioavailability to the low levels of zinc abundant in food. Also, they explained that phytate inhibiting effects might be more pronounced on zinc than iron. They also indicated that meals used in the absorption study contained cottage cheese which contributed additional calcium. Calcium and phytate have a strong combined inhibitory effect on zinc uptake (Gibson *et al.*, 2010). In contrast, increased zinc absorption was reported when rats were fed a fermented plant-based diet (Lazarte *et al.*, 2015a). The differences between the two studies may be due to the differences in the metabolic functions between rats and humans (as discussed in **section 2.14.4**), as no animal model can accurately simulate physiological functions in humans (La Frano *et al.*, 2014). Contradictory results concerning the bioaccessibility of zinc after fermentation could be indicative that each food products react differently to fermentation and also levels of antinutritional factors reduction may vary, hence there is no uniform trend of zinc bioavailability after fermentation.

Table 2-8: A summary of the effect of processing on the bioaccessibility of iron and zinc from plant-based foods

Processing method	Food product	Effect on antinutrients	Iron bioaccessibility	Zinc bioaccessibility	References
Soaking in acidic or alkaline medium	Pearl millet	Flavonoids (67%)* Phytic acid(80%)* Polyphenols (11-20%)*	Increased (2.5%)	Increased (35%)	Jha <i>et al.</i> (2015)
Soaking in water	Black bean	Polyphenols (30%)* Condensed tannins (20%)*	Increased (6.5-40.7%)	Increased (44.7%)	Feitosa <i>et al.</i> (2018)
Use of organic acids (Orange marmalade jam)	Bread and bread spreads	Antinutrients (-)	Increased (9.7%)	No change observed	Ting and Loh (2016)
Spontaneous fermentation	Batter of rice: black gram 2:1	Phytate (traces left) Tannin (traces left)	Increased (277%)	Increased (71%)	Hemalatha <i>et al.</i> (2007a)
Spontaneous fermentation	Kisra bread	Phytic acid (26.9%)* Oxalic acid (80.9%)* Tannins (46.5%)*	increased	increased	Ahmed <i>et al.</i> (2020)

()* percentage antinutrient reduction, (-) antinutrients not analysed

2.16 Conclusions

From the literature review, it is evident that there is a paucity of information on the nutritional status situation in Botswana; therefore, there is a need for more research to update the nutritional situation especially for essential minerals status. The available information shows that Botswana is facing a double burden of malnutrition with both over-nutrition and under-nutrition prevailing. There is lack of up-to-date data on the iron and zinc status of the population in Botswana.

. ALVs contain high amounts of iron and zinc, therefore their consumption could help to alleviate deficiencies of these minerals. However, there is limited information available on the bioaccessibility and bioavailability of these essential minerals from processed ALVs. The information on the bioaccessibility and bioavailability of minerals from ALVs will help in understanding the contribution these vegetables could make towards the diets of households that have limited access to haem iron and zinc sources from animal sources.

ALVs contains relatively high amounts of antinutrients mostly polyphenols and phytates that have been shown to impair iron and zinc absorption. There is limited research on strategies to decrease antinutrients in ALVs, hence the need to explore strategies that could reduce antinutrients, at the same time enhancing iron and zinc bioavailability from these vegetables. ALVs are seasonal and mostly consumed fresh when in season and dried when out of season for preservation. It may be useful to employ these traditional processing methods of blanching and spontaneous fermentation to achieve antinutrients reduction in order to improve iron and zinc bioavailability. Most of data on the bioavailability of iron and zinc from ALVs available is predicted using phytate-mineral molar ratios and mineral bioavailability does not solely depend on the inhibition effects of phytate hence this prediction of bioavailability may be inaccurate as it solely considers phytate as an inhibitor of a particular mineral neglecting other antinutritional factors (tannins and oxalates) present in the food. Therefore, this literature review supports the need for a study to be conducted that will evaluate the bioaccessibility of iron and zinc from processed ALVs and predict iron and zinc bioavailability *in vitro* to assess the influence of processing on mineral absorption.

CHAPTER 3: MATERIALS AND METHODS

3.1 Overview

This chapter gives an overview of the methodology, sample collection, laboratory analysis procedures and assays followed to obtain the results of this study.

Five ALVs selected for this research were cowpea leaves (*Vigna unguiculata*) wild jute (*Corchorus olitorius*), spider plant (*Cleome gynandra*) amaranth (*Amaranthus hybridus*) and Ethiopian mustard (*Brassica carinata* A. Baun) which was chosen as a control (commercialised ALV). In this study, the Ethiopian mustard will be referred to by its common name Ethiopian kale. The ALVs (non-commercialised) used in this study were purchased from a local farmer in Kgatleng district in Botswana, except for the already commercialised vegetable, which was purchased from the local market in Kanye, Botswana. The ALVs were subjected to three different processing methods; freshly cooked, blanched-dried and spontaneously fermented-dried (preservation types of methods) to simulate the state in which these vegetables will be commonly consumed (**Figure 1-1**). As stated, ALVs are available fresh in summer and are traditionally blanched or spontaneously fermented and subsequently dried for later use while out of season, however this study did not study ALVs preservation but rather processed ALVs similar to how they will be typically consumed when in and out of season. The mineral contents of ALVs were analysed using inductively coupled plasma optical emission spectroscopy (ICP-OES), and oxalates, phytates, tannins, and total phenolic content of ALVs were analysed to evaluate and compare whether cooked vegetables differ from blanched-dried and spontaneously fermented-dried ALVs. The *in vitro* dialysability of iron and zinc from differently processed ALVs were then evaluated to compare mineral bioaccessibility between processes. During the *in vitro* dialysability assay, the ALVs underwent simulated digestion as per the gastric and intestinal phases of the GI. In the intestinal phase, dialysis tubing was used to simulate the small intestine to measure mineral dialysability. ALVs with better bioaccessibility were chosen for the next stage of the study, that is, to predict bioavailability. Bioavailability of iron and zinc from differently processed ALVs were predicted using the *in vitro* digestion combined with the use of Caco-2 cells to simulate absorption in the small intestine. Iron absorption by the Caco-2 cells was measured in the form of ferritin formation. Zinc uptake was analysed using inductively coupled plasma mass spectrometry (ICP-MS). The Caco-2 cell model has been reported as the gold standard for evaluation of intestinal absorption *in vitro* studies (Hubatsch *et al.*, 2007). Therefore, data obtained from the cell line assay can be extrapolated to predict absorption in humans.

3.2 Materials and methods

3.2.1 Study design

Each ALV was divided into three sub-samples that were then processed per the three different processing methods. The ALVs were either freshly cooked, blanched-dried and spontaneously fermented-dried. The study was carried out in three phases (**Figure 3-1**). The first phase involved mineral and antinutrient contents analysis of the ALVs; the second phase was the assessment of iron and zinc bioaccessibility from processed ALVs using *in vitro* dialysability assay, and the last phase was the estimation of the bioavailability of iron and zinc from ALVs using a Caco-2 cell culture assay.

3.2.2 Study approval

The study was approved by the scientific committee of the Centre of Excellence for Nutrition in North-West University, South Africa. The study was exempted from ethics approval by the Health Research Ethics Committee (HREC) of the Faculty of Health Sciences, Ethics Office for Research, Training and Support in North-West University, South Africa, as it did not involve animals and human subjects. The exemption letter is attached as addendum 3.

3.2.3 Selection of ALVs for this study

The study selected five ALVs, i.e., four commonly consumed ALVs; spider plant, amaranth, wild jute and cowpea leaves, and for control purposes, Ethiopian kale (already commercialized ALV) were procured in Botswana. As previously stated, there is enough evidence on the consumption of these ALVs in sub-Saharan Africa (Faber *et al.*, 2010; Flyman & Afolayan, 2006b; Maroyi, 2011; Masarirambi *et al.*, 2010; Smith & Eyzaguirre, 2007; Steyn *et al.*, 2001; Uusiku *et al.*, 2010; Vorster *et al.*, 2009).

3.2.4 Procurement of ALVs

The young vegetable leaves were procured in December 2016 and January 2017. Approximately 25 kg of each vegetable was procured from the farmer in breathable woven bags and were transported to the laboratory on the same day for processing. To avoid wilting of the vegetables, it was arranged that one vegetable was supplied each day at the processing plant at the National Food Technology Research Centre in Botswana on the same day for processing. Two separate

samples from each ALV were processed. All reagents used in this study were of analytical grade and were purchased from Sigma-Aldrich, Johannesburg, South Africa unless stated otherwise.

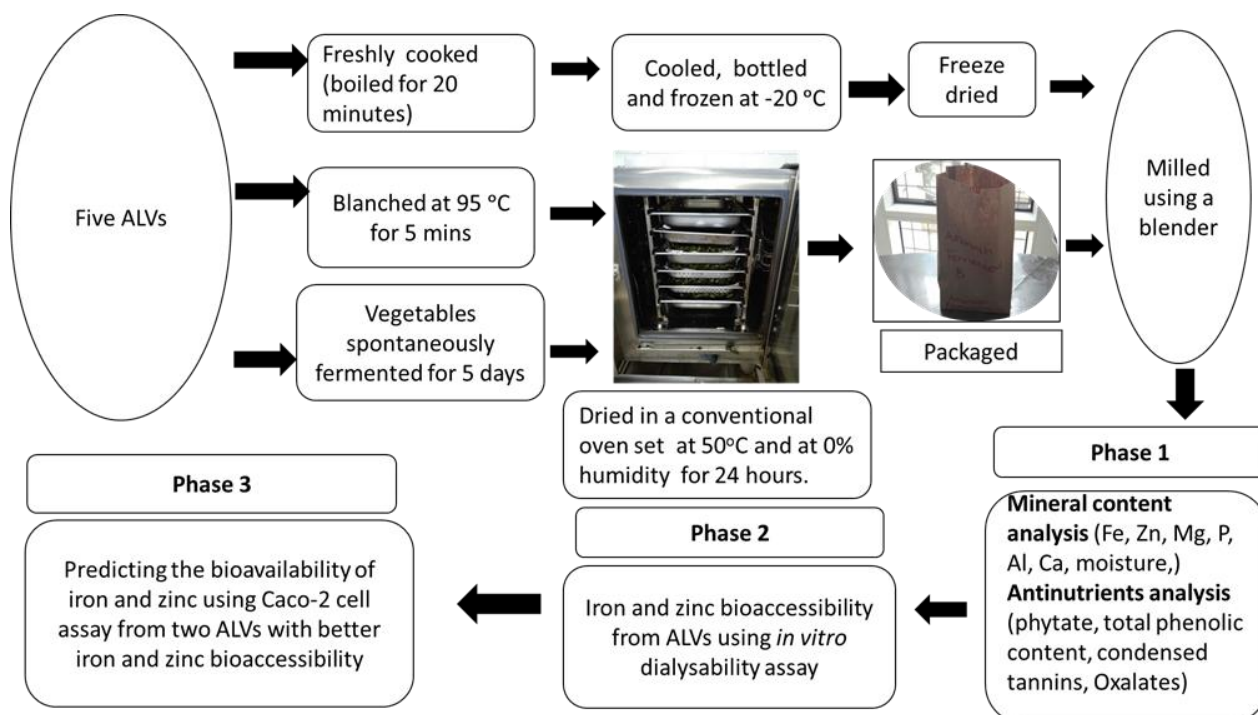


Figure 3-1: Study design

3.2.5 Vegetable sorting and washing

Upon delivery, the leaves were inspected for debris, fungal, and surface contaminants, colour and appearance, as outlined in the World Vegetable Centre manual (Acedo, 2010). Any debris from leaves was removed. Any visible stalks were removed from the leaves. Approximately 5 kg of each vegetable was washed twice in deionized water to remove any impurities and drained in a colander after which it had been divided into three equal batches for cooking, blanching and spontaneous fermentation (**Figure 3-2**). The ALVs were prepared as they would be normally consumed while fresh, and initial processing steps of preservation (blanched dried and fermented-dried) as they would undergo to be used later when out of season as illustrated by the experimental design (**Figure 3-1**). The storage, reconstitution and final cooking were not evaluated in this study. ALVs processing were all conducted in duplicates for each vegetable.

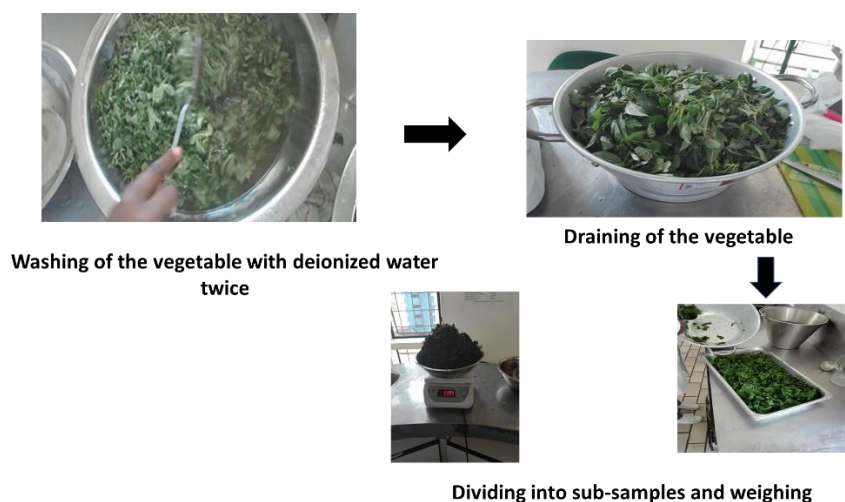


Figure 3-2: Vegetable sorting and washing.

3.2.6 The cooking of the vegetables

Each vegetable (500 g) was boiled in 100 ml of deionized water for 20 minutes as previously done (Matlhare *et al.*, 1999), cooled to room temperature and transferred with the cooking water into plastic jars with lids (**Figure 3-3**) and stored at -20°C until laboratory analysis. The cooked samples were then freeze-dried and milled to a fine powder using a laboratory miller (IKA A11, Staufen, Germany) and stored in zip-lock bags at -20°C before analysis.



Figure 3-3: Cooked vegetable packaging

3.2.7 Blanching of vegetables

For blanching, 1 kg of each vegetable was submerged into deionized boiling water (4 L) at 97°C for 5 minutes (Mosha *et al.*, 1995). The vegetables were drained and cooled in ice water for 5 minutes to stop the blanching process. The vegetables were then transferred into oven drying trays and dried in a conventional oven dryer at 50°C with 0% humidity for 24 hours (**Figure 3-4**). The dried samples were stored at 5 °C in the brown craft paper bags. The samples were then milled and stored as described above.



Figure 3-4: The drying process of blanched-dried and spontaneously fermented-dried African leafy vegetables

3.2.8 Fermentation of ALVs

The third batch of the vegetables was spontaneously fermented and dried according to the method adopted from previous studies (Ifesan *et al.*, 2014; Swain *et al.*, 2014). Each vegetable (1 kg) was spontaneously fermented with 30 g of salt as per the method described by the World Vegetable Centre (Acedo, 2010) and an addition of 30 g of glucose was added as adopted from the method used by Kasangi and colleagues (Kasangi *et al.*, 2010). ALVs need a fermentable carbohydrate to support the growth of LAB (Wafula *et al.*, 2016). Previous studies conducted on spider plant, amaranth and cowpea reported that these ALVs do not contain sufficient sugar to support the growth of a LAB population for active fermentation to occur (Muchoki *et al.*, 2007; Wafula *et al.*, 2016), hence the addition of sugar to ALVs in this study (**Figure 3-5**). For Ethiopian kale, no sugar was added as same studies reported that it contained adequate fermentable carbohydrate. The vegetables were placed in a 2.5 cm layer and sprinkled with the 50:50 salt-sugar mixture, on to which another layer of leaves was added and sprinkled with the salt-sugar mixture. This was repeated until all the 1 kg of leaves and 60 g of the 50:50 salt-sugar mixture was used. A cheese-cloth was placed over the vegetable and weight was added to compress the vegetable and assisted in the formation of brine (**Figure 3-6**). The vegetables were fermented for seven days at room temperature, with temperature and pH monitored throughout the fermentation period. A Crison basic 20 pH meter (Barcelona, Spain) was used. Acidity (**Figure 3-7**) and temperatures (**Figure 3-8**) of spontaneously fermented ALVs were plotted. At the completion of fermentation, the ALVs were weighed and placed in drying trays and the fermentable medium discarded. Spontaneously fermented vegetables were dried in a conventional dryer at 50 °C with

0% humidity for 24 hours. After drying, the vegetables were weighed and stored at 5 °C in sealed brown craft paper bags until laboratory analysis. The samples were then milled and stored as described above.

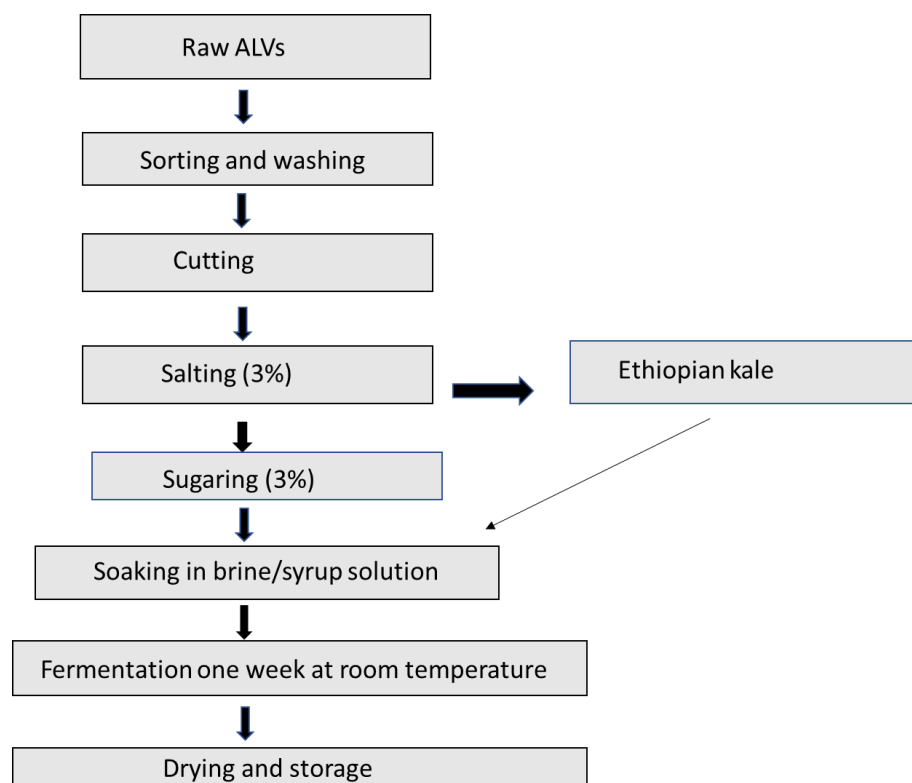


Figure 3-5: Spontaneous fermentation processing of vegetables



Figure 3-6: Brine formation in spontaneously fermented vegetables

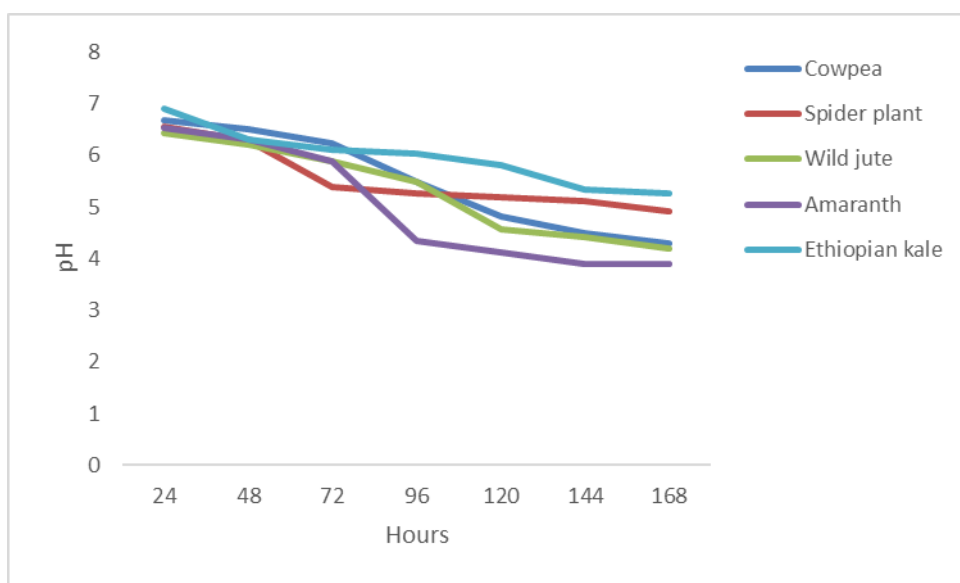


Figure 3-7: pH profiles of African leafy vegetables during the fermentation process

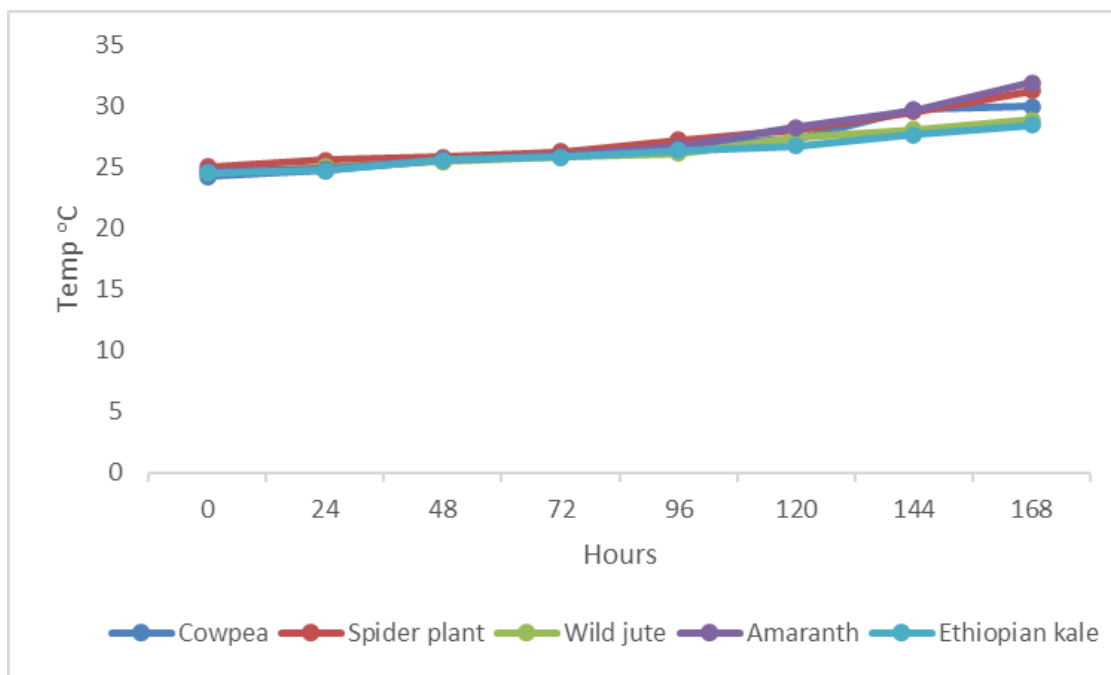


Figure 3-8: Temperature profiles of African leafy vegetables spontaneously fermented.

3.2.9 Mineral contents

Iron, zinc, magnesium, calcium, aluminium and phosphorus were analysed by the Central Analytical Facilities (CAF) at Stellenbosch University, a laboratory accredited by the South African National Accreditation System (SANAS). The ALV samples were digested by concentrated nitric acid and hydrogen peroxide-based on the USEPA method 3051A (Element, 2007). The concentration of minerals in the digested samples were determined by the ICP-OES (ICAP 6000 series, Thermo Fisher Scientific, Waltham, USA). Each element was analysed using a wavelength, 239.5 nm for iron, 206.7 nm for zinc, 315.8 nm for calcium, 285.2 nm for magnesium, 214.9 nm for phosphorus and 308.2 nm for aluminium. For quality assurances samples were analysed against National Institute for Standards and Technology traceable standards and independent quality controls. An internal standard technique was established, and a calibration acceptance criterion of $R^2 > 0.9995$ was used to ascertain results accuracy.

3.2.10 Moisture contents

The moisture contents of the ALVs were determined by an air oven, one stage, drying procedure method 44-15A which determines moisture by calculating loss in moisture as the percentage of original sample weight (AOC, 2000). A 5 g of ALV sample was weighed in a moisture dish in triplicates and placed in an oven and dried at 105 °C to constant weight for 6 hours. The moisture dish and contents were cooled in a desiccator and weighed. The percentage of moisture content was then determined by calculation.

$$\% \text{ moisture content} = \frac{W_1 - W_f}{W_1} \times 100$$

Where,

W₁ = initial weight of the sample before drying (g)

W_f = final weight of the dry sample (g)

3.2.11 Antinutrient contents

3.2.11.1 Total phenolic contents

Total phenolic content (TPC) was determined using a modified Folin-Ciocalteu method (Kaluza *et al.*, 1980) with some modifications. Blank samples were analysed for each sample to correct for the colour of the extract. Extracts from the samples were prepared using acidified methanol (1% HCl in methanol). The solvent (10 ml) was added to 0.3 g of the sample and covered. This mixture was stirred for 2 hours, centrifuged at 1200 g for 10 minutes and supernatant decanted. The sample further extracted with 10 ml of the solvent for 20 minutes, centrifuged and the supernatant decanted as described above. The pooled supernatants were combined, covered and stored at -20°C for further analysis. TPC was determined using the Folin-Ciocalteu phenol reagent. Concentrations of phenols were determined relative to a standard curve of catechin concentrations (mg/ml). Catechin (Sigma Cat no. C1788) was the standard used. 10 ml of deionised water, and 2.5 ml of Folin-Ciocalteu's phenol reagent was added to the catechin

standards and sample extracts, and mixed. Two minutes later, 7.5 ml of sodium carbonate (Na_2CO_3) (20 g /100 ml) was added, then the contents were mixed, and volume made up to 50 ml with deionised water. Blanks were prepared as above without the addition of Folin Ciocalteu's phenol reagent. The samples were incubated at ambient room temperature for 2 hours and the absorbance was measured at 760 nm. TPC were expressed as mg catechin equivalents per 100 g.

3.2.11.2 Condensed tannin contents

Condensed tannin content was determined by the modified Vanillin HCl assay (Price *et al.*, 1978) with some modifications. The absorbance of the reagent blank that corrected for the colour of the ALV extracts were included in the calculations. Condensed tannins were extracted with acidified methanol (25 ml, 1% conc. HCl in methanol) from 0.5 g of the vegetable sample. Condensed tannins were extracted at ambient room temperature with 5-minute intervals of vigorous shaking for 20 minutes. Extracts were centrifuged for 10 minutes at 1200 g and the supernatant collected. To determine the condensed tannin content from the extract, 1 ml of the extract/catechin standard (1.5 mg/ml) was transferred to test tubes containing 5 ml of mixed 8% HCl in methanol (solution B) and 1% vanillin reagent (solution A) mixed in equal volumes before use (20 ml of solution A mixed with 20 ml of solution B). The solution was vortexed and incubated at room temperature for 20 minutes. In the colour blank determination, 1 ml of the extract was transferred into test tubes containing 5 ml of 4% HCl in methanol instead of vanillin reagent, vortexed and incubated at room temperature for 20 mins. Both the extract and colour blanks were read at 500 nm with a spectrophotometer, using distilled water as the background. The method is founded on the theory that the reaction between condensed tannin and vanillin results in complexes which are coloured (Schofield *et al.*, 2001).

Results were calculated using the following equation,

$$CE = \frac{A500 \text{ Test} - A500 \text{ Colourblank}}{A500 \text{ Standard} - A500 \text{ Reagent blank}} * 1.5 \text{ mg /ml} * 5$$

Where:

CE = mg Catechin Equivalence per 100 mg sample

A_{500} = Absorbance read at 500 nm

The tannin content of samples was calculated by subtracting the mean colour blank tannin content from the extract tannin content and then expressed as mg catechin equivalents per 100 g of sample.

3.2.11.3 Phytate contents

Phytate content was determined through an indirect quantitative anion exchange method (Fruhbeck *et al.*, 1995), measuring the amount of organic phytate phosphorus. A milled 1 g sample was extracted with 20 ml of 2.4% HCl, and the mixture was shaken for two hours at 300 rpm. The extract was centrifuged at 1500 g for 60 minutes at 15 °C. The extract was repeatedly centrifuged to get a clear supernatant. 5 ml of the supernatant was collected and diluted with distilled water at a ratio of 2:5. The pH was then adjusted to 6 with 0.5 N NaOH, after which 0.5 g of anion exchange resin (Dowex 1; anion-exchange resin-AG 1 x 4, 4% cross-linkage, chloride form, 100-200 mesh from Sigma-Aldrich, Johannesburg South Africa) was added to vertically clamped Poly-Prep®Chromatography columns (Bio-Rad, Hercules, California, United States), and 10 ml of the diluted supernatant was added onto the columns. The inorganic phosphate was eluted with 15 ml 0.1 M NaCl, and the organic phosphate (phytate) was eluted with 15 ml 0.7 M NaCl. The pH of 3 ml of distilled water, sodium phytate standard solutions or the ALV purified samples were adjusted to 3, using 0.1 N HCl. Wade reagent (1 ml) (0.03% FeCl₃ and 0.3% sulfosalicylic acid) were added to each tube, vortexed for 5s and left to stand for 30 minutes at ambient room temperatures. The absorbance of the complex was read at 490 nm. Sodium phytate solutions were used to produce a standard curve. Concentrations of phytate were determined relative to a standard curve of phytic acid (µg phytate/ml diluted purified sample). Phytic acid sodium salt hydrate from rice (Sigma-Aldrich Johannesburg, South Africa) was the standard used. Phytate content was expressed as mg of phytate equivalents per 100 g.

The bioavailability of iron and zinc from ALVs was predicted using phytate to mineral molar ratios. Phytate to mineral molar ratios of Phy : Zn, Phy : Fe and Phy * Ca : Zn were calculated to predict mineral bioavailability as inhibited solely by phytate. (Wyatt & Triana-Tejas, 1994).

3.2.11.4 Oxalate contents

Oxalates contents was determined using the titration method as described in the official methods of analysis of 1999 (AOAC., 1999). For extraction of insoluble oxalates, 0.1 g of sample was weighed and mixed with 30 ml of 1 M HCl. The mixture was shaken in a water bath at 100 °C for 30 minutes. 0.5 ml of 5% calcium chloride was added and thoroughly mixed to precipitate out the calcium oxalate. The suspension was centrifuged at 1200 g for 15 minutes and the supernatant discarded. The pellets were washed twice, using 2 ml of 0.35 M NH₄OH and dissolved in 0.5 M H₂SO₄. The solution was titrated using a standard solution of 0.1 M KMNO₄. The temperature was maintained at 60°C throughout the titration until a faint violet colour (which lasted for at least 15 seconds) was observed. For soluble oxalates, the extraction was conducted using 30 ml of distilled water instead of 30 ml of 1M HCl and the same procedure was followed as for the insoluble oxalates. Oxalate content was calculated assuming 1 ml of 0.1 M KMNO₄ was equivalent to 2.2 mg of oxalate (Chinma & Igyor, 2007).

3.2.12 Mineral bioaccessibility by *in vitro* dialysability assay

To simulate human gastric and intestinal digestion, variously processed ALVs were subjected to *in vitro* digestion. The *in vitro* dialysability method described by Miller and co-workers was used (Miller *et al.*, 1981), with some minor modifications as previously published by Kruger and co-workers (Kruger *et al.*, 2012), by acidifying the dialysates with 0.002 ml 65% nitric acid/ml dialysate when decanted to avoid potential precipitation of minerals. The following enzymes were used in our study; pepsin (P-7000), bile extract (P-8631) and pancreatin (P-1750) (Sigma-Aldrich, Johannesburg, South Africa). In the intestinal phase, a dialysis tubing of Spectra/Por 7 (Ø = 20.4 mm) with a molecular mass cut-off (MMCO) of 10000 Da (Labretoria) was used (GIC Scientific, Johannesburg, South Africa).

40 g of deionized water was added to 5 g of the respective ALV samples in a 250 ml Erlenmeyer flask. The pH was adjusted to 2, using 6 M HCl. After 10 minutes, the pH was checked and readjusted to 2 if necessary. 3 g of freshly prepared pepsin solution (16 g pepsin in 100 ml of 0.1 M HCl) was added to the acidified vegetable samples and the mixture made up to 50 g with deionised water. Samples were incubated at 37°C in a shaking water bath (150 rpm, circular arm movement of 2 cm) for 120 minutes to simulate stomach digestion (gastric stage). The samples were cooled crushed ice bath while titratable acidity was measured.

A homogeneous 10 g of the gastric digest was taken out to measure titratable acidity, and the remainder was stored on ice for no more than 90 minutes. Freshly prepared pancreatic mixture (4g pancreatin, 25 g bile extract mixed with 1litre 0.1 M NaHCO₃) weighing 2.5 g was added to each sample and the pH was adjusted to 7.5 using 0.5 M NaOH. The number of moles of NaOH

used during titration to pH 7.5 was used to determine the equivalent moles of NaHCO₃ to be used in the dialysis tubing during the intestinal stage.

To simulate the intestinal stage of digestion, 10 g homogenized gastric digests were weighed into 250 ml Erlenmeyer flasks and heated in water bath at 37°C for 5 minutes. Segments of dialysis tubing (15 cm clamped both sides) containing 12.5 g of NaHCO₃ solution (equal in moles to the NaOH required during titration) were added into the Erlenmeyer flasks and incubated for 30 minutes in a shaking water bath at 37°C. After this incubation, 5 g of freshly prepared pancreatin mixture was added, followed by incubation at 37°C for a further two hours. The dialysis bags rinsed with deionised water and contents of each dialysis bag were transferred to sterilised tubes and acidified with 0.03 ml of 65% concentrated nitric acid to keep minerals in suspension, the tubes filled up to 15 ml with deionised water and frozen at -20 °C. Mineral contents of the dialysates were determined by the ICP-OES (ICAP 6000 series, Thermo Fisher Scientific, Waltham, USA) as described above as in mineral contents, without the digestion step. The bioaccessible (dialysable) minerals are expressed as the percentage of the total mineral contents.

$$\% \text{ bioaccessible mineral} = \frac{\text{dialysable mineral } (\mu\text{g})}{\text{Total mineral } (\mu\text{g})} \times 100$$

3.2.13 The potential contribution of ALVs in this study towards total absolute requirements for zinc and iron for women

Total absolute requirement (AR) is the total amount of bioavailable mineral required for growth, basal iron losses and, in women, also including menstrual losses (FAO & WHO, 2001). For adult women aged 19 to 50 years, the AR for iron is 1.46 mg/day (FAO & WHO, 2001) and for zinc, 1.75 mg/day (Sandstead, 2015). For this study, the contribution the bioaccessible mineral from one portion of processed vegetable could make towards the AR of women aged 19 to 50 years was calculated. A portion size was taken as 300 g (wet basis) as per the findings of a previous study (van der Hoeven *et al.*, 2013). The contribution each portion of processed ALV could make towards absolute iron and zinc requirements for women aged 19 to 50 years was calculated and expressed as percentages. Concentration of mineral on dry weight basis (mg/100 g) were first converted to wet weight basis (mg/100 g) using moisture content percentage conversion factor and calculated as shown below.

$$C_w = \left(\frac{100}{P_s} \right) \times 100 * C_d$$

Where;

C_d = concentration in dry weight

C_w = concentration in wet weight

P_s = Percentage solids (100 – percentage moisture)

absolute bioaccessible mineral = Amount of bioaccessible mineral $\times$ portion weight

$$\% \text{ contribution to absolute requirements} = \frac{\text{absolute accessible mineral (mg/100 g)}}{\text{Absolute requirements (mg/d)}} \times 100$$

3.2.14 Prediction of the bioavailability of iron and zinc using the Caco-2 cell uptake model

To predict the bioavailability of iron and zinc from selected ALVs, which had shown better bioaccessibility, ALVs were subjected to *in vitro* digestion to stimulate gastric digestion and the bioavailability of iron and zinc were estimated using the Caco-2 cell uptake assay.

3.2.14.1 *In vitro* digestion of vegetables

The ALVs were subjected to *in vitro* digestion to simulate human digestion as per the method previously used by Kruger and colleagues with some modifications (Kruger *et al.*, 2012), blank digestion without the samples but 10 ml deionized was conducted to measure any mineral from glassware. 10 ml of deionized water was added to 1 g of sample and the mixture was

homogenized by vigorously shaking. The pH of the vegetable mixtures and the blank sample were adjusted to pH 2 with 1 M HCl, and 20 µL of freshly prepared pepsin solution at a concentration of 20 µl/g sample (Sigma, P-7000, from porcine stomach mucosa) was added, and volume was adjusted to 40 ml. The mixture was then incubated at 37°C in a shaking water bath at 150 rpm for 2 hours to simulate gastric digestion. Digestion was then stopped by placing the sample in an ice bath for 10 minutes. To simulate intestinal digestion, the pH was adjusted to pH 7.0 with 1 M NaHCO₃, followed by the addition of 3.5 ml of the freshly prepared pancreatin-bile solution to provide 0.005 ml of pancreatin / g sample (Sigma, P-1750, from porcine pancreas) and 0.03 ml of bile salt / g sample (Sigma, B-8631, Porcine). Samples were incubated at 37°C in a shaking water bath at 150 rpm for 2 hours. After digestion, the samples were then placed in ice to denature and quench digestive enzymes. The pH was increased with 0.5 NaOH to pH 7.2. 40 g of the intestinal digests were centrifuged at 1500 g for 1 h at 4°C. The supernatants were collected and stored at -20°C. 10 g of the supernatant was analysed for soluble iron. Soluble iron contents were analysed as per the method described in mineral contents (**Section 3.2.9**) without the digestion stage. The soluble fraction was used in the Caco-2 cell model for the prediction of iron and zinc bioavailability.

3.2.14.2 Culturing conditions for Caco-2 cells

Caco-2 cells were obtained from the American Type Culture Collection (ATCC® HTB-37™) (ATCC, Manassas, VA, USA). These cells were cultured in Minimum Essential Medium (MEM) with Earle's Balanced Salts Solution (EBSS) (Hyclone, GE healthcare, South Logan, UT, USA), containing 10% foetal bovine serum (FBS), 1% penicillin/streptomycin, 1% 200 mM L-glutamine and 1% non-essential amino acids (Lonza, Basel, Switzerland). Cells were cultured at 37 °C in a 95% relative humidified atmosphere of 5% CO₂. Cells were maintained and grown under low iron conditions for iron experiments. To achieve this, MEM/EBSS was supplemented with FBS depleted of iron, obtained by titrating the FBS at pH 4.5 in the presence of 300 g/l Chelex®100 chelating resin (Bio-Rad, Hercules, CA, USA) (Viadel *et al.*, 2007).

3.2.15 The measurement of iron uptake by Caco-2 cells by ferritin determination

For the determination of iron bioavailability through ferritin determination, cells were seeded in six-well plates and allowed to differentiate by continuing to incubate cells for 14 days post 100% confluence. Cells were then exposed to the different extracts of ALVs (filtered through a 0.2 µm syringe filter); 50 µM FeSO₄ was included as a positive control (García-Nebot *et al.*, 2013). Before exposure of cells to the soluble fraction of the ALVs obtained after the *in vitro* digestion (section 3.5.1), 5 mM glucose and 50 mM HEPES 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer was added to each fraction to adjust the osmolality (García-Nebot *et al.*, 2009). Then 1 ml

of the respective soluble fractions were added to 1 ml of serum-free MEM/EBSS containing 1% penicillin/streptomycin, 1% 200 mM l-glutamine and 1% non-essential amino acids (Lonza, Basel, Switzerland). These mixtures were then added to the respective wells to expose the cells to the soluble fractions. Cells were incubated at 37 °C under 5% CO₂ with 95% relative humidity for 2 hours. Following 2 hours of exposure, cells were washed with phosphate-buffered saline (PBS), fresh serum-free MEM/EBSS was added, and the cells were incubated for a further 22 hours at 37 °C under 5% CO₂ with 95% relative humidity. Following the incubation period, cell monolayers were washed twice with PBS and cells were detached with trypsin-EDTA. Cell suspensions were then centrifuged at 1500 rpm for 5 min. The supernatant was discarded and cell pellet lysed by the addition of 500 µl of ice-cold lysis buffer (10 mM Tris, 130 mM NaCl, 1% Triton X-100 and a general-use water-soluble protease inhibitor cocktail (containing AEBSF, Aprotinin, Bestatin hydrochloride, E-64, EDTA and Leupeptin hemisulphate salt from Sigma-Aldrich Johannesburg, South Africa) the samples were vortexed and incubated at 4 °C for 1 hour. The lysate was then centrifuged, the resulting supernatant was stored at -20 °C, and the pellet discarded. The supernatant was used for protein determination and ferritin analysis. Ferritin measurements were performed making use of the Human FE (Ferritin) ELISA kit (Elabscience, Houston, TX, USA), following the manufacturer's instructions. Protein concentrations were determined by the bicinchoninic acid assay (BCA), making use of the Pierce BCA Protein Assay kit (Thermo Fisher Scientific, USA) and following the manufacturer's instructions. Results were expressed as ng ferritin/mg protein.

3.2.16 Measurement of predicted zinc bioavailability in Caco-2 cells

For the prediction of zinc bioavailability, Caco-2 cells were maintained as above in the ferritin assays; however, the cells were maintained in MEM / EBSS supplemented with FBS without iron depletion. Also, zinc (ZnSO₄, 50 µM) instead of iron was used as a positive control during experimentation. To evaluate zinc retention, transport and uptake, the method of García-Nebot *et al.* (2013). The cells were seeded onto polyester membrane chamber inserts (24 mm diameter, 0.4 µm pore size; Transwell® plates, (Corning® Transwell® Sigma-Aldrich, Johannesburg South Africa) at a density of 50,000 cells/well. Cells could differentiate on filters for 21 days. The medium was changed every two days. To assess the monolayer formation and tight junction maturation during the cell differentiation period, the measurement of the passage of phenol red across the monolayer was used, according to the method previously used by other authors in their study (Frontela *et al.*, 2009).

Experiments were performed 21 days after seeding the cells on the membranes. Before exposure of cells to the soluble fraction of the ALVs obtained after the *in vitro* digestion (section 3.5.1.), 5 mM glucose and 50 mM HEPES buffer were added to each fraction to adjust the osmolality

(García-Nebot *et al.*, 2009). Then the media in the basolateral chambers were removed and replaced with zinc transport buffer (130 mM NaCl, 10 mM KCl, 1 mM MgSO₄, 5 mM glucose and 50 mM HEPES buffer pH 7.4) and 750 µl of each fraction was added to 750 µl of serum-free MEM/EBSS containing 1% penicillin/streptomycin, 1% 200 mM L-glutamine and 1% non-essential amino acids (Lonza, Basel, Switzerland), which was then added to the apical chamber. Cells were incubated at 37 °C under 5% CO₂ with 95% relative humidity for 2 hours. After incubation, the apical compartment was aspirated, the insert removed, and the monolayer washed three times with a transport buffer at 4 °C to remove non-specifically bound mineral and residual medium. The cells were then lysed by adding 1 ml of 2% sodium dodecyl sulphate (SDS). The basolateral chamber solution was pipetted off for the determination of Zn transport across the monolayer. The zinc content of the cell monolayer (zinc retention) and basolateral chamber (zinc transport) was measured by ICP-OES method (S.O.P.C No 45) by the Central Analytical Facilities (CAF) at Stellenbosch University, South Africa. Protein concentrations (of the lysed cell fraction) were determined by the BCA assay, making use of the Pierce BCA Protein Assay kit (Thermo Fisher Scientific, USA) and following the manufacturer's instructions. Results were expressed as µg zinc/mg protein. Zinc uptake was calculated as the sum of zinc transport and retention.

3.3 Statistical data analysis

Compositional data for this study are given on a dry basis (db) except where stated. The experiments were repeated and compositional analyses (mineral contents) of the ALVs were conducted in duplicates and in triplicates for condensed tannins, total phenolic contents, total oxalate content and phytates. As a consequence of their relative complexity, antinutrients assays were replicated different number of times to obtain reasonable repeatability. For the bioaccessibility assay, each ALV was subjected to two independent dialysability experiments, with the gastric stages performed in duplicates and intestinal digestion step performed in triplicates, which in turn were analysed for mineral contents in duplicates. IBM SPSS statistics 25.0, Armonk, USA was used for data analysis. The statistical analysis performed in this study is outlined and described below.

- Data Normality was tested using the Shapiro-Wilk test.
- Outliers from the data were identified using the Interquartile range (IQR) method of outlier detection and outliers were removed from the data.
- Descriptive statistics were performed for minerals and antinutrients and presented as mean ± Standard Deviation (SD).
- Differences between the means were compared by one-way ANOVA.
- Significant differences between means of the various processing methods were determined by multifactor ANOVA and Tukey's HSD Post-hoc test was used to determine

significant differences between the means.

- A general linear model was performed to evaluate the interaction effects of processing methods and vegetable type.
- Principal Component Analysis (PCA) using SPSS was used to determine the relationship between minerals and antinutrients.
- Values of $P \leq 0.05$ were considered statistically significant for all the analysis except where stated.

CHAPTER 4: RESULTS AND DISCUSSION

4.1 Overview

The main contributors to malnutrition in sub-Saharan Africa are poverty, food insecurity and shifting of dietary patterns, which lead to micronutrient deficiencies. Sub-Saharan Africa has the highest rates of anaemia (46% to 71%) in children younger than five years of age (Stevens *et al.*, 2013), while 39% of women of reproductive age are anaemic (World Bank, 2016). In addition, previous population surveys conducted reported that the prevalence of inadequate zinc intake has been estimated to exceed 25% of the population, and the prevalence of low zinc serum concentrations is estimated to be greater than 20% of the population in Sub-Saharan Africa (Galetti, 2018).

Dietary diversification has been seen as a long-term sustainable strategy in improving dietary quality and mineral bioavailability, especially for populations in rural areas with limited access to haem and animal sources of zinc (Nair *et al.*, 2016). A strongly positive correlation between dietary diversity and mineral adequacy has been previously reported (Mekonnen *et al.*, 2020; Ruel, 2001). Dietary diversification encourages consumption of both local available and traditionally processed foods (Gibson & Anderson, 2009; Pritwani & Mathur, 2015), hence, traditional foods can help to contribute towards iron and zinc intake in sub-Saharan Africa thus including African leafy vegetables (ALVs). ALVs have high levels of essential minerals such as iron and zinc. However, as reviewed in chapter 2, these green leafy vegetables contain high amounts of antinutrients which inhibit the bioaccessibility and bioavailability of iron and zinc. Nevertheless, there is enough evidence that traditional processing methods such as blanching and spontaneous fermentation concurrently with drying have been effective in lowering antinutrients in these ALVs. Reduction of phytic acid, tannin, total phenolic content and oxalates has been previously reported in spontaneously fermented ALVs (Muchoki *et al.*, 2010; Wafula *et al.*, 2016). At a pH below 4.5, enzymatic degradation of phytate occurs through the activation of endogenous phytase present in plant-based foods resulting in reduced phytate levels (Hayta & Hendek Ertop, 2017). During spontaneous fermentation a pH below 4.5 is reached (Muchoki *et al.*, 2010). A pH speciation diagram for oxalic acid presented by Simpson and colleagues showed that both insoluble and soluble oxalates are reduced by the medium pH (Simpson *et al.*, 2009). During spontaneous fermentation, the fraction of fully deprotonated divalent oxalate ions decreases, reducing their ability to bind with divalent mineral cations to form insoluble oxalates (Wadamori *et al.*, 2014). Then LAB degrades soluble oxalates and uses it as energy and carbon source *in vitro* (Goldfarb *et al.*, 2007), hence contributing to their reduction.

Blanching can lead to a reduction of antinutrients when plant cell membranes ruptures and soluble compounds leach out into the blanching liquid (Sallau *et al.*, 2012). Both fermentation and blanching concurrently with drying are traditional processing methods used when preserving and prolonging the quality of ALVs in households (Elisha *et al.*, 2016; Kirigia *et al.*, 2017). However, this study did not evaluate ALV preservation but processed the vegetable through blanching and spontaneous fermentation concurrently with drying (preservation type method) as a way they will be customarily consumed when out of season.

It is critical to evaluate the solubility and absorption of iron and zinc from the processed ALVs because the concentration of minerals in food samples do not always translate to nutrient absorption and use by the body because of the presence of mineral inhibitors present in the vegetables. Therefore, the imbalance of mineral available and high levels of antinutrients in ALVs could undermine the nutritional value of ALVs. The *in vitro* dialysability assay measures the solubility and absorption of the minerals from food samples, as it stimulates the gastric and intestinal phases of human digestion (Etcheverry *et al.*, 2012). Bioavailability predicts the exact fraction of minerals absorbed and used by the body (Hunt *et al.*, 2008). It has been extensively investigated that mineral bioavailability can be affected by diet-related factors such as the structure of the food sample, antinutrient content, enhancers of nutrients and physiological factors such as the acidity of gastric secretions in the intestine, nutrient stores, infection and stress (Fairweather-Tait *et al.*, 2005). Though animal and human studies exactly give the actual amount of mineral bioavailable for absorption (Cardoso *et al.*, 2015); they are too expensive and laborious to conduct, hence it is advisable to estimate bioavailability using *in vitro* assays (Tan *et al.*, 2018). One way of estimating bioavailability combines the use of *in vitro* dialysability assay and Caco-2 cells model to simulate absorption in the small intestine (Cilla *et al.*, 2018). The current study aimed to compare mineral and antinutrients content of cooked ALVs to blanched-dried, and spontaneously fermented-dried ALVs and with the overall aim to evaluate the bioaccessibility of iron and zinc from these processed ALVs and to predict their iron and zinc bioavailability using Caco-2 cell model.

Results found in this investigation and discussion of the results are presented in this chapter. Mineral and antinutrient contents of blanched-dried and spontaneously fermented-dried ALVs are compared with those of their cooked counterparts. The bioaccessibility and bioavailability of iron and zinc from variously processed ALVs will also be presented and discussed.

4.2 Mineral and antinutrient content of cooked, blanched-dried and spontaneously fermented-dried ALVs

4.2.1 Mineral contents

Values are presented per 100 g of dry basis (db) unless otherwise stated. The iron content of cooked ALVs ranged from 13.4 mg/100 g (Ethiopian kale) to 61.7 mg/100 g (spider plant) (**Table 4-1**). ALVs in this study contained levels of iron which were within the ranges previous reported for similar cooked ALVs (32.9 to 116 mg /100 g) (Schönfeldt & Pretorius, 2011). Zinc levels of cooked ALVs in this study varied between 2.9 mg/100 g and 12.0 mg/100 g. Spider plant zinc levels were higher compared to other cooked ALVs ($p \leq 0.05$). The zinc values obtained in this study for cooked ALVs were within the ranges previously reported for the same type of ALVs cooked (5.4 to 8 mg/100 g) (**Chapter 2: Table 2-2, Table 2-3, Table 2-4,**

Table 2-5). There was a considerable variation in the magnesium content of cooked ALVs in this study. The content ranged from 301 to 1365 mg/100 g. The levels were within ranges previously reported in a study that evaluated magnesium in five ALVs which reported ranges between 379 to 1050 mg/100 g (Schönfeldt & Pretorius, 2011). Calcium content from cooked ALVs varied between 1422 mg/100 g to 2890 mg/100 g, and some of the values were within the ranges previously reported for these ALVs (1659 – 3310 mg/100 g) (Schönfeldt & Pretorius, 2011). The differences in mineral levels found between ALVs in this study can be attributed to genetic variation within vegetable species, environmental factors such as soil mineral variation in different locations and exposure to different climate (Bvenura & Afolayan, 2015; Uusiku *et al.*, 2010). The variation in cooking methodologies between studies could also attribute to mineral content differences found in the same ALV type.

This study compared the mineral value of cooked non-commercialised ALVs to a commercialised ALV (Ethiopian kale). When comparing the cooked commercialised ALV to other non-commercialised cooked ALVs, iron levels were significantly ($p \leq 0.05$) higher in the non-commercialised cooked ALVs (29.5 to 61.7 mg/100 g vs 13.4 mg/100 g) (**Table 1**). Significantly ($p \leq 0.05$) higher levels of zinc were found in cooked spider plant and amaranth (12.0 mg/100 g and 6.0 mg/100 g respectively) compared to cooked Ethiopian kale (4.1 mg/100 g). Also cooked amaranth had substantially higher levels ($p \leq 0.05$) of magnesium (1365 mg/100 g) compared to cooked Ethiopian kale (561 mg/100 g). However, all non-commercialised ALVs had lower calcium contents ($p \leq 0.05$) (1442 to 2456 mg/100 g) compared to cooked Ethiopian kale (2890 mg/100 g).

The current study evaluated levels of aluminium in variously processed ALVs. Aluminium levels of ALVs varied considerably (**Table 4-1**). Aluminium levels of cooked ALVs varied from 9.71 to 75.8 mg/100 g. However, the aluminium content of blanched-dried and spontaneously fermented-dried ALVs was significantly ($p \leq 0.05$) lower for all ALVs compared to their cooked counterparts except in Ethiopian kale which had significantly ($p \leq 0.05$) higher contents in the blanched-dried and spontaneously fermented-dried samples. Cooked spider plant had the highest aluminium content (75.8 mg/100 g) compared to other cooked ALVs. A high content of aluminium in plant-based foods could be an indication of soil contamination (Salvo *et al.*, 2018), and these could result in artificially high levels of essential minerals in food especially iron. Normal washing of vegetables was previously reported not to be effective in removing all impurities in ALVs (van Jaarsveld *et al.*, 2014). Therefore, additional soil impurities, which were not removed by normal cleaning, could have been removed and discarded with the blanching and fermentable mediums attributing to lower levels of aluminium found. Aluminium in cooked spider plant was higher than the permissible dose limit of aluminium for an adult which is 60 mg/day (Nordberg *et al.*, 2014). High levels of aluminium content observed in blanched-dried and spontaneously fermented-dried Ethiopian kale could be attributed to cross-contamination with aluminium foil, as it was observed that after drying Ethiopian kale stuck to the aluminium foil which lined the drying trays. To the best of my knowledge, the current study is the first study to evaluate aluminium content in processed ALVs.

Table 4-1 : Mineral contents of processed African leafy vegetables

Vegetable species		Iron	Zinc	Magnesium	Calcium	Phosphorus	Aluminium
mg/100 g, db [% diff]							
Cowpea	COO	29.7 ± 0.6 ^{b*}	2.9 ± 0.1 ^{a*}	301 ± 5 ^{a*}	1840 ± 33 ^{a*}	340 ± 6 ^{b*}	32.4 ± 0.6 ^{c*}
	BLA	18.1 ± 0.1 ^a [-39]	3.0 ± 0.3 ^a	268 ± 7 ^a	1850 ± 30 ^a	337 ± 4 ^b	13.5 ± 0.2 ^a [-58]
	FER	28.5 ± 0.5 ^b	3.4 ± 0.1 ^b [17]	343 ± 4 ^b [14]	2220 ± 33 ^b [21]	303 ± 4 ^a [-11]	25.6 ± 0.6 ^b [-21]
Spider plant	COO	61.7 ± 1.2 ^{c*}	12.0 ± 0.2 ^{b*}	437 ± 8 ^{b*}	2456 ± 43 ^{a*}	614 ± 11 ^a	75.8 ± 1.4 ^{c*}
	BLA	40.0 ± 0.5 ^a [-35]	11.8 ± 0.1 ^b	350 ± 1 ^a [-20]	2381 ± 29 ^a	617 ± 4 ^a	45.3 ± 0.1 ^a [-40]
	FER	52.8 ± 1.0 ^b [-14]	11.2 ± 0.1 ^a [-9]	377 ± 5 ^a [-14]	2660 ± 51 ^b [8]	601 ± 3 ^a	56.0 ± 1.1 ^b [-26]
Wild jute	COO	29.5 ± 0.4 ^{c*}	3.7 ± 0.0 ^a	573 ± 1 ^b	1422 ± 10 ^{a*}	648 ± 7 ^{b*}	30.8 ± 0.3 ^{c*}
	BLA	10.4 ± 0.4 ^a [-65]	3.5 ± 0.1 ^a	528 ± 12 ^a [-8]	2452 ± 93 ^b [72]	454 ± 10 ^a [-30]	6.4 ± 0.0 ^a [-79]
	FER	16.4 ± 0.3 ^b [-44]	4.5 ± 0.0 ^b [22]	640 ± 7 ^c [12]	2782 ± 29 ^c [96]	570 ± 6 ^c [12]	14.2 ± 0.3 ^b [-54]
Amaranth	COO	30.0 ± 0.6 ^{b*}	6.4 ± 0.0 ^{b*}	1365 ± 12 ^{b*}	1995 ± 21 ^{b*}	477 ± 3 ^{a*}	39.0 ± 0.4 ^{c*}
	BLA	26.4 ± 0.0 ^a [-12]	6.8 ± 0.1 ^c [6]	1556 ± 7 ^c [14]	1985 ± 11 ^b	570 ± 14 ^b [19]	26.2 ± 0.3 ^a [-33]
	FER	33.2 ± 0.7 ^c [11]	6.0 ± 0.1 ^a [-6]	1254 ± 16 ^a [-8]	1730 ± 28 ^a [-13]	476 ± 6 ^a	34.1 ± 0.1 ^b [-13]
Ethiopian kale	COO	13.4 ± 0.3 ^a	4.1 ± 0.1 ^a	561 ± 11 ^c	2890 ± 54 ^b	565 ± 11 ^b	9.7 ± 0.2 ^a
	BLA	20.0 ± 1.0 ^b [49]	5.1 ± 0.1 ^b [24]	421 ± 13 ^a [-25]	2216 ± 83 ^a [-23]	521 ± 2 ^a [-8]	42.3 ± 1.4 ^c [336]
	FER	24.5 ± 0.2 ^c [83]	5.2 ± 0.1 ^b [21]	521 ± 10 ^b [-7]	2928 ± 34 ^b	555 ± 14 ^b	21.2 ± 0.3 ^b [118]

Values are expressed as mean ± SD (n = 2). Mean values of the same type of vegetable in the same column with different superscripts are significantly different at $p \leq 0.05$, FER=Fermented, BLA = Blanched, COO = Cooked, [% diff] = (mineral content cooked in ALV - mineral content blanched-dried or spontaneously fermented-dried ALV) / (mineral content cooked in ALV) x 100, Values of cooked vegetable with a * are significantly different to the cooked Ethiopian kale at $p \leq 0.05$, db = dry basis

Compared to their cooked counterparts the mineral content of spontaneously fermented-dried ALVs was significantly ($p \leq 0.05$) lower for iron in wild jute (44%) and spider plant (14%); for magnesium in spider plant (14%), Ethiopian kale (7%) and amaranth (8%) and for calcium in amaranth (13%). In contrast, compared to the cooked ALVs, the mineral content of spontaneously fermented-dried ALVs was significantly ($p \leq 0.05$) higher for iron in amaranth (11%) and Ethiopian kale (83%); for zinc in cowpea leaves (17%), wild jute (22%) and Ethiopian kale (21%), for magnesium in wild jute (12%) and cowpea (14%) and for calcium in cowpea (21%), spider plant (8%) and wild jute (12%) (**Table 4-1**). Previously lower mineral contents after spontaneous fermentation were reported in spontaneously fermented-dried cowpea leaves (Kasangi *et al.*, 2010). Low magnesium contents after fermentation had been linked to the replacement of magnesium ions by hydrogen ions in the chlorophyll (Sanjuán *et al.*, 2001). The amount of chlorophyll in each vegetable type may have influenced the extent of the replacement of magnesium ions during fermentation hence the variation. In this study, the cooking water was not discarded before storage of the cooked ALVs. However, the sugar-salt solution used during fermentation was drained, which could have contributed to mineral losses through leaching (Swain *et al.*, 2014). Also, the addition of salt encourages the growth of lactic acid bacteria (LAB) which increases the osmolality and ionic strength of the fermentation medium, causing water and minerals to be extracted from vegetable tissues (Mutegi, 2002). On the other hand higher minerals found in the spontaneously fermented-dried ALVs could probably be due to release of bound minerals caused by LAB activation which releases minerals from their chelated complex compounds (Gabriel *et al.*, 2011). However, the localisation of the mineral in the vegetable leaf matrix determines how the mineral reacts to processing (Raes *et al.*, 2014). Minerals in the ALVs could have been located in different plant organelles attributing to variations in the influence of fermentation. Leaf structure and size could also have influenced the effect of fermentation, for instance, vegetables with small leaf structures (wild jute and spider plant) had low iron content than those with large broad leaves (amaranth and cowpea). As indicated in this study, some spontaneously fermented-dried ALVs had either higher or lower level of minerals compared to their cooked counterparts, hence, the present results contradict the conclusion that was made for cereal fermentation, which stated that fermentation does not have any consequence on mineral levels (Ejigui *et al.*, 2005).

Compared to their cooked counterparts the mineral content of blanched-dried ALVs was significantly ($p \leq 0.05$) lower for iron in cowpea (39%), spider plant (35%), wild jute (65%) and amaranth (12%), for magnesium in spider plant (14%) and Ethiopian kale (21%), for calcium in Ethiopian kale (23%), and for phosphorus in amaranth (30%) and Ethiopian kale (8%) (**Table 4-1**). In contrast, compared to cooked counterparts, mineral contents of blanched-dried ALVs

were significantly ($p \leq 0.05$) higher for iron in Ethiopian kale (49%), for zinc in Ethiopian kale (24%) and amaranth (6%), for calcium in wild jute (72%), phosphorus in amaranth (19%). There are no studies found which compared mineral content in blanched and cooked ALVs, however previous studies (Acho *et al.*, 2015; Mepba *et al.*, 2007) which compared raw and blanched ALVs reported lower levels of iron and zinc in blanched ALVs. In our study, ALVs were blanched for 5 minutes, which has been reported to be the maximum blanching time to minimise mineral losses through leaching (Acho *et al.*, 2015), however, some losses were still apparent. Therefore, the lower levels of mineral contents found in blanched ALVs compared to their cooked counterparts could be due to leaching out through the discarded blanching medium. The degree of heating, rate of leaching, duration of cooking and surface area exposed to water determines the degree in which minerals are lost through processing (Lešková *et al.*, 2006). The rate at which minerals are leached out into the blanching medium could have influenced the mineral content of each vegetable accounting for the variations observed. Leaf size and thickness of each ALV species could also have influenced how each vegetable reacts to blanching. ALVs, with wider and thicker leaves (amaranth and cowpea), seemed to have leached out less zinc, magnesium, calcium and phosphorus.

Except for Ethiopian kale, significantly ($p \leq 0.05$) lower levels of aluminium were observed in all other spontaneously fermented-dried ALVs compared to their cooked counterparts (**Table 4-1**). In addition, blanched-dried ALVs also had a lower aluminium content compared to their cooked counterparts except for Ethiopian kale. Lower contents of aluminium found in blanched-dried and spontaneously fermented-dried ALVs compared to cooked ALVs could be due to additional soil impurities, which were not removed by routine cleaning, being removed and discarded with the blanching and fermentation mediums before oven drying the vegetables. It has been suggested that aluminium might leach from the foil into vegetables during drying (Bassioni *et al.*, 2012). High levels of aluminium content observed in blanched-dried and spontaneously fermented-dried Ethiopian kale could be attributed to cross-contamination with aluminium foil, as it was observed that after drying Ethiopian kale stuck to the aluminium foil which lined the drying trays hence aluminium from foil possibly leached out into the ALV.

Table 4-2: A general linear model multivariate analysis of mineral content in African leafy vegetables controlling for processing

Vegetable species	mg/100 g, db					
	Iron	Zinc	Magnesium	Calcium	Phosphorus	Aluminium
Cowpea	25.41 ± 2.32 ^b	3.07 ± 0.09 ^a	304 ± 14 ^a	1970 ± 80 ^a	327 ± 8 ^a	23.85 ± 3.49 ^b
Spider plant	51.15 ± 4.20 ^d	11.66 ± 0.17 ^e	388 ± 16 ^b	2499 ± 54 ^c	611 ± 4 ^d	59.03 ± 5.66 ^d
Wild jute	18.75 ± 3.57 ^a	3.91 ± 0.19 ^b	580 ± 21 ^d	2219 ± 259 ^b	558 ± 36 ^c	17.13 ± 4.54 ^a
Amaranth	29.86 ± 1.25 ^c	6.39 ± 0.15 ^d	1392 ± 56 ^e	1903 ± 55 ^a	508 ± 20 ^b	33.09 ± 2.35 ^c
Ethiopian kale	19.29 ± 2.05 ^a	4.81 ± 0.22 ^c	501 ± 27 ^c	2678 ± 147 ^d	547 ± 9 ^c	24.39 ± 6.04 ^b

Values are expressed as means ± SE. Mean values in the same column with a different letter superscript are significant at $p \leq 0.05$. db = Dry basis

A general multivariate analysis of data in controlling for processing found that mineral contents were significantly ($p \leq 0.05$) different between ALV types (**Table 4-2**). Overall, spider plant contained the highest iron (51.2 mg/100 g), while wild jute (18.8 mg/100 g) and Ethiopian kale (19.3 mg/100 g) had the lowest levels. Spider plant also had the highest aluminium content (59.03 mg/100 g) compared to other ALVs. Significantly ($p \leq 0.05$) higher levels of zinc were also found in spider plant (11.7 mg/100 g) while low levels were observed in cowpea (3.07 mg/100 g). Amaranth contained the highest levels of magnesium compared to other ALVs. Ethiopian kale (2678 mg /100 g) significantly contained the highest calcium while cowpea (1970 mg/100 g) and amaranth (1903 mg /100g) had the lowest. High levels of aluminium found in spider plant could be due to its leaves which are near the ground surfaces which could result in soil contamination, which probably contributed to artificial iron content. Previously contamination has been reported in ALVs which were meticulously soaked and washed several times with changes of water (van Jaarsveld *et al.*, 2014). Therefore, as previously stated above, normal washing of ALVs as conducted in the current study may not have been effective in removing all the contaminants as indicated by high levels of aluminium in cooked ALVs (**Table 4-1**). Therefore, a non-commercialised ALV (spider plant) contained higher levels of essential minerals (iron and zinc) compared to the commercialised Ethiopian kale. Therefore, ALVs studied could be good sources of iron and zinc hence the need to promote their consumption and commercialisation for sustainable accessibility even when out of season.

In overall, a general multivariate analysis of the data controlling for vegetable type found that iron content of cooked ALVs (32.8 mg/100 g) was significantly higher ($p \leq 0.05$) than that of blanched-dried (22.8 mg/100 g) ALVs (**Table 4-3**). Though these vegetables were blanched at the maximum blanching time that is deemed to reduce mineral losses but still iron leached out. All other mineral contents were not significantly ($p > 0.05$) different between processing methods. Therefore, lower or higher levels of zinc, magnesium and calcium found in individual spontaneously fermented-dried and blanched-dried ALVs compared to their cooked counterparts (**Table 4-1**) could be due to other intrinsic characteristics of the ALVs such as matrix factors, leaf structure and propensity to contamination and not necessarily the processing methods. Therefore, some blanched-dried and spontaneously fermented-dried ALVs had either similar or significantly higher mineral contents than their cooked counterparts, thereby validating that blanched-dried and spontaneously fermented-dried vegetables are as nutritional good as freshly cooked ALVs.

Table 4-3: A general linear model multivariate analysis of different processing methods on the mineral content in African leafy vegetables controlling for vegetable type

Processing Method	Iron	Zinc	Magnesium	Calcium	Phosphorus	Aluminium
	mg / 100 g, db					
Cooked	32.80 ± 5.26 ^b	5.82 ± 1.10 ^a	647 ± 124 ^a	2121 ± 169 ^a	529 ± 37 ^a	36.55 ± 7.18 ^a
Blanched	22.77 ± 10.09 ^a	6.05 ± 1.06 ^a	625 ± 157 ^a	2176 ± 78 ^a	450 ± 33 ^a	26.76 ± 5.11 ^a
Fermented	31.07 ± 4.06 ^b	6.04 ± 0.90 ^a	627 ± 110 ^a	2465 ± 146 ^a	501 ± 36 ^a	31.18 ± 4.93 ^a

Values are expressed as means ± SE. Mean values in the same column with a different letter superscript are significant at $p \leq 0.05$. db = Dry basis

4.2.2 Antinutrient contents of ALVs

As previously stated in the review (**Chapter 2: Section 2.6**), antinutrients in ALVs are of significant importance as they inhibit mineral absorption by forming insoluble complexes with essential minerals rendering them unavailable for human absorption (Lazarte *et al.*, 2015a; Lucarini *et al.*, 2000; Shahidi & Ambigaipalan, 2015). This study evaluated levels of condensed tannin, total phenolic content (TPC), oxalates and phytates in variously processed ALVs.

4.2.2.1 Condensed Tannin contents of processed ALVs

The condensed tannin contents of differently processed ALVs varied (**Table 4-4**), the contents are expressed as mg catechin equivalents per 100 g of sample, dry weight basis. The tannin content for cooked, blanched-dried, and spontaneously fermented-dried ALVs varied between 137 to 220 mg CE/100 g, 116 to 174 mg CE/100 g and 58 to 223 mg CE/100 g, respectively. The variation of condensed tannin content in these ALVs may be a result of the genetic makeup of each vegetable and environmental factors such as sunlight, pollution, temperature, soil composition, water availability and seasonality (Blancquaert *et al.*, 2019; Holeski *et al.*, 2012). Also, biotic factors such as insects and pathogens attack during growth influences condensed tannin content (Ochoa-Velasco *et al.*, 2017). Due to various processing, condensed tannin content was significantly different across all vegetable types ($p \leq 0.05$). Condensed tannin content was significantly ($p \leq 0.05$) lower in spontaneously fermented-dried spider plant (14%), wild jute (40%), cowpea (58%) and amaranth (46%) than in their cooked counterparts. Significantly ($p \leq 0.05$) higher levels of condensed tannins were found in spontaneously fermented-dried Ethiopian kale (52%) than its cooked counterpart. Waluchio (2016), also found an 86% decrease in tannin content after spontaneous fermentation of cassava leaves. During fermentation microbial activity results in tannic acid being degraded into smaller compounds, and also fermentation results in the activation of the enzyme tannase (tannin acyl hydrolase), which hydrolyses the ester and depside bonds of tannic acid to form hydrolysable tannins (gallic acid) (Macedo *et al.*, 2011). The free tannins leach out into the fermentable medium, which is then discarded attributing to decreased tannin contents (Taylor & Duodu, 2015). Previous reviews concluded that during fermentation condensed tannins such as proanthocyanin are hydrolysed to phenols through the activation of Polyphenol oxidase that transforms tannins into smaller phenolic compounds, hence the reduction of tannin content and the increase in measured TPC (Emmambux & Taylor, 2003; Nkhata *et al.*, 2018), as observed in the current study.

Table 4-4: Condensed tannins and total phenolic contents of processed African leafy vegetables

Vegetable species		Condensed Tannins	Total Phenolic Content
		mg CE /100 g, db [%diff]	
Cowpea	COO	137 ± 14 ^b	2022 ± 83 ^{a*}
	BLA	174 ± 17 ^c [27]	2083 ± 155 ^a [3]
	FER	58 ± 5 ^a [-58]	2612 ± 55 ^b [29]
Spider plant	COO	172 ± 7 ^{b*}	2040 ± 25 ^{a*}
	BLA	140 ± 8 ^a [-19]	1951 ± 92 ^a [-4]
	FER	129 ± 9 ^a [-25]	3022 ± 293 ^b [48]
Wild jute	COO	220 ± 22 ^{b*}	2491 ± 222 ^{a*}
	BLA	116 ± 12 ^a [-47]	2106 ± 74 ^a [-15]
	FER	132 ± 13 ^a [-40]	2204 ± 176 ^a [-11]
Amaranth	COO	144 ± 16 ^b	1655 ± 95 ^{a*}
	BLA	132 ± 10 ^b	1857 ± 181 ^a
	FER	78 ± 8 ^a [-46]	1637 ± 110 ^a
Ethiopian kale	COO	147 ± 9 ^a	1103 ± 86 ^a
	BLA	135 ± 14 ^a	1020 ± 95 ^a
	FER	223 ± 18 ^b [52]	1168 ± 103 ^a

Values are expressed as mean ± SD (n = 4), Mean values of the same type of vegetable in the same column with different superscripts are significantly different at $p \leq 0.05$, FER = Fermented, BLA = Blanched, COO = Cooked, [% diff] = (amount of antinutrients in cooked ALV - antinutrients from blanched-dried or spontaneously fermented-dried ALV) / (antinutrients in cooked ALV) x 100, Values of cooked vegetable with a * are significantly different to the cooked Ethiopian kale at $p \leq 0.05$, db = dry basis, CE = catechin equivalents

Significantly ($p \leq 0.05$) lower contents of condensed tannin contents were found in blanched-dried spider plant (19%) and wild jute (47%) compared to their cooked counterparts while significantly ($p \leq 0.05$) higher contents were found in blanched-dried cowpea (27%) than in its cooked counterpart (**Table 4-4**). Lower levels by 61% of condensed tannins were also found in a study that blanch-dried ALVs at 97 °C for 15 minutes (Essack *et al.*, 2017). During blanching cell membranes rupture (Alonso *et al.*, 1998), and this probably leaches out some condensed tannins into the discarded blanching medium, contributing to their low levels. In addition, it has been reported that complete lowering of tannin content during blanching is time-dependent (Egbonu & Nzewi, 2016b), hence increasing blanching time will increase leaching out of tannins into the blanching medium and probably will also negatively affect mineral content. Lower levels of

condensed tannins in some ALVs are probably due to leaching out into the discarded blanching medium. In contrast, high levels were found in blanched-dried cowpea, and the current results are in agreement with a study that blanched-dried slender amaranth (*Amaranthus viridis*) and reported high levels of condensed tannins (Salau *et al.*, 2015). High temperatures break down hydrogen bonds that exist between tannin complexes hence increasing extraction of tannins during analysis (Peng & Jiang, 2004). A possible reason for the observed high levels of condensed tannins in blanched-dried cowpea could be due to its reaction towards heat exposure during blanching which acted on tannin complexes in it. Variation in condensed tannins between blanched-dried ALVs could be possibly due to differences in the distribution of tannins in each vegetable, environmental stresses and pollution faced during growth.

A general linear model multivariate analysis of the data controlling for processing showed that Ethiopian kale, wild jute and spider plant contained significantly higher levels of condensed tannin ($p \leq 0.05$) compared to other ALVs (**Table 4-5**). A general linear model multivariate analysis of the data controlling for vegetable type showed a significant difference ($p \leq 0.05$) in condensed tannin content between cooked vs blanched-dried and spontaneously fermented-dried ALVs (**Figure 4-1**). In overall, spontaneously fermented-dried (127 mg CE/100 g) and blanched-dried (139 mg CE/100 g) ALVs had significantly ($p \leq 0.05$) lower condensed tannin content compared to freshly cooked vegetables (164 mg CE/ 100 g).

Table 4-5: A general linear model multivariate analysis of tannin and total phenolic content in African leafy vegetables controlling for processing

Vegetable species	Tannin (mg CE /100g, db)	Total Phenolic (mg CE /100g, db)
Cowpea	123 ± 10 ^a	2239 ± 84 ^c
Spider plant	147 ± 7 ^b	2238 ± 153 ^c
Wild jute	156 ± 10 ^b	2267 ± 66 ^c
Amaranth	115 ± 38 ^a	1716 ± 46 ^b
Ethiopian kale	169 ± 42 ^b	1097 ± 31 ^a

Values are expressed as means ± SE. Mean values in the same column with a different letter superscript are significant at $p \leq 0.05$, db = dry basis,

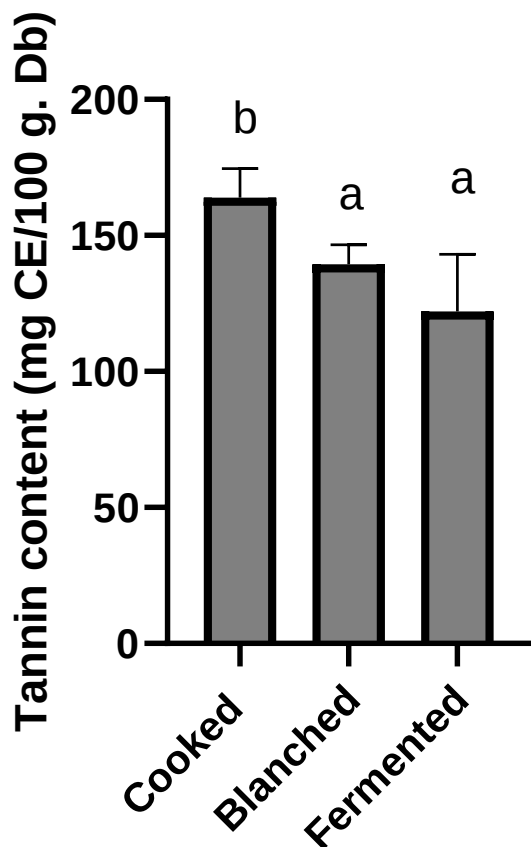


Figure 4-1: A general linear model multivariate analysis of tannin content in African leafy vegetables controlling for vegetable type. Values are expressed as means $\pm$ SE. Bars with a different letter superscript are significantly different at $p \leq 0.05$.

4.2.3 Total phenolic content of processed ALVs

The overall TPC of cooked, blanched-dried and spontaneously fermented-dried ALVs ranged between 1103 to 2491 mg CE/100 g, 1020 to 2106 mg CE/100 g, and 1168 to 3022 mg CE/100 g, respectively (**Table 4-4**). High levels of TPC ($p \leq 0.05$) were found in spontaneously fermented-dried cowpea and spider plant than in their cooked counterparts. Percentage differences in total phenolic content levels between spontaneously fermented-dried and cooked vegetables found for cowpea and spider plant were 29% and 48% respectively. In contrast, there were no significant ($p > 0.05$) differences found in total phenolic content of other spontaneously fermented-dried

ALVs (amaranth, wild jute and Ethiopian kale) compared to their cooked counterparts. The high levels of increase in TPC found in spontaneously fermented-dried ALVs could be attributed to the release of bound phenolic acids located in the cell walls (Gan *et al.*, 2016). The transformation of tannins into smaller phenolic compounds increases the total phenolic content measured (Nazarni *et al.*, 2016). Also, during fermentation the action of β -glucosidase hydrolyses phenolic glucosides to release free phenolics which could attribute to the increase in TPC measured (Adebo & Gabriela Medina-Meza, 2020). For example, high levels of condensed tannins in spontaneously fermented-dried Ethiopian kale (223 mg CE/100 g) compared to other vegetables may have been influenced by the rate of transformation of tannins into smaller phenolic compounds during fermentation. The variation in the TPC found in different vegetable species may be due to the genotype and maturity of each leafy vegetable. Abiotic factors influence the concentration of total phenolic contents (Blancquaert *et al.*, 2019).

On the other hand, no significant ($p > 0.05$) differences in total phenolic content in blanched-dried spider plant, wild jute, amaranth, cowpea and Ethiopian kale when compared to their cooked counterparts (**Table 4-4**). There are limited studies conducted on the effect of blanching on total phenolic content in ALVs, however previous studies conducted on sorghum observed similar results of a decrease in total phenolic compounds after blanching (Adebo *et al.*, 2018; Dlamini *et al.*, 2007). Phenolic compounds occurs in soluble forms and in combination with cell wall components (Francisco *et al.*, 2010). Therefore, lower levels found in blanched ALVs compared to cooked could be attributed of rupturing of cell membranes during blanching, which caused the release of phenolic compounds which leach out into the blanching water (Obboh & Rocha, 2007). The rate and proportion of cell membranes rupturing during blanching in each vegetable type could contribute to the variation in the amount of total phenolic content released into the discarded blanching mediums. A general linear model multivariate analysis of total phenolic content in processed ALVs controlling for vegetable type showed significant differences in total phenolic content ($p \leq 0.05$) between cooked and spontaneously fermented-dried ALVs (**Figure 4-2**). The overall total phenolic content found for the three different processes were 1861 mg CE/100 g, for cooking, 1804 mg CE/100 g, for blanching and 2129 mg CE/100 g, for fermentation, respectively. There were no significant differences found between the total phenolic content in cooked and blanched-dried ALVs ($p > 0.05$). These results of no changes after blanching are in agreement with a study which investigated the effect of blanching on the content of phenolic compounds in dill (herb), and reported that the total phenolic content was maintained when dill was blanched at 90°C for 30 seconds, cut into slices and dried (Galoburda *et al.*, 2012). It should be taken into consideration that each food product may require a different time for inactivation of peroxidase and polyphenol oxidase that degrades phenolic compounds (Xiao *et al.*, 2017). A general linear model multivariate analysis of vegetable type controlling for processing showed significant

differences ($p \leq 0.05$) in total phenolic content between each vegetable (**Table 4-5**). The lowest total phenolic content was observed in Ethiopian kale.

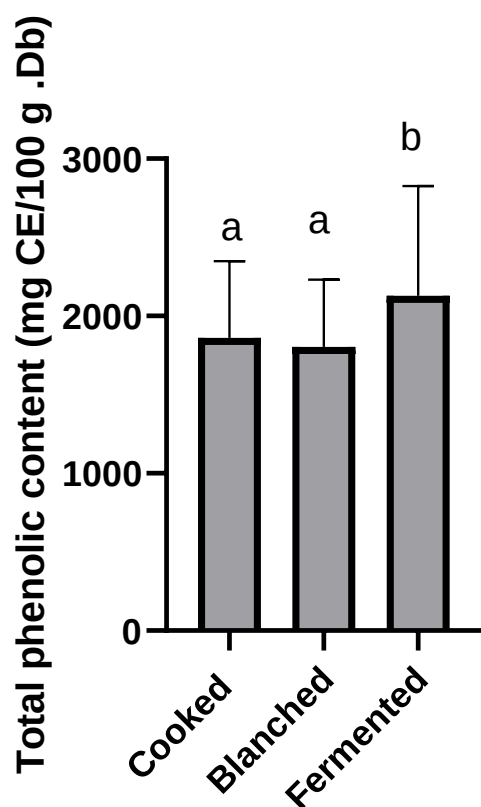


Figure 4-2: A general linear model multivariate analysis of phenolic content in African leafy vegetables. Values are expressed as means $\pm$ SE. Bars with a different letter superscript are significantly different at $p \leq 0.05$

4.2.4 Total Oxalate contents of processed ALVs

Concerning total oxalates, all spontaneously fermented-dried and blanched-dried ALVs had significantly ($p \leq 0.05$) lower total oxalates contents compared to their cooked counterparts (**Table 4-6**). The highest total oxalate content was found in cooked amaranth (4680 mg/100 g) and the lowest in spontaneously fermented-dried Ethiopian kale (843 mg/100 g). Values obtained for cooked ALVs in this study were much higher than the values previously reported for cooked amaranth (2883 mg/100 g), which was boiled for 15 minutes and drained (Mziray, 1999). The variations in the contents may be due to leaching out into the drained water in the previous study as in the current study the cooking water was not drained. Soluble oxalate content of processed ALVs ranged from 2334 to 4113 mg/100 g for cooked, 408 to 2039 mg/100 g for blanched-dried and 806 to 3219 mg/100 g for spontaneously fermented-dried, respectively. Significantly ($p \leq 0.05$) lower levels of soluble oxalates were found in all spontaneously fermented-dried ALVs compared to their cooked counterparts except for wild jute, in which levels were not significantly ($p > 0.05$) different. The salt-sugar brine solution was discarded after fermentation and probably contributed to low soluble oxalates levels due to leaching out into the discarded fermentable solution. Insoluble oxalate contents of cooked, blanched-dried and spontaneously fermented-dried ALVs varied substantially from 102 – 1361 mg/100 g, 187-1226 mg/100 g, and 37-196 mg/100 g, respectively. Spontaneously fermented-dried Ethiopian kale and wild jute had significantly ($p \leq 0.05$) lower insoluble oxalates contents than their cooked counterparts while in all other ALVs no significant ($p > 0.05$) differences were found. The reduction in pH that occurs during fermentation reduces the potential of oxalate to bind with divalent mineral cations to form insoluble oxalates (Simpson *et al.*, 2009) and this could account for the low levels of insoluble oxalates in the spontaneously fermented-dried vegetables. During fermentation LAB degrade oxalates, thereby reducing the levels (Goldfarb *et al.*, 2007; Okoye & Ene, 2018). However, there were no significant differences ($p > 0.05$) observed in other spontaneously fermented-dried ALVs (cowpea, spider plant and amaranth) when their insoluble oxalate content was compared to their cooked counterparts. Variation of insoluble oxalate levels between wild jute and other ALVs could be due to its appearance after fermentation. Wild jute became jelly-like after fermentation and less liquid was discarded before drying, which could account for the minor changes found in its soluble oxalates content. Wild jute has been found to contain pectin (Ahmed *et al.*, 2003), which probably influenced its processing to some extent. The type of food matrix influences the effectiveness of fermentation (Mota de Carvalho *et al.*, 2018). The findings of the current study showed some variations with some spontaneously fermented-dried ALVs having low levels of insoluble oxalate while others showed no significant differences when compared to their cooked counterparts, probable this may be due to how each vegetable matrix reacted to fermentation. However, a

general linear model multivariate analysis of insoluble oxalate content in ALVs in controlling for vegetable type showed substantially lower levels ($p \leq 0.05$) of insoluble oxalate in spontaneously fermented-dried than in cooked ALVs (**Figure 4-3**). The results of this study confirm the influence of each vegetable type on fermentation.

All blanched-dried ALVs had significantly lower levels ($p \leq 0.05$) of total oxalates and soluble oxalates compared to their cooked counterparts (**Table 4-6**). Total oxalates in all blanched-dried ALVs were 33 to 53% lower than in their cooked counterparts. During blanching soluble oxalates are leached out into the blanching medium resulting in low levels (Raes *et al.*, 2014), justifying the low levels of total oxalates found in blanched-dried ALVs in this investigation. Regarding insoluble oxalates, blanched-dried spider plant and Ethiopian kale had significantly ($p \leq 0.05$) higher contents than their cooked counterparts while no significant ($p > 0.05$) differences were found in other ALVs. Insoluble oxalates are only effectively reduced by prolonged thermal processing which transforms them into soluble constituents (Savage & Klunklin, 2018). Blanched-dried ALVs were not subjected to prolonged heat treatment, as stated vegetables were blanched at the shortest effective blanching time of maximum 5 minutes to lessen mineral losses through leaching. The current findings found that blanched-dried ALVs had high levels of insoluble oxalates (**Table 4-6**), cooked ALVs were subjected to heat for 15 minutes which may account for some insoluble oxalates transformed to soluble constituents reducing levels of insoluble oxalates in cooked ALVs compared to their blanched-dried counterparts. Insoluble oxalates are removed from the body through faeces excretion while soluble oxalates chelate with essential minerals forming insoluble complexes rendering these minerals unavailable for absorption (Natesh *et al.*, 2017). Dairy containing products are encouraged concurrently with food products with high levels of oxalates to alleviate the problems of kidney stones (Noonan & Savage, 1999).

Table 4-6: The oxalate contents of processed African leafy vegetables

Vegetable species	Processing method	Total oxalates	Soluble oxalates	Insoluble oxalates
mg/100 g, db [% diff]				
Cowpea	COO	3349 ± 224 ^{b*}	2967 ± 181 ^b (87)*	382 ± 68 ^a
	BLA	2226 ± 177 ^a [-33]	2039 ± 58 ^a (91)[-31]	187 ± 229 ^a
	FER	2111 ± 51 ^a [-37]	2031 ± 49 ^a (80)[-31]	80 ± 36 ^a
Spider plant	COO	2502 ± 55 ^c	2400 ± 64 ^c (96)	102 ± 107 ^a
	BLA	1862 ± 212 ^b [-26]	804 ± 24 ^a (43)[-67]	1058 ± 226 ^b
	FER	1272 ± 36 ^a [-50]	1234 ± 20 ^b (98)[-49]	37 ± 55 ^a
Wild jute	COO	3835 ± 403 ^{c*}	2470 ± 119 ^b (65)	1361 ± 409 ^{b*}
	BLA	1754 ± 178 ^a [-54]	812 ± 21 ^a (46)[-67]	943 ± 199 ^b
	FER	2629 ± 227 ^b [-34]	2433 ± 43 ^b (96)	196 ± 206 ^a
Amaranth	COO	4680 ± 242 ^{c*}	4113 ± 197 ^c (91)*	567 ± 45 ^a
	BLA	2126 ± 87 ^a [-53]	1640 ± 25 ^a (19)[-60]	485 ± 97 ^a
	FER	3408 ± 43 ^b [-26]	3219 ± 112 ^b (96)[-22]	189 ± 78 ^a
Ethiopian kale	COO	2627 ± 92 ^c	2334 ± 109 ^c (93)	293 ± 72 ^b
	BLA	1633 ± 24 ^b [-38]	408 ± 10 ^a (25)[-83]	1226 ± 32 ^c
	FER	843 ± 24 ^a [-68]	806 ± 17 ^b (96)[-67]	37 ± 25 ^a

Values are expressed as mean ± SD (n = 6), Mean values of the same type of vegetable in the same column with different superscripts are significantly different at p ≤ 0.05, FER = Fermented, BLA = Blanched, COO = Cooked, Insoluble oxalates = (Total oxalates – soluble oxalates), (%) = (soluble oxalates / Total oxalates) × 100, [% diff] = (Amount of total oxalate in cooked ALV – the amount of total oxalate from blanched-dried or spontaneously fermented-dried ALV) / (Amount of total oxalate in cooked ALV) × 100, Values of cooked vegetable with a * are significantly different to the cooked Ethiopian kale at p ≤ 0.05, db = dry basis

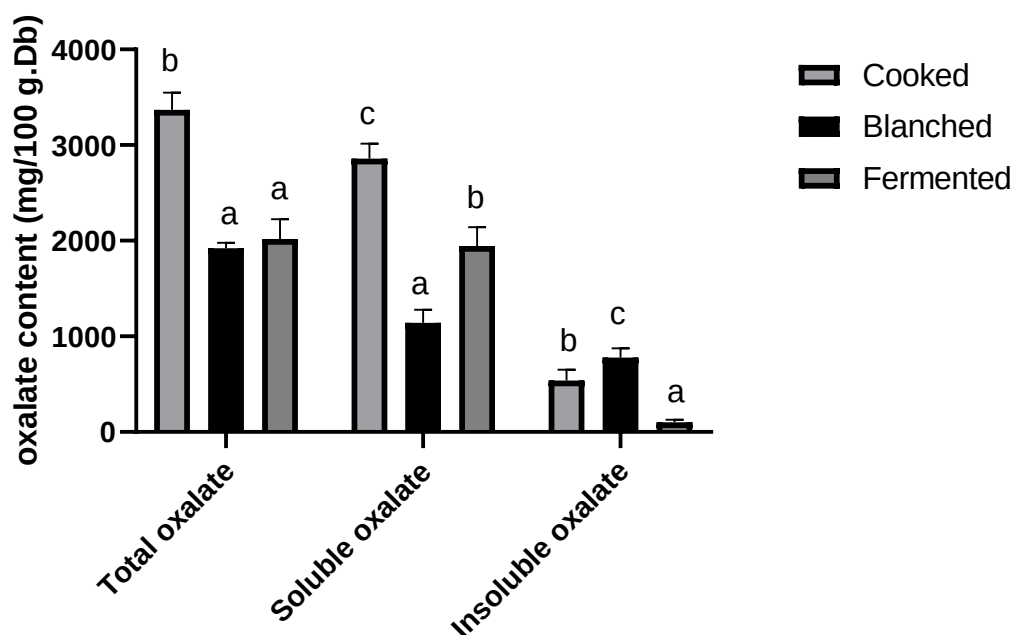


Figure 4-3: A general linear model multivariate analysis of oxalate content of processed African leafy vegetables in controlling for vegetable type. Bars corresponding to either total, soluble, or insoluble oxalates with a different letter superscript differ significantly at $p \leq 0.05$.

A general linear model multivariate analysis of total oxalate content controlling for vegetable type showed significant ($p \leq 0.05$) differences between spontaneously fermented-dried and cooked ALVs (**Figure 4-3**). Lower levels of total oxalates were observed in spontaneously fermented-dried ALV than in their cooked counterparts. A general linear model multivariate analysis of the total oxalate content in ALVs controlling for processing found significant ($p \leq 0.05$) differences (in total oxalate content between vegetable species (**Table 4-7**). Amaranth (3338 mg/100 g) and wild jute (2700 mg/100 g) had significantly ($p \leq 0.05$) higher amounts of total oxalates content compared to other ALVs. Low levels of total oxalates content were observed in spider plant and Ethiopian kale. However, total oxalates contents found in the current study for amaranth were lower than previously reported. High levels of total oxalate content were previously reported in raw amaranth (*Amaranthus hybridus*) (4860 – 6550 mg/100 g) (Onyango *et al.*, 2008). The current study and the previous study similarly used the same analytical method for oxalate determination, so differences in the results for amaranth could be influenced by some abiotic factors during growth. The study by Onyango and colleagues found an association between total oxalate content and amaranth samples from different locations.

Table 4-7: A general linear model multivariate analysis of oxalate content in African leafy vegetables controlling for processing.

Vegetable species	Total oxalates	Soluble oxalates	Insoluble oxalates
	mg/100 g, db		
Cowpea	2562 ± 603 ^b	2346 ± 470 ^{bc}	216 ± 181 ^a
Spider plant	1874 ± 543 ^{ab}	1479 ± 705 ^a	395 ± 308 ^{ab}
Wild jute	2700 ± 938 ^c	1905 ± 810 ^{ab}	796 ± 422 ^b
Amaranth	3338 ± 1044 ^c	2995 ± 1076 ^c	342 ± 245 ^{ab}
Ethiopian kale	1701 ± 763 ^a	1183 ± 869 ^a	519 ± 535 ^{ab}

Means ± SE, Insoluble oxalates = (Total oxalates – soluble oxalates), (%) = (soluble oxalates / Total oxalates) x 100, mean values in the same column with a different superscript are significantly different at $p \leq 0.05$, db = dry basis

4.2.5 Phytate content of processed ALVs

As stated, phytate is the principal storage form of phosphorus and inositol in plants (FAO/ZINC, 2018). Phytate also exists as a mixture of magnesium and potassium salts (Grases & Costa-Bauza, 2019). The phosphorus content of cooked ALVs varied substantially from 340 mg/100 g to 648 mg/100 g (**Table 4-8**). The ranges of phosphorus observed in the current study were higher than ranges previously reported (65 mg/100 g to 138 mg/100 g) (Schönfeldt & Pretorius, 2011). In a review conducted on ALVs it was reported that differences in mineral content of ALVs from different geographical locations might be due to variety of plant used, differences in stage of plant maturity and the method of analysis used (Uusiku *et al.*, 2010). It has been extensively researched that phytate binds with cations of zinc, calcium, magnesium and zinc (Gibson *et al.*, 2018). Insoluble complexes formed between phytate and iron, zinc and calcium exist (Lönnerdal, 2000). This section will focus on the phytate content (mg/100 g. db) of ALVs that underwent processing; however, the phytate-mineral molar ratios data were discussed with data for mineral bioaccessibility.

As stated, the presence of antinutrients in ALVs is of critical importance as they inhibit iron and zinc bioavailability (Kruger *et al.*, 2015). The phytate content varied substantially from 1977 to 4408 mg/100 g for cooked, 1751 to 3866 mg/100 g for blanched-dried and 1831 to 3429 mg/100 g for spontaneously fermented-dried ALVs, respectively (**Table 4-8**). Phytate content of cooked

ALVs ranged from 4408 mg/100 g (Ethiopian kale) to 1977 mg/100 g (amaranth). Values reported of the current study are higher than values previously reported by other studies. Previous studies which evaluated phytate in cooked cowpea and amaranth reported 731 mg/100 g of phytate content in a cowpea relish (Kruger *et al.*, 2018) and 2089 mg/100 g of phytate content in amaranth (Kruger *et al.*, 2015). A similar method for phytate determination was used by the current study and other studies (**Chapter 3**), therefore, variation in phytate content between studies may be due to variation in maturity stages of vegetables used. It has been previously reported that phytate content increases with maturity in amaranth and spider plant at stage 1 and stage 2 of maturity (Mwanri *et al.*, 2018). The exact maturation stage of the vegetables in the current study was not determined, but the ALVs were harvested at a more matured stage (December to March) which is the time that they are normally consumed in households giving a true picture of what is normally consumed.

Phytate contents of blanched-dried and spontaneously fermented-dried vegetables were different across all vegetables ($p \leq 0.05$). Significantly ($p \leq 0.05$) lower levels of phytate were observed in spontaneously fermented-dried amaranth (22% lower) and spider plant (25% lower) compared to their cooked counterparts. On the other hand, there were no significant ($p > 0.05$) differences found in phytate contents between other spontaneously fermented-dried vegetables (cowpea, wild jute, and Ethiopian kale) compared to their cooked counterparts. Similarly, lower levels of phytate after ALV fermentation were reported in a study which spontaneously fermented spider plant for 48 hours (Akello, 2014). During fermentation, endogenous phytase is activated which degrades phytate (Coulibaly *et al.*, 2011). Phytase degrades phytate (InsP6, myo-inositol 1, 2, 3, 4,5,6,-hexakisphosphate) into lower forms of phytates which have lower binding capacity to minerals (Coulibaly *et al.*, 2011). LAB produces lactic acid which decreases the pH to the optimal range for phytase activity increasing phytate degradation (Gupta *et al.*, 2015), so the extent of reduction of inositol phosphate levels varies during fermentation of plant products due to the amount of lactic acid produced. The pH of the ALVs at the end of fermentation period varied (5.3 to 3.9) (**Figure 3-7**). The amount of lactic acid produced during fermentation of ALVs in the current study varied in each vegetable and influenced the action of phytase; hence the variation in phytate levels found each ALV. In addition, there is a possibility that high levels of condensed tannin found in spontaneously fermented ALVs (**Table 4-4**) influenced the extent of fermentation in each ALV. Condensed tannins are associated with formation of insoluble complexes with proteins inhibiting digestive enzymes (Shahidi & Ambigaipalan, 2015). There is a likelihood that the action of phytase was probably inhibited by high levels of condensed tannins resulting in less degradation of phytate. The presence of high levels of phytate in spontaneously fermented-dried ALVs is a concern predominantly that it binds to iron and zinc forming insoluble complexes.

Table 4-8: Phytate contents and phytate-mineral molar ratios of processed African leafy vegetables

Vegetable species		Phytate	PA : Zn ¹	PA : Fe ²	PA x Ca : Zn ³
		(mg/100 g, db) [% diff]			
Cowpea	COO	2263 ± 189 ^a	78.1 ± 6.5 ^b	6.5 ± 0.5 ^a	3585 ± 299 ^a
	BLA	2131 ± 297 ^a [-6]	69.1 ± 9.8 ^{ab}	9.8 ± 1.4 ^b	3187 ± 453 ^a
	FER	2025 ± 263 ^a [-11]	59.9 ± 7.8 ^a	6.0 ± 0.8 ^a	3316 ± 430 ^a
Spider Plant	COO	2437 ± 272 ^b	20.1 ± 2.2 ^a	3.4 ± 0.4 ^a	1232 ± 137 ^a
	BLA	1848 ± 272 ^a [-24]	15.5 ± 2.3 ^a	3.9 ± 0.6 ^a	921 ± 135 ^a
	FER	1831 ± 246 ^a [-25]	14.8 ± 1.7 ^a	2.7 ± 3.2 ^a	982 ± 109 ^a
Wild jute	COO	2140 ± 408 ^a	48.3 ± 10.9 ^a	6.2 ± 1.2 ^a	2038 ± 388 ^a
	BLA	2296 ± 386 ^a [7]	57.5 ± 10.8 ^b	18.7 ± 3.2 ^c	3930 ± 660 ^b
	FER	2194 ± 418 ^a [3]	48.3 ± 9.2 ^a	11.4 ± 2.2 ^b	3352 ± 638 ^b
Amaranth	COO	4408 ± 213 ^b	68.5 ± 3.3 ^a	12.5 ± 0.6 ^b	3406 ± 164 ^b
	BLA	3866 ± 538 ^{a b} [-12]	56.3 ± 7.8 ^a	12.4 ± 1.7 ^b	2789 ± 389 ^a
	FER	3429 ± 532 ^a [-22]	56.6 ± 8.8 ^a	8.8 ± 1.4 ^a	2443 ± 379 ^a
Ethiopian kale	COO	1977 ± 90 ^a	47.4 ± 2.4 ^b	12.5 ± 0.6 ^b	3419 ± 155 ^c
	BLA	1751 ± 126 ^a [-11]	34.0 ± 2.5 ^a	7.4 ± 0.7 ^a	1876 ± 135 ^a
	FER	1831 ± 246 ^a [-7]	34.9 ± 4.7 ^a	6.3 ± 0.9 ^a	2547 ± 343 ^b

Values are expressed as mean ± SD (n = 6), Mean values of the same type of vegetable in the same column with different superscripts are significantly different at $p \leq 0.05$, FER = Fermented, BLA = Blanched, COO = Cooked, [% diff] = (phytate content in cooked ALV - phytate content in blanched-dried or spontaneously fermented-dried ALV) / (phytate content in cooked ALV) x 100). Values of cooked vegetable with a * are significantly different from the cooked Ethiopian kale at $p \leq 0.05$, db = dry basis

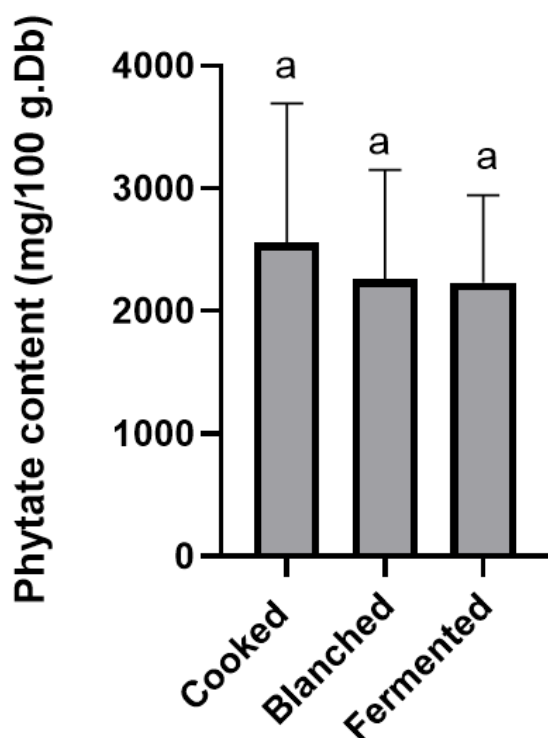


Figure 4-4: A general linear model multivariate analysis of phytate content in African leafy vegetables controlling for vegetable type. Values are expressed as means $\pm$ SE. Bars with different letter superscripts are significantly different at $p \leq 0.05$.

There were no significant differences ($p > 0.05$) found between phytate contents of all blanched-dried ALVs and their cooked counterparts except for spider plant which had 24% lower phytate content than its cooked counterpart (**Table 4-8**). Prolonged heating has been reported to be effective in degrading phytate (Deol & Bains, 2010) contribution to the reduction in levels. Previously, a study reported no significant effect in phytate levels after blanching *Moringa oleifera* leaves for 15 minutes (Sallau *et al.*, 2012). Therefore, the lack of differences observed between blanched-dried and cooked ALVs could be accounted to the duration of heat exposure which probably was not favourable for phytate degradation. A general linear model analysis of the phytate content in processed ALVs controlling for vegetable species exhibited no significant ($p > 0.05$) differences between the processing methods (**Figure 4-4**). Despite processing, Ethiopian kale (1850 mg/100 g) had the lowest phytate content while the highest phytate content was observed in amaranth (3979 mg/100 g) (**Table 4-9**). ALVs in this study contained high levels of

phytate, which possibly have a negative effect on iron and zinc bioavailability. Therefore, other processing methods should be explored to ensure substantial reduction of phytate levels in ALVs.

Table 4-9: A general linear model multivariate analysis of phytate content in African leafy vegetables controlling for processing.

Vegetable species	Phytate (mg/100 g, db)
Cowpea	2130 ± 260 ^{ab}
Spider plant	2004 ± 410 ^{a b}
Wild jute	2208 ± 387 ^b
Amaranth	3979 ± 575 ^c
Ethiopian kale	1850 ± 177 ^a

Means ± SE, mean values in the same column with a different superscript are significantly different with $p \leq 0.05$, db = Dry basis.

4.2.6 The relationship of minerals and antinutrients in processed African leafy vegetables

The principal component analysis (PCA) was constructed to structure and visually display the relationship/correlation between mineral and antinutrients contents of traditionally processed ALVs (**Figure 4-5**). The PCA shows that total oxalates, soluble salts, and magnesium were positively correlated which could be an indication that oxalates were complexed with magnesium than with calcium resulting in oxalates in these ALVs predominantly in the form of magnesium oxalate. Magnesium has a higher affinity to oxalates, thus when dietary magnesium levels are higher than calcium levels, it reduces the ability of oxalate to crystallise into stones (Kmieciak *et al.*, 1997). Insoluble calcium oxalates pose a health risk as it can accumulate in the renal glomeruli and form kidney stones (Massey, 2007). It has been reported that magnesium consumed in combination with oxalates decreased the absorption of oxalates leading to its excretion through urine (Zimmermann *et al.*, 2005a). As evidenced in this study, ALVs had high levels of oxalates (**Table 4-6**) and magnesium (**Table 4-1**), having magnesium complexing with oxalate could be beneficial in avoiding kidney stones formation after consumption of these ALVs. Therefore, high oxalate contents found in this study could not be detrimental to human health considering its form.

Phytate primarily is found in plant-based foods as a complex salt of non-valent and divalent cations of magnesium, calcium and potassium (Coulibaly *et al.*, 2011). The PCA graph displayed a strong positive correlation between phytate and magnesium contents, which could be an indication that phytate is predominantly present in the form of magnesium salt. Magnesium phytate salts chelates with metal ions especially zinc, iron and calcium forming insoluble complexes that are not absorbable for human utilization (Gibson *et al.*, 2010). A strong negative association shown between calcium, magnesium and phytate by the PCA graph overrides the possibility of phytate existing in the form of phytin (mixture of calcium and magnesium) in ALVs in the current study. Another factor that affects the solubility of phytate is pH. Most phytate salts are more soluble at low pH values (Greiner & Konietzny, 2006). The solubility of phytate-mineral complexes increases at pH values below 5.5 to 6.0 for calcium and 7.2 to 8.0 for magnesium respectively (Nissar *et al.*, 2017). In the current investigation, lower levels of phytate were observed in spontaneously fermented-dried spider plant (pH 4.9) and amaranth (pH 3.9) (**Figure 3-7**) compared to their cooked counterparts (**Table 4-8**), which could infer that some phytate in these spontaneously fermented-dried ALVs solubilised and leached out into the fermentable mediums which was discarded before drying the vegetables resulting in low levels of phytate found.

The PCA graph also showed that iron, zinc, and aluminium were closely associated. As stated, high aluminium content in ALVs (**Table 4-1**) could be associated with soil contamination. Therefore, the strong correlation observed between aluminium, zinc and iron may strongly suggest that a portion of iron and zinc from these ALVs may be from soil/dust contamination resulting in artificially high levels of these minerals in the vegetables. A review concluded that human studies should be conducted to ascertain the bioavailability of contaminant iron as previous studies reported contradictory results of low and no bioavailability of contaminant iron (Teklu, 2017). The presence of soil contamination in these ALVs may give inaccurate results on the intrinsic content of these essential minerals. Therefore, estimating the bioavailability of iron and zinc from ALVs referring only to their mineral concentration and phytate mineral molar ratios may give misleading results as it does not consider artificial minerals contributed by soil/dust which may be unabsorbable.

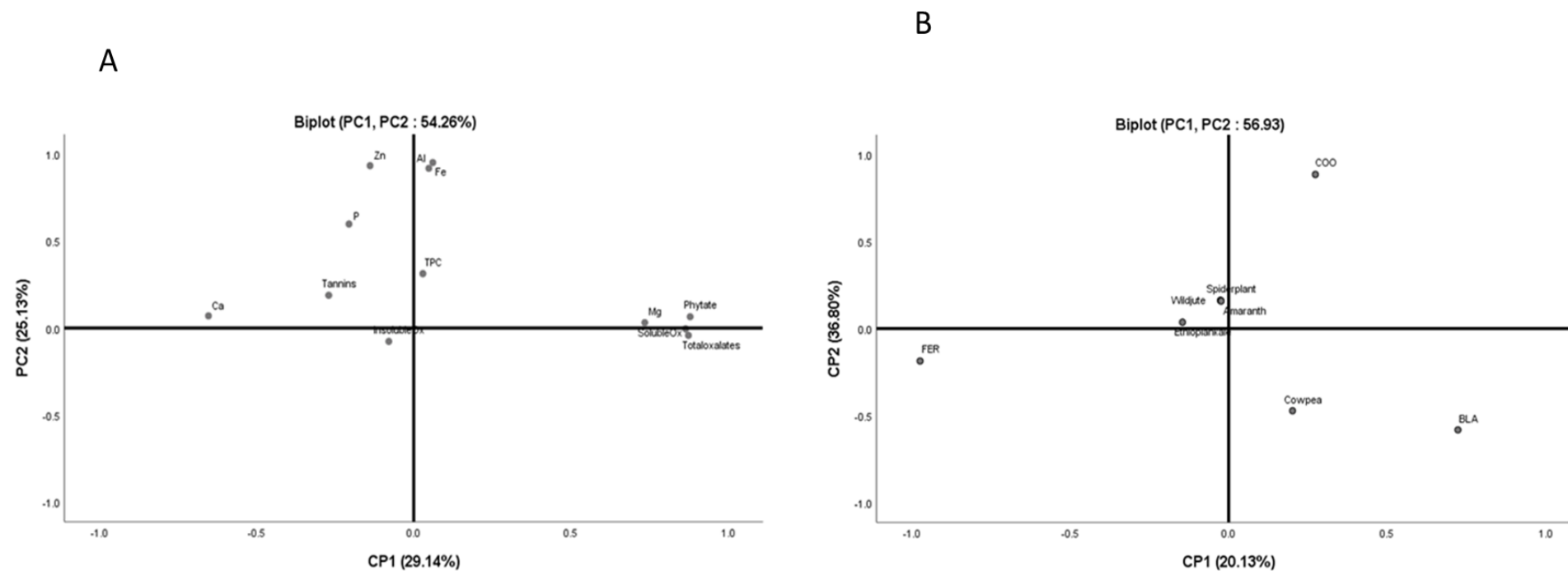


Figure 4-5: Principal component analysis of minerals and antinutrients of processed African leafy vegetables

- A. PCA loadings; phytate, total oxalate, soluble oxalate, insoluble oxalate total phenolic content (TPC), tannins, iron, zinc, phosphorus, magnesium, calcium, aluminium.
- B. African leafy vegetable species; spider plant, cowpea, wild jute, amaranth, Ethiopian kale and processing methods (COO = cooked, BLA = Blended, FER = Fermented).

4.3 Bioaccessibility of iron and zinc from processed African leafy vegetables

Bioaccessibility estimates the amount of extractable mineral from the food matrix available for absorption in the gut (Jenzer, 2016). Bioaccessibility includes the digestive transformation of food matrix and nutrient absorption into the intestinal epithelium cells (Fernández-García *et al.*, 2009). It is evaluated *in vitro*, by simulating the stomach and small intestine conditions (Etcheverry *et al.*, 2012). ALVs in the current investigation had high levels of antinutrients that may inhibit the bioaccessibility of iron and zinc. As previously stated, ALVs investigated were processed similar to how they are usually prepared when consumed fresh and consumed when out of season (**Figure 1-1**). Both the percentage and amount of mineral bioavailable are crucial in the evaluation of mineral bioaccessibility. The two measures give a good indication of the total amount of nutrient content and nutrient bioaccessibility as influenced by inhibitors and enhancers (Kruger *et al.*, 2015). The percentage mineral bioaccessibility gives the percentage bioaccessible amount of mineral of the total mineral content and the amount of mineral bioaccessible gives the actual mineral value available for absorption. Both percentage mineral bioaccessibility and amount of bioaccessible mineral were evaluated. Values of bioaccessibility are presented per 100 g of dry matter.

4.3.1 Iron bioaccessibility from processed African leafy vegetables

As stated, high mineral content in the food matrix does not always translate into higher mineral bioaccessibility and bioavailability (Carbonell-Capella *et al.*, 2014). The bioaccessibility of iron from plant-based diets (non-haem) iron is naturally low due to presence of antinutrients that inhibits non-haem iron absorption (Milman, 2020). The percentage iron bioaccessibility and amount of iron bioaccessible in blanched-dried and spontaneously fermented-dried ALVs were significantly ($p \leq 0.05$) different across all vegetable types. The percentage iron bioaccessible and amount of iron bioaccessible from ALVs in this study varied from 1.6 to 15.3% and 0.47 to 3.20 mg/100 g for cooked vegetables (**Table 4-10**). The values of percentage iron bioaccessibility (10.7%) and amount of iron bioaccessible (3.20 mg/100 g) from cooked amaranth in the current study were within values previously reported in amaranth in a study that evaluated amaranth composited with other ALVs (Kruger *et al.*, 2015). The previous study found 9.7% and 1.60 mg/100 g of percentage iron bioaccessibility and amount of iron bioaccessible in amaranth, respectively.

Table 4-10: The bioaccessibilities of iron and zinc of processed African leafy vegetables

		Bioaccessibility		Amount bioaccessible	
Vegetable Species [#]		($\%$)		mg/100 g, db [% difference]	
		Fe	Zn	Fe	Zn
Cowpea	COO	3.9 $\pm$ 0.5 ^a	35.7 $\pm$ 4.4 ^a	1.15 $\pm$ 0.14 ^a	1.04 $\pm$ 0.13 ^a
	BLA	2.1 $\pm$ 0.9 ^a	24.2 $\pm$ 10.8 ^a	0.38 $\pm$ 0.17 ^a [-67]	0.73 $\pm$ 0.32 ^a [-30]
	FER	3.7 $\pm$ 1.3 ^a	30.8 $\pm$ 5.4 ^a	1.06 $\pm$ 0.86 ^a [-8]	1.02 $\pm$ 0.66 ^a [-2]
Spider plant	COO	2.1 $\pm$ 0.7 ^a	15.7 $\pm$ 5.2 ^a	1.30 $\pm$ 0.42 ^a	1.88 $\pm$ 0.62 ^a
	BLA	1.8 $\pm$ 0.3 ^a	18.2 $\pm$ 5.3 ^a	0.71 $\pm$ 0.12 ^a [-45]	2.14 $\pm$ 0.62 ^a [14]
	FER	1.5 $\pm$ 0.3 ^a	17.0 $\pm$ 3.0 ^a	0.80 $\pm$ 0.16 ^a [-38]	1.90 $\pm$ 0.33 ^a [1]
Wild jute	COO	1.6 $\pm$ 0.6 ^a	22.5 $\pm$ 10.0 ^a	0.47 $\pm$ 0.19 ^{a*}	0.83 $\pm$ 0.37 ^a
	BLA	4.4 $\pm$ 0.6 ^a	25.1 $\pm$ 7.9 ^a	0.46 $\pm$ 0.06 ^a [-2]	0.89 $\pm$ 0.28 ^a [7]
	FER	2.3 $\pm$ 0.2 ^a	12.2 $\pm$ 1.3 ^a	0.37 $\pm$ 0.03 ^a [-21]	0.55 $\pm$ 0.05 ^a [-34]
Amaranth	COO	10.7 $\pm$ 2.3 ^a	8.4 $\pm$ 4.6 ^a	3.20 $\pm$ 0.68 ^{b*}	0.54 $\pm$ 0.30 ^a
	BLA	7.8 $\pm$ 0.4 ^a	18.0 $\pm$ 16.3 ^b	2.06 $\pm$ 0.64 ^a [-36]	1.23 $\pm$ 1.10 ^a [128]
	FER	11.5 $\pm$ 4.7 ^a	16.6 $\pm$ 10.1 ^b	3.81 $\pm$ 1.26 ^b [19]	1.00 $\pm$ 0.61 ^a [85]
Ethiopian kale	COO	15.3 $\pm$ 3.6 ^a	40.9 $\pm$ 8.6 ^a	2.05 $\pm$ 0.38 ^b	1.67 $\pm$ 0.35 ^a
	BLA	2.5 $\pm$ 1.9 ^a	16.6 $\pm$ 13.5 ^a	0.51 $\pm$ 0.37 ^a [-75]	0.85 $\pm$ 0.68 ^a [-49]
	FER	2.6 $\pm$ 1.1 ^a	22.3 $\pm$ 7.0 ^a	0.63 $\pm$ 0.26 ^a [-69]	1.16 $\pm$ 0.36 ^a [-31]

Values are expressed as mean $\pm$ SD (n = 4), Mean values of the same type of vegetable in the same column with different superscripts are significantly different at $p \leq 0.05$, FER = Fermented, BLA = Blanched, COO = Cooked, [% diff] = (amount of mineral bioaccessible in cooked ALV - the amount of mineral bioaccessible in blanched-dried or spontaneously fermented-dried ALV) / (amount of mineral bioaccessible in cooked ALV) $\times$ 100). Values of cooked vegetable with a * are significantly different to the cooked Ethiopian kale at $p \leq 0.05$, # mineral availabilities expressed in two ways; mineral content in dialysate as a % of the total mineral in a vegetable, as well as the amount of minerals in the dialysate, db = dry basis

The percentage iron bioaccessibility and amount of bioaccessible iron in blanched-dried and spontaneously fermented-dried ALVs ranged from 1.8% to 7.8%, 0.38 to 2.06 mg/100 g and 1.4% to 10.2%, 0.37 to 3.81 mg/100 g, respectively (**Table 4-10**). Significantly ($p \leq 0.05$) higher percentage of iron bioaccessibility was found in spontaneously fermented-dried Ethiopian kale compared to its cooked counterpart while no significant ($p > 0.05$) differences were found between other spontaneously fermented-dried ALVs and their cooked counterparts. Compared to cooked counterparts, percentage iron bioaccessibility and amount of iron bioaccessible in all the blanched-dried ALVs showed no significant ($p > 0.05$) differences except for Ethiopian kale. Significantly ($p \leq 0.05$) lower amount of bioaccessible iron was found in blanched-dried amaranth compared to its cooked counterpart. Ethiopian kale has been previously found to contain high levels of β -carotene (Odongo *et al.*, 2017). β -carotene is an enhancer of iron bioaccessibility (Gautam *et al.*, 2010b; Kruger *et al.*, 2018). Drying results in losses of β -carotene and ascorbic acid in ALVs (Mutuli & Mbugue, 2018), which probably affected iron bioaccessibility content in spontaneously fermented-dried and blanched-dried Ethiopian kale compared to its cooked counterpart. A recent study reported that the presence of β -carotene counteracted the inhibitory effect of phytate on non-haem iron, making it absorbable (Butler & Ghugre, 2020). However, the current study did not assess β -carotene content of variously processed ALVs, therefore, this study could not draw a direct conclusion that β -carotene content in Ethiopian kale influenced its iron bioaccessibility positively. Blanched-dried amaranth had lower iron contents (26.4 mg/100 g) compared to its cooked counterpart (33.2 mg/100 g) (**Table 4-1**) which could have attributed to the variation in the amount of iron bioaccessible between the amaranth samples.

The lack of significant differences in the percentage iron bioaccessibility found in spontaneously fermented-dried and blanched-dried ALVs compared to their cooked counterparts could be due to high levels of antinutrients found in all processed ALVs (**Table 4-4, Table 4-8**), which forms complexes with iron rendering it unabsorbable. High levels of phytates found in all processed ALVs possibly had a negative effect on iron bioaccessibility. Phytate forms insoluble phytate-iron complexes which affect iron solubility (Dasa & Abera, 2018). Phytate and magnesium were positively correlated (**Figure 4-5**) and as stated this form of phytate binds with iron forming insoluble complexes rendering iron unabsorbable and could contribute to the low iron bioaccessibility found. The high phytate/iron molar ratios found also indicate that phytate had a more substantial inhibitory effect on iron bioaccessibility. Phytate-iron molar ratios of all processed ALVs ranged between 2.7 to 18.7 (**Table 4-8**) and were above the critical level of less than 1 which estimate iron bioavailability that is strongly impaired (Hurrell, 2004). The inhibitory effect of phytate on iron is dose-dependent and occurs even at lower concentrations. It was previously reported that 2 mg of phytate inhibited iron absorption

by 18%, 25 mg by 64% and 250 mg by 82% respectively (Hallberg *et al.*, 1989). To achieve four to five fold increases in non-haem iron bioaccessibility, phytate should be completely degraded (Gibson *et al.*, 2010) or the phytate/iron molar ratios should be reduced to less than 1 to increase iron absorption (Hurrell, 2004). Therefore, with high phytate levels found in the current study, the inhibitory effect of phytate on iron was more pronounced.

High levels of condensed tannins and total phenolic contents were found in all processed ALVs in this study (**Table 4-4**). Condensed tannins' inhibitory effect on iron bioaccessibility is even more pronounced than that of phytate (Greffeuille *et al.*, 2011), hence any reduction in phytate levels during processing may not be significant in the presence of condensed tannins. Condensed tannins are associated with the formation of insoluble complexes with iron and also binding with protein forming tannin-protein complexes inhibiting digestive enzymes activation that are crucial in the breaking down of food matrix to release minerals in the lumen (Shahidi & Ambigaipalan, 2015). A study that evaluated the inhibitory effect of ten flavonoids on pepsin *in vitro* found that all flavonoids bound directly into the enzyme cavity size and reduced the enzyme activity (Zeng *et al.*, 2015). In addition, high phenolic contents found in ALVs in the current study had an inhibitory effect on iron bioaccessibility which could have occurred through iron-phenolic complex formation which would inhibit iron bioaccessibility. It has been demonstrated previously that iron-polyphenol complexes inhibit the bioaccessibility of iron (Dueik *et al.*, 2017). Both condensed tannins and total phenolic content had inhibitory effects on iron bioaccessibility in this study.

A strong association between iron and aluminium contents was observed (**Figure 4-5**) indicating that some of the iron contents could be from extrinsic contamination, affecting the overall iron bioaccessibility. There is controversy as to the bioavailability of contaminant iron and therefore human studies should be conducted to ascertain the bioavailability of contaminant iron (Teklu, 2017). Hence it may suggest that a portion of iron in ALVs was from soil/dust contamination which was less bioavailable attributing to low iron bioaccessibility.

A general linear model multivariate analysis of percentage iron bioaccessibility from processed ALVs controlling for vegetable species found no significant ($p > 0.05$) differences in the percentage iron bioaccessibility between cooked and spontaneously fermented-dried vegetables, while significantly ($p \leq 0.05$) lower percentage iron bioaccessibility was found in blanched-dried vegetables compared to cooked vegetables (**Figure 4-6**). However, a general linear model multivariate analysis of percentage iron bioaccessibility and amount of iron dialysable controlling for vegetable species showed no significant ($p > 0.05$) differences in the amount of bioaccessible iron between cooked and spontaneously fermented-dried ALVs

(Figure 4-7). Overall, the amounts of iron bioaccessible from blanched-dried and cooked ALVs were significantly ($p \leq 0.05$) lower than from spontaneously fermented-dried ALVs. Blanched-dried ALVs had a lower total iron content which possibly influenced the overall percentage of iron bioaccessibility. Therefore, spontaneously fermented-dried ALVs could be as good as cooked ALVs in terms of percentage iron bioaccessibility. Overall, despite processing significant ($p \leq 0.05$) high levels of iron bioaccessible were found in amaranth (2.98 mg/100 g) (Table 4-11), though this is a relative estimate not the actual value of mineral available for absorption (Etcheverry *et al.*, 2012), it can be anticipated that amaranth is a good source of iron and can be promoted in nutritional interventions for rural communities who may have limited access to haem iron sources to alleviate iron deficiencies but human studies are needed to confirm bioavailability.

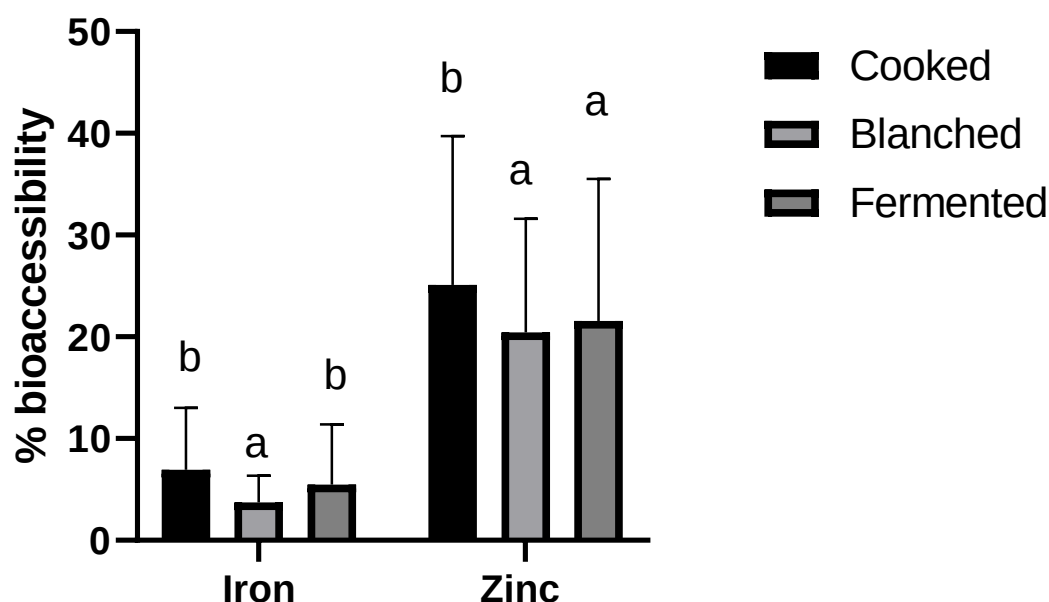


Figure 4-6: A general linear model multivariate analysis of different processing methods on the percentage amount of iron and zinc dialysable from African leafy vegetables in controlling for vegetable type. Values are expressed as means $\pm$ SE. Bars with a different letter superscript are significantly different at $p \leq 0.05$.

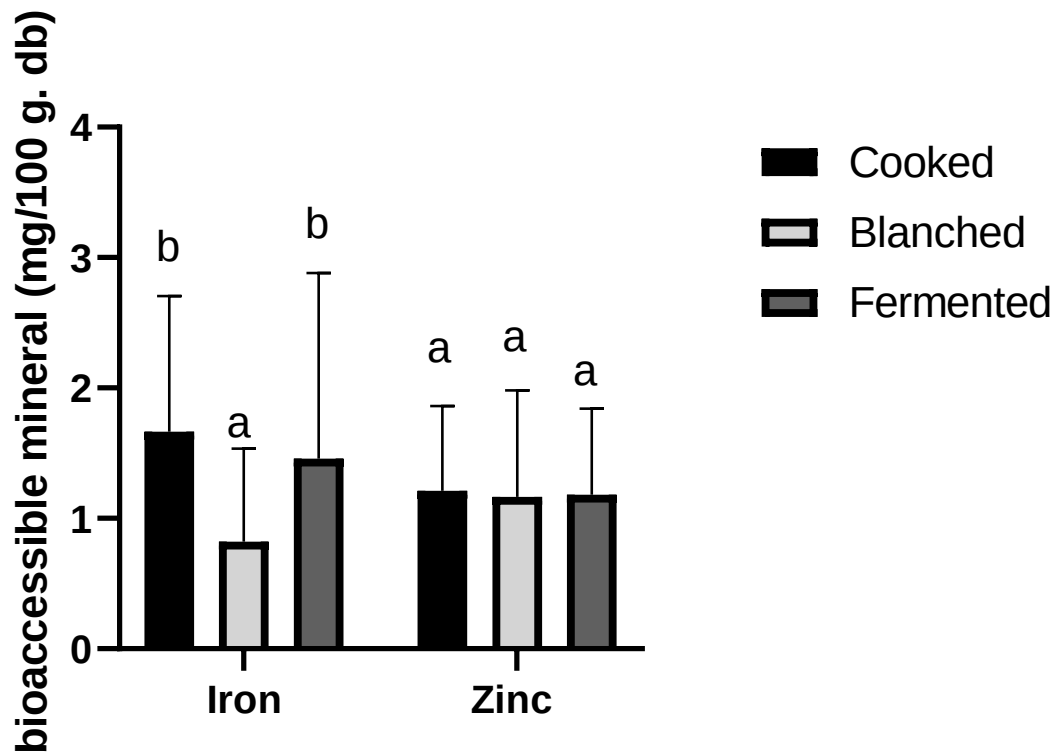


Figure 4-7: A general linear model multivariate analysis of different processing methods on the amount of iron and zinc dialysable from African leafy vegetables in controlling for vegetable type. Values are expressed as means $\pm$ SE. Bars with a different letter superscript are significantly different at $p \leq 0.05$.

Table 4-11: A general linear model multivariate analysis of the bioaccessibility of iron and zinc from African leafy vegetables in controlling for processing

Vegetable species	Bioaccessibility (%)		Amount bioavailable (mg /100 g, db)	
	Fe	Zn	Fe	Zn
Cowpea	3.2 ± 1.8 ^a	30.2 ± 12.9 ^b	0.85 ± 0.58 ^a	0.92 ± 0.41 ^a
Spider plant	1.8 ± 0.5 ^a	17.0 ± 4.5 ^a	0.94 ± 0.37 ^a	1.98 ± 0.53 ^b
Wild jute	2.8 ± 1.4 ^a	21.5 ± 9.1 ^b	0.44 ± 0.12 ^a	0.79 ± 0.30 ^a
Amaranth	9.9 ± 3.1 ^c	14.2 ± 2.8 ^a	2.98 ± 1.1 ^b	0.92 ± 0.77 ^a
Ethiopian kale	6.0 ± 1.7 ^b	25.0 ± 14.33 ^b	0.97 ± 0.77 ^a	1.17 ± 0.69 ^a

Values are expressed as mean ± SE, mean values in the same column corresponding to the same mineral with a different superscript differ significantly at $p \leq 0.05$. db = Dry basis,

Table 4-12: Correlations between antinutrients and mineral bioaccessibility of African leafy vegetables

Antinutrient	% Fe bioaccessible	Amount of Fe bioaccessible	% Zn bioaccessible	Amount of Zn bioaccessible
Phytate	0.390**	0.656**	-0.416**	-0.405**
Total phenolic content	0.472**	0.276	-0.158	-0.016
Tannin	-0.369**	-0.424**	-0.150	-0.013
Soluble oxalates	0.457**	0.599**	-0.145	-0.321
Insoluble oxalates	0.183	0.057	0.762	0.793
Total oxalates	0.378**	-0.486**	-0.169	-0.348**

** Correlation is significant at $p \leq 0.01$ level (2-tailed), N = 56, Degree of freedom = 54

Iron bioaccessibility in differently processed ALVs was correlated to antinutrients content and correlation was deemed to be significant at $p \leq 0.01$. There was a weak positive association demonstrated between phytate contents and percentage iron bioaccessibility ($r = 0.390$, $p \leq 0.01$) while a strong positive association was demonstrated between the amount of bioavailable iron and phytate contents ($r = 0.656$, $p \leq 0.01$) (**Table 4-12**). Not only phytate but also other vegetable components (enhancers and other antinutrients) could have influenced iron bioaccessibility. A weak inverse association between the amount of bioavailable iron and tannin content was also observed ($r = -0.424$, $p \leq 0.01$). Condensed tannins have been found to have highly inhibitory effects on the bioaccessibility of iron (Kruger *et al.*, 2012), in the current study their inhibitory effect may not have been evident indicating that other factors together with condensed tannins influenced the amount of iron that is bioaccessible. In addition, a positive weak correlation existed between total phenolic content and iron bioaccessibility in the current investigation ($r = 0.472$, $p \leq 0.01$), this weak positive association possibly does not exempt the inhibitory effect of total phenolic content on iron bioaccessibility from ALVs. Therefore, these factors together with other ALVs components not evaluated in this study (levels of enhancers such as organic acids and β -carotene) could have played a role in lowering the bioaccessibility of iron in ALVs.

4.3.2 Zinc bioaccessibility from processed African leafy vegetables

The percentage bioaccessibility of zinc in spontaneously fermented-dried and blanched-dried ALVs differed between vegetable species ($p \leq 0.05$). The percentage zinc bioaccessibility and amount of bioaccessible zinc in cooked ALVs ranged between 8.4 to 40.9% and 0.54 to 1.88 mg/100 g respectively (**Table 4-10**). The values were within ranges of zinc bioaccessibility previously reported in cooked amaranth (7%) and ALVs composited with each other (7.3 - 8.1%) (Kruger *et al.*, 2015). In contrast, the current study observed higher zinc bioaccessibility from cooked cowpea (35%) compared to a study which observed lower contents (10%) (Kruger *et al.*, 2018). The differences in the levels of condensed tannin and total phenolic contents between this study and the other study possibly caused the variation in the percentage of zinc bioaccessibility between the two studies. Cooked cowpea had 137 mg CE/100 g of condensed tannins and 2022 mg CE/100 g of total phenolic content respectively (**Table 4-4**) while the study by Kruger *et al.* (2018), reported 1021 mg CE/100 g of condensed tannins and 5295 mg CE/100 g of total phenolic content. Both condensed tannins and total phenolic contents have some inhibitory effects on zinc bioaccessibility (Kim *et al.*, 2011); hence their levels will account for the variations in levels between the two studies.

There were no significant ($p > 0.05$) differences found in percentage zinc bioaccessibility between the same type of cooked and spontaneously fermented-dried ALVs (**Table 4-10**).

Significantly ($p \leq 0.05$) higher amounts of bioaccessible zinc were found in blanched-dried amaranth (14.5%) than in its cooked counterpart. On the other hand, there were no significant differences ($p > 0.05$) found in the percentages of zinc bioaccessibility from all other blanched-dried ALVs compared to their cooked counterparts. Blanched-dried amaranth sample had 50% lower total oxalate content compared to its cooked counterpart sample (**Table 4-6**) which probably attributed to the variation between samples. Oxalates impair zinc absorption due to poor solubility of zinc oxalate (Mills, 2013).

High levels of phytates were found in all processed ALVs this study (**Table 4-8**), which also possibly contributed to the low zinc bioaccessibility found. Phytate has an inhibitory effect on zinc bioaccessibility (Gibson *et al.*, 2018), it binds to zinc forming insoluble complexes making the mineral unabsorbable. The phytate/zinc molar ratios for ALVs in the current study were very high (14.8-78.1). Phytate/zinc molar ratios values above the critical level of 15 predict zinc bioavailability that is seriously impaired (FAO & WHO, 2005). Values of phytate/zinc molar ratios found in these ALVs were two to four times higher than the critical level value of 15 except for spider plant, attributing to a more pronounced phytate inhibitory effect on zinc bioaccessibility. However, spider plant phytate/zinc molar ratios were also high (14.8 to 20.1).

Calcium-phytate/zinc molar ratios accurately predict zinc bioavailability than phytate/zinc molar ratios (Bindra *et al.*, 1986). Calcium has the affinity to form complexes with phytate and zinc that are insoluble and consequently has an inhibitory effect on zinc absorption (Fordyce *et al.*, 1987). Phytate-calcium/zinc molar ratios for which zinc bioavailability is seriously impaired are higher than the critical value of 200 (Bindra *et al.*, 1986). The phytate-calcium/zinc molar ratios in all processed ALVs were very high (921 to 3930) (**Table 4-8**). These molar ratios were four to nineteen times more than the critical value, which reveals that the combined inhibitory effect of phytate and calcium on zinc was strong. Therefore, considering high calcium and phytate contents in all processed ALVs found in this study, their combined inhibitory effect on zinc bioaccessibility cannot be ignored.

High levels of tannins and total phenolic content observed in the current investigation (**Table 4-4**) probably contributed to no significant differences found between zinc bioaccessibility in cooked ALVs compared to spontaneously fermented-dried or blanched-dried same species of ALVs. Though some blanched-dried and spontaneously fermented-dried ALVs had lower condensed tannins (spider plant and wild jute) than cooked counterparts, their levels were still high to probably have some inhibitory effects on zinc bioaccessibility. Both condensed tannin and total phenolic content binds to zinc and hinders its bioaccessibility (Kim *et al.*, 2011). Total

phenolic content complexes with zinc, consequently resulting in insoluble compounds which inhibit zinc bioaccessibility (Călinoiu & Vodnar, 2018). Therefore, high levels of total phenolic content found in the spontaneously fermented-dried samples (1168 to 3022 mg CE/100 g) contributed to low zinc bioaccessibility from spontaneously fermented-dried ALVs. The structure and type of phenolic compounds present also determine how it chelates zinc (Gabaza, 2018). It has been reported that phenolic compounds with catechol and galloyl groups, even when present in small amounts, negatively impact on mineral bioaccessibility (Towo *et al.*, 2006). This study measured total phenolic content; hence there is a need to quantify and identify the type of phenolic compounds in these ALVs in future.

The overall percentage zinc bioaccessibility in cooked, blanched-dried and spontaneously fermented-dried ALVs were 24.7%, 19.1% and 20.4% respectively (**Figure 4-6**), with significantly lower percentages of zinc bioaccessibility observed in spontaneously fermented-dried and blanched-dried ALVs compared to their cooked counterparts. There were no significant ($p > 0.05$) differences observed in the amount of zinc bioaccessible between spontaneously fermented-dried and cooked same type of ALV. Although the bioaccessibility of zinc in spontaneously fermented ALVs has not been previously reported, previous studies reported its positive effect of fermentation in cereals (Gabaza, 2018; Kruger *et al.*, 2018). This has been attributed to decreases in antinutrient contents. Though overall low condensed tannins (**Figure 4-1**) and total oxalates (**Figure 4-3**) levels were found in the spontaneously fermented-dried ALVs in the current study, these did not positively influence zinc bioaccessibility. The bioaccessibility of zinc was not significantly correlated with antinutrient contents ($p > 0.01$) (**Table 4-12**). Therefore, this may suggest that other ALV components such as calcium contents had more pronounced inhibitory effects on zinc bioaccessibility. As stated, calcium in the presence of phytates had a combined inhibitory effect on zinc bioavailability (Lönnerdal, 2000). Organic acids also found in vegetables such as citric and malic had been reported to influence zinc bioavailability (Lönnerdal, 2000). During ALV processing organic acids could have been lost and contributed to low zinc bioaccessibility found. Further studies should be conducted to ascertain the extent of loss of enhancers of zinc during processing of ALVs.

A general linear model multivariate analysis of the amount of zinc bioaccessible in ALVs controlling for processing found significant ($p \leq 0.05$) differences in the amount of zinc bioaccessible between vegetable species (**Table 4-11**). Spider plant had significantly higher amounts of bioaccessible zinc (1.78 mg/100 g). There are few good sources of zinc from plant sources, therefore, spider plant could contribute to zinc requirements in humans than other ALVs.

4.3.3 The potential contribution of processed ALVs towards absolute requirements for zinc and iron for women of aged 19 to 50 years

Total absolute requirement (AR) is the total amount of mineral required for growth, basal iron losses and, in women, also includes menstrual losses (FAO & WHO, 2001). For women aged 19 to 50 years, the absolute requirement for iron is 1.46 mg/day (FAO & WHO, 2001) and for zinc, 1.75 mg/day (Sandstead, 2015). The contribution that each processed ALV could make towards iron or zinc AR for women aged 19 to 50 years were calculated based on when a standard portion of 300 g (as it is eaten) of ALV is consumed per day as per the findings of a previous study (van der Hoeven *et al.*, 2013). However, *in vitro* dialysability data should not be interpreted as an actual value but as a relative because the assay does not simulate all conditions that happen *in vivo* (Fairweather-Tait *et al.*, 2005).

The contribution that 300 g (as consumed) of ALV could make towards AR for women aged 19-50 years was calculated using bioaccessibility data. The moisture contents of processed ALVs were used to convert the dry weight basis to wet weight basis (**Table 4-13**) in order to calculate mineral contribution. The percentage iron contribution estimation that could be made by cooked, blanched-dried and spontaneously fermented-dried ALVs towards iron AR were 14.5% to 50.3%, 6.1% to 40.6% and 3.9% to 48.1%, respectively (**Figure 4-8**). Higher contributions were observed in amaranth irrespective of the type of processing. Amaranth has the potential to contribute over 40% towards AR for iron towards women aged 19 to 50 years who have limited access to haem iron, but it should be highlighted that this is only an estimate not actual mineral bioavailability as uptake was not evaluated *in vivo*. *In vitro* dialysability assays does not entirely simulate all factors involved in human digestion because host factors such as age, nutritional status, physiological conditions (pregnancy, obesity), presence of chronic and acute infections can influence mineral absorption in humans (Etcheverry *et al.*, 2012), hence the measured magnitude of the response may differ with that in humans.

Table 4-13: Moisture contents of processed African leafy vegetables

Processing	Cowpea	Spider plant	Wild jute	Amaranth	Ethiopian kale
	Moisture (%)				
COO	93.9 ± 2.6 ^a	81.9 ± 0.4 ^a	92.9 ± 2.6 ^c	95.5 ± 0.5 ^c	83.1 ± 3.7 ^a
BLA	85.7 ± 0.3 ^a	85.9 ± 0.3 ^a	64.7 ± 7.4 ^a	69.6 ± 0.1 ^a	94.1 ± 0.5 ^b
FER	86.8 ± 0.3 ^a	82.5 ± 0.1 ^a	76.2 ± 0.7 ^b	80.3 ± 0.8 ^b	96.8 ± 0.1 ^b

Values are expressed as mean ± SD. n=2. Mean values in the same column corresponding to same vegetable species with different superscripts were considered significant at $p \leq 0.05$, FER=Fermented, BLA = Blanched, COO = Cooked

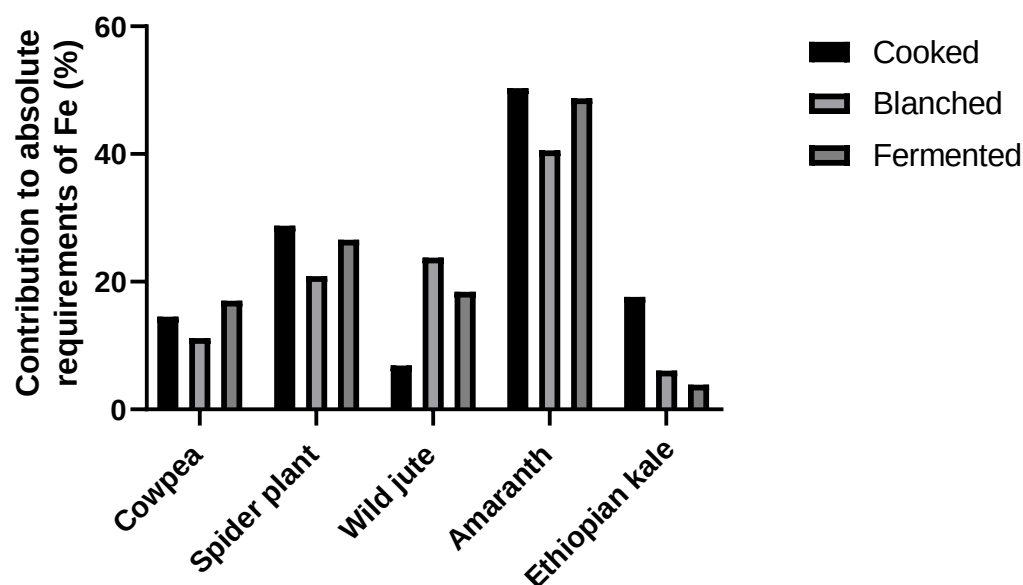


Figure 4-8: Contribution (%) of a 300 g portion of processed African leafy vegetables towards absolute iron requirements of women aged 19 to 50 years

The percentage zinc contributions that 300 g of cooked, blanched-dried, and spontaneously fermented-dried ALVs could make towards zinc AR for women aged 19 to 50 years estimated

were 2.9% to 34.9%, 8.5% to 51.9% and 6.0% to 57.2%, respectively (**Figure 4-9**). Spider plant could make the most considerable contribution towards the zinc AR for a woman aged 19 to 50 years, but intervention studies are needed to evaluate whether it can improve status as the bioaccessibility estimated using *in vitro* dialysability assays cannot be substituted for situations in humans (Etcheverry *et al.*, 2012).

Though this investigation revealed that ALVs could possibly contribute over 40.6% towards iron AR (amaranth) and over 34.9% towards zinc AR (spider plant) for women aged 19 to 50 years, these values only give an estimate or direction of bioavailability which needs to be validated *in vivo*. Unlike estimating mineral contribution using Recommended Dietary Allowances (RDA) relative to mineral concentration, which does not take into account mineral inhibitory factors (FAO & WHO, 2005), the current results account for mineral inhibition hence are a better estimate of mineral contribution. Though the iron and zinc contributions by these ALVs towards AR for iron and zinc for women aged 19 to 50 years may look promising, ALVs are usually consumed with staples such as maize, millet and sorghum, which also contains high levels of antinutrients that inhibit iron and zinc absorption. Food needs to be prepared in a similar manner in which they are generally consumed to be more applicable to human bioavailability (Fairweather-Tait *et al.*, 2005). A study that composited ALVs with refined maize porridge found an increase in iron and zinc bioaccessibility because of low antinutrient contents in refined maize (Kruger *et al.*, 2015). Therefore, there is a need for reduction of antinutrients in staple foods through processing when consumed concurrently with ALVs otherwise ALVs mineral contribution could be overrated.

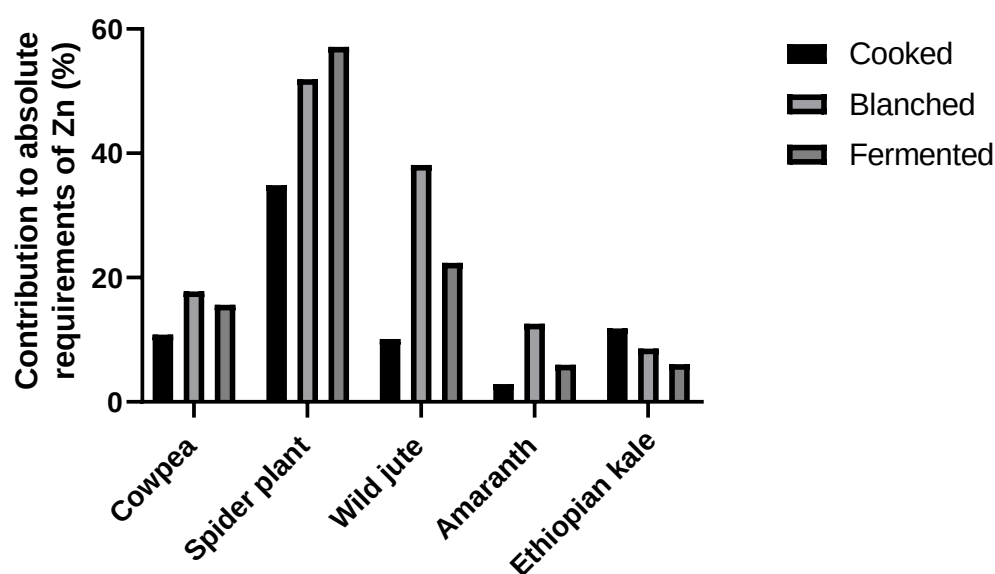


Figure 4-9: Contribution (%) of a 300 g portion of processed African leafy vegetables towards absolute zinc requirements for women aged 19 to 50 years

The bioaccessibility of iron and zinc from ALVs have been previously investigated (Kruger *et al.*, 2018; Kruger *et al.*, 2015) but none of these studies has addressed both the bioaccessibility and intestinal uptake of iron and zinc hence this study further evaluated iron and zinc uptake from differently processed ALVs using Caco-2 cells. Mineral dialysability cannot be directly translated to bioavailability as it does not measure the uptake of minerals (Perales *et al.*, 2005). Therefore, there is a need to use the Caco-2 cell line, which simulates the mineral uptake in the small intestine, transport and utilisation (Etcheverry *et al.*, 2012).

4.4 Cellular uptake of iron and zinc by Caco-2 cells from differently processed ALVs

Bioavailability is the amount of mineral that reaches the system circulation and used by the body (Carbonell-Capella *et al.*, 2014). Bioavailability estimation *in vitro* is often determined by measuring the uptake and transport of nutrients by Caco-2 cells (Etcheverry *et al.*, 2012). An understanding of the estimation of bioavailability of iron and zinc from ALVs is crucial as these vegetables are consumed in households everyday by people living in rural communities in sub-Saharan Africa, usually as a relish that accompanies staple foods. Caco-2 cells, which are the human intestinal adenocarcinoma cells, exhibit the same biochemical and morphological characteristics of small intestinal enterocytes and have been used together with simulated gastrointestinal digestion to estimate mineral bioavailability (Etcheverry *et al.*, 2012; Perales *et al.*, 2005). Uptake by Caco-2 cells is an important indicator of mineral bioavailability as it measures the amount of mineral retained and transported into the cell (García-Nebot *et al.*, 2013).

The third phase of the current study (estimation of bioavailability) was conducted based on the dialysability assay results, which served as a measure of bioaccessibility of iron and zinc available for absorption. Bioavailability cannot be measured using *in vitro* assays because it has a metabolic or physiological end point (Etcheverry *et al.*, 2012), therefore it can only be estimated. Mineral absorption in humans can be influenced by the nutritional status of the body, age, gender, physiological factors such as pregnancy, obesity or lactation and presence of acute and chronic diseases (Etcheverry *et al.*, 2012). Thus, the *in vitro* uptake is estimated to provide information on possible interactions between food components and minerals, the

effect of food processing on mineral absorption and the potential of minerals to be absorbed from food (Sandberg, 2005). An advantage of conducting both the *in vitro* human cell model together with the simulated gastrointestinal model for predicting bioavailability is that it is a more accurate representation of the *in vivo* conditions than using solubility studies (Cilla *et al.*, 2018). *In vitro*/Caco-2 models are handy screening tools in ranking mineral bioavailability from food products and promising results can be followed up using *in vivo* models.

Studies on the influence of blanching and fermentation on the uptake of iron and zinc are limited. Previous studies evaluated the bioaccessibility of iron and zinc in cooked ALVs (Kruger *et al.*, 2018; Kruger *et al.*, 2015) but none of these studies has concurrently addressed bioaccessibility and predict bioavailability (cellular uptake) of essential minerals of processed ALVs. Two ALVs, amaranth and spider plant, which had the highest amounts of bioaccessible iron and zinc were selected for further evaluation to predict the uptake of iron and zinc using *in vitro* Caco-2 cell model.

4.4.1 Estimation of iron bioavailability in processed African leafy vegetables measured by ferritin formation in Caco-2 cells

Caco-2 cell ferritin is a sensitive marker of iron uptake by the cells, when intracellular iron increases, cell ferritin production also increases (Ariza-Nieto *et al.*, 2007). Ferritin formation in the Caco-2 cells exposed to differently processed spider plant and amaranth was evaluated. As previously discussed, ALVs had high iron contents (2.9 to 12.0 mg/100 g, cooked, 3.0 to 11.8 mg/100 g, blanched-dried and 3.4 to 11.2 mg/100 g, spontaneously fermented-dried, respectively) (**Table 4-1**). However, high concentrations of minerals do not always translate to high bioavailability (Chiocchetti *et al.*, 2018). The ferritin formation in Caco-2 cells was measured in the negative (serum-free MEM/EBSS) and positive controls (50 μ M FeSO₄), which demonstrated the capacity of the model to predict ferritin formation (**Figure 4-10**).

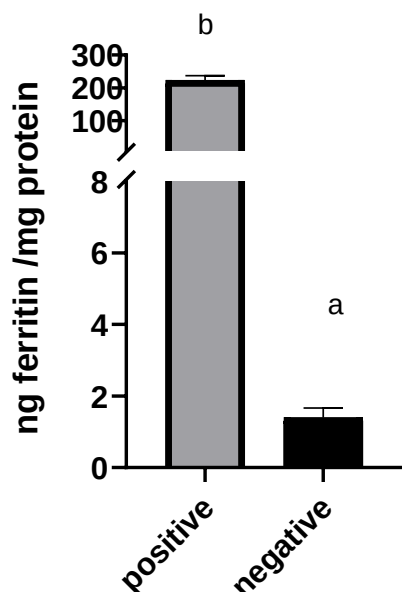


Figure 4-10: Ferritin formation in CaCo2 cells controls. Where, the positive control was 50 μ M ZnSO₄ and the negative control was serum free MEM/EBSS. Values expressed as mean $\pm$ SD (n=6), values on the bars with different superscripts differ significantly ($p \leq 0.05$).

The formation of ferritin in Caco-2 cells following exposure to the differently processed ALVs ranged from 0.31 ng ferritin/mg cell protein to 2.35 ng ferritin/mg cell protein (**Figure 4-11**). Generally, the ferritin formation in Caco-2 cells following exposure to differently processed ALVs were lower than ranges previously reported. A study that evaluated iron uptake in boiled green leafy vegetables commonly consumed in Ghana, reported between 6 to 10 ng ferritin/mg protein formation by Caco-2 cells (Amagloh *et al.*, 2017). The variation between the results could be attributed to levels of antinutrient content found in each study. The current study found high levels of total phenolic content in amaranth and spider plant (1637 to 3022 mg CE /100 g) while in the previous study's total phenolic content ranged from 196 to 1646 mg GAE/100 g. Therefore, higher levels of total phenolic content in the current study possibly contributed to the low ferritin measured in the cells. Total phenolic content reduces non-haem absorption by complexing it with Fe³⁺ with greater stability than Fe²⁺ making iron unabsorbable (Perron & Brumaghim, 2009). Also, the study above did not evaluate phytate, oxalate and tannin contents of the leafy vegetables and as stated ALVs in this study had high levels of tannins, oxalates and phytates (**Table 4-4, Table 4-6, Table 4-8**). These antinutrients have an inhibitory effect on iron absorption. AA content of influences iron uptake by Caco-2 cells. (Owade *et al.*, 2020). As stated, drying has some adverse effects on AA content (Naidu, 2003) which could have led to losses thereof in the spontaneously fermented-dried and blanched-dried samples. In the study above, Moringa was ranked highest in iron bioavailability (10 ng

ferritin/mg protein) with high levels of AA (46 mg/100 g) (Amagloh *et al.*, 2017). AA acts as a reducing agent for apical membrane-bound ferrereductase which reduces Fe^{3+} iron to a more absorbable Fe^{2+} iron (Teucher *et al.*, 2004). It has been concluded that AA is effective in enhancing iron bioavailability (Heffernan *et al.*, 2017). AA regulates cell iron uptake; it modulates the metabolism of iron by stimulating ferritin synthesis, preventing lysosomal ferritin degradation and reducing cell iron efflux (Lane & Richardson, 2014). ALVs in the current study were dried after blanching and fermentation which possibly degraded AA in ALVs. A review summed up that blanched and fermented dried vegetables contain lower levels of AA compared to raw vegetables (Owade *et al.*, 2020). AA is sensitive to oxygen, heat and light (Naidu, 2003). Therefore, processing techniques that reduce losses of iron enhancers should be explored.

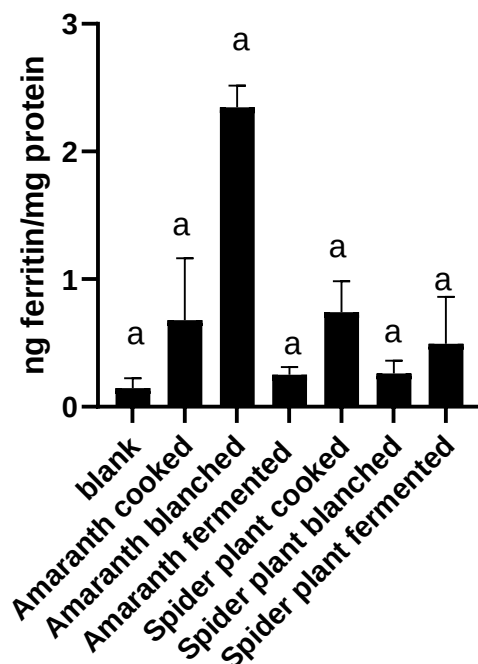


Figure 4-11: Ferritin formation in CaCo2 cells following the exposure of cells to differently processed African leafy vegetables. Values expressed as mean $\pm$ SD (n=6), values on the bars with different superscripts differ significantly ($p \leq 0.05$), Blank = no sample added

There were no significant ($p > 0.05$) differences found in the ferritin formation in Caco-2 cells between the same type of ALV differently processed (**Figure 4-11**). Both spontaneously fermented-dried and blanched-dried amaranth and spider plant had high levels of phytate (1831 to 3866 mg/100 g) (**Table 4-8**), condensed tannin (78 to 140 mg/100 g) and total phenolic contents (1637 to 3022 mg/100 g) (**Table 4-4**), and the presences of these antinutrients possibly affected ferritin formation in the Caco-2 cells negatively. As indicated, the phytate-iron molar ratios of both spider plant (2.7 – 3.4) and amaranth (8.8 – 12.5) were double to twelve times more than the critical value of 1 which is associated with iron that is seriously impaired (Hurrell, 2004). Phytates interfere with iron uptake through the formation of insoluble complexes with iron which make it less absorbable (Nissar *et al.*, 2017), contributing to low ferritin formation in Caco-2 cells. Phytates in these ALVs predominantly existed as magnesium phytate salts (**Figure 4-5**), this phytate salt strongly binds to iron making it unabsorbable by chelating with iron ions forming insoluble complexes that are less available for absorption (Gibson *et al.*, 2010). Moreover, the inhibitory effect of phytate on iron absorption is dose-dependent and occurs even at lower concentrations, with 2 mg of phytate inhibiting iron absorption by 18%, 25 mg by 64% and 250 mg by 82%, respectively (Hallberg *et al.*, 1989). Hence, high phytate contents found in amaranth and spider plant in the current study shows that iron uptake by the Caco-2 cells was seriously impaired by phytate contents which possibly contributed to low ferritin formation in the cells.

Total phenolic contents and condensed tannins have some inhibitory effects on iron uptake. Condensed tannins are associated with the formation of insoluble complexes with iron and also binding with protein forming tannin-protein complexes inhibiting digestive enzymes activation that are crucial in the breaking down of food matrix to release minerals (Delimont *et al.*, 2017; Shahidi & Ambigaipalan, 2015), which probably contributed to the low ferritin formation found in Caco-2 cells exposed to processed ALVs. In addition, total phenolic content forms insoluble complexes with Fe^{3+} reducing iron uptake (Perron & Brumaghim, 2009), consequently resulting in low ferritin formation in the cell line. The inhibitory effect of both condensed tannins and total phenolic content on iron uptake are dose-dependent (Delimont *et al.*, 2017; Shahidi & Ambigaipalan, 2015). A study by Hurrell *et al.* (1999), revealed that drinks containing 20 to 30 mg and 100 to 400 mg of total polyphenols per serving reduced iron absorption by 50-70% and 20 to 80%, respectively. As previously indicated, processed amaranth and spider plant had high levels of total phenolic content and condensed tannins (**Table 4-4**) which possibly contributed to iron inhibitory effects resulting in low ferritin formation in Caco-2 cells.

Low ferritin formation in Caco-2 cell exposed to simulated gastrointestinal digestates of processed ALVs could also be linked to the presence of artificial iron. Substantially high levels of aluminium were found in processed amaranth (26.2 to 39.0 mg/100 g) and spider plant with highest contents (56.0 to 75.8 mg/100 g) (**Table 4-1**). As stated, high levels of aluminium in plant-based foods could indicate soil contamination. The strong association between iron and aluminium found (**Figure 4-5**) possibly indicated the presence of high levels of artificial iron in these ALVs. There is controversy regarding the bioavailability of contaminant iron. Previous studies that assessed the bioaccessibility of contaminant iron using *in vitro* dialysability assays reported that contaminant iron is insoluble and unavailable for absorption (Icard-Vernière *et al.*, 2013; Seim *et al.*, 2013) while other studies reported that some fractions of contaminant iron is soluble in the GI and available for absorption (Hooda *et al.*, 2004; Kikouama *et al.*, 2009; Smith *et al.*, 2000). With contradictory results, a review concluded that rat haemoglobin repletion efficiency assays should be conducted before drawing conclusions on the bioavailability of contaminant iron as *in vitro* bioavailability assays may not entirely address all multiple effects of contaminant iron bioavailability (Teklu, 2017). Therefore, the current study could not ascertain that artificial iron present in the vegetables as indicated by high contents of aluminium was available for uptake.

Another factor that could have influenced low iron bioaccessibility in the current study is high calcium content in ALVs (1422 to 2928 mg/100 g). Calcium, either given as dairy products or calcium salts, inhibits iron absorption in humans (Lönnerdal, 2010). However, the mechanism in which calcium inhibit iron absorption is unknown (Lynch, 2000).

As stated, oxalates in plant-based foods may be present as insoluble magnesium or calcium oxalates and its influence on iron absorption rely on the chemical state of oxalate in the food (Milman, 2020). Oxalates in this study predominantly appeared to be in the form of magnesium oxalate salt (**Figure 4-5**). Substantially high levels of total oxalates were found in amaranth (2120 to 4680 mg/100 g) and spider plant (1272 to 2502 mg/100 g). Oxalates form stable salts with Fe^{3+} and Fe^{2+} (Genannt Bonsmann *et al.*, 2008), and Fe^{2+} oxalate is insoluble (Savage *et al.*, 2000). Oxalates complexes with Fe^{2+} blocking its absorption by the epithelial cells (Salovaara *et al.*, 2002), making iron unavailable for absorption. To some extent the presence of oxalates in these ALVs impaired the absorption on non-haem iron attributing to the low ferritin formation found in cells exposed to simulated gastrointestinal digestates of processed ALVs.

In the current study, vegetables were spontaneously fermented for one week without the addition of starter cultures. ALVs were spontaneously fermented typically the way they are traditionally fermented in households (Wafula *et al.*, 2016). The enhancing effect of

fermentation on iron availability has been reported to be attributable to acidification by natural lactic acid production rather than antinutrient reduction (Proulx & Reddy, 2007). A study that reported an increase in ferritin formation in spontaneously fermented-dried vegetables used a starter culture of *Lactobacillus plantarum* fermenting a mixture of vegetables (carrot, turnips, white cabbage, celery and onion) for one week at 20 °C (Scheers *et al.*, 2016). It is possible that spontaneous fermentation did not produce adequate lactic acid for enhanced iron absorption hence low ferritin formation in Caco-2 cells. However, the temperature of all vegetables increased as the fermentation progressed (24 to 32 °C) (**Figure 3-8**), while pH decreased for all vegetables as fermentation progresses (**Figure 3-7**). As stated, LAB needs an optimum temperature for lactic acid production. Further studies that involve inoculation with LAB should be explored.

The predicted bioavailability of iron from processed ALVs in the current study was similar regardless of the different processing methods. This gives a direction that iron bioavailability from differently processed ALVs could also be similar in humans. Therefore, iron solubility does not always predict uptake as interactions between iron and other food components such as antinutritional factors impair the absorption of non-haem iron. Though amaranth in the current study was found to have high iron content, its iron uptake by Caco-2 cells could have been seriously impaired by phytates, condensed tannins, total phenolic content and calcium. One of the limitations of the current study is that we did not measure levels of known enhancers of iron in ALVs after processing which possibly also influenced iron uptake to some degree.

4.4.2 Cellular uptake, retention and transport of zinc from differently processed African leafy vegetables by Caco-2 cells

Zinc retention, transport and uptake (retention plus transport) by Caco-2 cells from differently processed ALVs (spider plant and amaranth) were measured using soluble fractions of the simulated digestions (**Table 4-14**). Results are expressed as the percentage of mineral retention in the Caco-2 cells, transport across the Caco-2 cells and uptake by the Caco-2 cells (calculated as the sum of cell retention and transport). For quality purposes, controls were included in the current study (**Figure 4-12**). There were significantly ($p \leq 0.05$) higher retention and uptake of zinc from the positive controls (50 μM ZnSO_4) compared to the negative controls (serum-free MEM/EBSS).

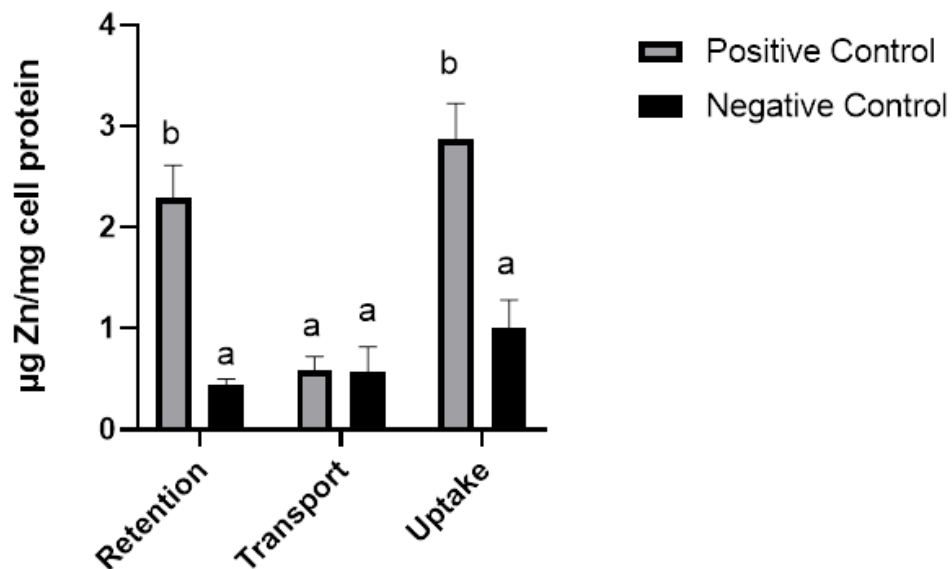


Figure 4-12: The retention, transport and uptake of zinc from control samples by Caco-2 cells. Where, the positive control was 50 μM ZnSO_4 and the negative control was serum-free MEM/EBSS. Values expressed as mean $\pm$ SD ($n=6$), values on the bars relating to the same title with different letter superscripts differ significantly at $p \leq 0.05$.

Table 4-14: Zinc retention, transport and uptake by Caco-2 cells from differently processed African leafy vegetables

Vegetable type		Solubility	Retention		Transport		Uptake ³		Transport Efficiency	Uptake Efficiency
		%	(µg Zn/mg cell protein)	% ¹	(µg Zn/mg cell protein)	% ²	(µg Zn/mg cell protein)	% ⁴	% ⁵	% ⁶
Amaranth	COO	3.1 ± 0.3 ^a	0.61 ± 0.45 ^a	31.2 ± 9.2 ^b	0.22 ± 0.08 ^a	10.9 ± 4.0 ^a	0.83 ± 0.48 ^a	42.1 ± 26.3 ^b	0.34 ± 0.12 ^a	1.28 ± 0.73 ^a
	BLA	12.0 ± 11.2 ^b	0.36 ± 0.12 ^a	3.1 ± 0.9 ^a	0.56 ± 0.01 ^a	4.9 ± 1.7 ^a	0.93 ± 0.01 ^a	8.0 ± 2.6 ^a	0.83 ± 0.01 ^b	1.36 ± 0.01 ^a
	FER	2.3 ± 1.4 ^a	0.39 ± 0.15 ^a	22.8 ± 8.6 ^b	0.28 ± 0.15 ^a	15.8 ± 11.4 ^a	0.65 ± 0.29 ^a	38.6 ± 18.4 ^b	0.43 ± 0.28 ^a	1.08 ± 0.46 ^a
Spider plant	COO	12.2 ± 1.4 ^b	0.48 ± 0.15 ^a	3.3 ± 1.3 ^a	0.29 ± 0.19 ^a	2.1 ± 1.6 ^a	0.77 ± 0.30 ^a	5.5 ± 2.6 ^a	0.24 ± 0.16 ^a	0.64 ± 0.25 ^a
	BLA	6.4 ± 5.4 ^a	0.45 ± 0.11 ^a	9.9 ± 6.5 ^a	0.17 ± 0.34 ^a	4.3 ± 3.1 ^a	0.62 ± 0.10 ^a	14.2 ± 9.5 ^a	0.15 ± 0.03 ^a	0.52 ± 0.08 ^a
	FER	16.5 ± 2.3 ^b	0.45 ± 0.12 ^a	2.5 ± 0.8 ^a	0.32 ± 0.17 ^a	1.8 ± 1.5 ^a	0.77 ± 0.21 ^a	4.3 ± 1.6 ^a	0.28 ± 0.15 ^a	0.69 ± 0.19 ^a

Values expressed as mean ± SD (n=6), values in the same column with different letter superscripts differ significantly (p ≤ 0.05). COO = Cooked, BLA = Blanched, FER = Fermented. ¹ % Retention = mineral retention / mineral soluble x 100, ² % Transport = Cellular transport / mineral soluble x 100, ³ Cell uptakes = cell retention plus transport, ⁴ % Uptake = mineral uptake / mineral soluble x 100, ⁵ transport efficiency = (% solubility x % transport) / 100, ⁶ uptake efficiency = (% solubility x % uptake) / 100

Cooked amaranth (31.2%) had significantly ($p \leq 0.05$) higher percentages of zinc retained by Caco-2 cells compared to blanched-dried amaranth (3.1%) (**Table 4-14**). On the other hand, zinc retained by Caco-2 cells from spontaneously fermented-dried amaranth was not significantly ($p > 0.05$) different from zinc retained in the cooked counterpart. In addition, no significant ($p > 0.05$) differences found in percentage zinc retention by cells between differently processed spider plants. The localisation of minerals and antinutrients in plant foods influences the effect of processing and mineral absorption (Raes *et al.*, 2014). The variation between the two ALVs could be attributed to different cellular locations of minerals and antinutrients factors in each vegetable after processing. Percentage zinc retained by the cells showed a negative correlation with zinc solubility ($p \leq 0.01$, $r = -0.633$) (**Table 4-15**), which could infer that zinc solubility influenced the amount of zinc retained by cells.

Zinc transport percentages in Caco-2 cells varied from 1.8 to 15.8% (**Table 4-14**). There were no significant ($p > 0.05$) differences found in zinc transported by the cells between spontaneously fermented-dried and blanched-dried ALVs compared to their cooked counterparts. However, the highest percentage zinc transport efficiency corresponded to blanched-dried amaranth (0.83%). A negative correlation was established between zinc transport efficiency and total phenolic content ($p \leq 0.01$, $r = -0.529$). ALVs contained substantially higher levels of total phenolic content (**Table 4-4**), as stated total phenolic content inhibits zinc absorption and possibly influenced zinc transport efficiency. The highest zinc solubility was observed in the blanched-dried amaranth sample, which probably influenced transport efficiency to some extent. Zinc transport efficiency across the cells was positively correlated with percentage zinc retention ($p \leq 0.01$, $r = 0.623$). A negative correlation was found between percentage zinc solubility and percentage zinc transport ($p \leq 0.01$, $r = -0.621$). Data discussion is difficult to compare to other studies due to a few studies available that have been published on retention, transport and uptake of zinc by Caco-2 cells in processed ALVs. The negative correlation observed between zinc solubility and percentage transport found in the current study confirms an observation also found by Camara *et al.* (2005) that solubility and bioavailability predicted by Caco-2 cells do not always show parallel trends. Zinc uptake (retention and transport) by the cells varied between the two ALVs (**Table 4-14**). Significantly ($p \leq 0.05$) higher percentage of zinc uptake by the cells was observed in cooked amaranth (42.1%) than in cooked spider plant (5.5%). The amount of zinc uptake by Caco-2 cells in these vegetables was $0.83 \mu\text{g Zn/mg protein}$ and $0.77 \mu\text{g Zn/mg protein}$ for cooked amaranth and cooked spider plant, respectively. Though cooked amaranth showed higher percentage of iron uptake than cooked spider plant, the amount of zinc uptake between the vegetables was not significant ($p > 0.05$). In addition, the uptake of zinc by Caco-2 cells is not parallel to zinc bioaccessibility (solubility) results obtained for these ALVs (**Section 4.3**). Cooked amaranth percentage zinc bioaccessibility and amount of zinc bioaccessible were 10.7% and 0.54 mg/100

g, and the percentage zinc bioaccessibility and amount of zinc bioaccessible for cooked spider plant were 15.7% and 1.88 mg/100 g, respectively (**Table 4-10**). For cooked spider plant the amount of zinc dialysable was three folds more than that of cooked amaranth, but zinc uptake by the cells was almost similar hence no significant ($p > 0.05$) differences found between the cooked amaranth and cooked spider plant. Mineral dialysability and cell uptake do not always represent parallel trends (Perales *et al.*, 2005). One possible reason may be because dialysability assay does not take into consideration minerals or food component competition at the site of absorption (Etcheverry *et al.*, 2012). High levels of one mineral could lower the absorption level of the other mineral. It has been reported that iron inhibits zinc absorption when the micronutrients are ingested in solution (Lim *et al.*, 2013). In the current study, the supernatant was applied to cells. Therefore, competition between iron and zinc at the point of absorption probably caused low zinc uptake by the cells from these ALVs. Interactions of zinc with other minerals at the site of absorption probably influenced zinc uptake in these ALVs. Mineral uptake is influenced by interactions between minerals and other food components, and it has been concluded that the inhibitory effect of calcium on zinc intestinal absorption is more pronounced in the presence of high phytate levels (Maares & Haase, 2020). High phytates and calcium levels were found in amaranth and spider plant (**Table 4-1**, **Table 4-8**) and could have influenced zinc uptake by Caco-2 cells. As stated, the predicted combined inhibitory effect of calcium and phytate on zinc showed zinc bioavailability that is seriously impaired. Phytate-calcium x zinc molar ratios were above the critical level of 200 which predict zinc that is seriously impaired (Bindra *et al.*, 1986), and this possibly could account for the overall low zinc uptake by cells found in this study. Data on Caco-2 cell uptake of zinc in processed ALVs is very scarce. To the best of my knowledge, limited research has been conducted on this subject though zinc deficiency is a worldwide problem, calling for more research.

Despite low levels of condensed tannins (**Table 4-4**), total oxalate (**Table 4-6**) and phytate (**Table 4-8**) found in both spontaneously fermented-dried spider plant and amaranth compared to their cooked counterparts, there was no significant ($p > 0.05$) differences found between their percentage zinc uptakes (**Table 4-14**). However, high levels of zinc uptake were observed in cooked amaranth (42.1%) and spontaneously fermented-dried amaranth (38.6%). The differences in the levels of zinc uptake by these ALVs could be attributed to variations in calcium contents in each vegetable. Amaranth had lower levels of calcium (1730 to 1995 mg/100 g) compared to spider plant (2456 to 2660 mg/100 g) (**Table 4-1**). As stated, calcium is highly inhibitory when levels of phytate are high through the formation of strong insoluble complexes with phytate and zinc.

Blanched-dried amaranth had significantly ($p \leq 0.05$) lower percentage zinc uptake (approximately five times less) compared to cooked amaranth (**Table 4-14**). There were no significant ($p > 0.05$) differences between levels of phytate, tannins and total phenolic content (**Table 4-4, Table 4-8**) of blanched-dried amaranth and cooked amaranth. However, total oxalates contents (**Table 4-6**) were significantly ($p \leq 0.05$) lower in blanched-dried amaranth than in cooked amaranth. Other amaranth components such as organic acids and zinc interaction with other minerals at the point of absorption possibly influenced the variation in zinc uptake found between amaranth samples. The negative effect of iron on zinc absorption is exerted at very high ratios of iron to zinc and in water solution (Lönnerdal, 2000). Iron to zinc ratios for cooked, blanched-dried and spontaneously fermented-dried amaranth found in the current study were 5:1, 4:1 and 5:1, respectively. Previously, zinc absorption was found to decrease along with increases in iron to zinc ratios up to the level of 5:1 (Pérès *et al.*, 2001), hence to some extent iron interacted with zinc inhibiting its uptake in the current study.

On the other hand, no significant ($p > 0.05$) differences were found in percentage zinc uptake by cells between blanched-dried spider plant and its cooked counterpart. The amount of zinc uptake in both spontaneously fermented-dried amaranth and spider plant compared to their cooked counterpart was not significant ($p > 0.05$). In this study, percentage zinc uptake was positively correlated with phytate ($p \leq 0.01$, $r = 0.694$) and total oxalates ($p \leq 0.01$, $r = 0.708$) contents (**Table 4-15**). Previous studies reported that fermentation enhances zinc absorption due to the presence of organic acids (lactic and citric) which forms soluble ligands with zinc preventing it to form zinc-phytate complexes which are insoluble (Platel & Srinivasan, 2016; Thompson & Amoroso, 2011). Possibly the impact of organic acids in the formation of soluble ligands with zinc might not have been enough to counteract the formation of insoluble phytate-zinc complexes hence no enhancement in zinc uptake observed in spontaneously fermented-dried ALVs compared to their cooked counterparts. The unusually positive correlation found between zinc uptake and antinutrients could associate zinc uptake with other ALVs components such as enhancers and zinc interactions with other minerals at the point of absorption.

There are limitations in using *in vitro* models to predict zinc bioavailability. The Caco-2 cell model evaluates zinc bioavailability in three dimensions, i.e., the amount of mineral in the apical and basolateral compartments, amount of mineral in the cell and amount actual transported by the cells to the blood side (Richardson *et al.*, 2018). The transportation of mineral from the basolateral to the luminal site of the intestinal epithelium has not been observed in humans (Finley *et al.*, 1995). These models do not have intestinal and blood fluid flow as well as peristaltic motions (Gefeller *et al.*, 2015), which has an influence mineral uptake *in vivo*. The intestinal peristalsis enables chyme movement along the intestine which enhances mechanical food component

degradation which influences zinc solubility and absorption (Maares & Haase, 2020). Also, the in Caco-2 cell model zinc is added as a liquid solution to the cells without food matrix while in vivo meals contains dietary ligands that influence zinc bioavailability (Lönnerdal, 2000). Therefore, inaccurate predictions on the direction of zinc bioavailability may occur *in vitro*. Despite limitations of the in vitro/Caco-2 model used in this study, data obtained allow for the establishment of the direction and ranking of zinc uptake from processed ALVs.

Table 4-15: Correlations between zinc solubility, retention, uptake, efficiency and antinutrients

	Total Phenolic content	Tannin	Phytates	Total Oxalates	% zinc retention	Uptake efficiency	% zinc transport
% zinc solubility	0.640**	-	-	- 0.640**	- 0.633**	-	0.621**
% zinc uptake	-		0.694**	0.708**	0.967**	0.862**	0.795**
Zinc transport efficiency	-0.529**		-	-	-	0.623*	0.636**
% zinc retention	-	-	0.685**	0.668**	-	0.862**	-
Uptake efficiency	-	-	0.719**	-	0.862**	-	0.619**

** Correlation is significant at $p \leq 0.01$ (2 tailed), N =18, degree of freedom = 16

4.5 Conclusions

The study presents novel information on the mineral and antinutrients composition of processed ALVs, and the influence of processing on the bioaccessibility and cellular uptake of iron and zinc using *in vitro* assays. Though this study found that cooked non-commercialised ALVs had higher iron and zinc contents than the already commercialised ALV (Ethiopian kale), it cannot be confirmed that this will be the case with ALVs in other locations as soil nutrients, climate and amount of rainfall received has an impact on iron and zinc content. With such high levels of iron

and zinc found in ALVs in this study, there is a need to advocate for the consumption of ALVs for rural communities that have limited access to exotic vegetables and are at risk of iron and zinc deficiencies. Spontaneously fermented-dried ALVs had similar levels of iron, zinc, and phosphorus as cooked vegetables, and this could suggest that processing ALVs by fermentation could provide ALVs with similar mineral values as freshly cooked vegetables. Blanching and fermentation in combination with drying further removed some of the soil contamination from the ALVs which remained after routine cleaning; hence these methods could be explored further when cooking fresh ALVs.

Though blanched-dried and spontaneously fermented-dried ALVs had lower contents of antinutrients it did not translate to enhanced iron and zinc bioaccessibility compared to their cooked counterparts because levels of antinutrients present still had some inhibitory factors towards iron and zinc dialysability, also other ALVs components probably influenced mineral bioaccessibility to some extent. In addition, the uptake of iron and zinc from processed ALVs were ranked low. Most antinutrients are dose-dependent, hence levels which were present after processing still had some inhibitory effects on iron and zinc uptake by the cells. Irrespective of processing it is estimated that amaranth could contribute the highest amount of iron while spider plant could contribute the highest percentage of zinc towards AR for women aged 19 to 50 years compared to other ALVs, but these are not actual values but estimates that predicts the direction of response as *in vitro* dialysability assays cannot measure the actual rate of mineral absorption and transport compared to human studies. Due to high levels of antinutrients in these ALVs, consumption of ALVs can be encouraged concurrently with other locally available foods which are rich sources of enhancers of iron and zinc (tomatoes, oranges and Mopani worms) to influence iron and zinc absorption. There is a need to deepen knowledge on the bioavailability of iron and zinc from ALVs and the magnitude of effects of antinutrients on iron and zinc bioavailability mostly when ALVs are consumed concurrently with cereal staples as it is the norm. Both dietary inhibitors and enhancers of iron and zinc, and other food components that influence iron and zinc absorption should be identified. The findings of the current *in vitro* study can only be used to predict the direction of iron and zinc bioavailability from processed ALVs, as *in vitro* models cannot simulate all *in vivo* conditions.

CHAPTER 5: GENERAL SUMMARY AND RECOMMENDATIONS

5.1 Overview

Micronutrient deficiencies especially essential minerals such as iron and zinc are a major health problem globally with 1.2 billion people being iron deficient causing 50% of anaemia cases worldwide (Gardner & Kassebaum, 2020) and 17.3% of the global population has inadequate intakes of zinc with the highest estimates found in Africa and Asia (Bailey *et al.*, 2015). The highest prevalence of iron deficiency is found in Africa, with approximately 68% in children and 57% in pregnant women (Lopez *et al.*, 2016). The major causes of iron and zinc deficiencies are inadequate dietary intakes and poor absorption or bioavailability (Gibson *et al.*, 2010). Plant-based diets have low iron and zinc bioavailability due to its antinutritional factors such as phytates, total phenolic contents, tannins and oxalates (Gibson *et al.*, 2010; La Frano *et al.*, 2014; Zimmermann & Hurrell, 2007). African leafy vegetables (ALVs) have been part of food systems in many African countries for many generations (Muhanji *et al.*, 2011), and have been found to play a significant role in the livelihoods of Africans (Dube *et al.*, 2018). In most cases, ALVs are cooked and eaten as a relish accompanying staples such as maize, millet or sorghum porridges (Bvenura & Afolayan, 2015; Kruger *et al.*, 2015). Various ALVs analysed have been reported to be good sources of protein, fibre, vitamin C, magnesium, iron, zinc and calcium (Ndlovu & Afolayan, 2008; Schönfeldt & Pretorius, 2011; van Jaarsveld *et al.*, 2014). Due to urbanisation, there have been the introduction, production and consumption of exotic vegetables which declined the utilisation of ALVs (Matenge *et al.*, 2012; van der Hoeven *et al.*, 2013). However, ALVs are as good as exotic vegetables in regard to their essential mineral contents (Mungofa *et al.*, 2018). ALVs have been compared with commercial exotic vegetables such as spinach, kale and cabbage and emerged superior in mineral content (Ndlovu & Afolayan, 2008; Orech *et al.*, 2007).

The most challenging factor with ALVs is their seasonality shortages and perishability state. Several studies conducted in South Africa and Kenya on ALVs reported that these vegetables are available in summer during rainy seasons (Dweba & Mearns, 2011; Faber *et al.*, 2010; Maseko *et al.*, 2018; Smith & Eyzaguirre, 2007). Nevertheless, to overcome perishability and seasonality shortages in households, traditional processing methods such as fermentation in combination with drying and blanching in combination with drying have been used (Gogo *et al.*, 2018; Kirakou *et al.*, 2017; Maseko *et al.*, 2018; Wafula *et al.*, 2016). Though ALVs contains high levels of iron and zinc, they also contain high amounts of inhibitors of these essential minerals when it comes to absorption (Yang & Keding, 2009). Previous studies observed high levels of phytate, total oxalates, condensed tannin and total phenolic contents in ALVs (Essack *et al.*, 2017;

Kruger *et al.*, 2015; Muchoki *et al.*, 2010; Vilakati *et al.*, 2016). These antinutritional factors in ALVs inhibit the bioavailability of iron and zinc (Natesh *et al.*, 2017). A study that evaluated the bioaccessibility of iron and zinc from ALVs reported that the presence of phytates influenced the amount of iron and zinc accessible to the body (Kruger *et al.*, 2015). Nevertheless, previous studies reported that traditional processing methods such as blanching and fermentation are effective in reducing inhibitory factors of essential minerals in ALVs (Akello, 2014; Elisha *et al.*, 2016; Fadupin *et al.*, 2014; Wafula *et al.*, 2016). This study investigated the potential of blanching and fermentation to reduce the levels of antinutrients in ALVs, hence influencing iron and zinc bioaccessibility and bioavailability. Though this study did not assess preservation of ALVs, the vegetables were dried after fermentation and blanching to simulate how they are typically processed, stored and consumed when out of season. This PhD aimed to compare mineral and antinutrients contents of cooked ALVs to blanched-dried and spontaneously fermented-dried counterparts, with the overall aim to evaluate the bioaccessibility of iron and zinc from these processed ALVs and to predict their iron and zinc bioavailability using the Caco-2 cell assay.

The following were the objectives of this PhD research:

- To compare the mineral and antinutrient content of cooked, blanched-dried, and spontaneously fermented-dried ALVs.
- To compare the bioaccessibility of iron and zinc in cooked, blanched-dried, and spontaneously fermented-dried ALVs using *in vitro* dialysability assay.
- To compare the estimated bioavailability of iron and zinc from cooked, blanched-dried and spontaneously fermented-dried ALVs using an *in vitro* Caco-2 cell culture assay.

This chapter aims to discuss the main findings of the study, the methodological considerations, strengths, and weakness of the study and offer recommendations for future studies. It also outlines the significance of this research from a public health perspective.

5.2 Comparisons of mineral and antinutrient contents of differently processed African leafy vegetables

The study found that, except for calcium, non-commercialised ALVs had high mineral contents compared to commercialized Ethiopian kale (**Chapter 4:Table 4-1**). Therefore, ALVs could be explored for commercialisation. Spontaneously fermented-dried ALVs had higher levels of iron, zinc and calcium compared to their cooked counterparts. Blanched-dried ALVs had lower iron and higher calcium levels compared to their cooked counterparts. At the same time, for other minerals, there were no significant differences found between cooked and blanched-dried vegetables. It can be concluded that blanching and spontaneous fermenting ALVs were as good

as cooking as minimum losses of essential minerals were observed. The consumption of these ALVs could increase essential mineral intake and increase dietary diversity. Both blanched-dried and spontaneously fermented-dried vegetables had lower aluminium contents compared to their cooked counterparts (**Chapter 4: Table 4-1**). As stated, high levels of aluminium in plant-based foods could indicate soil contamination (Salvo *et al.*, 2018). Blanching and fermentable mediums were discarded before drying the vegetables which probably contributed to low levels of aluminium through leaching out into the discarded mediums. Therefore, these processing methods could be beneficial in further removing soil/dust contamination in ALVs that had not been removed by regular washing. The PCA (**Chapter 4:Figure 4-5**) found that iron and zinc were positively correlated with aluminium, so it could be suspected that high iron and zinc levels observed may be due to artificial mineral from the soil contamination. ALVs in this study were thoroughly cleaned to remove any impurities before processing. Contamination of ALVs with soil has been previously reported despite thorough cleaning (van Jaarsveld *et al.*, 2014). There is contradictory information on the bioavailability of contaminant iron. Previous studies observed that a fraction of contaminant iron is bioavailable (Hooda *et al.*, 2004; Kikouama *et al.*, 2009; Smith *et al.*, 2000) while others reported that it is unavailable for absorption (Icard-Vernière *et al.*, 2013; Seim *et al.*, 2013). A review of the bioavailability of contaminant iron recommends that *in vivo* studies using rat HRE assay should be conducted before conclusions could be drawn on the bioavailability of contaminant iron as most previous studies were conducted using *in vitro* assays (Teklu, 2017). On the other hand, artificial zinc from soil contamination was previously reported unabsorbable (Intawongse & Dean, 2006). Irrespective of processing, spider plant (51.2 mg/100 g) and amaranth (29.9 mg/100 g) had higher iron levels compared to other ALVs. The highest zinc contents were found in spider plant (11.7 mg/100 g) and amaranth (6.4 mg/100 g) (**Chapter 4:Table 4-2**). Overall, blanched-dried and spontaneously fermented-dried ALVs had lower contents of phytates, oxalates and condensed tannins compared to their cooked counterparts (**Chapter 4: Figure 4-1, Figure 4-3, Figure 4-4**). Fermentation activates tannase (tannic acyl hydrolase) which hydrolyses the ester and depside bonds of tannic acid to form hydrolysable tannins (Macedo *et al.*, 2011), which are soluble in water. In the current study the free tannins could have been lost through leaching into the salt-sugar fermentation solution that was discarded before drying the ALVs. LAB degrades oxalates for carbon and energy which decreases the levels of total oxalates content during fermentation (Okoye & Ene, 2018). In addition, during fermentation, the activation of the endogenous phytase causes the degradation of phytate (Coulibaly *et al.*, 2011) resulting in decreasing its levels in the spontaneously fermented-dried vegetables. Lower antinutrient contents in blanched-dried ALVs compared to their cooked counterparts found in the current study were linked to leaching out into the discarded blanching water. However, the mineral phytate-mineral ratio for all differently processed ALVs (**Chapter 4:Table 4-8**) was above the critical level values at which iron and zinc bioavailability are impaired.

Phytate-iron molar ratios above the critical limit of 1 predict iron bioavailability that is seriously impaired (Hurrell & Egli, 2010). The phytate-iron molar ratios of processed ALVs were between 2.7 to 18.7 and these values were double to eighteen times more than the critical level indicating a more pronounced phytate inhibitory effects on iron hence predicting iron bioaccessibility that is seriously impaired. The phytate-zinc molar ratios of the ALVs ranged from 14.8 to 78.1. These values were also mostly (except for spontaneously fermented-dried spider plant) above the set critical limit of 15 in which zinc bioaccessibility is known to be seriously impaired (FAO & WHO, 2005). With high phytate content in these ALVs its inhibitory effects on iron and zinc cannot be ignored.

As previously stated, the combined inhibitory effect of phytate and calcium on zinc has been reported to be a better predictor of zinc bioavailability (Hailu & Addis, 2016). Phytate-calcium/zinc molar ratios above a critical value of 200, predicts zinc bioavailability that is impaired (Bindra *et al.*, 1986). The phytate-calcium/zinc molar ratios of ALVs were above the critical limit value (> 200). Values were six hundred times more than the critical limit value (1232 and 1385) (**Chapter 4: Table 4-8**), which is associated with low zinc bioavailability. The phytate-mineral molar ratios found in the current study predict iron and zinc bioavailability that is impaired. However, phytate-mineral molar ratios only predict the inhibitory effect of phytate and do not take into consideration other antinutrients (condensed tannins and total phenolic contents) which also influences iron and zinc bioaccessibility. Spontaneously fermented-dried ALVs had higher levels of total phenolic content compared to their cooked counterparts. This was probably associated with the activation of PPO during fermentation which transforms tannins into smaller phenolic compounds which increased the total phenolic compounds (Emmambux & Taylor, 2003; Nkhata *et al.*, 2018).

5.3 The bioaccessibility of iron and zinc from differently processed African leafy vegetables using *in vitro* dialysability assay

The percentage iron bioaccessibility from ALVs ranged from 1.4% to 16.4% (**Chapter 4: Table 4-10**) and the average percentage iron bioaccessibility of cooked, blanched-dried and spontaneously fermented-dried ALVs were 6.8%, 3.4% and 5.3%, respectively (**Chapter 4: Figure 4-6**). Percentages iron and zinc bioaccessibility of cooked ALVs in the current study were within ranges reported previously (Kruger *et al.*, 2018; Kruger *et al.*, 2015). The highest iron content was found in spider plant (40 mg/100 g to 61.7 mg/100 g) (**Chapter 4: Table 4-1**) but its percentage iron bioaccessibility was low (1.5% to 2.1%) (**Chapter 4: Table 4-10**). High iron contents in the spider plant did not directly translate into enhanced iron bioaccessibility probably due to some inhibitory factors of iron present in the vegetable. Thus, estimating mineral bioaccessibility based on mineral concentration could be misleading. However, dialysability

assays can only be used as a relative but not a measure of the actual value of mineral bioavailability (Fairweather-Tait *et al.*, 2005).

Results of this study indicated that there were no significant differences found in iron and zinc bioaccessibility between spontaneously fermented-dried and cooked vegetables of the same type except for lower percentage iron bioaccessibility in spontaneously fermented-dried Ethiopian kale and higher percentage zinc bioaccessibility in spontaneously fermented-dried amaranth (**Chapter 4: Table 4-10**). On the other hand, significantly lower percentages of iron bioaccessibility were found in blanched-dried Ethiopian kale than in its cooked counterpart, while high percentages of zinc bioaccessibility were found in blanched-dried amaranth compared to its cooked counterpart.

The present study found lower levels of phytates, condensed tannins and oxalates in spontaneously fermented-dried and blanched-dried ALVs compared to their cooked counterparts (**Chapter 4: Table 4-4, Table 4-6, Table 4-8**). However, these levels perhaps were still very high and probably continued exerting their inhibitory effects on these essential minerals resulting in low iron and zinc bioaccessibility. It should be noted that phytate-mineral ratios of blanched-dried and spontaneously fermented-dried ALVs (except for spider plant) were more than the critical levels of iron (> 1) and zinc (> 15) predicting iron and zinc bioavailability that is impaired. In addition, high levels of total phenolic content found in spontaneously fermented-dried ALVs due to transformation of condensed tannins into total phenolic contents, could also have counteracted the positive influence of fermentation in enhancing iron and zinc bioaccessibility in plant-based foods. Also, high levels of condensed tannins were found in these ALVs which also had some inhibitory effect on iron and zinc. Condensed tannins bind to proteins affecting digestive enzymes activity (Shahidi & Ambigaipalan, 2015) that are crucial in the breaking down of food matrix to release minerals in the lumen for absorption. Therefore, high levels of condensed tannins found in this study probably negatively influenced iron and zinc bioaccessibility.

Based on the calculations made on the potential of ALVs contributing iron and zinc towards absolute requirements (AR) for women aged 19 to 50 years, the estimated amounts of iron the processed ALVs could contribute towards AR of iron for women aged 19 to 50 years were between 14.5 to 50.3% for cooked, 6.1 to 40.6% for blanched-dried and 3.9 to 48.1% for spontaneously fermented-dried, respectively. Regardless of processing amaranth could contribute the highest amount of iron towards AR for women aged 19 to 50 years compared to other ALVs. Percentage contributions that could be made by cooked, blanched, and spontaneously fermented-dried ALVs towards zinc AR for women of age 19 to 50 years were

2.9% to 34.9%, 8.5% to 51.9% and 6.0% to 57.2%, respectively. However, it should be highlighted that these are not actual contributions but estimates, as *in vitro* assays will never simulate all conditions in the human digestion process (Etcheverry *et al.*, 2012). From the dialysability data obtained from this study, it shows that ALVs are good sources of iron and zinc, and they could contribute towards iron and zinc AR of women aged 19 to 50 years. The concept of dialysability is that the dialysable compounds are available for intestinal absorption (Etcheverry *et al.*, 2012). The reliability of *in vitro* dialysability data remains questionable as small polyphenol iron complexes are dialysable while high molecular weight protein ferritin that is bioavailable are excluded (La Frano *et al.*, 2014), this could result in underestimation of iron bioaccessibility. So, *in vitro* dialysability assays may not always be accurate in the prediction of the direction of mineral absorption. The predicted iron and zinc bioavailability from the current study needs to be validated with human intervention studies (Etcheverry *et al.*, 2012). However, human studies are expensive and laborious to conduct, hence *in vitro* dialysability assays combined with Caco-2 cell assays can be used to predict iron and zinc bioavailability. Compared to the other ALVs amaranth and spider plant had the best amount of iron and zinc bioaccessible, and these two ALVs, were further evaluated to predict iron and zinc bioavailability using Caco-2 cells model.

5.4 Iron uptake from ALVs measured by ferritin formation in Caco-2 cells

Ferritin formation in Caco-2 cells exposed to simulated gastrointestinal digestates of differently processed ALVs ranged from 0.31 ng ferritin/mg cell protein to 2.35 ng ferritin/mg cell protein (**Chapter 4: Figure 4-11**). Lack of differences between iron uptake by cells from fermented, blanched-dried and cooked ALVs was associated with high antinutrient contents found in this study (**Chapter 4: Section 4.2.2**). Phytates in these ALVs were associated with magnesium, indicating that phytates in these ALVs could be predominantly existing as magnesium phytate salt (**Chapter 4: Figure 4-5**). As stated, magnesium phytate salt strongly binds to iron, making it unabsorbable and less available for absorption (Gibson *et al.*, 2010). Phytate-iron molar ratios of all processed ALVs were double to twelve times more than the critical limit (> 1) predicting iron bioavailability that is seriously impaired (Hurrell, 2004). As stated, condensed tannins form insoluble complexes with iron and also binds with protein forming tannin-protein complexes inhibiting digestive enzymes activation that are crucial in the breaking down of food matrix to release minerals for absorption (Delimont *et al.*, 2017; Shahidi & Ambigaipalan, 2015), which probably contributed to the low ferritin formation found in Caco-2 cells exposed to processed ALVs. Also, total phenolic content forms insoluble complexes with Fe^{3+} reducing iron uptake by the cells (Perron & Brumaghim, 2009). High phenolic content, condensed tannins and phytate levels found in both amaranth and spider plant in this study, consequently resulted in low ferritin formation in the cell line. As

previously mentioned, the inhibitory effect of phytate, condensed tannins and total phenolic content on iron absorption are dose dependent and occur even at very much lower concentrations (Delimont *et al.*, 2017; Hallberg *et al.*, 1989; Shahidi & Ambigaipalan, 2015). Though levels of antinutrients in spontaneously fermented-dried and blanched-dried were lower than in their cooked counterparts, the levels were still adequate to cause iron inhibition rendering iron unabsorbable which also contributed to low ferritin formation found in the Caco-2 cells.

Low ferritin formation in Caco-2 cell exposed to simulated gastrointestinal digestates of processed ALVs could also be linked to the presence of artificial iron. Substantially high levels of aluminium were found in processed amaranth (26.2 to 39.0 mg/100 g) and spider plant (56.0 to 75.8 mg/100 g) (**Chapter 4: Table 4-1**). As stated, high levels of aluminium in plant-based foods could indicate soil contamination. There was a strong association between iron and aluminium (**Chapter 4:Figure 4-5**), indicating that a portion of iron from these ALVs was from contamination. The bioavailability of contaminant iron is questionable and rat HRE assays are recommended to ascertain its bioavailability (Teklu, 2017). So, the current study could not ascertain the presence of artificial iron in the vegetables as indicated by the high content of aluminium.

As stated, ALVs were spontaneously fermented-dried for one week without the addition of starter culture, typical the way they are traditionally fermented in the households (Wafula *et al.*, 2016). The enhancing effect of fermentation on iron availability has been reported to be attributed to acidification by natural lactic acid production rather than antinutrient reduction (Proulx & Reddy, 2007). The pH of the ALVs was monitored throughout spontaneous fermentation (**Chapter 3: Figure 3-7**) It is possible that spontaneous fermentation did not produce adequate lactic acid for enhancing iron absorption hence the low ferritin formation in Caco-2 cells.

The predicted bioavailability of iron from processed ALVs in the current study showed no significant differences. Iron solubility does not always predict uptake as interactions between iron and other food components affect the absorption of non-haem iron. The dialysability assay found high levels of bioaccessible iron from differently processed amaranth (2.06 – 3.81 mg/100 g) (**Chapter 4: Table 4-10**) compared to other ALVs, but its iron uptake probably was seriously impaired by the presence of inhibitors such as phytates, condensed tannins, total phenolic content, oxalates and calcium. Therefore, using only dialysability data to predict iron bioavailability could give a false impression that amaranth is a good source of iron that can improve iron status in humans.

5.5 Zinc uptake, retention and transport from differently processed African leafy vegetables by Caco-2 cells

The uptake of zinc by Caco-2 cells ranged from 5.5% to 42.1% for cooked, 8.0% to 14.2% for blanched-dried and 4.3% to 38.6% for spontaneously fermented-dried ALVs respectively (**Chapter 4:Table 4-14**). The highest zinc uptakes were observed in cooked amaranth (42%) and spontaneously fermented-dried amaranth (38%). Though the amount of zinc bioaccessible from amaranth and spider plant were high (0.54 mg/100g to 2.14 mg/100 g) (**Chapter 4:Table 4-10**), uptake of zinc by the Caco-2 cells was very low (0.62 µg Zn/mg cell protein to 0.93 µg Zn/mg cell protein). According to Perales et al. (2005), mineral dialysability and cell uptake do not always represent parallel trends. Cooked spider plant had double the amount of bioaccessible zinc (1.88 mg/100 g) compared to cooked amaranth (0.54 mg/100 g), but the percentage zinc uptake (5.5%) by the cells was lower compared to the uptake found in cooked amaranth (42.1%). (**Chapter 4:Table 4-14**). High aluminium contents in spider plant (75.8 mg/100 g) which was suspected to be from soil contamination (**Chapter 4:Table 4-1**), was strongly associated with zinc (**Chapter 4:Figure 4-5**) could account for the low zinc uptake found in spider plant. Artificial zinc from soil contamination was previously reported unabsorbable (Intawongse & Dean, 2006). Hence, it could be suspected that some portion of zinc is mainly from extrinsic sources which were not available for absorption.

Zinc uptake from amaranth and spider plant probably was seriously impaired by the combined inhibitory effect of phytate and calcium. High molar ratios of phytate-calcium/zinc were observed (921-3406) (**Chapter 4:Table 4-8**). Phytate-calcium/zinc molar ratios above the critical level of 200 predict zinc bioavailability that is impaired (Bindra *et al.*, 1986). These ALVs also contained high levels of calcium (**Chapter 4:Table 4-1**). Calcium in the presence of phytate forms strong insoluble complexes with zinc, consequently rendering it unabsorbable (Lönnerdal, 2000). This probably confirms the magnitude of the combined inhibitory effect of calcium and phytate on zinc bioavailability, which could have been more pronounced in these ALVs.

There were no significant differences found in zinc transported by the cells between spontaneously fermented-dried and blanched-dried ALVs compared to their cooked counterparts (**Chapter 4:Table 4-14**). A negative correlation was found between percentage zinc solubility and percentage zinc transport (**Chapter 4:Table 4-15**). The results of the current investigation confirm that solubility and bioavailability predicted by Caco-2 cells do not always show parallel trends. Transport efficiency in amaranth was higher in the blanched-dried sample than in the cooked and spontaneously fermented-dried samples. The highest zinc

solubility was found in the blanched-dried sample which probably influenced transport efficiency to some extent.

A negative correlation between zinc transport efficiency and total phenolic content was also found (**Chapter 4: Table 4-15**). Both amaranth and spider plant total phenolic contents were high (1637 to 3022 mg CE/100 g)(**Chapter 4:Table 4-4**), as stated total phenolic content inhibits zinc uptake and it possibly influenced zinc transport efficiency in the cells. Phenolic compounds with catechol and galloyl groups, even if present in smaller quantities, negatively inhibit zinc absorption (Towo *et al.*, 2006).

5.6 Study hypotheses

Hypothesis 1

The hypothesis that ALVs blanched-dried for a maximum time of five minutes will have similar mineral contents compared to cooked counterparts is rejected.

Hypothesis 1 was made based on the assumption that the shortest blanching time would result in negligible loss of minerals due to leaching out into the discarded blanching medium and minerals leached out from the cooked ALVs will not be lost as the cooking medium was not discarded. However, in the current study some blanched-dried ALVs had lower iron, magnesium, calcium and phosphorus contents than their cooked counterparts while higher zinc, iron, calcium and phosphorus contents were also found in some blanched-dried ALVs compared to their cooked counterparts.

Hypothesis 2

The hypothesis that spontaneously fermented-dried ALVs will have substantially lower mineral contents compared to their cooked counterparts is rejected.

Some spontaneously fermented-dried ALVs had higher mineral contents than their cooked counterparts while others had lower contents.

Hypothesis 3

The hypothesis that blanched-dried ALVs will have similar levels of condensed tannins, phytates, total phenolic content and total oxalates as their cooked counterparts is rejected.

There was no uniform pattern on the levels of condensed tannins, total phenolic contents and total oxalates in blanched-dried ALVs compared to their cooked counterparts. Compared to their

cooked counterparts, lower levels of condensed tannins were found in some blanched-dried ALVs, while all blanched-dried ALVs had lower levels of total oxalates and soluble oxalates. Total phenolic content of blanched-dried ALVs compared to cooked counterparts was similar.

Hypothesis 4

The hypothesis that spontaneously fermented-dried ALVs will have substantially lower levels of condensed tannins, total phenolic content, total oxalates and phytates than their cooked counterparts is rejected.

Some spontaneously fermented-dried ALVs had lower levels of condensed tannins, total phenolic content, total oxalates and phytates while others had higher levels compared to their cooked counterparts. Moreover, there were no significant changes found in phytates levels for other spontaneously fermented-dried ALVs compared to their cooked counterparts.

Hypothesis 5

The hypothesis that blanched-dried ALVs will substantially have similar estimated iron and zinc availability as cooked counterparts is accepted.

Iron and zinc uptakes were not influenced by blanching, even though some antinutrients levels were lower in blanched-dried ALVs than their cooked counterparts. In the present study, there were no significant differences found in percentage iron bioaccessibility among all blanched-dried ALVs and cooked counterparts except for Ethiopian kale. There were also no significant differences found in percentage zinc bioaccessibility between all blanched-dried ALVs and their cooked counterparts except for amaranth. Regarding iron and zinc uptake by the Caco-2 cells, there were no significant differences found in ferritin formation in Caco-2 cells (iron uptake) between cooked and blanched-dried ALVs and zinc uptake was also not significant between the two processes.

Hypothesis 6

The hypothesis that spontaneously fermented-dried ALVs will substantially have higher estimated iron and zinc bioavailability compared to their cooked counterparts is rejected.

In this study, fermentation had no significant effect on percentage iron bioaccessibility in any ALVs except for Ethiopian kale. Moreover, no significant differences were found in the zinc bioaccessibility between the same type of cooked and spontaneously fermented-dried ALV. Ferritin formation and zinc uptake in Caco-2 cells exposed to simulated gastrointestinal digestates

of spontaneously fermented-dried and cooked ALVs were also not significantly different. Spontaneously fermented-dried ALVs did not show enhanced iron and zinc uptake compared to their cooked counterparts.

5.7 Review of the methodology and considerations

The mineral content of ALVs in the current study varied (**Chapter 4:Table 4-1**). The variation may be due to abiotic and biotic factors. Environmental factors such as sunlight, pollution, temperature, soil composition, water availability and seasonality have an influence on mineral contents (Martínez-Ballesta *et al.*, 2010; Mitova *et al.*, 2017). Also, biotic factors such as insects and pathogens that attack ALVs during growth influences the levels of minerals (Ochoa-Velasco *et al.*, 2017). Amaranth, cowpea, wild jute and spider plant were purchased from the same farmer while Ethiopian kale was purchased from street vendors. Future studies should consider growing ALVs in a controlled environment to limit variations in the abiotic and biotic factors. A review concluded that the type of soil and climate in which the ALV had grown influence its mineral content (Uusiku *et al.*, 2010). Therefore, there is a need to determine the mineral content of the soil to get an in-depth understanding of the mineral content of plants grown in different locations. However, this was beyond the scope of this study.

As stated, location in which vegetables grows influences their nutrient contents, therefore, results of the current study limit the conclusions to only ALVs found in that location. Non-commercialised ALVs were found to contain high levels of iron and zinc compared to commercialised Ethiopian kale. However, it may not be the case in other locations due to some environmental conditions that may differ. ALVs from this study are from one location, hence their mineral content could be different from other ALVs grown in other locations. Future studies should be conducted with ALVs grown at the same time in different locations to have a truer representation of how these environmental factors influences mineral contents.

The results of this study showed high levels of aluminium content in ALVs. This probably indicates possibly soil/dust contamination. The vegetables were thoroughly washed before processing. However, it has been previously reported that meticulous cleaning of the ALVs did not remove all the soil from the vegetables (van Jaarsveld *et al.*, 2014). The bioavailability of contaminant iron is questionable (Teklu, 2017), while zinc from contamination is not absorbable (Intawongse & Dean, 2006). Therefore, there is a need to evaluate the degree of contamination of ALVs by soil/dust because artificial mineral contents could give a false impression that ALVs are good sources of essential minerals while in reality these minerals are less or not available for absorption.

The Folin-Ciocalteu method used to determine total phenolic content in the current study is non-specific and other reducing substances such as AA and fructose present in the sample may interfere with the assay (Magalhães *et al.*, 2010). The reaction of the Folin-Ciocalteu with other reducing substances could result in overestimating total phenolic contents (Sánchez-Rangel *et al.*, 2013). Folin-Ciocalteu method is an oxidation-reduction method hence any substance present in the sample capable of being oxidized will produce the reduced form of the reagent and could be assumed as total phenolic content (Waterman & Mole, 1994). A clean-up procedure using solid-phase extraction has been proposed to separate phenolic compounds and eliminating oxidizing substances (Magalhães *et al.*, 2010). The current study adopted the Folin-Ciocalteu method to determine total phenolic content as it was the standardized used method at the institution where the ALVs were analysed, but some precautions were considered to reduce the oxidizing effect. Nevertheless, the inhibitory effect through oxidation was avoided by adding the Folin-Ciocalteu before the addition of an alkali. The current study did not use the solid-phase extraction but corrected the absorbance of samples in the absence of Folin-Ciocalteu reagent. A blank subtraction was conducted to eliminate non-phenolic compounds that may give positive reactions and overestimate total phenolic contents. Concentrations of AA higher than 1.0 mg/L have been reported to interfere with Folin-Ciocalteu assay, resulting in an additive effect (Magalhães *et al.*, 2010). Therefore, there is a need for corrective measures based on AA interference with Folin-Ciocalteu reagent. Though our study did not evaluate AA content in ALVs, other studies reported high levels of AA in ALVs (Goweke *et al.*, 2019; Mamboleo, 2015). The interference of AA reducing the Folin-Ciocalteu possibly overestimated the total phenolic content. Methods that specifically measure total phenolic content such as liquid chromatograph-mass spectrometry could be used in future to analyze metabolic extracts from plants as they allow for profiling of phenolic compounds (Leone *et al.*, 2015).

During spontaneous fermentation of ALVs LAB convert carbohydrate contents in vegetables into lactic acid which decreases the pH of the vegetables (Wafula *et al.*, 2016). Previous studies reported addition of fermentable carbohydrates in ALVs to support the growth of LAB (Kasangi *et al.*, 2010; Muchoki *et al.*, 2010). So, the decrease in pH as LAB produces lactic acid activates the endogenous phytase which degrades phytate in the vegetables enhancing mineral absorption (Kumar *et al.*, 2010b). The enzyme phytase is activated at acidic pH conditions of 4.5 to 5.5 (Sanz-Penella *et al.*, 2012). In acidic conditions PPO condensed tannins such as proanthocyanidins are hydrolysed (Emmambux & Taylor, 2003), and pH lower than 6 reduces the proportion of insoluble oxalates salt formed which reduces oxalates binding effect on minerals (Simpson *et al.*, 2009). The pH of the spontaneously fermented ALVs in this study ranged from 5.3 to 3.9 which could be conducive for the activation of phytase and PPO in some vegetables, however the current study did not measure the amount of lactic acid produced in each vegetable. If lactic acid produced is

not enough for activation of enzymes, then future fermentation of ALVs could be explored inoculating with strains of LAB.

The age and developmental stage of a plant influence its phytate content (Mamboleo, 2015; Sheetal *et al.*, 2006). In this study, ALVs were harvested at the time when they were abundant (Jan-April), and this is generally the time that most households have access to them. This study used non-domesticated ALVs in Botswana (spider plant, wild jute and amaranth) and the farmer did not have control over the plant age. This could suggest that the results given in this study are a good reflection of the actual mineral value of the ALVs as they are typically consumed in the households.

From the literature review, there is variation in data obtained from the *in vitro* dialysability assay (**Chapter 2**). Variations in methodology in the *in vitro* dialysability assay have been reported as a challenge when comparing results between different research laboratories (Minekus *et al.*, 2014). A result variation of between 2 to 55% was reported in an inter-laboratory trial between nine laboratories which assessed the bioaccessibility of iron in three different meals using the *in vitro* dialysability assay (Luten *et al.*, 1996). Variation in results from the *in vitro* dialysability assay had been linked to sample handling due to multiple stages in the assay contributing to possibilities of making errors during the experiment (Fairweather-Tait *et al.*, 2007). To help compare results, it has been proposed that standardized and static digestion methods should be adopted, which include specific digestion fluids both in the gastric and intestinal phases (Minekus *et al.*, 2014). In the current study, the *in vitro* gastric digestion was carried out at pH 2, which is assumed to be that of the stomach (Hunt *et al.*, 2015). However, other studies suggest that the stomach pH should be 4 (Kalantzi *et al.*, 2006; Simonian *et al.*, 2005). Also, the pH in the stomach of infants is assumed to be 4 (Kalgaonkar & Lönnerdal, 2008). *In vitro* iron uptake has been reported to be different at pH 2 and 4 (Kalgaonkar & Lönnerdal, 2008). Future studies should, therefore, compare bioavailability at both pH 2 and pH 4.

It has also been reported that the final iron content in the dialysate or solubility supernatant may be affected by digestion time and speed of shaking during incubation (Shen, 1994). Although it is easier to control time and incubator shaking speed, it is not always easy to control the pH of the gastric and intestinal phases. Even working with due diligence, factors such as buffering capacity and consistency of the samples, different pH time gradients are difficult to control (Shen, 1994). The pH used in the gastric and intestinal phase is critical as it influences the overall iron solubility (Dias *et al.*, 2018). The changes in pH in the dialysability assay simulates the changes that occur in the gastrointestinal tract (GI). The GI has mineral transporters that aid iron and zinc uptake (Abboud & Haile, 2000; Krebs, 2000). In the dialysability assay, zinc and iron could cling to the glass surface. At pH 4, precipitation of minerals out of the dialysate/supernatant were reported

(Kruger, 2012). In the current study, the dialysates were acidified to reduce the pH and to ensure minerals remained in the solution until analysis.

Contamination from glassware and chemicals added during *in vitro* dialysability assays have been reported to cause variation in mineral data (Luten *et al.*, 1996). In the current study, all glassware was washed in 10% nitric acid to solubilize any minerals and rinsed with deionized water. ALVs were found to have high levels of aluminium (**Chapter 4:Table 4-1**) which could be an indication of soil contamination. Surprisingly, the PCA results (**Chapter 4:Figure 4-5**) showed that aluminium was associated with iron and zinc, this may result in high levels of artificial essential minerals. There is a debate on the bioavailability of contaminant iron (Teklu, 2017). ALVs in this research were thoroughly cleaned to remove any soil/dust. There is a need to investigate artificial iron and zinc content in ALVs which could be from soil contamination.

There are phenolic compounds found in tea and spinach that bind with iron to form soluble complexes (Moran *et al.*, 1997). These compounds could also be present in ALVs. These compounds can diffuse through the dialysis membrane into the dialysate and be considered as bioaccessible iron (Luten *et al.*, 1996). The soluble iron bound to phenolic compounds is not available for absorption (Dueik *et al.*, 2017). Hence, this may result in overestimation of bioaccessible iron. There is a need to characterize these phenolic compounds in ALVs.

AA promotes iron uptake by the human intestinal cells. The mechanism by which it enhances iron uptake is the reduction of ferric iron to ferrous iron (Scheers & Sandberg, 2008). There is evidence that AA can result in ferritin formation in Caco-2 cells in the absence of iron. Uptake of AA in the absence of iron was found to result in an increase of 19% of ferritin formation in Caco-2 cells (Scheers & Sandberg, 2008). Therefore, it may not be accurate to assume that iron uptake from food products is directly proportional to ferritin formation as ferritin may be formed due to the presence of AA and not necessarily of iron uptake. In the current study, the AA content in the ALVs was not analysed as it was beyond the scope of the current study.

The current study found that processed ALVs with high levels of iron and zinc (**Chapter 4: Table 4-1**) had low percentages of iron and zinc bioaccessible (**Chapter 4:Table 4-10**), indicating that these essential minerals were more dialysable in ALVs with less total mineral contents compared to ALVs with higher minerals contents. As previously stated, (**Chapter 2: Section 2.14.1.2**) that the reliability of *in vitro* assays is questionable. Therefore, it is crucial to regard the dialysability assay as a relative but not as the actual amount of mineral available for absorption (Fairweather-Tait *et al.*, 2005). *In vitro* assays do not simulate all factors involved in human digestion (Etcheverry *et al.*, 2012). The amount of iron and zinc that processed ALVs could contribute towards AR for iron and zinc of women aged 19-50 years should be interpreted with caution. The

measured magnitude of the effect may differ with the magnitude effect response observed in humans or predicts the wrong direction of response (Fairweather-Tait *et al.*, 2005). The physiological status of the individual consuming a meal is critical as it influences the mineral absorption (Dasa & Abera, 2018). Hence, absorption of iron is low in individuals with infections (Hurrell, 2012). Though dialysability assays can be used to screen food to identify differences in mineral availability due to dietary composition and changes that occurs during processing, there is a need to conduct *in vivo* assays to validate results from dialysability assays.

5.8 Strengths and limitations of the study

The *in vitro* dialysability assay and Caco-2 assays results obtained from this research estimate iron and zinc bioavailability from spontaneously fermented-dried and blanched-dried ALVs. Although the Caco-2 cells model cannot be translated directly to *in vivo* situations as the assay does not simulate all physiological and metabolic responses as in humans (Dias *et al.*, 2018). The results from the current study can be used for screening and identifying ALVs which have the potential to contribute towards iron and zinc nutrition. Also, the *in vitro* dialysability assays determines the direction of the influence of an inhibitor or enhancer towards mineral bioavailability (Fairweather-Tait *et al.*, 2007). Therefore, *in vitro* dialysability data can only be interpreted as an estimate for bioavailability but not actual value. There is a need for *in vivo* assays to validate and confirm results from *in vitro* dialysability assays.

The current study used Caco-2 cell model to predict iron and zinc uptake by the cells. Caco-2 cells are of human origin and have intestinal epithelium characteristics, causing their mineral uptake to be physiologically close to that of humans (García-Nebot *et al.*, 2013). Caco-2 cell model is very crucial in the simulation of digestion in humans and predicting the amount of minerals that can be absorbed by cells from the food matrix (Maares & Haase, 2020). As it is not always easy to conduct *in vivo* bioavailability studies, *in vitro* results can be used to predict the direction of the absorption. So, results obtained from this research can be used to predict the direction of bioavailability of iron and zinc from processed ALVs.

The strengths of this study include the use of an accredited laboratory for both mineral bioaccessibility and bioavailability content. Standardized methods and quality controls were used for laboratory experiments.

In addition, the study is first of its kind to assess zinc and iron content, bioaccessibility and estimated bioavailability from ALVs processed from three different methods, making the approach of this research more comprehensive and systematic.

There were several limitations identified in the current study. The study concentrated only on the antinutrients that inhibit iron and zinc but did not investigate the interaction between these two minerals. Soluble iron has been reported to inhibit zinc absorption (Lönnerdal, 2000; Pérès *et al.*, 2001).

The study compared cooked ALVs with blanched-dried or spontaneously fermented-dried ALVs. Usually, ALVs are spontaneously fermented-dried or blanched-dried and later reconstituted and cooked when out of season (Masarirambi *et al.*, 2010; Matenge *et al.*, 2012; Wafula *et al.*, 2016). This study did not assess the preservation of ALVs, but rather ALVs were processed as they will be typically consumed when in season, and also undergone the first initial steps of preservation normally used when ALVs are out of season (**Figure 1-1**). It needs to be mentioned that storage, reconstitution and final drying of the ALVs were not done in this study. However, these vegetables were not further cooked before analysis as in real situation. So, the results of the spontaneously fermented-dried and blanched-dried ALVs may not present the actual situation after reconstitution and further cooking. Therefore, future studies could evaluate the uptake of iron and zinc by Caco-2 cells in preserved ALVs.

The bioaccessibility assay cannot assess mineral uptake, transport kinetics or utilization of the mineral and evaluate nutrient uptake at the site of absorption (Etcheverry *et al.*, 2012). As a result, the bioaccessibility assay can result in underestimating the amount of bioaccessible iron. However, these limitations were taken into consideration in the current study, therefore, the use of the *in vitro* Caco-2 cell model to estimate iron and zinc uptake by the cells was used in conjunction with the *in vitro* dialysability assay method to estimate bioaccessibility of zinc and iron from ALVs.

ALVs are normally consumed in meals together with cereals and other vegetables (Kruger *et al.*, 2015), and therefore, the predicted uptake of iron and zinc in Caco-2 cells may not be the same as in meals. Results from the current study, therefore, predict bioavailability in the relish but when these ALVs are taken with staples, as is the norm (taken accompanying maize meal, millet and sorghum), other food components and antinutrients from staples may influence the uptake of these essential minerals from ALVs. Therefore, there is a need to determine the magnitude effects of antinutrients on iron and zinc bioavailability from ALVs when taken with other foods.

Furthermore, the bioavailability of iron is affected by host status, *in vitro* assays, there is no host (Hurrell & Egli, 2010). In Caco-2 cell assay's iron uptake differs from the *in vivo* situation. Iron accumulates in the cell and there is no blood circulation system for mineral transport (Sandberg, 2010). In the Caco-2 cell model, there are no organs involved in the regulation of mineral absorption in humans (Scheers *et al.*, 2014). There is a poor agreement in results obtained by

different researchers using Caco-2 cells (Dias *et al.*, 2018). Caco-2 cell assays need experienced personnel and experienced laboratories to conduct (Sandberg, 2010). More standardized methods and simpler procedures should be adopted. Assessing iron and zinc bioavailability using Caco-2 cells has been validated, but the magnitude effect is not always accurate (Dias *et al.*, 2018). Results obtained through *in vitro* models such as the use of the Caco-2 cell model to estimate iron and zinc bioavailability should always be confirmed through *in vivo* studies.

This study did not evaluate the degree of reduction of enhancers of iron and zinc during processing which probably influenced the outcome of the predicted iron and zinc bioavailability. ALVs in the current study were blanched-dried and spontaneously fermented-dried. These two processes have a negative effect on AA (Heffernan *et al.*, 2017). AA is an enhancer for non-haem iron as it reduces Fe^{3+} to Fe^{2+} facilitating its intestinal absorption (Scheers *et al.*, 2016). So, alternative processing methods of ALVs that could lessen AA losses need to be explored. Also, there is a need for research to evaluate AA content before and after processing ALVs. Lack of differences in iron uptake between cooked, spontaneously fermented-dried and blanched-dried ALVs could be linked to AA levels in each vegetable type.

5.9 The significance of the research from a public health perspective

The main contributors to malnutrition in sub-Saharan Africa are micronutrient deficiencies, food insecurity and shifting dietary patterns. Sub-Saharan Africa has the highest rates of anaemia (46%-71%) and Vitamin A deficiency (48%) prevalence in children less than five years of age (Stevens *et al.*, 2016; Stevens *et al.*, 2013). Also, 39% of women of reproductive age are anaemic (World Bank, 2016). The prevalence of iodine deficiency is reported at 42% and estimated inadequate zinc nutrition (25%) is also high in sub-Saharan Africa (Andersson *et al.*, 2005; Muthayya *et al.*, 2013). ALVs investigated in the current study could play a significant role in contributing to iron and zinc demands to improve the nutritional status of people in sub-Saharan Africa.

RDAs consider only the mineral content of the food, not the bioaccessibility of the mineral (FAO & WHO, 2001). Estimating the contribution of iron and zinc that differently processed ALVs could contribute towards the RDAs using mineral contents could give misleading expectations as their bioavailability is affected by inhibitors of iron and zinc. ALVs contained high levels of condensed tannins, total phenolic content, phytates and oxalates (**Chapter 4:Table 4-4, Table 4-6, Table 4-8**). Considering the inhibitory effects of these antinutrients, the nutritional contribution of these ALVs should be predicted using dialysability data and be based on the contribution they could make towards AR of iron and zinc. Total absolute requirements (AR) are the total amount of mineral required for growth, basal iron losses and, in women, also menstrual losses (FAO & WHO,

2001). For women of aged 19 to 50 years, the absolute requirement for iron is 1.46 mg/day (FAO & WHO, 2001) and for zinc, 1.75 mg/day (Sandstead, 2015). The contribution of each processed vegetable was calculated based on consumption of a standard portion of 300 g (as it is consumed) of ALV per day (van der Hoeven *et al.*, 2013).

Differently processed ALVs in the current study have the potential to contribute between 3.9% (0.06 mg/day) and 50.3% (0.73 mg/day) towards the iron AR for women aged 19 to 50 years (**Chapter 4:Figure 4-8**). However, the estimated contribution that spontaneously fermented-dried and blanched-dried ALVs could make towards iron AR of women was not different from the cooked ALVs. As a result, processing ALVs by fermentation and blanching could be as good as freshly cooked hence the methods should be encouraged. Amaranth had the potential to contribute over 40% (0.58 mg/day) towards the iron AR irrespective of how it was processed. Women aged 19 to 50 years who have limited access to animal sources of protein could be encouraged to consume amaranth as an alternative source of dietary iron.

ALVs in the current study are estimated to contribute between 2.9% (0.05 mg/day) to 57% (1.0 mg/day) towards zinc AR for women aged 19 to 50 years (**Chapter 4:Figure 4-9**). Spider plant had the highest contribution compared to other ALVs. There are limited good plant sources of zinc, and ALVs could potentially contribute zinc in diets which have limited animal sources of zinc. However, intervention studies are needed to validate these results in humans as the bioaccessibility of zinc in this study was conducted *in vitro*. When recommending these ALVs, it should be kept in mind that these ALVs are consumed accompanying staples such as maize meal porridge, sorghum porridge and millet and these staples contain high levels of antinutrients which possibly will contribute to the inhibition of iron and zinc reducing their bioavailability from these ALVs.

Low iron uptake (measured in the form of ferritin formation by Caco-2 cells) was found in processed amaranth and spider plant (**Chapter 4:Figure 4-11**). Highest zinc uptake by Caco-2 cells was observed in amaranth (**Chapter 4:Table 4-14**). However, the predicted iron and zinc bioavailability data from this research cannot be directly translated to the human situation because the *in vitro* dialysability assays do not simulate all *in vivo* conditions (Etcheverry *et al.*, 2012). The results could be used to predict the direction of iron and zinc bioavailability. Human studies should be conducted to confirm the bioavailability of iron and zinc from differently processed ALVs.

5.10 Recommendations for future research

In vitro dialysability assays simulate oral, gastric and intestinal digestion through the application of a series of different digestive enzymes and pH conditions to assess mineral bioaccessibility (Etcheverry *et al.*, 2012). The method is often combined with Caco-2 cell model to assess mineral absorption (Galanakis, 2017; Maares & Haase, 2020). These simulated assays cannot always be used to substitute human studies because they cannot simulate all conditions *in vivo* (La Frano *et al.*, 2014). The iron and zinc dialysability and iron and zinc uptake from processed ALVs obtained from this research should be validated *in vivo*. The bioavailability of iron and zinc from processed ALVs can be assessed using animal or human studies. However, *in vivo* assays are expensive to conduct and require ethical approval to conduct (Carbonell-Capella *et al.*, 2014). Human studies accurately assess mineral absorption (La Frano *et al.*, 2014), but it is not always easy to conduct human studies. Therefore, this may determine the choice of method to be used in determining bioavailability. Animal studies have been used previously to study iron and zinc bioavailability (Delgado-Andrade *et al.*, 2004; Della Lucia *et al.*, 2014; Fairweather-Tait *et al.*, 1991; Junior *et al.*, 2014; Lazarte *et al.*, 2015b). Though animal models allow for whole body assessment of mineral absorption as tissues are dissected and analysed they could not precisely simulate physiological responses in human (Carbonell-Capella *et al.*, 2014). Data obtained from animal studies can provide useful information about the situation in humans.

Non-commercialised ALVs in this study (amaranth, cowpea, wild jute and spider plant) had high levels of iron and zinc, with amaranth and spider plant having higher zinc contents than already commercialised Ethiopian kale. In addition, higher iron contents were found in cowpea, spider plant and amaranth compared to Ethiopian kale. Therefore, these ALVs should be marketed and commercialised to increase their availability throughout the year. This will promote their availability in the markets and utilisation by households in augmenting their iron and zinc needs.

ALVs contain high amounts of antinutrients which have some inhibitory effects on the bioavailability of iron and zinc. More research is needed that focuses on the production and processing of ALVs with low levels of antinutritional factors but with high concentrations of iron and zinc. Biofortification involves breeding crops to increase their mineral contents through genetic modification and the use of enriched fertilizers (Kumar *et al.*, 2019). It has been previously reported that biofortification of wheat, rice and maize with iron and zinc positively reduced their deficiencies (Bouis, 2018; Brinch-Pedersen *et al.*, 2007). Therefore, biofortification strategies of producing ALVs with high levels iron and zinc and low levels of antinutrients should be explored.

The bioaccessibility of iron and zinc should be investigated on processed ALVs consumed with iron and zinc enhancers. Combining ALVs with other vegetables that are rich in β -carotene could improve iron bioavailability. For example, the positive influence of β -carotene on iron bioavailability was reported in a study that investigated the addition of orange-fleshed sweet potatoes to cereals (Kruger *et al.*, 2018). Furthermore, the use of organic acids such as AA has been reported to improve mineral bioavailability (Etcheverry *et al.*, 2012). Iron bioavailability has been reported to be improved in the presence of AA (Singh *et al.*, 2016b). In a study by Engle-Stone and colleagues, which evaluated the iron bioavailability from an iron-phytic acid solution (1:20 iron-phytate molar ratio) in which different amounts of AA were added, an increase in iron uptake by Caco-2 cells was reported (Engle-Stone *et al.*, 2005). Beiseigel *et al.* (2007), evaluated the bioavailability of iron both *in vitro* and *in vivo*, the study found that addition of AA from orange juice to maize samples increased iron uptake by Caco-2 cells from 2% to 7%. Therefore, the use of AA, compositing these ALVs with orange flesh potatoes and the addition of tomatoes to enhance iron absorption from ALVs should be explored.

Also, the consumption of ALVs in combination with meat, poultry, and fish should be encouraged. Animal flesh such as meat, fish and poultry increases the absorption of non-haem iron and zinc from plant-based diets (Gibson, 2007; Maares & Haase, 2020). Though animal protein may be expensive for households which are food insecure, the use of other available local protein such as mopani worms in combination with processed ALVs should be evaluated. Mopani worms are high in protein and available for consumption in rural communities (Baiyegunhi *et al.*, 2016; Ndlovu *et al.*, 2019). (Teucher *et al.*, 2004)

In the current study, it was observed that spontaneously fermented-dried ALVs had high amounts of total phenolic content compared to their cooked counterparts. Therefore, other processing methods that can reduce the content of total phenolic content to enhance zinc and iron zinc bioavailability should be explored. In this study high levels of phytates were observed in ALVs, so the use of enzymes such as phytase should be explored to degrade phytate. A study which dephytinized infant cereals using exogenous phytase reported an increase in cell uptake efficiency (from 0.66% - 6.05% to 3.93% - 13%) of iron and (5.0% - 35.4% to 7.3% - 41.6%) of zinc (Frontela *et al.*, 2009).

As stated, blanching and fermentation in combination with drying have a negative effect on the contents of iron enhancers such as AA. AA is water-soluble, sensitive to light, oxygen and heat (Singh *et al.*, 2016a), hence it is easily degraded during processing and elevated temperatures. During drying, ALVs were subjected to air and light which probably leads to oxidation of AA and β -carotene (Naidu, 2003), reducing its levels. The loss of AA and β -carotene during blanching

and fermentation could be attributed to leaching out of AA into the discarded mediums during processing and oxidation of AA and β -carotene into other compounds. Consequently, other processing methods that could reduce antinutritional factors but at the same time minimising losses of iron enhancers such as AA and β -carotene should be explored.

5.11 Conclusions

ALVs in this study have high levels of iron and zinc, as they are widely used in sub-Saharan Africa their consumption could be beneficial in the livelihoods of rural communities who have limited access to animal sources of iron and zinc and are at risk of their deficiencies. ALVs studied could be marketed and commercialised to increase their accessibility in markets. Iron and zinc uptake by Caco-2 cells was not enhanced due to presence of high antinutrients content in processed ALVs, hence, consumption of ALVs should be encouraged with locally available foods which are good sources of enhancers of iron and zinc (tomatoes, oranges and mopani worms) to influence these essential minerals absorption. Also, other processing methods that can completely degrade antinutrients in AVs such as the use of exogenous enzymes (phytases) should be explored. Though it is always accurate to study bioavailability in humans, factors such as costs, time and ethical clearances may be a hindrance hence the need to predict mineral bioavailability by *in vitro* assays. The findings of this study provide an estimation on the direction of iron and zinc bioavailability from processed ALVs. ALVs are used mostly in food security strategies targeting vulnerable groups who are at risk of iron and zinc deficiency, therefore, these findings could provide a guide in selecting appropriate nutrition interventions. The findings from the current *in vitro* study can only be used to predict the direction of iron and zinc bioavailability from processed ALVs, as *in vitro* models cannot simulate all *in vivo* conditions, hence, dietary intervention studies should be conducted on processed ALVs to ascertain iron and zinc bioavailability.

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ADDENDUM 1: MANUSCRIPT UNDER REVIEW

Food Science and Technology



**MINERALS, ANTINUTRIENTS CONTENT AND THE
BIOACCESSIBILITY OF IRON AND ZINC IN COOKED,
SPONTANEOUSLY FERMENTED-DRIED, AND BLANCHED-
DRIED DARK GREEN LEAFY VEGETABLES COMMONLY
CONSUMED IN SUB-SAHARAN AFRICA**

Journal:	<i>Food Science and Technology</i>
Manuscript ID	Draft
Manuscript Type:	Original Article
Keyword:	Traditional leafy vegetables, dialysability, mineral, preservation, nutritive value

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Topic Relevance: This manuscript is relevant for public health nutrition interventions and industries in sub-Saharan Africa as they can adopt technologies of fermentation and blanching combined with drying African leafy vegetables to curb post-harvest losses and promote their consumption.

MINERALS, ANTINUTRIENTS CONTENT AND THE BIOACCESSIBILITY OF IRON AND ZINC IN COOKED, SPONTANEOUSLY FERMENTED-DRIED, AND BLANCHED-DRIED DARK GREEN LEAFY VEGETABLES COMMONLY CONSUMED IN SUB-SAHARAN AFRICA

BLANCHING AND FERMENTATION ON AFRICAN LEAFY VEGETABLES AND MINERAL BIOACCESSIBILITY

ABSTRACT: This study aimed at determining mineral, antinutrient contents of differently processed ALVs, and the bioaccessibility of iron and zinc from cooked, blanched-dried, as well as spontaneously fermented-dried ALVs. Iron, zinc, magnesium, and calcium contents of the ALVs were high. Compared to cooked vegetables, both spontaneously fermented-dried and blanched-dried vegetables had significantly lower levels of condensed tannins, while significantly lower total phenolic contents were found in blanched-dried vegetables. Compared to cooked counterparts, percentage iron and zinc bioaccessibilities in spontaneously fermented-dried and blanched-dried ALVs showed no significant ($p > 0.05$) differences except for iron in Ethiopian kale and zinc in amaranth. Consumption of ALVs should be encouraged concurrently with iron and zinc enhancers to enhance mineral bioaccessibility.

Keywords: Traditional leafy vegetables; dialysability; mineral; preservation; nutritive value

Practical Application

Improving in vitro bioaccessibility of vegetables through blanching and fermentation

INTRODUCTION

The prevalence of anaemia is estimated at 22.8% globally with sub-Saharan Africa as one of the regions bearing the greatest burden (Gardner and Kassebaum, 2020). Iron deficiency anaemia (IDA) is associated with inadequate dietary intake, increase in iron body demand, chronic loss of blood, and poor iron absorption (Chaparro and Suchdev, 2019, Gardner and Kassebaum, 2020). The prevalence of inadequate zinc intake was estimated to exceed 25% in the sub-Saharan Africa region (Wessells and Brown, 2012). Iron and zinc deficiencies in sub-Saharan Africa are mainly caused by inadequate dietary intake of animal sources of iron and zinc due to most households relying mainly on cereal-based staple foods, which have low iron and zinc bioavailability (Fanzo, 2012). Consumption of African leafy vegetables (ALVs) can be effective in alleviating iron and zinc deficiencies by increasing dietary diversification and improving household nutrition security (Yang and Tsou, 2006, Elisha et al., 2016). ALVs are mostly utilized by households in rural areas, cooked fresh and consumed as a relish accompanying staple foods (Uusiku et al., 2010, van der Hoeven et al., 2016, Bvenura and Afolayan, 2015). Major challenges in the sustainable incorporation of ALVs into diets are their seasonality and post-harvest losses due to their high perishability (Uusiku et al., 2010, Gogo et al., 2018).

However, traditional processing methods such as blanching and spontaneous fermentation in combination with drying have been previously used to reduce postharvest losses (Wafula et al., 2016, Masarirambi et al., 2010, Natesh et al., 2017). ALVs also contains high levels of antinutrients in ALVs (Kruger et al., 2015, Essack et al., 2017). Blanching and spontaneous fermentation have been reported to be effective in reducing antinutrients levels in ALVs (Wafula et al., 2016, Kirakou et al., 2017). To the best of our knowledge, no research has been published on the bioaccessibility of iron and zinc from blanched-dried and spontaneously fermented-dried ALVs and compared to

their cooked counterparts. This study aimed to determine the mineral and antinutrient contents of processed ALVs, and the iron and zinc bioaccessibility of processed ALVs.

MATERIALS AND METHODS

Plant Materials

Five commonly consumed ALVs *Cleome gynandra* (spider plant), *Amaranthus hybridus* (amaranth), *Corchorus olitorius* (wild jute) and *Vigna unguiculata* (cowpea leaves) and *Brassica Carinata* A. Baun (Ethiopian kale) were procured in Botswana. Two separate samples from each ALV were processed. All reagents were of analytical grade and were purchased from Sigma-Aldrich, Johannesburg, South Africa unless stated otherwise.

Vegetable sorting and washing

ALVs were inspected for fungal contamination, surface contaminants, debris as well as defective colour and appearance as outlined in the world vegetable centre manual (Acedo, 2010). Approximately 5 kg of each vegetable was washed twice in deionized water to remove any impurities and was drained after which it was divided into three equal batches for cooking, blanching, and spontaneous fermentation. The ALVs were prepared the way in which they are normally prepared in households when in season and out of season (Figure 1).

Cooking

ALVs (500 g) were boiled in 100 ml of deionized water for 20 minutes as per households methods previously described (Matlhare et al., 1999), cooled to room temperature, and transferred along with the cooking water into plastic jars with lids and stored at –20 °C for a month until laboratory analysis. To prepare for laboratory analysis, the cooked samples were then freeze-dried

and milled to a fine powder using a laboratory miller (IKA A11, Staufen, Germany) and stored in zip-lock bags for two to three days at -20 °C before analysis. Cooking was used as a control in this study.

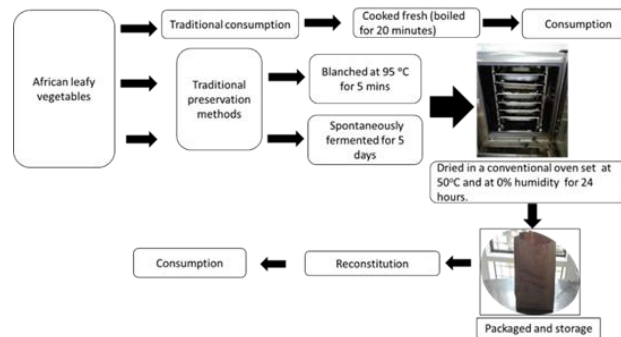


Figure 1: Experimental design illustrating traditional processing methods of African leafy vegetables

Blanching

For blanching, 1 kg of ALVs were submerged into deionized boiling water (4L) at 97 °C for five minutes as previously described (Mosha et al., 1995). The vegetables were then transferred into oven drying trays and dried in a conventional oven dryer at 50 °C with 0% humidity for 24 hours (ClimaPlus Combi, Germany). The dried samples were stored at 5 °C in the brown craft paper bags for a month. For laboratory analysis, samples were milled and stored as described for cooked samples.

Spontaneous fermentation

The third batch of ALVs were fermented according to the method by (Ifesan et al., 2014). Each vegetable (1 kg) was fermented with 30 g of salt as per the method described by the World Vegetable Centre (Acedo, 2010). An additional 30 g of sugar was added as adopted from the method used by (Kasangi et al., 2010) with some modifications. The ALVs were placed in a 2.5

cm layer and sprinkled with the 50:50 salt-sugar mixture, on to which another layer of leaves was added and sprinkled with the salt-sugar mixture. This was repeated until the whole 1 kg of leaves and 60 g of the 50:50 salt-sugar mixture was used. A cheesecloth was placed over the ALVs, and weight was added to compress the vegetable and assisted in the formation of brine. The vegetables were left to ferment for seven days at room temperature. Temperature and pH of the fermented ALVs were monitored and measured daily. A Crison basic 20, Barcelona, Spain pH meter and a handheld digital thermometer (-50 to +200 °C, P300 Temp, France) were used. After fermentation, the vegetables were weighed and placed in drying trays and the fermentation liquid was discarded. Fermented ALVs were dried as described for blanched samples. After drying, the vegetables were weighed and stored at 5 °C in sealed, brown craft paper bags for a month. For laboratory analysis, samples were milled and stored as described for cooked samples.

Mineral contents

Iron (Fe), zinc (Zn), magnesium (Mg), calcium (Ca), aluminium (Al), and phosphorus (P) contents of the ALVs were analysed at the Central Analytical Facilities (CAF) in Stellenbosch University laboratory, which is accredited by the South African National Accreditation System (SANAS). The ALV samples were digested by concentrated nitric acid and hydrogen peroxide by adopting the United Nations Environment Protection Agency (USEPA) method 3051A (Element, 2007). The concentration of minerals in the digested samples were determined by using Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES) (ICAP 6000 series, Thermo Fisher Scientific, Waltham, USA). Elements were analysed in the following wavelengths, 239.5nm for iron, 206.7nm for zinc, 315.8nm for calcium, 285.2nm for magnesium, 214.9nm for phosphorus, and 308.2nm for aluminium. For quality assurance, samples were analysed against National Institute for Standards and Technology traceable standards and independent quality

controls. An internal standard technique was established, and a calibration acceptance criterion of $R^2 > 0.9995$ was used to ascertain the accuracy of the result.

Total phenolic content, condensed tannins, phytates, and oxalate contents

Total phenolic content (TPC) was determined using a modified Folin-Ciocalteu method (Kaluza et al., 1980). Condensed tannins were determined by the Vanillin- HCl method with slight modifications of the protocol (Price et al., 1978). For both TPC and tannin contents, blank samples without the addition of the reagent were analysed for each sample to correct for the colour of the extracts. The absorbance for TPC was measured at 760nm and for condensed tannins at 500nm. Phytate content was determined by anion exchange chromatography, an indirect quantitative analysis by measuring the organic phytate-phosphorus (inositol -1 to 6- phosphate) (Fruhbeck et al., 1995). Purification of the extracts was conducted using Dowex1-anion-exchange resin-AG 1 x 4 (4% Cross-linkage, chloride form, 100-200 mesh (74-149 μ m) in a glass barrel Econo-column (Poly-Prep®Chromatography columns (Bio-Rad, Hercules, California, United States). Purified extracts were reacted with the Wade reagent and the absorbance was measured at 500nm. Oxalates were determined using the titration method and calculated assuming that 1 ml of 0.1 M KMnO_4 was equivalent to 2.2 mg of oxalate (AOAC., 1999).

Iron and zinc bioaccessibility

The *in vitro* dialysability method described by (Miller et al., 1981) was used, with some minor modifications. The dialysates were acidified with 0.002 ml 65% nitric acid/ml dialysate when decanted to avoid potential precipitation of minerals (Kruger et al., 2012). The following enzymes were used: pepsin (P-7000), bile extract (P-8631) and pancreatin (P-1750) (Sigma-Aldrich, Johannesburg, South Africa). In the intestinal phase, a dialysis tubing of Spectra/Por 7 (Ø

= 20.4 mm) with a molecular mass cut-off (MMCO) of 10000 Da (Labretoria) was used (GIC Scientific, Johannesburg, South Africa). Mineral contents of the dialysates were determined by the ICP-OES (ICAP 6000 series, Thermo Fisher Scientific, Waltham, USA) as described above on mineral contents analysis but without the digestion step.

The potential contribution of the vegetables towards total absolute requirements

The contribution that each processed ALV could make towards iron and zinc AR of women aged 19 to 50 years were calculated using bioaccessibility data assuming that a standard portion size of ALV (300 g wet basis) was consumed (van der Hoeven et al., 2013). For women aged 19 to 50 years, the AR for iron and zinc is 1.46 mg/day and 1.75 mg/day, respectively (Sandstead, 2015; FAO and WHO, 2001). The contributions were expressed as a percentage.

$$\% \text{ contribution} = \frac{\text{absolute accessible mineral(mg/100g)}}{\text{absolute requirements (mg/day)}} \times 100$$

Statistical analysis

The composition analyses on the ALVs were conducted in duplicates with each antinutrient analysis repeated on two separate days. Independent dialysability experiments were conducted in duplicates with the intestinal digestion step for each time performed in triplicates. IBM SPSS Statistics 25.0, Armonk, USA was used for data analysis and Analysis of Variance (ANOVA) and Tukey's HSD Post-hoc test was applied to determine significant differences between the means. Values of $p \leq 0.05$ were considered statistically significant for all the analysis. A Principal Component Analysis (PCA) was performed to determine the correlation patterns and variation between mineral and anti-nutrient content in ALVs.

RESULTS AND DISCUSSION

Compositional data for this study are given on a dry basis (db) except where otherwise

131 stated.

133 **Mineral contents of traditionally processed African leafy vegetables**

134 Compared to their cooked counterparts, the mineral content of fermented ALVs was
135 significantly ($p \leq 0.05$) lower for iron in wild jute and spider plant; for magnesium in spider plant,
136 Ethiopian kale and amaranth; and for calcium in amaranth (Table 1). In contrast, compared to the
137 cooked ALVs, the mineral content of fermented ALVs was significantly ($p \leq 0.05$) higher for iron
138 in amaranth and Ethiopian kale; for zinc in cowpea leaves, wild jute and Ethiopian kale; for
139 magnesium in wild jute and cowpea; and for calcium in cowpea, spider plant and wild jute. In this
140 study, the cooking water was not discarded minerals leached out remained in cooked ALVs.
141 However, the sugar-salt solution used for fermentation was drained out, which could have
142 contributed to mineral losses through leaching. Also, the addition of salt encourages the growth of
143 lactic acid bacteria (LAB) which increases the osmolarity and ionic strength of the fermentation
144 medium, causing water and minerals to be extracted from vegetable tissues (Swain et al., 2014).
145 The localisation of the mineral in the vegetable leaf matrix determines how the mineral reacts to
146 processing (Raes et al., 2014). Minerals in the ALVs could have been located in different plant
147 organelles attributing to variations in the influence of fermentation.

148 Compared to their cooked counterparts the mineral content of blanched ALVs was
149 significantly ($p \leq 0.05$) lower for iron in cowpea, spider plant, wild jute and amaranth; for
150 magnesium in spider plant and Ethiopian kale; for calcium in Ethiopian kale; and for phosphorous
151 in amaranth and Ethiopian kale (Table 1). In contrast, compared to cooked counterparts, mineral
152 contents of blanched ALVs were significantly ($p \leq 0.05$) higher for iron in Ethiopian kale; for zinc
153 in Ethiopian kale and amaranth; for calcium in wild jute; and phosphorous in amaranth.

There are no studies available which compared mineral contents in blanched and cooked ALVs, however, previous studies which compared raw and blanched ALVs reported lower levels of iron and zinc in blanched ALVs (Acho et al., 2015, Mepba et al., 2007). ALVs were blanched for five minutes in the current investigation, which has been reported to be effective maximum blanching time to minimise mineral losses through leaching (Acho et al., 2015), however, some losses were still apparent. The degree of heating, rate of leaching, duration of cooking, and surface area exposed to water determines the degree in which minerals are lost through processing (Lešková et al., 2006).

Table 1: Mineral contents of traditionally processed African green leafy vegetable

		Fe	Zn	Mg	Ca	P	Al
		mg/100 g, db					
CP	COO	29.7 ± 0.6 ^{b*}	2.9 ± 0.1 ^{a*}	301 ± 5 ^{a*}	1840 ± 33 ^{a*}	340 ± 6 ^{b*}	32.4 ± 0.6 ^{c*}
	BLA	18.1 ± 0.1 ^a	3.0 ± 0.3 ^a	268 ± 7 ^a	1850 ± 30 ^a	337 ± 4 ^b	13.5 ± 0.2 ^a
	FER	28.5 ± 0.5 ^b	3.4 ± 0.1 ^b	343 ± 4 ^b	2220 ± 33 ^b	303 ± 4 ^a	25.6 ± 0.6 ^b
SP	COO	61.7 ± 1.2 ^{c*}	12.0 ± 0.2 ^{b*}	437 ± 8 ^{b*}	2456 ± 43 ^{a*}	614 ± 11 ^a	75.8 ± 1.4 ^{c*}
	BLA	40.0 ± 0.5 ^a	11.8 ± 0.1 ^b	350 ± 1 ^a	2381 ± 29 ^a	617 ± 4 ^a	45.3 ± 0.1 ^a
	FER	52.8 ± 1.0 ^b	11.2 ± 0.1 ^a	377 ± 5 ^a	2660 ± 51 ^b	601 ± 3 ^a	56.0 ± 1.1 ^b
WJ	COO	29.5 ± 0.4 ^{c*}	3.7 ± 0.0 ^a	573 ± 1 ^b	1422 ± 10 ^{a*}	648 ± 7 ^{b*}	30.8 ± 0.3 ^{c*}
	BLA	10.4 ± 0.4 ^a	3.5 ± 0.1 ^a	528 ± 12 ^a	2452 ± 93 ^b	454 ± 10 ^a	6.4 ± 0.0 ^a
	FER	16.4 ± 0.3 ^b	4.5 ± 0.0 ^b	640 ± 7 ^c	2782 ± 29 ^c	570 ± 6 ^c	14.2 ± 0.3 ^b
A	COO	30.0 ± 0.6 ^{b*}	6.4 ± 0.0 ^{b*}	1365 ± 12 ^{b*}	1995 ± 21 ^{b*}	477 ± 3 ^{a*}	39.0 ± 0.4 ^{c*}
	BLA	26.4 ± 0.0 ^a	6.8 ± 0.1 ^c	1556 ± 7 ^c	1985 ± 11 ^b	570 ± 14 ^b	26.2 ± 0.3 ^a
	FER	33.2 ± 0.7 ^c	6.0 ± 0.1 ^a	1254 ± 16 ^a	1730 ± 28 ^a	476 ± 6 ^a	34.1 ± 0.1 ^b
EK	COO	13.4 ± 0.3 ^a	4.1 ± 0.1 ^a	561 ± 11 ^c	2890 ± 54 ^b	565 ± 11 ^b	9.7 ± 0.2 ^a
	BLA	20.0 ± 1.0 ^b	5.1 ± 0.1 ^b	421 ± 13 ^a	2216 ± 83 ^a	521 ± 2 ^a	42.3 ± 1.4 ^c
	FER	24.5 ± 0.2 ^c	5.2 ± 0.1 ^b	521 ± 10 ^b	2928 ± 34 ^b	555 ± 14 ^b	21.2 ± 0.3 ^b

Values are expressed as mean ± SD (n = 2). Mean values of each vegetable in the same column with different superscripts are significantly different at $p \leq 0.05$, CP= Cowpea, SP= Spider plant, WJ = Wild jute, A= Amaranth, EK = Ethiopian kale, FER=Fermented, BLA = Blanched, COO = Cooked, Values of cooked vegetable with a * are significantly different to the cooked Ethiopian kale at $p \leq 0.05$, db = dry basis

Compared to cooked counterparts, aluminium content of all blanched and fermented ALVs were significantly ($p \leq 0.05$) lower, except for Ethiopian kale with higher contents (Table 1). Spider plant had the highest aluminium contents. High content of aluminium in plant-based foods could be an indication of soil contamination (Salvo et al., 2018) and result in artificially high levels of essential minerals in food, especially iron. Previously, it was reported that normal cleaning before processing ALVs did not remove all impurities (van Jaarsveld et al., 2014). Additional soil impurities which were not removed by normal cleaning could have been removed through leaching out into the blanching and fermentation mediums. Spider plant's leaves generally grow nearer to the ground surfaces which makes it susceptible to soil contamination. High levels of aluminium content found in blanched and fermented Ethiopian kale could be attributed to cross-contamination with aluminium foil as the vegetable stuck to aluminium foil which lined the drying trays.

Antinutrient contents of traditionally processed African green leafy vegetables

Compared to cooked counterparts, condensed tannins were significantly ($p \leq 0.05$) lower in all fermented ALVs except Ethiopian kale (Table 2). Waluchio (2016) also found a decrease of 86% of tannin contents after spontaneous fermentation of cassava leaves. During spontaneous fermentation, microbial activity results in tannic acid degradation into smaller compounds, and also the activation of the tannin acyl hydrolase hydrolyses the ester and depside bonds of tannic acid to form hydrolysable tannins (Macedo et al., 2011), free tannins leaches out into the fermentable medium which was discarded attributing to low levels.

Compared to cooked counterparts, condensed tannins were significantly ($p \leq 0.05$) lower in blanched spider plant and wild jute while higher contents were found in blanched cowpea. Lower levels of condensed tannins in some ALVs could probably be due to leaching out into the blanching medium. High temperatures break down hydrogen bonds that exists between tannin

complexes and, hence, increase the extraction of tannins during analysis (Peng and Jiang, 2004), which may be a possible reason for the observed high levels of condensed tannins in blanched cowpea.

TPC was found to be significantly higher ($p \leq 0.05$) in fermented cowpea and spider plant than in their cooked counterparts. During fermentation, condensed tannins such as proanthocyanin are hydrolysed to phenols through the activation of polyphenol oxidase (Nkhata et al., 2018, Emmambux and Taylor, 2003). The transformation of tannins to phenols that occurs during fermentation increases phenolic contents (Taylor and Duodu, 2015).

Compared to cooked counterparts, significantly lower ($p \leq 0.05$) total oxalates and soluble contents were found in all fermented and blanched ALVs except fermented wild jute with no significant changes while blanched spider plant and Ethiopian kale had higher insoluble oxalates content (Table 2). During fermentation LAB degrade oxalates, thereby reducing the levels (Okoye and Ene, 2018, Goldfarb et al., 2007). Wild jute contain pectin (Ahmed et al., 2003), which probably influenced its processing to some extent as less medium was discarded before drying as it turned jelly-like possibly accounting for the inconsequential changes found in its soluble oxalate contents. During blanching, soluble oxalates are lost through leaching out into the blanching medium (Raes et al., 2014). Insoluble oxalates are only effectively reduced by prolonged thermal processing, (Savage and Klunklin, 2018)

Compared to cooked counterparts, blanched spider plant, fermented amaranth and spider plant had significantly ($p \leq 0.05$) lower phytate contents (Table 2). Similarly, lower levels of phytates were reported in a study in which spider plant was fermented for 48 hours (Akello, 2014). In contrast to the current study, significantly higher phytate levels were found in blanched *Moringa*

oleifera leaves than in the cooked sample (Sallau et al., 2012). ALVs were blanched at the maximum blanching time of five minutes which served to minimise mineral losses and were further dried at 60 °C for 24 hours, which to some degree degraded some phytates in spider plant. During fermentation, endogenous phytase is activated resulting in degradation of phytate (Coulibaly et al., 2011), and LAB produces lactic acid, which decreases the pH to the optimal range for phytase activity increasing phytate degradation (Gupta et al., 2015). The extent of reduction of phytates likely was due to the amount of lactic acid produced in each ALV.

Relationship between minerals and antinutrients in traditionally processed African green leafy vegetables

The principal component analysis (PCA) (Figure 2) shows that total oxalates, soluble salts, and magnesium were positively correlated, indicating that oxalates in these ALVs probably predominantly existed in the form of magnesium oxalate. Magnesium oxalate has been associated with more solubility than calcium oxalate (Laranjinha et al., 2019). Insoluble calcium oxalates pose a health risk as it can accumulate in the renal glomeruli and forms kidney stones (Massey, 2007). Also, the strong association found between iron, zinc, and aluminium may suggest that a portion of iron and zinc from these ALVs may be from soil/dust contamination.

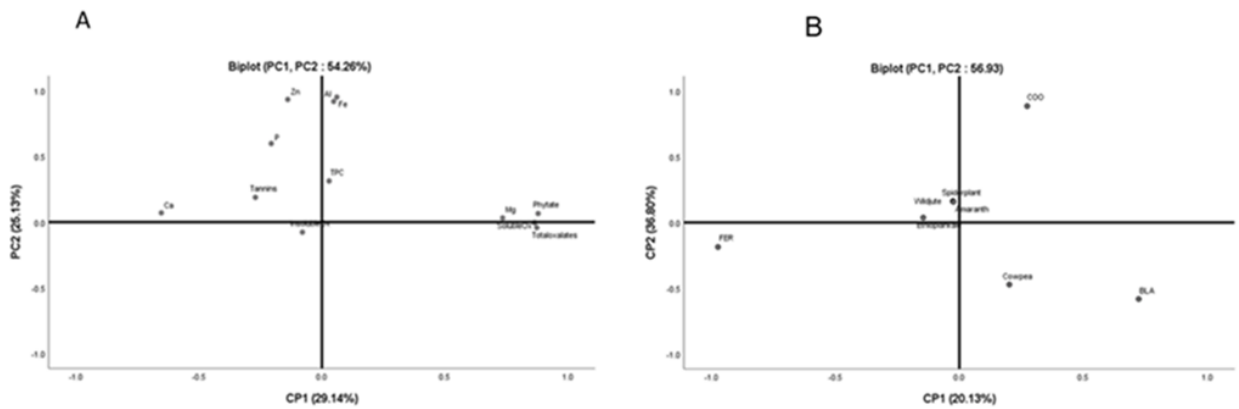


Figure 2: Principal component analysis of minerals and antinutrients of traditionally processed African leafy vegetables. **(A).** PCA loadings; phytate, total oxalate, soluble oxalate, insoluble oxalate total phenolic content (TPC), tannins, iron, zinc, phosphorus, magnesium, calcium, aluminium. **(B).** Green leafy vegetable species; spider plant, cowpea, wild jute, amaranth, Ethiopian kale and processing methods (COO = cooked, BLA = Blanched, FER = Fermented)

Bioaccessibility of iron and zinc from processed African leafy green vegetables

Compared to cooked counterparts, percentage iron bioaccessibility and the amount of bioaccessible iron in all the fermented and blanched ALVs showed no significant ($p > 0.05$) differences except for Ethiopian kale and blanched amaranth with lower contents (Table 4). Blanched amaranth had lower iron contents (Table 1), which possibly influenced the amount of bioaccessible iron between the samples. Ethiopian kale has been found to contain high levels of β -carotene (Odongo et al., 2017), which enhances iron bioaccessibility (Gautam et al., 2010, Kruger et al., 2018). Drying result in losses of β -carotene and ascorbic acid in ALVs (Mutuli and Mbuge, 2018), which probably affected iron bioaccessibility in fermented and blanched samples. All processed ALVs had high levels of antinutrients (Table 2) which possibly influenced iron bioaccessibility. Phytate binds to iron making it unavailable for absorption (Gibson et al., 2010). Phytate/iron molar ratios of all processed ALVs ranged between 2.7 to 18.7 (Table 3) and were above the critical level of less than 1, which estimates iron bioavailability that is strongly impaired (Hurrell, 2004). ALVs had high levels of condensed tannins (58-220 mg CE/100 g) found in this study (Table 2) also had some inhibitory effects on iron bioaccessibility. Condensed tannins forms insoluble complexes with iron making unavailable for absorption, and also binds with protein forming tannin-protein complexes inhibiting digestive enzyme activation that are crucial in breaking down food matrixes to release minerals in the lumen for absorption (Shahidi and Ambigaipalan, 2015). TPC form insoluble complexes with Fe^{3+} reducing the bioaccessibility of non-haem iron (Perron and Brumaghim, 2009), negatively influencing iron bioaccessibility, Iron from these ALVs probably was not available for absorption due to a strong positive association

between iron and aluminium contents (Figure 2) displaying that some of the iron could be from extrinsic contamination which its bioavailability questionable. There is a controversy on the bioavailability of contaminant iron, human studies should be conducted to ascertain its bioavailability (Teklu, 2017).

Concerning zinc bioaccessibility, there were no significant ($p > 0.05$) differences found in percentage zinc bioaccessibility between cooked and fermented same type of ALVs while higher amount of bioaccessible zinc was observed in blanched amaranth, attributing to 50% lower total oxalate contents found in the blanched sample (Table 2). Oxalates binds to zinc forming an insoluble salt which are less absorbable and impairs zinc absorption (Lonnerdal, 2013). Phytate also has an inhibitory effect on zinc bioaccessibility (Gibson et al., 2010), it forms insoluble complexes with zinc making the mineral unabsorbable. Phytate/zinc molar ratios of ALVs were way above the critical level of 15 (four times higher) (Table 3), which predicts that zinc absorption from these ALVs is seriously impaired (FAO and WHO, 2005). Calcium has the affinity to form complexes with phytate and zinc that are insoluble (Gibson et al., 2010). Phytate-calcium/zinc molar ratios for which zinc bioavailability is seriously impaired, are higher than the critical limit of 200 (Bindra et al., 1986). The phytate-calcium/zinc molar ratios in all processed ALVs were very high (921 to 3930) (Table 4), four to nineteen times higher than the critical limit of 200 which shows that the combined inhibitory effect of phytate and calcium on zinc was more pronounced.

The potential contribution of ALVs in this study towards absolute requirements for zinc and iron in women

For women aged 19 to 50 years a standard portion of 300 g (as consumed) of amaranth has the potential to contribute over 40% towards iron AR irrespective of processing (Figure 3 A) while spider plant could make the largest contribution than other ALVs. However, *in vitro* dialysability

assays does not entirely simulate all factors involved in human digestion because bioavailability has a physiological endpoint (Etcheverry et al., 2012), therefore these are not actual values but estimates.

Limitations and strengths of the study

These findings provide valuable information on predicting the direction of bioavailability of iron and zinc from processed ALVs to a wide audience in sub-Saharan Africa. ALVs are reconstituted and cooked for consumption when out of season, so the results of this study only simulated the situation only after processing but not after preservation. ALVs were processed in duplicates, hence low number of samples used could be a weakness of this study.

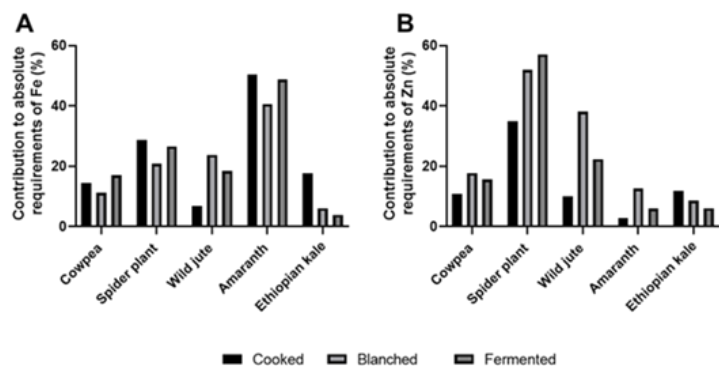


Figure 3: Contribution (%) of traditionally processed African leafy vegetables towards (A) absolute iron and (B) absolute zinc requirements of women aged 19 to 50 years

294 **Table 3: Phytate-mineral ratios of processed green leafy vegetables**

Molar ratios		CP	SP	WJ	A	EK
PA : Zn¹	COO	78	20	48	69	47
	BLA	69	16	58	56	34
	FER	60	15	48	57	35
PA : Fe²	COO	7	3	6	13	13
	BLA	10	4	19	12	7
	FER	6	3	11	9	6
PA x Ca/ Zn³	COO	3585	1232	2038	3406	3419
	BLA	3187	921	3930	2789	1876
	FER	3316	982	3352	2443	2547

CP= Cowpea, SP= Spider plant, WJ = Wild jute, A= Amaranth, EK = Ethiopian kale, FER = Fermented, BLA = Blanched, COO = Cooked ¹ (mg phytate/MW of phytate : mgZn/MW of Zn), ² (mg phytate/MW of phytate : mgFe/MW of Fe), ³ (mg phytate/MW of phytate x mg Ca/MW of Ca / mg Zn/MW of Zn).

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296 **Table 2: Antinutrients content of traditionally processed African green leafy vegetables**
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		Condensed Tannins	Total Phenolics	Total oxalates	Soluble oxalates	Insoluble oxalates	Phytates
Vegetable		mg CE /100 g, db		mg/100 g, db			
CP	COO	137 ± 14 ^b	2022 ± 83 ^{a*}	3349 ± 224 ^{b*}	2967 ± 181 ^{b*}	382 ± 68 ^a	2263 ± 189 ^a
	BLA	174 ± 17 ^c	2083 ± 155 ^a	2226 ± 177 ^a	2039 ± 58 ^a	187 ± 229 ^a	2131 ± 297 ^a
	FER	58 ± 5 ^a	2612 ± 55 ^b	2111 ± 51 ^a	2031 ± 49 ^a	80 ± 36 ^a	2025 ± 263 ^a
SP	COO	172 ± 7 ^{b*}	2040 ± 25 ^{a*}	2502 ± 55 ^c	2400 ± 64 ^c	102 ± 107 ^a	2437 ± 272 ^b
	BLA	140 ± 8 ^a	1951 ± 92 ^a	1862 ± 212 ^b	804 ± 24 ^a	1058 ± 226 ^b	1848 ± 272 ^a
	FER	129 ± 9 ^a	3022 ± 293 ^b	1272 ± 36 ^a	1234 ± 20 ^b	37 ± 55 ^a	1831 ± 246 ^a
WJ	COO	220 ± 22 ^{b*}	2491 ± 222 ^{a*}	3835 ± 403 ^{c*}	2470 ± 119 ^b	1361 ± 409 ^{b*}	2140 ± 408 ^a
	BLA	116 ± 12 ^a	2106 ± 74 ^a	1754 ± 178 ^a	812 ± 21 ^a	943 ± 199 ^b	2296 ± 386 ^a
	FER	132 ± 13 ^a	2204 ± 176 ^a	2629 ± 227 ^b	2433 ± 43 ^b	196 ± 206 ^a	2194 ± 418 ^a
AM	COO	144 ± 16 ^b	1655 ± 95 ^{a*}	4680 ± 242 ^{c*}	4113 ± 197 ^c	567 ± 45 ^a	4408 ± 213 ^b
	BLA	132 ± 10 ^b	1857 ± 181 ^a	2126 ± 87 ^a	1640 ± 25 ^a	485 ± 97 ^a	3866 ± 538 ^{ab}
	FER	78 ± 8 ^a	1637 ± 110 ^a	3408 ± 43 ^b	3219 ± 112 ^b	189 ± 78 ^a	3429 ± 532 ^a
EK	COO	147 ± 9 ^a	1103 ± 86 ^a	2627 ± 92 ^c	2334 ± 109 ^c	293 ± 72 ^b	1977 ± 90 ^a
	BLA	135 ± 14 ^a	1020 ± 95 ^a	1633 ± 24 ^b	408 ± 10 ^a	1226 ± 32 ^c	1751 ± 126 ^a
	FER	223 ± 18 ^b	1168 ± 103 ^a	843 ± 24 ^a	806 ± 17 ^b	37 ± 25 ^a	1831 ± 246 ^a

Values are expressed as mean ± SD (n = 4 for condensed tannins and phenolics, n = 6 for oxalates and phytates), Mean values of each vegetable in the same column with different superscripts are significantly different at $p \leq 0.05$, CP= Cowpea, SP= Spider plant, WJ= Wild jute, A= Amaranth, EK = Ethiopian kale, FER = Fermented, BLA = Blanched, COO = Cooked, Insoluble oxalates = (Total oxalates – soluble oxalates), (%) = (soluble oxalates / Total oxalates) x 100, Values of cooked vegetable with a * are significantly different to the cooked Ethiopian kale at $p \leq 0.05$, db = dry basis, CE = catechin equivalents.

Table 4: The bioaccessibilities of iron and zinc of traditionally processed African green leafy vegetables

Vegetable #		Bioaccessibility (%)		Amount bioaccessible (mg/100 g, db)	
		Fe	Zn	Fe	Zn
CP	COO	3.9 ± 0.5 ^a	35.7 ± 4.4 ^a	1.15 ± 0.14 ^a	1.04 ± 0.13 ^a
	BLA	2.1 ± 0.9 ^a	24.2 ± 10.8 ^a	0.38 ± 0.17 ^a	0.73 ± 0.32 ^a
	FER	3.7 ± 1.3 ^a	30.8 ± 5.4 ^a	1.06 ± 0.86 ^a	1.02 ± 0.66 ^a
SP	COO	2.1 ± 0.7 ^a	15.7 ± 5.2 ^a	1.30 ± 0.42 ^a	1.88 ± 0.62 ^a
	BLA	1.8 ± 0.3 ^a	18.2 ± 5.3 ^a	0.71 ± 0.12 ^a	2.14 ± 0.62 ^a
	FER	1.5 ± 0.3 ^a	17.0 ± 3.0 ^a	0.80 ± 0.16 ^a	1.90 ± 0.33 ^a
WJ	COO	1.6 ± 0.6 ^a	22.5 ± 10.0 ^a	0.47 ± 0.19 ^{a*}	0.83 ± 0.37 ^a
	BLA	4.4 ± 0.6 ^a	25.1 ± 7.9 ^a	0.46 ± 0.06 ^a	0.89 ± 0.28 ^a
	FER	2.3 ± 0.2 ^a	12.2 ± 1.3 ^a	0.37 ± 0.03 ^a	0.55 ± 0.05 ^a
A	COO	10.7 ± 2.3 ^a	8.4 ± 4.6 ^a	3.20 ± 0.68 ^{b*}	0.54 ± 0.30 ^a
	BLA	7.8 ± 0.4 ^a	18.0 ± 16.3 ^b	2.06 ± 0.64 ^a	1.23 ± 1.10 ^a
	FER	11.5 ± 4.7 ^a	16.6 ± 10.1 ^b	3.81 ± 1.26 ^b	1.00 ± 0.61 ^a
EK	COO	15.3 ± 3.6 ^b	40.9 ± 8.6 ^a	2.05 ± 0.38 ^b	1.67 ± 0.35 ^a
	BLA	2.5 ± 1.9 ^a	16.6 ± 13.5 ^a	0.51 ± 0.37 ^a	0.85 ± 0.68 ^a
	FER	2.6 ± 1.1 ^a	22.3 ± 7.0 ^a	0.63 ± 0.26 ^a	1.16 ± 0.36 ^a

Values are expressed as mean ± SD (n = 4), Mean values of each vegetable in the same column with different superscripts are significantly different at $p \leq 0.05$, CP= Cowpea, SP= Spider plant, WJ = Wild jute, A= Amaranth, EK = Ethiopian kale, FER = Fermented, BLA = Blanched, COO = Cooked, Values of cooked vegetable with a * are significantly different to the cooked Ethiopian kale at $p \leq 0.05$, # mineral availabilities expressed in two ways; mineral content in dialysate as a % of the total mineral in a vegetable, as well as the amount of minerals in the dialysate, db = dry basis.

Conclusions

ALVs have relatively high levels of essential minerals and could contribute to the alleviation of iron and zinc deficiencies in sub-Saharan Africa. Due to low iron and zinc bioaccessibility found, consumption of ALVs should be encouraged concurrently with other locally available foods rich in enhancers of iron and zinc (tomatoes, oranges, and Mopani worms) to enhance these essential minerals bioavailability.

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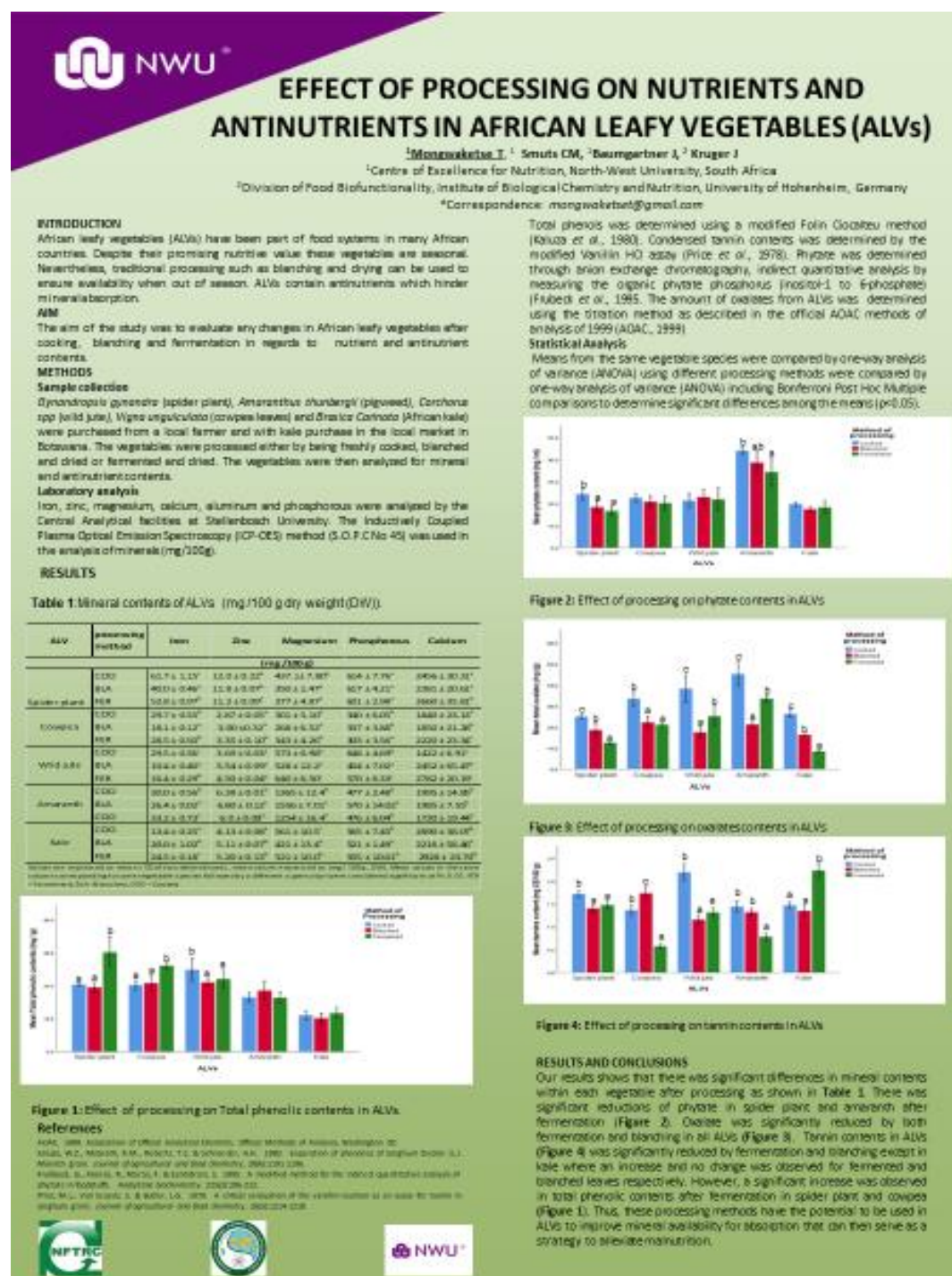
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ADDENDUM 2: POSTER PRESENTED AT THE NUTRITION CONGRESS

Mongwaketse T., Smuts CM., Baumgartner J., Kruger J. (2018). Effects of processing on nutrients and antinutrients in African leafy vegetable. *South African Journal of Clinical Nutrition*. 31(3);supplement. <http://www.sajcn.co.za/index.php/SAJCN/article/view/1354>



ADDENDUM 3: ETHICS EXEMPTION



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**Health Sciences Ethics Office for Research,
Training and Support**

**North-West University Health Research Ethics
Committee (NWU-HREC)**

Tel: 018-285 2291
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21 August 2019

Dear Prof Smuts

PROOF THAT THE FOLLOWING STUDY DOES NOT REQUIRE ETHICAL APPROVAL

Study title: The potential of fermentation and blanching in improving bioaccessibility and bioavailability of iron and zinc in African leafy vegetables

Study title: The potential of fermentation and blanching in improving bioaccessibility and bioavailability of iron and zinc in African leafy vegetables

Study leader: Prof CM Smuts

Student: Ms Mongwaketse - 24759457

Following review of a notification received from Higher Degrees Administration by the North-West University Health Research Ethics Committee (NWU-HREC), it was determined that no ethical approval was required as this study does not:

- Involve any human participants or their data/information
- Involve any human samples
- Involve any animals
- Involve any animal samples
- Have any possible environmental impact

The study involves laboratory work and the possible risks to researchers are covered by NWU-HREC approved Standard Operating Procedures for:

- Good laboratory practice
- Appropriate waste management
- Appropriate safety measures and protection of laboratory workers

Following review of this notification, the NWU-HREC is in agreement that the aforementioned study does not require ethical approval.

Yours sincerely

Digitally signed by Wayne
Towers
Date: 2019.08.21
15:34:18 +02'00'

Prof Wayne Towers
NWU-HREC Chairperson

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Prof Minrie Greeff
Head of Health Sciences Ethics
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Support

ADDENDUM 4: CERTIFICATE FOR LANGUAGE EDITING

This is to declare that I, Annette L Combrink, accredited language editor and translator of the South African Translators' Institute, have language-edited the dissertation by

TC Mongwaketse



orcid.org/000-0003-4850-9685

With the title

**The potential of fermentation and blanching in improving bioaccessibility
and bioavailability of iron and zinc in African leafy vegetables**

A handwritten signature in purple ink, appearing to read 'Annette L Combrink'.

Prof Annette L Combrink

Accredited translator and language editor

South African Translators' Institute

Membership No. 1000356

Date: 14 December 2020