FORMULATION OF AN IRON BIOFORTIFIED BEAN-SILVERFISH INSTANT SAUCE FOR PREGNANT WOMEN

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DECLARATION

I, Diana Musabi (Reg. No. 19/U/GMFT/18938/PD), declare that this is my original work and it has never been submitted anywhere for an academic award.

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Date.....11/11/2022.....

APPROVAL

We verify that the information presented in this dissertation titled "Formulation of an Iron Biofortified Bean-Silverfish Instant Sauce for Pregnant Women during Pregnancy" was written under our guidance and observation, and we hereby forward it to Graduate Board for examination.

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DEDICATION

I dedicate this work to my lovely parents Mr. Bahane Paul and Mrs. Bahane Ruth. If it wasn't for you, this milestone in my life wouldn't have been possible.

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LIST OF ACRONYMS

LBW-Low birth weight

PROM-Premature rupture of membranes

IUGR-Intrauterine growth restriction

IDD-Iodine deficiency disorders

GDM-Gestational diabetes mellitus

SGA-small-for-gestational-age

RDA- Recommended Daily Allowances

IDA-Iron Deficiency Anaemia

UBOS-Uganda Bureau of Statistics

WHO-World Health Organisation

AOAC-Association of Analytical Chemists

ICF-International Classification of Functioning, Disability and Health- FAO-Food and Agricultural Organisation.

FAOSTAT- Food and Agricultural Organisation Statistics

NABE-NARO Bean

NaFIRRI-National Fisheries Resources Research Institute

NARO- National Agricultural Research Organisation

IUGR-Intrauterine growth restriction

UNBSUganda National Bureau of Standards

g/dL-grams per decilitres

IDDs-iodine deficiency disorders

DEFINITION OF TERMINOLOGIES

Anaemia: A condition in which the number of red blood cells or their oxygen-carrying capacity is Insufficient to meet the body's physiological needs.

Maternal: A synonym referring to a mother.

Iron-rich beans: Bean varieties contain higher levels of the essential mineral nutrient iron.

Iron bio-fortified beans: Bean varieties whose iron contents have been increased through biotechnology techniques.

(25(OH) D): Hydroxylated vitamin D which is mainly used for quantifying the amounts of vitamin D in the body.

(1, 25(OH) 2D3): 1, 25-dihydroxyvitamin D is formed in the kidney and it is the form of Vitamin D with a biological effect in the body.

ABSTRACT

In Uganda, there is an increasing burden of anaemia especially among pregnant women and children aged 6-59 months. Some of the readily available and affordable food to pregnant mothers are beans which have relative amounts of iron but also high levels of phytates which reduce Fe bio-availability. Bio-fortification of beans enhances their iron content which is further complemented with the iron in the silver fish. This study aimed at developing a safe and acceptable iron bio-fortified bean - silver fish composite sauce to prevent anaemia among pregnant women through improved micronutrient intake. Design expert software was used to generate four composite formulations of iron biofortified bean-silver fish in the ratios of 90:10, 80:20, 70:30 and 100:0, respectively, each contributing more than 70% Recommended Daily Allowance of iron for pregnant women of reproductive age. Commonly used seasonings and spices (tomatoes, garlic, onions and Mchuzi mix) were added in equal proportions to each of the composite formulations. The instant composite flour was produced using extrusion technology and analysed for moisture, crude protein, crude oil, crude ash, carbohydrates, selected minerals, folate, total phytates and aflatoxin levels. Microbiological analysis considered total plate counts, total coliforms, *Escherichia coli*, yeast and moulds, *Salmonella* and *Staphylococcus aureus*. The composite flour was reconstituted into instant sauce with boiling water and evaluated for sensory properties using a panel of 50 semi-trained analysts.

Extrusion of iron bio-fortified beans-silverfish instant sauce with the ratio of 80:20 had increased moisture content from 5.84 to 10.33% compared to the raw un-extruded beans. Fortification of extruded NABE beans composited with silverfish (NABE 1 (90:10); NABE 5C (70:30); NABE 5C (100:0); NABE 1 (70:30); NABE 1 (80:20), respectively) increased the iron, calcium, zinc, magnesium and folic acid content from 41 to 336 mg/kg, 447 to 15214 mg/kg, 36 to 276 mg/kg, 875 to 1113 mg/kg, and 7.75 to 47.47 mg/kg, respectively compared to the non-fortified raw beans. The treatments reduced total phytate content from 17.36 mg/g in raw beans to 12.92 mg/g in the extruded product. The iron bio-fortified beans – silverfish composite instant sauce had low levels of total aflatoxin, aflatoxin B1 and aflatoxin B2, and undetectable levels of *Salmonella* spp., *E. coli*, and *Staphylococcus aureus* indicating that the product is safe for human consumption. Addition of silver fish to the beans enhanced the sensory acceptability of the instant sauce with the sample containing NABE 5C (80:20) being the most preferred overall ($p>0.05$). From the findings, it was shown that addition of silver fish increases the nutrient content of an iron bio-fortified beans – silverfish instant composite sauce. The addition of silver fish also increases iron and folic acid content of the product and could therefore, reduce cases of anaemia among pregnant mothers. It was recommended that consumption of about 50g per serving of the instant sauce (80:20) twice a day can complement the iron-folic acid (IFA) from the diet as well as IFA supplements given out at antenatal centres to meet the recommended daily allowances for these micronutrients in pregnant women of reproductive age.

CHAPTER ONE: INTRODUCTION

1.1 Background

Globally, undernutrition is a challenge, with emphasis on pregnant women in developing countries whose nutritional demands are enhanced due to the pregnancy (Lartey, 2008). The low intake of essential nutrients by pregnant mothers can cause dentrimental health effects among newborns (Aya, 2019). Deficiencies for iron, folate, iodine, vitamin B12, vitamin A, vitamin D, and zinc are the commonest during pregnancy (Ahmed et al., 2012).

Anaemia is a medical condition whereby the ability of the red blood cells to transport oxygen within the body is below the body requirement for a healthy individual (WHO, 2011). This condition victimizes about 2 billion people globally especially in developing countries in which 30% of the victims are reproductive mothers between 15-49 years of age while 47% are children under the age of 5 years (WHO, 2014). The disease contributes to three quarters of mortality in Africa and Southeast Asia (Osungbade and Oladunjoye, 2012). The prevalence of anaemia in Uganda has been reported to increase in the recent years among children (from 50% to 53% in 2011 to 2016, respectively) and women (from 23% to 32% in 2011 to 2016, respectively) (Nankinga, Aguta and Kabahuma, 2019). There a number of predisponsing factors to anaemia including poor feeding leading to nutrient (especially vitamin A and iron) deficiency, hereditary factors as well as infections (Nynke, 2003; Maaz, Tariq, Bhatti and Ikram, 2019; American Society for Nutrition, 2020).

In view of the above factors, various efforts have been developed to mitigate iron deficiency anaemia (IDA) and these include blending and fortification of staple foods using locally available food materials with emphasis on reducing anti-nutrient factors during processing (Nnam, 2002; Badamosi, 1995). This has been reported to enhance intake of micronutrients through consumption of complementary foods leading to reduction of complications associated with anaemia (Badamosi, 1995). However, some of the food products being used in formulation of complementary foods such as beans, soy beans and sorghum contain anti-nutrient compounds including phytates and tannins which reduce the bioavailability of micronutrients such as iron, folate and calcium (Murekatete and Irakoze, 2010; Obiakor-Okeke et al., 2001; Satter et al., 2013; Vimala et al., 1990). Efforts in Uganda involve use of bio-fortified crops (such as *Phaseolus vulgaris*) NABE 1,

NABE 2 and NABE 5C) since beans, which are a good source of iron, are commonly consumed almost in every region of Uganda (Petry, Boy, Wirth and Hurrell, 2015; Akande, Nakimbugwe and Mukisa, 2017; Nuwahereza, 2019). However, a study in Isingiro District-Uganda indicated that children between 6-59 months who never consumed iron-biofortified beans (39.3%) were associated with anaemia with a 26.3% prevalence (Nuwahereza, 2019). This, therefore, calls for value addition and sensitisation of populations for adoption of these bean varieties. Consumption of beans high in iron could increase serum iron levels (Mgawe, 2009; Ogonda et al., 2014).

Despite the initiatives in Uganda, little is known about the prospects of using iron bio-fortified beans in combination with animal ingredients such as fish in formulation of instant sauce. Silver fish is among the underutilized animal foods in Uganda, probably due to its strong smell. Whereas beans are generally low in iron, silver fish is rich in proteins and micronutrients such as calcium, iron and folate as well as omega3 fatty acids (Ogonda et al., 2014; Kawarazuka and Béné, 2011). Therefore, addition of silver fish to bean-based products could improve micronutrient intake for pregnant mothers whose iron and protein requirements are particularly high (WHO, 2018). Therefore, the aim of this study was to formulate an instant sauce using silver fish and iron bio-fortified beans to alleviate iron deficiency anaemia and improve micronutrient intake among pregnant women of reproductive age.

1.2 Problem statement

Micronutrient deficiencies, mainly folic acid and IDA increase the risk of infections, reduce physical performance, increase maternal mortality, lead to poor birth outcomes and reduce work productivity (UBOS and ICF, 2018). In Uganda, anaemia mainly affects children 6-59 months (53% prevalence) and pregnant women aged 15-49 years (32% prevalence) (UBOS, 2016; Nuwahereza, 2019). Majority of pregnant women in Uganda are fed on plant-based foods such as legumes, plantain, starchy roots and cereals (UBOS and WFP, 2013). Such foods have low amounts of nutrients in terms of protein, iron and other micronutrients (Anigo, Ameh, Ibrahim and Danbauchi, 2010). Legumes such as soybeans, peas, beans and peanuts are a common source of nutrients in Uganda but have poor quality protein (limiting in some essential amino acids), have non-haeme iron with low bioavailability and contain anti-nutritional factors. The anti-nutritional factors restrict the way nutrients are digested and absorbed in the body resulting into inadequate intake. Thus, consumption of foods low in iron could be associated with high levels of anaemia

amongst pregnant women of reproductive age in Uganda which currently stands at 29% (UBOS and ICF, 2017).

About 22.8% of the people worldwide are affected by anaemia (WHO, 2019). Despite the fact that iron defienciency anaemia is victimizing an estimated number of 1.74 billion people worldwide, researchers have reported promising results of bean biofortification which could provide high iron intake (Goyal, Zheng, Albenberg, Stoner, Hart, Alkhouri and Grossman, 2020). However, the fortification programme has not completely addressed the prevalence of anaemia (Benoist et al., 2008). If left unattended to, iron deficiency anaemia among children and women of reproductive age could have several consequences such as low mental and physical performance, fatigue, maternal & perinatal death and depression (Haas and Brownlie, 2001; Stoltzfus, 2003; McClung and Murray-Kolb, 2013).

In line with the Uganda Nutrition Action Plan II 2020-2025 of ensuring proper nutrition and food secure families for all Ugandans, formulating an iron bio-fortified bean and silver fish instant sauce could be one of the innovative solutions for alleviating IDA in Uganda. Moreover, beans and silver fish are some of the most commonly consumed foods in Uganda, and thus have potential to reach a wider population. Given the above factors, this study aimed to develop an iron rich instant sauce using iron bio-fortified beans and silver fish composite flour. Being partially cooked, i.e., instant in nature, the sauce would be convenient to prepare, reducing maternal workload and would consequently contribute to anaemia and other micronutrient deficiencies reduction in pregnant women and women of reproductive age.

1.3 Justification

Anaemia is a national health problem among children and pregnant women of reproductive age in Uganda due to lack of complementary foods and poverty as some of the key factors (Nankinga et al., 2019). Moreover, history of chronic diseases by pregnant mothers could predispose their babies to anaemia. If a pregnant mother is anaemic, the baby is most likely to suffer from pre-term delivery, low birth weight, and even death (Michaelsen et al., 2009). Therefore, encouraging pregnant mothers to feed on readily available cheap nutritious foods such as silver fish and beans could be an ultimate solution. Silver fish and beans could contribute to high protein, folate, sodium, calcium, potassium, magnesium and iron intake reducing the incidences of anaemia among

pregnant mothers in the long run since silverfish is rich in protein, iron and folate while beans are rich in protein and vitamin A (Khush, Lee, Cho and Jeon, 2012). However, beans are generally low in iron. Therefore, incorporation of beans bio-fortified with iron and an iron-rich animal food such as silver fish in formulation of a complementary instant sauce could be a promising alternative in combating anaemia among this target group.

Moreover, silver fish does not contain anti-nutritional factors that form complexes with micronutrients reducing their bioavailability, whereas both the fish and beans are cheap and contain high quality protein. Dried beans are hard to cook requiring long cooking hours. Since most pregnant mothers are reluctant at taking iron and folic acid tablets, use of dietary approach could be a more promising alternative. This approach would help pregnant women who find it hard to take these tablets by developing an innovative formulated product from pre-cooked composite mixture of iron-bio-fortified beans and silver fish flours. This would be more convenient for preparation since it would require shorter preparation time. Therefore, the results of this study would contribute to anaemia reduction in Uganda hence improving the nutritional status of pregnant women of reproductive age in the country.

1.4 Significance of the study

The study could avail an inexpensive, convenient and effective solution to anaemia among children, women and pregnant mothers since the existing food-based solutions are either expensive or insufficient in essential nutrients. The instant sauce also reduces maternal workload during food preparation. Results of this study also add more information to support research and development, increasing knowledge on the benefits of fortification, supporting advances in technology and innovations for future research.

1.5 Objectives of the study

1.5.1 General objective

To formulate an iron biofortified bean-silverfish instant sauce for pregnant women.

1.5.2 Specific objectives

1) Analyse the chemical composition of the iron bio-fortified bean - silver fish composite flour.

2) Assess the microbiological safety of an iron bio-fortified bean - silver fish composite flour.

3) Assess the total aflatoxin content of an iron bio-fortified bean - silver fish composite flour.

4) Evaluate the sensory acceptability of an iron bio-fortified bean - silver fish instant sauce.

5) Determine bioavailability of iron in the iron biofortified bean - silver fish composite flour.

1.6 Hypotheses

1) Incorporation of silver fish in iron-bio fortified beans has a significant effect on the chemical and mineral composition of the composite flour.
2) Iron bio-fortified beans-silver fish instant sauce is microbiologically safe.
3) Iron bio-fortified beans-silver fish instant sauce free from aflatoxin.
4) Iron bio-fortified beans-silver fish instant sauce is sensory appealing or acceptable.
5) Supplementing Iron boi-fortified beans with silverfish and fortifying with Iron significantly increases the Iron bioavailability of the instant sauce.

1.7 Study indicators

The study indicators included;

(i) Microbial counts including:
   a) Total plate counts
   b) Total coliforms
   c) *E. coli*
   d) Yeast and moulds
   e) *Salmonella*
   f) *Staphylococcus aureus*.

(ii) Chemical parameters including total phytates, proximate and mineral content including: a) Iron
   b) Zinc
   c) Calcium
   d) Phosphorus
   e) Potassium

f) Sodium

g) Magnesium

CHAPTER TWO: LITERATURE REVIEW

2.1 Description of anaemia

Anaemia is a clinical condition that manifests when the blood haemoglobin levels goes below 12 g/dl for men and below 11 g/dl for women. It is classified as severe, when the haemoglobin levels in blood fall below 7 g/dl, moderate if the haemoglobin levels are in the range of 7 - 9.9 g/dl or mild if the levels of haemoglobin in blood fall below 10 g/dl (WHO, 2001). In Africa, cases of anaemia are approximated between 21.1% to 64.4% especially among women of reproductive age because of poor feeding, menstrual losses and high physiological requirements for iron (Mukaya, Ddungu, Ssali, O'Shea and Crowther, 2009; Adam, Khamis and Elbashir, 2005; Asobayire, Adou, Davidsson, Cook and Hurrell, 2001; Morris, Bird and Nell, 1989).

The Uganda Demographic Health Survey (UDHS) carried out in 2016, indicated that 53% of children between 0.5-5 years of age and women of reproductive age in Uganda were anaemic (UBOS, 2016). Other authors reported anaemia amongest children below 60 months at 46.6% prevalence in Gulu-district, Uganda (Ocan et al, 2018).

This is evidence for the prevalence of iron deficiency with or without anaemia among children and women aged 15-49 years of age in Uganda which could be associated with poor feeding practices, menstrual losses and the high physiological requirements for iron. However, iron is readily obtained from the diet and it exists in two forms including heme and non-heme (Hurrell and Egli, 2010). Heme iron is readily available in animal tissues as haemoglobin and myoglobin and can be obtained through animal diets such as fish, meat and poultry products. On the other hand, non-heme iron is available in foods of plant origin such as fruits and vegetables, legumes and pulses, and cereals (FAO, 2001). However, presence of antinutrient factors lower the bioavailability of the non-heme iron to 2 to 20% unlike the heme form which has a relatively higher bioavailability (15%-35%) hence, its absorption is not so much affected by antinutrient factors (Hurrell and Egli, 2010). Iron as a micronutrient is essential in the body as in the blood transportation mechanisms where it is part of the iron transporting proteins myoglobin and hemoglobin as well as a co-enzyme in the body (Hurrell, 1997; McDowell, 2003). However, inhibitory factors such as, calcium, phytates, tannins, and egg proteins reduce iron bioavailability in the body (Siegenberg, Baynes, Bothwell, Macfarlane, Lamparelli and Car, 1991; Hallberg, Rossander-Hulthen, Brune and Gleerup, 1993; Cook and Monsen, 1976; Hurrell, Reddy and Cook, 1999).

Consequences of iron deficiency include decreased physical performance and physical activity, decreased cognitive performance, depression and fatigue (McClung and Murray-Kolb, 2013; Haas and Brownlie, 2001). Social and economic consequences include increased childhood, maternal and perinatal mortality, low work productivity, increased energy needs and lost disability-adjusted life-years (Stoltzfus, 2003). Nutritional iron deficiency arises when physiological requirements cannot be met by iron absorption from the diet (Larocque, Casapia, Gotuzzo and Gyorkos, 2005). Dietary iron bioavailability is low in populations consuming monotonous plant-based diets with little meat (Larocque, Casapia, Gotuzzo and Gyorkos, 2005). In many developing countries, plantbased weaning-foods are rarely fortified with iron, and the frequency of anaemia exceeds 50% in children younger than 4 years (WHO, 2001).

Globally, anaemia is responsible for about 17,000 deaths every year (WHO, 2012). The global burden of anaemia is more pronounced in developing countries because there is less consumption of heme iron in developing countries. For instance, the levels of anaemia in the North east parts of Ethiopia were reported at 47.4% (Woldie, Kebede and Tariku, 2015); 51.8% in Cape Verde (Semedo, Santos, Baião, Luiz and da Veiga, 2014); 28.8% in Kenya (Ngesa and Mwambi, 2014); 45% in southern Cameroon and 43% in DRC (Cornet, Le Hesran and Fievet, 1998). Ghana and Tanzania have reported relatively the highest prevalences of anaemia at 78.4% and 77.2%, respectively (Ewusie, Ahiadeke, Beyene and Hamid, 2014; Simbauranga, Kamugisha, Hokororo, Kidenya and Makani, 2015). In Gulu district, northern Uganda, the prevalence of severe, moderate, and mild anaemia among children below five years was reported at 11.9%, 58.8% and 29.4%, respectively (Ocan et al., 2018).

Therefore, to avoid iron deficiency anaemia in children below 60 months old and in pregnant women, their dietary intake of iron should be adequate so that their haemoglobin level does not go below 120 g/l (WHO, 2011). This could be better implemented through the dietary approach.

Anaemia manifests as a result of poor feeding and health and is common in Uganda especially among women of reproductive age (UNICEF/WHO/World Bank, 2017). The disease compromises the body immunity system and it is also associated with low birth weights, premature deliveries, reduced cognitive and physical development, child and maternal death, as well as negative impacts on social and economic development (Michaelsen et al., 2009).

Anaemia has a devastating cost to individual and national productivity with resultant loss of work output. The annual health sector performance report of 2013/14 pointed out that anaemia was ranked fourth among the main causes of deaths and many hospital cases in populations of all ages in Uganda (Ministry of Health, 2014).

Poor feeding leads to deficiency of major essential nutrients which may not only affects the pregnancy but also cause dentrimental effects on the newly born such as low birthweights, rduced mental and physical growth that could even be transferred to other generations (Anderson, 2001; De-Regil, Peña-Rosas, Fernández-Gaxiola and RaycoSolon, 2015; Li, Zhao, Song, Zhang, Tang, Xin and 2015). Therefore, various interventions have been developed to meet the nutritional demands of pregnant women. These include foods sumplemented with B-group vitamins such as vitamin B1, vitamin B2 & vitambin B3, and production of fortified foods such as iron fortified beans and iodised salt among other (Gernand, Schulze, Stewart, West and Christian, 2015).

The global Sustainable Development Goal No. 2 focuses on sector development planning guidelines for sustainable food security. Unfortunately, a report from Uganda Bureau of Statistics (UBOS) indicated that on average, four out of every ten Ugandans are unable to meet the required dietary iron intake (UBOS and ICF, 2017) due to high reliance on the less productive staples for caloric intake. The reports also revealed that diets of most people are lacking in food safety, diversity, adequacy and availability of the essential nutrients. Reports by the Uganda Demographic and Health Survey conducted in 2011 and 2016, showed that there was an increase (23% to 32%, respectively) in the cases of anaemia among women aged 15-49 years (UBOS and ICF, 2018). This is associated with: (i) limited consumption of animal based foods which are rich in micronutrients such as iron, (ii) lack of portable water and (iii) poverty (UBOS and ICF, 2018). Other causes of anaemia include consumption of plant based foods which are rich in energy but limited in biologically available micronutrient and, excessive blood losses during menstrual periods and low iron bioavailability (Nigeria Nutrition Network, 2000). Moreover, the convenience of preparation of low iron ready-to-eat snacks reduces the maternal workload leading to adoption of modern lifestyles such as change in their food preparation habits which results in low iron and other micronutrient intakes.

2.2 Beans production, consumption, processing and bio-fortification

2.2.1 Bean production and consumption

Beans (*Phaseolus vulgaris*) are leguminous crops. They contribute to household food security and income including provision of protein especially in developing countries (CIAT, 1998). The world production of beans stands at 24,221,252 tons (FAOSTAT, 2017). Within the sub-Saharan Africa, the East African region is the leading producer of beans at 4,778,206 tons per year with Kenya, Uganda and Democratic Republic of Congo as the chief producers (FAOSTAT, 2017). Annual productions in Uganda, is reported at 627, 000 tones (UBOS, 2020).

Beans are nutritionally essential mainly for their dietary proteins and carbohydartes (Pachico, 1993). The plant seeds can be eaten fresh or dried and mixed with *Matooke,* cassava, potatoes to produce *Katogo,* a local dish. The leaves can be steamed in banana leaves with *Matooke* and eaten as vegetables. In Uganda, beans are mainly produced for home consumption due to their short maturity period, ease of handling and storability with only about 40% of production used for commercial purposes in retail shops (Wortmann et al., 1999).

Over dependence of the East African community on beans is threatened by declining soil fertility, rapidly increasing populations, and emerging pests & diseases (Saltzman et al., 2017). For this reason, new hybrid varieties such as bush variety K132, K131, and K20 were introduced in Uganda in 1968 to address issues of malnutrition especially among children and pregnant mothers (David, Kirkby and Kasozi, 2000). These varieties are preferred for their marketability and yield stability and resistance to bean mosaic virus. Nutritionally, beans are rich in macro nutrients such as proteins (17.96-31.59 g/100 g), lipids (0.67–1.19 g/100 g), crude fibre (4.66–5.95 g/100 g), ash (2.86–4.26 g/100 g), carbohydrates (56.53–61.56 g/100 g), and micro nutrients for example calcium (136-1929.77 mg/kg), zinc (2.1-28.22 mg/kg), iron (5.3-7.5 mg/100 g), and phosphorus (374-453 mg/100 g) (Shimelis and Rakshit, 2005; Koehler et al., 1987). Efforts in Uganda include production of iron bio-fortified bean varieties such as NABE 1, NABE 2 and NABE 5C which are officially released bean hybrids from National Crops Resources Research Institute (NaCCRI), Namulonge and have been reported to be high-yielding, bioaccumulate iron, have short cooking time and good farmers' preference (Akande, Nakimbugwe and Mukisa, 2017). Thus, consumption of iron bio-fortified beans could complement other strategies to address anaemia among pregnant women of reproductive age. Moreover, iron biofortified beans are a cheap source of iron and protein to majority of Ugandan.

2.2.2 Proximate composition of bio-fortified beans

Beans are a good source of protein, dietary fibre and some minerals needed to meet the body nutritional requirements (Hu et al., 2006). Research has shown that the protein content of beans on a dry basis range 22 to 26%, 23.38 to 31.59%, for raw treatment beans and 22.24 to 33.10% for macerated/cooked beans (Esteves, 2000). In a study on beans prepared in a pressure cooker, Oliveira et al. (2001a) reported protein values of 19.8% in beans cooked without maceration and 19.2% and 19.3% in macerated and cooked beans with and without the maceration water, respectively; thus, replacing the maceration water did not affect these values (Oliveira et al., 2001b).

Moisture content of beans is reported in the range of 13.89 to 15.62% dry weight basis in raw beans and 76.96 to 81.40% in macerated/cooked beans (Brigide, Canniatti-Brazaca and Oliveira Silva, 2014). The elevated moisture content of macerated beans can be explained by the hydration process of the grains, which explains their high moisture content. Oliveira et al. (2008) and Ramírez-Cárdenasi et al. (2008), also reported moisture content of beans in the range of 9 to 11% on a dry basis in raw beans. Beans are low in lipids with lipid values of raw beans varying from 1.66 to 2.13% and from 1.77 to 2.22% in cooked beans (Barampama and Simard, 1993; Brigide, et al., 2014). Carbohydrates and proteins are major components of dry beans. The carbohydrate content of beans is in the range of 16.18 to 22.68% in raw beans and 25.07 to 40.63% in cooked beans (Sathe, 2002; Brigide and Canniatti-Brazaca, 2006; Brigide, et al., 2014).

The total dietary fibre of beans ranges from 30.32 to 34.01% in raw beans and from 27.80 to 31.78% in cooked beans (Brigide, et al., 2014). The decrease in dietary fibre of beans on cooking is attributed to the disruption of the cellular components of beans (cellulose, hemicellulose, lignin, pectin and gums) (Gonzales, 2000). The cooking process also results in interactions between proteins and lipids, which causes qualitative and quantitative changes in the composition of total dietary fibre of cooked foods compared to that of raw foods (Gonzales, 2000).

On the basis of mineral content, beans are rich in total minerals with values for ash content varying between 4.1 to 4.57% in raw beans and 3.79 to 4.82% in cooked beans (Costa et al., 2006; Brigide, et al., 2014). For instance, beans are a good source of phosphorus (0.37 to 0.54 g 100 g^{-1}), potassium (25.27 to 33.86 mg kg^{-1}), calcium (0.06 g to 0.28 g 100 g^{-1}), magnesium (140 mg

$100g^{-1}$ for kidney bean, to 222 mg 100 g^{-1} for yellow bean), sulphur (2.04 to 2.36 mg kg^{-1} in raw beans and 1.45 to 2.66 mg kg^{-1} for cooked beans), copper (5.2 to 11.07 73 mg kg^{-1} and 4.96 to 13.3373 mg kg^{-1} for raw and macerated/cooked beans) iron (61.12 and 80.82 mg kg^{-1}) and zinc (20.20 to 36 mg kg^{-1}) (Esteves, 2000; Oliveira et al., 2001b; Universidade Estadual de Campinas, 2011; United States Department of Agriculture, 2013). However, despite the fact that beans are rich in minerals, their bioavailability depends on the biochemical composition of the grain, such as phytic acid, fibre, amino acids, and proteins that can chelate minerals easily (Erdman Jr., 1981).

2.2.3 Bean processing

Beans are processed to enhance their sensory qualities, reduce the antinutrient factors thereby increasing their nutrient bioavailability and digestability (Xu and Chang, 2009). This includes processes like soaking, germination and cooking of the beans among others.

It is a common practice to soak beans in water not only to reduce their hard to cook tendence but also to leach out the water soluble polyphenols (EL-Adawy, 2002; Khattak et al., 2007).

Exposing beans to 48 h of sprouting enhances their protein quality due to extensive breakdown of their proteins to amino acids and peptides. It also enhances the digestability of beans with limited tendancy to form gas in the stomach (Khattak et al., 2007). It has also been noted to increase the nutritive value of seeds with minimal energy and technical requirements.

Cooking legumes such as beans at 50-100^{o}C for atleast half an hour to 2 hours increases their sensory attributes, enhances nutrient bioavailabilty due to destruction of tannins and phytates that tend to bind micronutrients as well as improving starch digestability (Ramakrishna et al., 2008; Tavano and Neves, 2008; Ranilla et al., 2009).

2.2.4 Food fortification

In order to supplement the nutritive value of agricultural food products, biotechnologists normally work together with food scientists to eliminate micronutrient deficiencies for instance iron, phosphorus, zinc, vitamin A and calcium deficiencies. This is mainly achieved by either fortifying food product after processing or through bio-fortification using staple foods including sweet potatoes, bananas, beans, millet, wheat, and cassava as the vehicles (Khush et al., 2012). Bio-fortification aims at developing crop hybrids naturally enriched with micronutrients so as to meet

the dietary nutrient requirements for the less privileged communities in developing countries worldwide (Saltzman et al., 2013).

Some of the biofortified foods include the orange-fleshed potatoes, 'golden rice' fortified with β-carotene, iron biofortified beans, vitamin A biofortified bananas and wheat enriched with zinc (Low et al., 2007; Cakmak, 2008; Fungo and Pillay, 2011; Paul et al., 2017).

2.2.5 Bio-fortification of beans

Biofortification, the process of increasing the bioavailable concentrations of essential elements in edible portions of crop plants through agronomic intervention or genetic selection, may be the solution to malnutrition or hidden hunger mitigation (Ummed, Praharaj, & Chaturvedi, 2016).

Biofortification can be achieved by agronomic intervention, plant breeding, or genetic engineering, whereas only plant breeding and genetic engineering can influence mineral bioavailability. Plant breeding and genetic engineering are often compared because, in contrast to agronomic interventions, both involve changing the genotype of a target crop. The two processes are similar in aim, albeit different in scope. Both attempt to create plant lines carrying genes that favor the most efficient accumulation of bioavailable minerals—plant breeding achieves this by crossing the best performing plants and selecting those with favorable traits over many generations—whereas genetic engineering accesses genes from any source and introduces them directly into the crop. Plant breeding is limited to genes that can be sourced from sexually compatible plants, whereas genetic engineering has no taxonomic constraints and even artificial genes can be used. The main advantage of genetic engine (Ummed, Praharaj, & Chaturvedi, 2016).

These modalities of biofortification can be combined. Crops biofortified with iron (e.g., high-iron pearl millet, high-iron beans) or biofortified with vitamin A (e.g., orange-fleshed sweet potato and pro-vitamin A maize and cassava) can reduce iron deficiency and vitamin A deficiency, respectively (Haas et al. 2011) (Haas, Salvador, Raymond, Tere, & Erick, 2011)

In Rwanda, iron bio-fortified beans resulted into increased haemoglobin levels by 3.8 g/L compared to the control population which was fed on ordinary beans after 128 days of consumption (Petry et al., 2015). From the same study, it was also reported that haemoglobin levels increased by 4.2 g/L on behalf of every 1 g of iron consumed from beans over the 128-day study. However, there are short comings to bio-fortification. For instance, when beans are bio-fortified with iron,

there are constraints of low bioavailability due to antinutrient factors such as phytates, tannic acid and fibre which chelate minerals (Petry et al., 2014).

2.3 Fish production in Uganda

2.3.1 Capture fish

The annual fish production in Uganda stand at 112,343 tonnes of fish from the fresh water lakes and farmed fish ponds and cages in swamps across the country (FAO FishStat, 2017). The country is gifted with many fresh water lakes including Lake Victoria, Lake Kyoga, and Lake Albert among others. These lakes harbour fresh water fish including silver fish locally known as *Mukene*, Nile tilapia and Nile perch which dominate the fish catch in the country (National Fisheries Resources Research Institute (NaFIRRI), 2006). Among these predominant types of fish, catches are dominated mainly by silver fish (656,268 million tonnes, 43 %), Nile perch (377,219 million tonnes, 25 %) and Nile tilapia (132,782 million tonnes, 9%) according to the 2015 catch assessment survey (NARO and NaFIRRI, 2006). Table 2.1 indicates the annual production statistics of different fish species in Uganda.

Table 2. 1Trends in annual fish yield (000 tonnes) of the major commercial fish species on Lake Victoria, Uganda (2005-2015)

Year	Nile perch	Nile tilapia	Silver fish	Other species	Overall catch
2005	95	29	106	8	239
2006	91	27	96	2	216
2007	87	24	114	3	227
2008	81	20	70	2	173
2010	85	17	59	2	163
2011	70	19	89	4	182
2014	67	21	166	15	270
2015	37	13	65	34	149

Source: (NARO and NaFIRRI, 2016)

2.3.2 Other fish species produced in Uganda

The commonest types of fish in Uganda are silver fish (*Rastrineobola argentea*), Nile perch (*Lates niloticus*) and Nile tilapia (*Oreochromis niloticus*) which are the dominant fish species in the country (NaFIRRI, 2006). Nile tilapia is preferred due to its good sensory attributes when cooked, its easy to multiply on farm and grows very fast (FAO, 2005). There are other species for instance the North African catfish (*Clarias gariepinus*) which is mainly grown in swamps by rural farmers attributed to its flexibility in feeding since its capable of feeding on a range of organic matter and it is easy to multiply since it is a fast growing species (Brummett and Randall, 2002). The North African catfish is the dominant cultured species in Uganda followed by the Nile tilapia (FAO, 2020). However, the farming of the catfish in Uganda is challenged by the low quantity of seed fish and the low quality of the breeds of catfish in the country (MAAIF, 2000).

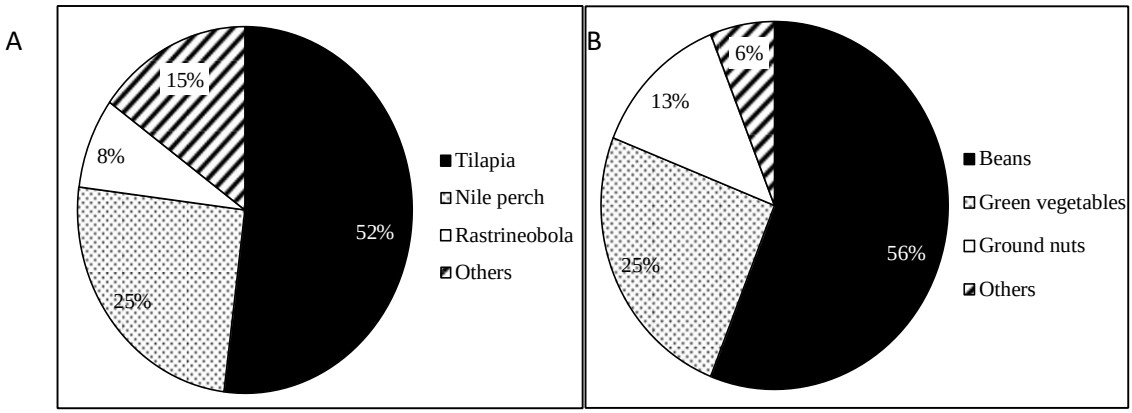
Tilapia zilli and *Oreochromis leucostictus* are also commonly farmed in ponds and cages mainly on Lake Albert, Lakes Kyoga and Lake Victoria respectively (Directorate of Fisheries Resources, DFiR, 2005).

The black bass (*Tilapia rendalli*), giant river prawn (*Macrobrochium rosenbergii*) and the red swamp crawfish (*Procambarus clarkii*) are foregin to Uganda and they were imported into the country's fresh water lakes such as Lake Bunyonyi and Kajjansi Aquaculture Research and Development Center as larvae way back from the 1940s to enhance the diversity of fish species in the country (Brummett and Randall, 2002).

2.4 Fish consumption in Uganda

In Uganda, it is estimated that on average every person consumes 12.5 kg of fish per year (FAO, 2020). However, the per capita consumption of fish in Uganda is even higher than that for Africa (10.1 kg) since majority of the catch is consumed locally contributing to household food security (UBOS, 2010; FAO, 2020).

Nile tilapia is the most consumed fish followed by Nile perch and silver fish (*Mukene*) in that order (Figure 1). Food substitutes to fish products include beans, vegetables and other meat types.



Source: (NARO and NaFIRRI, 2016)

Figure 2. 1Fish consumption by species for fish products in Uganda (A) and alternative products consumed (B)**.**

Silver fish is mainly consumed by low-income earners and fed to children due to its low cost of purchase regardless of its high nutrient content such as protein (18.77%), calcium (700 mg/100 g), iron (0.7 mg/100 g), zinc (2.7 mg/100 g), and lipids (4.9%) (Ogonda et al., 2014; Kawarazuka and Béné, 2011). Silver fish has therefore been applied in enrichment of cassava flour and it has been reported that the levels of calcium, zinc, iron and phosphorus increase from 0.56 to 1.16 %, 0.0% to 0.05%, 0.04% to 0.12 % and 0.06 to 0.93 %, respectively (Elolu et al., 2016).

Silver fish is mainly sundried for preservation and is distributed in local and regional markets yielding a dry weight output of about 35% (Mgawe, 2009). Extreme physical and quality losses in silver fish stand at 5-27% of the total value, and in terms of weight, about 3,660 tons of dried silver fish is lost as physical losses regardless of its low cost and high nutrient content (Elolu et al., 2016; Mgawe, 2009; Ogonda et al., 2014).

2.5 Description and production of silver fish (*Rastrineobola argentea*) in Uganda

2.5.1 Silver fish catches and contributions to Uganda

Silver fish is locally reffered to as *Mukene* in Uganda and it is commonly harvested in the fresh water lakes of Kyonga and Victoria as well as River (East African Standard., 2014). Silver fish is nutritious since its rich in a number of macro and micronutrients incuding proteins, fats and minerals (Ogonda et al., 2014). It is also rich in the essential polyunsaturated fatty acids such as eicosapentaenoic acid and docosahexaenoic acid (Mwanja et al., 2010).

Silver fish catch is ranked third among the predominant fish species from Lake Victoria and it contributes 42 % (70,000 tonnes) of the total annual fish catches in Uganda (DFR, 2012). The

silver fish is usually sun dried after harvest and it consumed as a protein rich diet among Ugandan consumers since it is cheap and readily available (Owaga et al., 2010). Some efforts have been made to process silver fish into baby formulas and animal feeds in combination with other food crops for instance soy beans (Bucyanayandi, 2011). Silver fish farming and trading has also provided employment opportunities to millions of Ugandans especially the women who are mainly engaged in this agribusiness (Muhoozi and Mbabazi, 2010; Bucyanayandi, 2011).

2.5.2 Nutrient composition of silver fish

Silver fish is a good source of nutrients. There are substantial amounts of proteins, calcium and polyunsaturated fatty acids in silver fish (Mwanja et al., 2010; Kabahenda et al., 2011; Masette and Kwetegyeka, 2013). The proteins in the fish are a rich source of essential amino acids including lysine, methionine, cysteine, threonine and tryptophan (Usydus et al., 2008). Silver fish is also a good source of micro and macro elements (calcium, phosphorous, fluorine, iodine, iron) and fats (Table 2.2) which are valuable sources of energy, fat soluble vitamins (A, D, E, and K) and unsaturated fatty acids that are vital for the healthy functioning of the body (Usydus et al., 2008). Therefore, its consumption could help to reduce malnutrition in Uganda (Kabahenda et al., 2011; Kawarazuka and Béné, 2011; Neumann et al., 2014). Silver fish could therefore be used to supplement relatively low nutrient-dense foods such as beans and could contribute to the fight against malnutrition in Uganda.

Table 2. 2 Nutrient composition of silver fish

Nutrient	**Composition**
Energy (Kcal/g)	5.01
Protein (%)	58 – 86
Fat (%)	12.54 – 13.6
Water (%)	12.5 – 78.4
Calcium (mg/100 g)	1556.39
Iron (mg/100 g)	10.68
Zinc (mg/100 g)	10.25
Folate (μg/100 g)	125.0

Source: (Mwanja, Nyende, Kagoda and Munguti, 2010; Kabahenda et al., 2011; Masette and Kwetegyeka, 2013 Nölle, et al., 2020)

2.6 Micronutrients in pregnancy

There is usually increased maternal nutritional demand during pregnancy in order to support normal physiological processes in the mothers's body as well as ensure proper growth of the foetus (Baker, De Angelis, Holland, GittensWilliams and Barrett, 2002). Among the essential nutrients required during pregnancy is folic acid, which is readily available in green leafy vegetables. Folic acid is mainly needed for cell divion and tissue growth by the growing foetus and its deficiency is associated with defects in the foetal neural tube (Berry, Li, Erickson, Li, Moore, Wang, Mulinare, Zhao, Wong and Gindler, 1999; De-Regil, et al., 2015). Therefore, in prevention of this, pregnant women are advised to take 4 to 5 mg of folic acid per day (de Benoist, 2008).

Vitamin A is essential for the copying of gene base sequencies during cell division and tissue growth as well as enhancing the immune system of the foetus (McCauley, van den Broek, Dou and Othman, 2015). Pregnant women with low dietary intake of vitamin A are prone to becoming blind, anaemic and even producing babies with low birth weights (Black et al., 2013).

B-group vitamins are essential in the synthesis of genetic material, energy production and in the formation of foetal red blood cells and blood platelets (Ang et al., 2008; Sukumar et al., 2016). B-group vitamin deficiencies may result into preterm delivery, retarded growth, heart defects and an impared nervous system of the newly born which may last the entire lifetime of the baby (Dias et al., 2017).

Vitamin C is essential in the release of iron from the maternal reserves thereby protecting the mother and baby against anaemia therefore, women are advised to take 7 to 10 mg of vitamin C for normal formation of the baby's collagen tissues as well as guarding pregnant mothers against anaemia and preterm deliveries (Roberts et al., 1990; Kingdom et al., 2000; Woods et al., 2001; Rumbold et al., 2015).

Vitamin D is needed in gene synthesis, bone growth and development, boosting the immune system and balancing the calcium levels in the body (Mousa et al., 2015). Vitamin D deficiencies are associated with rickets among the babies (Theodoratou et al., 2014; Mousa et al., 2017).

Calcium is an essential for bone formation and nervous transmissions and therefore, pregnant women have recommended daily allowances of calcium of 1.2g per day to safe guard pregnant

mothers from excessive calcium losses from their bones as well as ensuring proper bone growth of the foetus (Hofmeyr et al., 2014; Buppasiri et al., 2015). Iodine is important in the formation of thyroxine hormone which is a growth promoting hormones and deficiencies of iodine are associated with goiter, impared nervous and physical growth of the newborn babies (Prado and Dewey, 2014; Harding et al., 2017).

Iron is essential in the formation of red blood cells which are needed in the transportation of oxygen around the body for proper energy generation in the body to meet the body's physiological demands (Cairo et al., 2006; Milman, 2006). Therefore, pregnant mothers are expected to consume 27 mg of iron per day to meet the foetal and maternal needs against anaemia (Beard, 2000).

Zinc is needed in cell and tissue growth for normal physiological processes for both the mother and her newly born baby against delayed delivery, impared nervous and immune system, delayed physical growth, maternal and child deaths (Ota et al., 2015). It is therefore, recommended that pregnant women consider having 15 mg of zinc per day during pregnancy (Goldenberg et al., 1995; Parr, 1996; Caulfield et al., 1998).

2.7 Extrusion process

Extrusion cooking involves the application of shear force and limited heating to push a product through a tube under pressure (Szczygiel et al., 2014). The process involves steeping followed by a short exposure to boiling water for enzyme inactivation and microbial load reduction then steam-cooking of the beans. The cooked beans are then dried and milled. Extrusion is ecomonical in terms of water and heat needed and it enhances the digestability of beans by reducing flatulence agents such as raffinose, maltose and polyphenols including tannins which chelate mineral nutrients, enhances flavor, texture and sensory characteristics of extruded products (Nyombaire et al., 2011; Siddiq et al., 2011). However, extrusion forms lipid complexes with proteins and carbohydrates which interferes with oxidation of lipids thereby reducing the tendancy of forming off flavours (Simons, 2004; Siddiq, Butt and Sultan (2011).

2.7.1 Mechanism of extrusion process

During extrusion, raw materials are forced to flow under controlled conditions along the length of the extruder barrel and through a shaped opening (called die assembly) at a defined throughput. First, raw materials are commonly ground to the preferred particle size. Frequently they are passed through a preconditioner in which other ingredients are added and steam may be injected. During

extrusion the product is cooked and mixed by three separate energy sources: mechanical energy (shear caused by the screw elements), thermal energy that comes from the heating system, and self-heating due to the melt viscosity in the barrel. As the rheological behavior of the dough in the barrel greatly affects finished product quality, it is very important to control temperatures and process times to optimize food quality and heat transfer (Tara, 2017).

2.7.2 Categories extrusion process and theirs conditions

There are four main categories of extrusion processes: cold, hot, steam-induced and co-extrusion. Cold extrusion is used to gently mix and shape dough without direct heating or cooking within the extruder. It is used mainly for producing pasta and dough. Hot extrusion thermomechanical transforms raw materials through short-time and high-temperature conditions under pressure. This type of extrusion is used mainly to cook raw materials to produce textured food and feed products. Steam-induced expansion defines the melt expansion at the die exit due to water flashing off, leading to highly expanded products. Subsequent processing then determines the textural attributes of extruded products. Examples of products produced using this type of extrusion are expanded snacks and breakfast cereals. Expanded co-extrusion combines steam-induced expansion and filling injection for expanded products with dual textures (usually crispy shell and soft filling) (Tara, 2017).

2.7.3 Advantages of extrusion process

It includes the following; It is adaptable in accommodating consumers demand for new foods. A variety of commercially available such conventional/traditional foods and snacks can be made with extrusion. A variety of sizes, shapes, textures, colors and appearance can be produced. These are not possible in the other production methods. Also used in flour production. Since extrusion is high temperature short time, heating process, a high quality and consistency of products can be got. It improves the digestibility of proteins and starches and destroys the anti-nutritional factors in food. Extrusion has lower processing cost and saves raw materials, labor cost and capital investment. It requires less space for unit operation, man power and energy cost is low. High productivity and continuous production. No waste and effluent generation-Extrusion does not produce waste matter and effluents and no disposal problems. Therapeutic foods and fabricated foods: Foods such as low calorie, high fibre, high protein and nutritious food supplements can be produced (Tara, 2017).

CHAPTER THREE: MATERIALS AND METHODS

3.1 Materials and methods

Five kilograms for each of the three varieties of iron bio-fortified beans (NABE 1, NABE 2 and NABE 5C) were purchased from National Crops Resources Research Institute (NaCCRI), Namulonge. These varieties are high-yielding, bio-accumulate iron, have short cooking time and have good farmers' preference (Akande, Nakimbugwe and Mukisa, 2017; Wabusa, 2021). Fresh silver fish was bought from Katosi landing site in Mukono district and placed in a clean disinfected cooler box containing ice. The box was transported to National Agricultural Research Organisation (NARO) Food Bioscience laboratory, Kawanda within 8 h of collection.

The fish was washed three times in running potable water, placed in a clean disinfected oven tray and dried in a dryer (JW-1350ED, Jin woo Electronics, Korea) at 55˚C for 48 hours. Product formulation was conducted at NARO Food Bioscience laboratory, Kawanda and chemical analyses at NARO Analytical Laboratory, UNBS Analytical Laboratory and Government Analytical Laboratory, Wandegeya. Finally, sensory evaluation was done at Kyambogo University Food Science Laboratory and iron bioavailability was done in USDA-ARS, Robert W. Holley Center for Agriculture and Health, Cornell University, Ithaca, NY 14853, USA laboratory.

3.2 Sample preparation

The process of sample preparation is indicated in Figure 3.1. The beans and silver fish were sorted manually to remove stones, defected and broken pieces and other foreign matter. The cleaned fish was washed in portable water and re-dried for 48 hours to constant weight in a dryer to facilitate the roasting process (JW-1350ED, Jin Woo Electronics, Korea) at 55^{o}C. The dried fish was then roasted in a drum roaster (THDRE, So Sejin, Korea) for 15 minutes at 150°C, cooled for 15 minutes at ambient temperature and milled into grits (2.5 mm screen size) using a hammer mill (SFSP60X3545, Hengshiu Electrical Motors, China). The beans were soaked for 12 hours in water and dried overnight at 55^{o}C in the same dryer until a moisture content of 12% and milled into grits using a roller mill (THGCR-100, So Sejin, Korea).

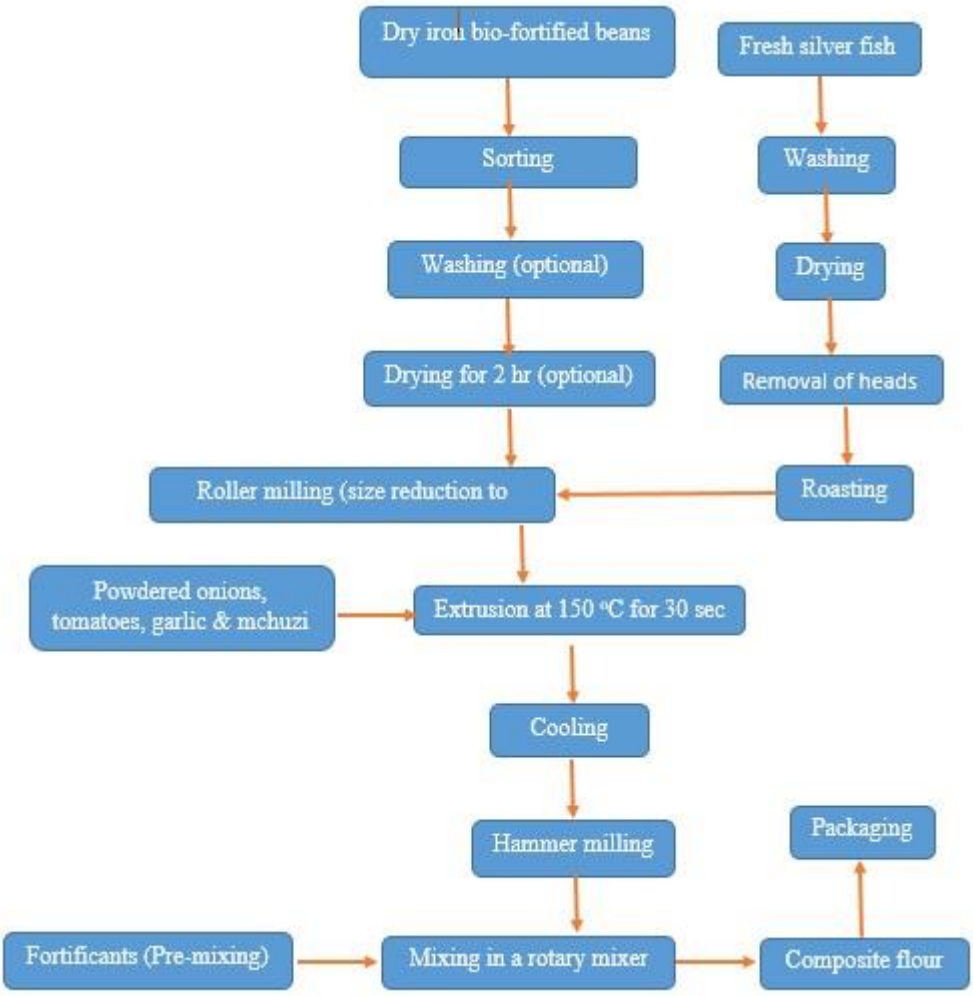


Figure 3. 1Sample preparation and production of the iron bio-fortified bean-silver fish composite sauce

3.3 Preparation of the instant iron-bio-fortified bean – silver fish composite flour

A total of four (04) instant sauce formulations (A-D, Table 3.1) whose composites contribute more than 70% iron of the recommended dietary intake for pregnant mothers of reproductive age (RDA of 0.8 to 7.5 mg/day) (Beard, 2000) was generated using Design expert software version 12

Table 3. 1The iron-bio-fortified bean - silver fish composite formulations

Formulations	**Level (%) of ingredients used in formulation of the composite flour**			
	A	**B**	**C**	**D**
	90:10 (g)	**80:20 (g)**	**70:30 (g)**	**100 (g)**
Bean	3,000	3,000	3,000	3,000
Silver fish	333.3	750	1,286	0
Tomato powder	40	40	40	40
Onion powder	30	30	30	30
Garlic powder	30	30	30	30
Mchuzi mix	30	30	30	30
Folate + Iron (dry powder)	0.0145+0.003	0.01625+ 0.00333	0.0185+ 0.0038	0.013+0.0027
Total	3,463.3175	3,880.01958	4,416.0223	3,130.0157

3.3.1 Production of instant bean-silverfish composite flour using extrusion processing

The instant sauce was developed following the flow diagram (Figure 3.2).

a) Moisture adjustment

The dried beans and roasted silverfish were ground separately into individual grits using a corrugated Roller Mill (THGCR – 100, SO Sejin, Korea) and then stored in an airtight container before extrusion as indicated in Fig. 3.1 above. Extrusion method of Abd-Ellatif, Ibrahim and Ragab (2017) was adopted. Moisture content of the composite flour was first manipulated so as to obtain the right amount of water to bring 1000 g of samples to the desired moisture content during the extrusion process. The following equation adopted from AACC (2010) was used.

$$Water\ added\ (g)\ = \frac{moisture\ desired\ (\%) - \ sample\ moisture\ (\%)}{100 - desired\ moisture}\ x\ 10^3$$

Water was gradually added to the sample and mixed for 5 minutes (THMW-25, SO Sejin, Republic of Korea). The sample was then sealed in a polyethylene bag and kept for one hour at ambient temperatures for moisture equilibration (El Dash, 1985).

b) Extruder operation

The bean flour was mixed with silver fish flour. Powdered onions, tomatoes, garlic and Mchuzi mix were purchased from Kireka shopping centre, which is a local supermarket in Kireka-Wakiso District. The mixture was then placed in a humidifying mixer (THMW-25, Sosejin, Korea) for mixing and extrusion at 150^{o}C for 30 seconds. A Bartender single-screw extruder (THEX-100, Sosejin, Korea) was used for extrusion process. Operating conditions were moisture content, 15%, barrel temperature, 150°C and screw speed, 150 rpm. A 3:1 compression ratio screw was used at a feed rate of 60 grams per minute. Extruder temperature was controlled by heating and cooling using compressed air. At desired temperature of extruder zones, a 200-300 g sample (15% moisture) was placed in the feed hopper. Feeding rate was initated with a screw speed of 25-30 rpm. At the extruder steady state, the final product (extrudate) was collected.

The extrudate was cooled to room temperature and milled (Hengshiu Electrical Motors) into fine flour using a 0.5 mm screen size. The flour was then mixed with fortificants (pre-mix) in a ribbon mixer (THRM, Sosejin, Korea). The final product was packaged in a polyethylene primary package and a pre-labelled paper box secondary package.

3.4 Production of instant sauce using iron-bio-fortified bean – silver fish composite flour

The composite flour was reconstituted into a sauce following the procedure demonstrated in Figure 3.2. For reconstitution, 100 ml boiling water was added to 50 g of the instant sauce mix (Rahman, Saifullah and Islam, 2012). The mixture was continuously agitated. The heat on the hotplate was reduced and cooking continued for a further 5 min to obtain a thick sauce. The sauce was subjected to analysis for sensory acceptability as described in section 3.8.

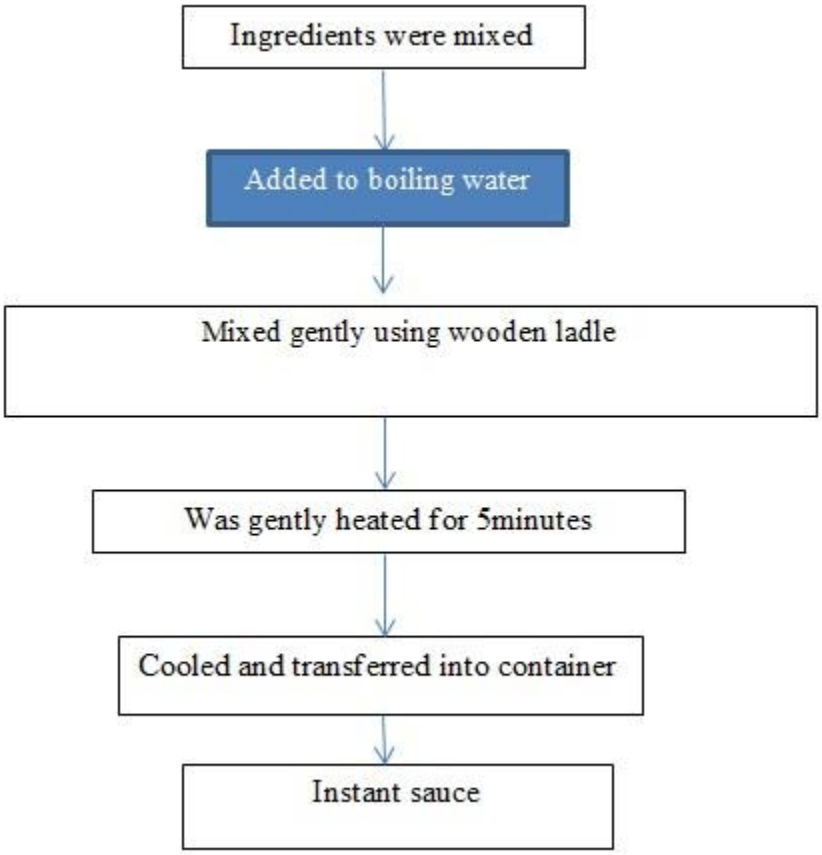


Figure 3. 2 Flow diagram for production of instant bean-silver fish composite sauce

3.5 Chemical analyses

Chemical analyses were conducted at the NARO analytical laboratory, Uganda National Bureau of Standards (UNBS) analytical laboratory and The Directorate of Government Analytical Laboratory (DGAL), Wandegeya. Analytical-grade chemicals were used in the study.

3.5.1 Moisture content

This was achieved using the oven drying method according to the protocol of AOAC (2016) method no. 44-15A. A 5g sample was weighed on a clean preweighed petridish (W_1) and the weight of dish plus sample recorded (W_2). The dish and sample were oven dried at 105^{o}C for 3 hours and subsequently cooled in a desiccator for about 30 min. The weight of the dish plus the dried sample (W_3) was recorded. Moisture content was calculated following the formula below:

$$\% \, Moisture = \frac{W_2 - W_3}{W_1} x \, 100$$

3.5.2 Crude protein

Protein content was determined using the Kjeldahl method according to AOAC (2001) method no.11. One gram of the sample was weighed into a digesting tube, and 12 ml of concentrated sulphuric acid and one Copper (II) Sulphate tablet catalyst were added to the tube. The sample was then digested at 420^{o}C on a heating block. Digestion was ended when the solution became clear. The sample was heated for another 20 min and cooled. Then, 50 ml of distilled water was added to the tube and loaded onto the distillation unit. A blank, was also be prepared and run at the same time. Then, 50 ml of 40% sodium hydroxide was added into each of the blank and sample tubes and the liberated ammonia distilled in excess of 2% boric acid. The distillate was titrated against 0.05 N hydrochloric acid to determine the ammonia absorbed by boric acid. Protein content was calculated using the following formula:

% Crude Protein = (V_2-V_1) M x 14 x F X 100 / W

Where, V_1 = Volume of HCl required for the blank, V_2 = volume of HCl required for the sample

M = morality of HCl, 14 = atomic mass of nitrogen, W = weight of test sample

F = 6.24 = Nitrogen conversion factor

3.5.3 Crude oil

The crude oil content of the samples was determined using the Soxhlet method according to the protocol of AOAC (2000), method no. 920.39. Three grams of the sample were weighed into a pre-dried extraction thimble lined with a Whatman No. 1 filter paper. The thimble was covered and fixed in the Soxhlet extraction system, and 60 ml of petroleum ether measured into a pre-weighed dry aluminium cup and fixed in the Soxhlet unit. The thimble with the samples were dipped in boiling water for 15 min. The sample was rinsed for 30 min and the excess solvent evaporated using compressed air. The extracted oil was dried for 30 min in an air oven at 105^{o}C and weighed. Crude oil content was calculated using the following formula:

$$\% \, Crude \, oil = \frac{W_1 - W_3}{W_2 - W_1} x \, 100$$

Where W_1 is weight of empty beaker and sample, W_2 is weight of the empty beaker.

3.5.4 Crude ash

Ash content was determined using the protocol of AOAC (2000), method no. 923.03. Three grams of the dried sample in section 3.5.1 was weighed in a porcelain crucible, and carbonated by charring in in a muffle furnace, for 2 hours at 550^{o}C to form ash. The ash was cooled in a desiccator for 30 min and weighed. Ash content was expressed as a percentage using the following formula:

% Ash = W_3-W_1 / W2-W3 x 100%

Where W_1 = weight of crucible, W_2 = weight of sample and crucible, W_3 = weight of ash plus crucible

3.5.5 Carbohydrate content

This was established calorimetrically using the Anthrone method (David, 1990). Different volumes of glucose solution were pipetted into a 15 test tubes from the stock solution (200 µg /ml) and made up the volume to 1 ml with distilled water. To each tube, 5 ml of the Anthrone reagent was added and mixed by vortexing. The tubes were cooled on ice, covered with marbles/caps and incubated at 90^{o}C for 17 min. Tubes were cooled to room temperature and the absorbance of these compounds was measured by a spectrophotometer (Uv-1800, SCHIMAD ZU, Japan) at 630 nm against a blank. Carbohydrate content was extrapolated and calculated from the plotted standard curve of absorbance vs. µg glucose in the standard solutions.

3.5.6 Determination of selected mineral content

Selected minerals were analysed using the closed tube digestion method and Microwave Plasma Atomic Emission Spectrophotometer (MP-AES), (Agilent Technologies G8003A MY16460002, Australia).

Approximately 250 mg of the powdered sample were weighed into a new Greiner's tube. Then 2 ml of nitric acid (69% concentration) and 500 µl of H_2O_2 (30% concentration) were added into each tube. The cap was tightened on each tube and agitated by vortexing for thorough mixing. The sample was pre-digested overnight at room temperature and placed into the digestion block and heated at 80^{o}C for 30 min, then at 125^{o}C for 2 hours in a fume hood. The sample was cooled to about 25^{o}C and volume made to 25 ml using distilled water. The tube was then re-sealed and the sample agitated by vortexing for 5 min. The sample was allowed to settle, decanted into a 4.5 ml

polystyrene sample tube and taken for analysis by MP-AES. The concentration of the selected minerals in each sample was obtained and expressed in parts per million (ppm).

3.5.7 Determination of phytic acid content

Total phytate content was analysed using the calorimetric method according to the protocol of (Eskin, 1980). A 1 g sample was mixed with 10 ml of 2.4% HCl mixed with sodium chloride solution. The mixture was macerated in a mortar for 5 min to extract phytic acid. The extract was filtered into a 50 ml volumetric flask using Whatman No 1 filter paper and made to the 100 ml mark using distilled water. Then, 1 ml of the clear top supernatant was diluted 25-fold using distilled water and collected for colour development. A 3 ml aliquot was mixed with Wade reagent and vortexed for about 5 seconds. Standard solutions containing 0, 1.12, 2.24, 3.36, 5.6, 7.84 and 11.2 mg/l sodium phytate was prepared. Absorbance of colour reaction products for the sample and standards was read at 500 nm using a UV spectrophotometer (Uv-1800, SCHIMAD ZU, Japan). Phytates were quantified using the formula below:

$$Phytate\ content\ (g/100\ g)\ =\ \frac{C\ x\ d}{P\ x\ W}$$

$Where\ C\ =\ concentration\ of\ phosphorus\ \left(\frac{mg}{l}\right)\ from\ the\ calibration\ curve,$

$d\ =\ dilution\ factor\ of\ NaCl\ in\ the\ supernatant,$

$P\ =\ \%\ of\ phosphorous\ in\ phytic\ acid,$

$W\ =\ weight\ of\ sample$

3.5.8 Quantification of folate

The quantification of folate was conducted at Government Analytical Laboratory, Wandegeya. Folate content was determined using reversed-phase high-performance liquid chromatographic procedure described by Ekinci and Kadakal (2005). Deionized water (20 g) was added to 5 g of instant flour. The mixture was homogenized (400 Circulator, Seward, UK) for 1 min. The homogenate was spinned (Sigma, Bioblock Scientific 2-16) at 14,000 g for 10 min. Solid Phase Extraction protocol described by Cho et al. (2005) was used to extract folic acid. In order to activate the stationary phase, it was flushed with 10 ml methanol and 10 ml water and pH adjusted to 4.2. Then, a homogenized spinned sample (10 ml) was injected into the column. Acidified water was

prepared by adding a 0.005 M HCl solution until a pH value of 4.2. The sample was then eluted with 5 ml of the acidified water followed by 10 ml methanol at a flow rate of 1 ml per minute. The eluent was collected in a flask and evaporated to dryness. The residue was dissolved in mobile phase. Before HPLC analysis, all samples were filtered through 0.45 µm pore size FP30/45 CA-S filters (Schleicher and Schuell, Darmstadt, Germany) at 7 bar max. Samples (20 µl) of solutions of folic acid were injected into the HPLC column.

The column eluate was monitored with a photodiode-array detector at 282 nm for folic acid. The mobile phase was filtered through a 0.45 µm membrane and degassed by sonication before use. The mobile phase was 0.1 mol $L^{-1}KH_2PO_4$ (pH 7)-methanol, 90:10. The flow rate was 0.7 ml min^1. Chromatographic peak data were integrated up to 39 min. Identification of folic acid was obtained by comparing its retention time and UV spectra with those of standards in the data bank. Concentration of folic acid was obtained from integrated areas of the sample and the corresponding standards.

3.6 Microbial analyses

Microbial analysis was conducted at the Uganda National Bureau of Standards analytical laboratory and the product was tested for each of the following parameters: total viable counts, total coliforms, *E. coli,* yeast and moulds, *Salmonella* and *Staphylococcus aureus*.

3.6.1 Culture Media

Rose Bengal Chloramphenicol (RBC), Nutrient agar, Eosin methylene blue agar (EMB), Chromogenic media for *E. coli*, *Salmonella* and *S. aureus* were used for selective enumeration of total viable counts, total coliforms, *E. coli*, *Salmonella* and *S. aureus*, respectively. All media were obtained from Oxoid (United Kingdom), prepared according to manufacturer's instructions and stored in a cold room at 4^{o}C until use.

3.6.2 Sample preparation and enumeration of microbial populations

The composite flour sample (25 g) was weighed into a stomacher bag, diluted in 225 ml of sterile peptone water and mixed using a stomacher machine (400 Circulator, Seward, UK) at 230 rpm for 2 min to obtain a sample with dilution factor 10^{-1}. Further 10-fold dilutions were prepared up to 10^{-7}.

3.6.3 Microbial enumeration

a) Total plate counts

Total plate counts were enumerated by pour plate technique according to ISO 4833-1:2013 Horizontal method. Diluted samples (1 ml) from section 3.6.2 were inoculated on sterile Petri dishes using a sterile pipette. Then, 15 ml of plate count agar held at 44 - 47^{o}C was added into each petri dish and carefully mixed with the inoculum by rotating the petri dishes. The mixture was allowed to solidify by leaving the petri dishes standing on a cool horizontal surface at room temperature for 15 min. After complete solidification, the plates were incubated (N750, Memmert, Germany) at 30±1^{o}C for 72±3 h to form colonies. Plates with colonies in the range 30-300 were enumerated using a colony counter (SC5, Stuart Scientific, UK) and used to calculate the total plate counts using the following formula:

$N = c/ (V \cdot d)$ Where; m- is the arithmetic mean of the counts obtained from the duplicate plates on single dilution v - is the volume of inoculum applied to each dish, in millilitres, and d - is the dilution factor

b) Total coliforms

Total coliforms were enumerated by pour plate technique according to IS0 4832:2006 Horizontal method. From each of the diluted samples in section 3.6.2, 1 ml was transferred to the centre of a sterile petri dish using a sterile pipette. Then, 15 ml of molten violet red bile lactose agar (VRBL) at 44 - 47ºC was added into each petri dish. The inoculum was carefully mixed with the medium and allowed the mixture to solidify. After complete solidification, 4 ml of molten VRBL agar at 44-47ºC was poured onto the surface of the inoculated medium and allowed to solidify as described above. All plates were incubated at 37ºC for 24±2 hours. After 24 hours of incubation, petri dishes containing 30-150 purplish red/pink colonies were enumerated using a colony counter and counts used to obtain the total coliforms using the following formula:

$N = c/ (V \cdot d)$ Where; m- is the arithmetic mean of the counts obtained from the duplicate plates on single dilution v - is the volume of inoculum applied to each dish, in millilitres d - is the dilution factor

c) Enumeration of *Escherichia coli* (*E. coli*)

E. coli counts were enumerated by pour plate technique according to ISO 16649-2:2001 Horizontal method for beta-glucuronidase-positive *Escherichia coli*. For each of the diluted samples, 1 ml was transferred to sterile duplicate plates using a sterile pipette. Approximately 15 ml of molten Eosin methylene blue agar at 46ºC was added and carefully mixed with the inoculum. The plates were allowed to solidify on a cool horizontal surface, and incubated at 44°C for 21±3 h. After 24 h incubation, plates containing up to 150 blue colonies were enumerated using a colony counter and used to calculate total *E. coli* counts using the following formula:

$N = c/ (V \cdot d)$ Where; m- is the arithmetic mean of the counts obtained from the duplicate plates on single dilution v - is the volume of inoculum applied to each dish, in millilitres

E. coli and *Enterococcus faecalis* were used as the positive and negative controls, respectively

d) Yeast and moulds

Yeast and moulds were enumerated by spread plate technique according to ISO 21527-2:2008 Horizontal method for the enumeration of the yeast and moulds. Each of the diluted samples (0.1 ml) was transferred onto Rose Bengal Chloramphenicol (RBC) agar plates in duplicate. The sample was spread over the surface of the agar with a sterile spreader. Plates were incubated aerobically (IN160, Memmert, Germany) at 25±1˚C for 5 days. Dishes containing up to 150 colonies were counted and used to calculate the yeast and mould count using the following formula:

$N = c/ (V \cdot d)$ Where; m- is the arithmetic mean of the counts obtained from the duplicate plates on single dilution v - is the volume of inoculum applied to each dish, in millilitres

e) Detection of *Salmonella*

Salmonella spp. were detected according to method AOAC 967.25,26,27 (967.25 Preparation of the culture media and reagents, 967.26 Detection of *Salmonella* in processed foods and 967.27 *Salmonella* in foods identification). The sample (23.8-26.3 g) was aseptically weighed into a stomacher bag, 214.2-236.7 ml of peptone water added and homogenised for 2 min. The stomacher bag was sealed and incubated at 35ºC for 24 hours for pre-enrichment. The mixture was gently shaken, and 1 ml portion transferred to 10 ml of selenite cystine broth (SCB) and 0.1 ml to 10 ml of Rappaport-Vassiliadis soya peptone broth (RVS) broth separately. Selenite cystine broth was

incubated at 35ºC and RVS at 42ºC for 24±2 hours for selective enrichment. The tubes were vortexed and a loopful of the culture from each tube separately streaked into (1) bismuth sulphite agar (BSA), (2) Hektoen enteric agar (HEA) and Xylose desoxycholate agar (XLD).

All plates were incubated at 35ºC for 24±2 hours. BSA plates were examined after 48 hours. Plates were examined for suspect *Salmonella* colonies having the following features: BSA: brown, grey, or black colonies with or without metallic sheen; HEA: blue or blue green colonies with or without black centres; XLD: pink colonies (or atypically yellow) with or without black centres. Two typical *Salmonella* suspect colonies from each selective agar were inoculated into Triple sugar iron (TSI) agar and Lysine iron (LI) agar slants by stabbing the butt and streaking slant. The TSI slants were incubated with loose caps at 35ºC for 24±2 hours and observed for red slants (alkaline) and yellow butt (acid) colour with or without (H_2S) blackening of agar.

The presumptive *Salmonella* cultures from TSI agar were inoculated into urea broth and incubated at 35ºC for 24±2 hours. *Salmonella* was observed for absence of a purple-red urease reaction. *Salmonella* was further confirmed by inoculating positive culture from TSI agar into tryptone broth followed by incubation at 35ºC for 24±2 hours. Then, 0.3 ml of Kovacs' reagent was added to the 24-hour tryptophan tube and observed for a pink colour at the surface.

f) Enumeration of *Staphylococcus aureus*

Staphylococcus aureus were enumerated by spread plate technique according to EN ISO standard methods 6888-1 (1999). Horizontal method for the enumeration of coagulase-positive staphylococci. The diluted sample (0.1 ml) in section 3.6.2 was transferred on the surface of two Baird Parker agar (BPA). The inoculum was carefully spread over the surface of the agar plate using a sterile spreader. The plates were allowed to dry for about 15 min at laboratory temperature, inverted and incubated for 48±2 hours at 37°C. After incubation, plates containing 30-300 *Staphyococcus* typical colonies (black or grey, shining and convex and surrounded by a clear zone) were enumerated and used to calculate the staphylococcal counts:

$N = c/ (V \cdot d)$ Where; m- is the arithmetic mean of the counts obtained from the duplicate plates on single dilution v - is the volume of inoculum applied to each dish, in millilitres

Staphylococcus aureus and *E. coli* were used as the positive and negative control, respectively.

3.7 Total Aflatoxin content

Total aflatoxin content was determined using HPLC. A 25 g sample and 5 g of sodium chloride were weighed into the reagent bottle and 100 ml methanol: water (70:30) was added and blended at 25,000 rpm for 3 min. The extract was centrifuged at 4000 rpm for 5 min and filtered into a clean 50 ml Falcon tube. Then, 7.5 ml of the filtrate was pipetted into another clean 50 ml Falcon tube and diluted with 15 ml of distilled water. The diluted mixture was vortexed for 1 min and filtered through a glass microfibre filter into a clean 50 ml Falcon tube.

To perform sample clean-up and elution, the immuno affinity column (Aflatest) was attached to a reservoir syringe and placed into a vacuum manifold. Then, 7.5 ml of the filtered diluted extract was completely passed through the column at a rate of 1-2 drops/second until air came through the column. Distilled water (10 ml) was then passed through the column at a rate of 2 drops/second; the step was repeated with another 10 ml of distilled water. Excess water was removed from the system by suction and the glass tube placed into the manifold. Then, 2 ml HPLC grade methanol was added into the immuno-affinity column, allowed 3-4 drops of methanol into the glass tubes and then closed the tap of the manifold. Methanol was allowed to soak for 5 min and eluted 1-2 drops/s into glass tubes. Excess methanol was removed by suction, the glass tubes were removed from the manifold and 2 ml HPLC water were added. The tube was capped, mixed for 1 min then filtered with a 0.45 µm PTFE microfilter into the auto sampler vial. The extract (40 µl) was injected onto HPLC system. HPLC conditions were as follows: oven temperature (40^{o}C), flow rate (1 ml/min), mobile phase (methanol: water), and the column was C18.

3.8 Sensory evaluation

Samples of the different formulations were used to prepare different instant sauces as described in section 3.5. The sauce samples were assigned different random numeric codes. The acceptability of different sensory attributes including appearance, colour, taste, flavour, texture, mouthfeel and general acceptability were scored by 50 untrained panelists of Food Technology at Kyambogo University. The sensory attributes were scored on a 9-point hedonic scale where 1 = extremely disliked and 9 = extremely liked. The panellists were chosen on the basis of their availability and because they are learned to constitute a semi trained panel.

3.9 Iron bioavailability (Caco-2 cell bioassay)

This was achieved according to the method of Wiesinger (2021). The bioassay works according to the following principle: in response to increases in cellular iron concentrations, Caco-2 cells produce more ferritin protein, therefore, iron bioavailability was determined as the increase in Caco-2 cell ferritin production expressed as a ratio to total Caco-2 cell protein (ng ferritin per mg of total cell protein) after exposure to a digested sample. Ferritin was measured by enzyme linked immunoassay (Human Ferritin ELISA kit S-22, Ramco Laboratories Inc., Stafford, TX, USA) and total cell protein concentrations were quantified using the Bio-Rad DC™ protein assay kit (Bio-Rad Laboratories Inc., Hercules, CA, USA).

Each Caco-2 experiment was run with three control samples to ensure the quality of bioassay. The first control included a blank-digest with FeCl3 (66 μM), blank-digest of FeCl3 (66 μM) and a 1.3 mM ascorbic acid. Ferritin values averaged 41.4 ± 2.8 and 474 ± 36 ng ferritin/mg cell protein for the FeCl3 digest and the FeCl3 digest with ascorbic acid, respectively.

Two reference standards with similar food matrix properties as the cooked beans and cooked bean pasta were also run with each experiment to confirm the responsiveness of the bioassay. The first reference standard was a cooked, lyophilized and milled navy bean (cultivar name: Merlin). Ferritin values for the navy bean reference standard were 12.2 ± 0.09 ng/mg cell protein (mean ± SD) over the course of three experiments. The second reference standard was cooked, lyophilized and milled navy bean pasta (cultivar name: Merlin) extruded as fresh pasta before boiling. Ferritin values for the navy bean pasta reference standard were 38.7 ± 2.5 ng/mg cell protein (mean ± SD, n = 3). The iron bioavailability of each sample was measured in triplicate.

3.10 Data analysis

All data were analysed for variance (One-way ANOVA) using XLSTAT version 2019.2.2.59614 to obtain the means and standard deviations for each study variable (proximate composition, mineral analysis, folic acid, phytates, aflatoxin content, microbiological safety and sensory acceptability of the iron bio-fortified bean - silver fish instant composite sauce). Means were separated using the least significant difference (LSD) test at $p < 0.05$. Correlations were established using the XLSTAT Principal Component Analysis (PCA) correlation matrix.

3.11 Ethical considerations

The study was approved by Kyambogo University Directorate of Research and Graduate Training and ethical approval was sought from the Makerere University School of Health Science (Ref: MAKSHREC-2020-8) and Uganda National Council for Science and Technology (Ref: HS828ES) to develop the product for trial on pregnant women. The product quality assurance testing and clearance was provided by the UNBS.

CHAPTER FOUR: RESULTS AND DISCUSSION

4.1 Chemical composition of the iron bio-fortified bean - silver fish composite flour

4.1.1 Mineral analysis

The mineral content (zinc, iron, potassium, magnesium, sodium and calcium) of the selected bean varieties under different preparation treatments is represented below (Table 4.1). Iron composition ranged from 41 mg/kg to 336 mg/kg with the final instant iron bio-fortified product of NABE 1 (90:10) having significantly ($p<0.05$) the highest content while extruded NABE 5C had the lowest (Table 4.1). Calcium content was highest (15214 mg/kg) in extruded NABE 5C composited with silver fish (70:30) and lowest in extruded NABE 1 (447 mg/kg).

Zinc content was highest in extruded NABE 5C (276 mg/kg) and lowest in extruded NABE 2 with spices (36 mg/kg). Potassium was highest (6806 mg/kg) in the final instant iron bio-fortified product of NABE 1 (90:10) and lowest (5036 mg/kg) in extruded NABE 5C with spices. Magnesium was highest (1113 mg/kg) in extruded NABE 1 composited with silver fish (70:30) and lowest in extruded NABE 2 composited with silver fish (80:20) (875 mg/kg).

Sodium content ranged from 3437 mg/kg to 10210 mg/kg with the NABE 1 with spices having significantly ($p<0.05$) the highest content and extruded NABE 1 with the lowest. Extruded NABE 5C significantly had the highest ($p<0.05$) phosphorus content (4180 mg/kg) while extruded NABE 1 with spices had the lowest (922 mg/kg).

Table 4. 1 Changes in mineral composition (dry weight) of selected bean varieties under different preparation treatments

Bean Variety	Treatment method	P (mg/kg)	Zn (mg/kg)	Fe (mg/kg)	K (mg/kg)	Mg (mg/kg)	Na (mg/kg)	Ca (mg/kg)
NABE 1	Extruded	1008±11^{i}	38±2^{f}	83±0.4^{d}	6256±454^{cdef}	1017±19^{abcdef}	5639±86^{ghij}	1447±20^{ghi}
NABE 2	Extruded	1453±26^{jk}	42±0.5^{f}	54±2^{efg}	5274±9^{hi}	975±36b^{cdef}	3437±44^{efghij}	7756±33^{hi}
NABE 5C	Extruded	4180±72^{a}	276±19^{a}	41±2^{g}	6086±8^{fg}	1054±64^{abde}	6822±19^{cdefghi}	1617±25^{cdef}
NABE 1	Extruded + spices	922±15^{i}	41±3^{f}	66±0.8^{e}	6567±278^{bcdef}	1014±62^{abcdef}	10210±215^{a}	1340±68^{hi}
NABE 2	Extruded + spices	973±18^{i}	36±0.5^{f}	56±2^{ef}	6495±399^{bcdef}	988±99^{bcdef}	7380±239^{bcdef}	1720±442^{ghi}
NABE 5C	Extruded + spices	1332±13^{k}	44±3^{f}	56±2^{ef}	5204±273^{hi}	936±54^{ef}	7500±506^{bcdef}	1660±117^{ghi}
NABE 1	Beans:silver fish (90:10) +Spices	1332±18^{k}	67±2^{e}	61±2^{ef}	6547±185^{bcdef}	1098±30^{abc}	6769±74^{bcdefg}	4867±57^{fghi}
NABE 1	Beans:silver fish (80:20) +Spices	1777±89^{fgh}	91±2^{d}	64±1^{ef}	6153±206^{efg}	981±12b^{cdef}	6331±22^{cdefgh}i	7477±59^{cdef}
NABE 1	Beans:silver fish (70:30) +Spices	2225±49^{c}	117±2^{b}	58±1^{ef}	6536±216^{bcdef}	1113±9^{ab}	5783±30^{efghij}	10522±94^{abcd}
NABE 2	Beans:silver fish (90:10) +Spices	1393±60^{k}	62±0.4^{e}	55±3^{ef}	6066±13^{fg}	960±94^{cdef}	4428±29^{ghij}	5363±37^{efghi}
NABE 2	Beans: silver fish (80:20) +Spices	1724±78^{ghi}	89±3^{d}	52±7^{fg}	5401±173^{hi}	875±50^{f}	4049±40^{hij}	7667±52^{cdef}
NABE 2	Beans:silver fish (70:30) +Spices	2242±28^{c}	118±7^{b}	60±4^{ef}	6526±485^{bcdef}	1054±96^{abcde}	3750±47^{ij}	11334±27^{abc}
NABE 5C	Beans:silver fish (90:10) +Spices	1648±87^{ghi}	69±8^{e}	58±2^{ef}	5036±418^{i}	935±9^{ef}	5880±38^{defghij}	7005±26^{cdef}
NABE 5C	Beans:silver fish (80:20) +Spices	2047±72de	93±2^{d}	60±2^{ef}	5215±208^{hi}	948±27d^{ef}	5046±30^{fghij}	9499±25^{cdef}
NABE 5C	Beans:silver fish (70:30) +Spices	2414±58^{b}	121±4^{b}	58±1^{ef}	5347±108^{hi}	1001±5^{abdef}	5045±49^{eghij}	15214±96^{a}
NABE 1	Iron fortified product (90:10) +Spices	1469±23^{jk}	68±36^{e}	336±3^{a}	6806±84^{abc}	1085±56^{abcd}	8530±89^{abcd}	5656±76^{defgh}
NABE 1	Iron fortified product (80:20) +Spices	1721±58^{hi}	89±1^{d}	312±16^{b}	6441±170^{cdef}	1010±93^{abcdef}	8290±37^{abcd}	7971±47^{cdef}
NABE 1	Iron fortified product (70:30) +Spices	1955±86^{c}	109±65b^{c}	286±0.1^{c}	6415±325^{cdef}	1005±34^{abcdef}	8660±59^{abc}	11171±65^{abcd}
NABE 2	Iron fortified product (90:10) +Spices	1460±3^{hjk}	62±0.7^{e}	294±16^{c}	6271±198^{cdef}	969±43^{bcdef}	6326±88^{cdefghi}	6462±30^{cdefg}
NABE 2	Iron fortified product (80:20) +Spices	1885±42^{fg}	88±1^{d}	315±2^{b}	6211±65^{defg}	974±37^{bcdef}	5612±24^{fghij}	9190±19^{cdef}
NABE 2	Iron fortified product (70:30) +Spices	2224±102^{c}	113±7^{b}	282±9^{c}	6112±111^{fg}	1012±42^{abdef}	6455±37^{cdefgh}	14883±16^{ab}
NABE 5C	Iron fortified product (90:10) +Spices	1667±186^{hi}	70±3^{e}	291±4^{c}	5327±142^{hi}	961±15^{cdef}	8204±43^{abcde}	7218±37^{cdefcd}
NABE 5C	Iron fortified product (80:20) +Spices	2082±95^{cde}	98±6^{cd}	291±7^{c}	5679±298^{gh}	1031±14^{abcde}	9329±27^{ab}	8463±61^{cdef}
NABE 5C	Iron fortified product (70:30) +Spices	2208±14^{cd}	119±2^{b}	287±11^{c}	5275±45^{hi}	925±28^{ef}	6275±12^{cdfhi}	10080±63^{bcde}

Values are means of two replicates ± standard deviations of the means. Values in columns with the same superscript letters are not significantly different (p>0.05)

Iron values in this study were higher than those reported by Brigide *et al*. (2014) who reported iron levels for bio-fortified beans in the range of 61.12 mg/kg to 80.82 mg/kg. The higher levels of iron in the present study could be explained by differences in the fortification process and cultivar variations. Iron helps in the production of haemoglobin, the oxygen carrying chemical in the body's red blood cells reducing physiological disorders related to anaemia during pregnancy (Martinez-Navarrete et al., 2002). Low dietary intake of iron during pregnant women leads to postpartum and breast-feeding anaemia up to six months after delivery, pre-eclampsia, preterm labour and premature rupture of membranes (Milman, 2006). Therefore, iron fortification had a positive effect on the iron levels of the instant bean product. Exponential increase of the iron content in the final product due to the fact that fortificants contained high amount of iron. NABE 1 had the highest iron content among the three bean varieties. Therefore, consumption of the composite instant product made from NABE 1 could increase the iron content in pregnant mothers to meet the body recommended daily allowance (RDA) of 0.8 to 7.5 mg/day (Beard, 2000).

Calcium plays essential roles during foetus development such as tissues construction, cell signalling and bone development (Gharibzahedi and Jafari, 2017). Therefore, there is a need for increased calcium uptake from 1000 mg/day to 1300 mg/day during pregnancy to stabilize calcium homeostatis in pregnant women (Berti, Biesalski, Gärtner, Lapillonne, Pietrzik and Poston, 2011; Smolin and Grosvenor, 2011). NABE 5C composited with silverfish (70:30) had the highest calcium content among the three bean varieties and could therefore be recommended as the best formulation for calcium uptake in pregnant women to meet their RDA of 0.3–2.0 g/day (Buppasiri, et al., 2015). Addition of silver fish to beans increased their calcium content. This could be explained by the high calcium content of silver fish (Masette and Kwetegyeka, 2013).

Zinc and potassium act as antioxidants in the body and prevent digestive disorders such as diarrhoea, appetite loss, and taste variations (Badii, Nekouei, Fazilati, Shahedi and Badiei, 2012). Zinc also plays an important role in pregnancy and lactation, including foetal development and milk secretion. Severe zinc deficiency is associated with long-term labour, teratogenicity and foetal or embryonic mortality, abortion, dwarfism, and infants with low birth weight (Caulfield, Zavaleta, Shankar and Merialdi, 1998; King, 2011). NABE 5C extruded had the highest zinc content among the three bean cultivars while addition of silverfish significantly elevated the zinc content in the samples compared to other treatments in the study.

Magnesium plays an important role of building bones in the growing baby in pregnant women and plays part in regulation of pressure by causing relaxation of muscles within the vessel walls and thus, vasodilatation and decreased vascular resistance as well as decreased vasospasm and blood pressure which are beneficial during pregnancy (Smolin and Grosvenor, 2011; Gharibzahedi and Jafari, 2017). Magnesium deficiency during pregnancy is associated with hypertension, preeclampsia and preterm birth in pregnancy (Jain, Sharma, Kulshreshtha, Mohan and Singh, 2010). NABE 1 had the highest content of magnesium and therefore could recommended for consumption by pregnant women.

Sodium is needed for balancing the osmotic potential in the body (Palfrey and Rao, 1983). NABE 1 had the highest sodium content among the three bean varieties while the sodium content did not significantly vary ($p>0.05$) with the treatments in the study. Phosphorus is usually bound by phytates which reduce its bioavailability in the body by forming chelates and complexes (Gharibzahedi and Jafari, 2017). Deficiency of this mineral, although rare, but if occurs, may have symptoms such as anaemia, myasthenia, bone pain, rickets, Genu varum, anorexia, vertigo, confusion and possibly can cause death (Ross, Caballero, Cousins, Tucker and Ziegler, 2014). NABE 5C had the highest phosphorous content while the different treatments did not affect the phosphorous content of the composite bean samples.

4.1.2 Proximate composition

The proximate composition (moisture, ash, fat, protein and carbohydrates) of selected bean varieties under different preparation treatments are presented in Table 4.2. Moisture content significantly varied ($p=0.0001$) from 5.84 to 10.33% with the extruded NABE 5C composited with silver fish (80:20) having the highest moisture content while extruded NABE 2 had the lowest. The moisture content was slighty lower than the reported moisture content of beans of 13.89 to 15.62% dry weight basis offereing a more shelf stable product (Brigide et al., 2014). Ash content ranged from 4.25% to 8.37%. Extruded NABE 1 composited with silver fish (70:30) had the highest ash content of 8.37% while NABE 5C extruded had the lowest values of 4.25%.

The fat content ranged 0.54% to 5.45% with the extruded NABE 5C composited with silver fish (70:30) and the instant iron bio-fortified product of NABE 2 (80:20) significantly ($p<0.05$) having the highest content compared with extruded NABE 2 with spices and extruded NABE 1. Results of the findings which were higher than previous studies for fat content of beans which were in the

ranges of 1.66 to 2.22% in cooked beans (Barampama and Simard, 1993; Brigide, et al., 2014) indicating that addition of silver fish enhanced the fat content of the biofortified beans.

Crude protein content varied from 15.86% to 31.90% (Table 4.2). Extruded NABE 5C composited with silver fish (70:30) and the final instant iron bio-fortified product of NABE 5C significantly had the highest crude protein content ($p<0.05$) while extruded NABE 5C with spices and extruded NABE 5C composited with silver fish (90:10) had the lowest. Protein content of the sample were higher than the reported protein content of beans which are in the range of 19.2% to 19.8% (Oliveira et al. 2001a, b) indicating that addition of silver fish to biofortified beans enhanced their protein content.

Carbohydrate content ranged from 20.66% to 27.36%. Extruded NABE 2 composited with silver fish (90:10) and extruded NABE 2 composited with silver fish (80:20) had the highest carbohydrate content (in terms of composite flour) while the final instant iron bio-fortified product of NABE 5C and extruded NABE 1 composited with silver fish (80:20) had the lowest. These results were in agreement with the reported carbohydarates content which were in the ranges of 16.18 40.63% (Sathe, 2002; Brigide and Canniatti-Brazaca, 2006; Brigide, et al., 2014). Overall, NABE 2 had the highest carbohydrate content among the three bean varieties and, the carbohydrates content of the beans did not significantly vary with the treatments.

Table 4. 2 Changes in proximate composition (dry weight) of selected bean varieties under different preparation treatments

Bean Variety	Treatment method	Moisture (%)	Ash (%)	Fat (%)	Protein (%N)	Carbohydrates (%)
NABE 1	Extruded	6.260 ± 0.01^{c}	5.158 ± 0.19^{b}	0.541 ± 0.14^{c}	24.815 ± 1.02^{bc}	25.968 ± 0.73^{ab}
NABE 2	Extruded	5.833 ± 0.12^{c}	4.311 ± 0.03^{bc}	0.915 ± 0.12^{c}	17.745 ± 0.18^{cd}	26.841 ± 0.31^{ab}
NABE 5C	Extruded	7.326 ± 0.51^{bc}	4.251 ± 0.05^{bc}	1.928 ± 0.01^{bc}	23.995 ± 2.24^{bc}	24.599 ± 0.15^{b}
NABE 1	Extruded + spices	7.379 ± 0.33^{bc}	5.787 ± 0.13^{b}	0.671 ± 0.01^{c}	24.491 ± 0.45^{bc}	27.007 ± 0.16^{ab}
NABE 2	Extruded + spices	7.726 ± 0.19^{bc}	5.147 ± 0.03^{b}	1.048 ± 0.24^{bc}	27.101 ± 1.43^{b}	26.180 ± 0.23^{ab}
NABE 5C	Extruded + spices	9.293 ± 0.16^{ab}	5.090 ± 0.14^{b}	1.983 ± 0.05^{bc}	18.027 ± 4.52^{cd}	25.071 ± 0.35^{ab}
NABE 1	Beans:silver fish (90:10) + spices	8.683 ± 1.09^{b}	6.663 ± 0.07^{ab}	1.756 ± 0.06^{bc}	26.039 ± 0.70^{b}	26.204 ± 0.00^{ab}
NABE 1	Beans:silver fish (80:20) + spices	8.183 ± 0.35^{b}	7.281 ± 0.12^{ab}	3.184 ± 0.04^{b}	27.869 ± 1.22^{b}	20.656 ± 5.31^{d}
NABE 1	Beans: silver fish (70:30)+ spices	8.010±0.25^{b}	8.342 ±0.06 ^{a}	4.697 ± 0.09^{ab}	26.008 ± 0.07^{b}	24.599 ± 0.27^{b}
NABE 2	Beans:silver fish (90:10)+ spices	8.131 ± 0.34^{b}	5.114 ± 0.03^{b}	1.106 ± 0.18^{bc}	21.287 ± 1.94^{c}	27.361 ± 0.08^{a}
NABE 2	Beans: silver fish (80:20+ spices)	7.589 ± 0.02^{bc}	6.021 ± 0.02^{b}	3.149 ± 0.35^{b}	24.710 ± 1.87^{bc}	26.936 ± 0.64^{ab}
NABE 2	Beans:silver fish (70:30)+ spices	7.805 ± 0.02^{bc}	6.884± 0.03^{ab}	4.298± 0.01^{ab}	27.555± 0.44^{b}	25.826 ± 0.16^{ab}
NABE 5C	Beans:silver fish (90:10) + spices	9.204 ± 0.09^{ab}	6.474 ± 0.17^{ab}	2.818 ± 0.20^{b}	15.860 ± 13.74^{d}	24.764 ± 0.32^{b}
NABE 5C	Beans:silver fish (80:20)+ spices	10.329 ± 1.78^{a}	6.657 ± 0.34^{ab}	4.229 ± 0.16^{ab}	29.913 ± 0.14^{ab}	24.363 ± 0.26^{b}
NABE 5C	Beans:silver fish (70:30) + spices	7.079 ± 1.76^{bc}	7.361 ± 0.22^{ab}	5.452 ± 0.02^{a}	31.930 ± 1.08^{a}	23.584 ± 0.98^{bc}
NABE 1	Instant iron fortified product (90:10) + spices	8.630 ± 0.62^{b}	6.573 ± 0.17^{ab}	2.584 ± 0.14^{b}	26.008 ± 0.07^{b}	25.992 ± 0.12^{ab}
NABE 1	Instant iron fortified product (80:20) + spices	8.105 ± 0.11^{b}	7.289 ± 0.09^{ab}	3.027 ± 0.69^{b}	21.721 ± 2.56^{cd}	26.700 ± 0.55^{ab}
NABE 1	Instant iron fortified product (70:30) + spices	8.531 ± 0.47^{b}	8.276 ± 0.04^{a}	3.868 ± 0.06^{ab}	22.436 ± 4.30^{bc}	26.310 ± 0.39^{ab}
NABE 2	Instant iron fortified product (90:10) + spices	8.636 ± 0.34^{b}	5.357 ± 0.83^{b}	1.790 ± 013^{bc}	24.029 ± 0.18^{bc}	25.449 ± 0.35^{ab}
NABE 2	Instant iron fortified product (80:20) + spices	7.784 ± 0.13^{bc}	6.682 ± 0.17^{ab}	5.050 ± 1.39^{a}	25.768 ± 0.82^{bc}	26.346 ± 0.19^{ab}
NABE 2	Instant iron fortified product (70:30) + spices	8.531 ± 0.47^{b}	8.276 ± 0.04^{a}	3.868 ± 0.06^{ab}	22.436 ± 4.30^{bc}	26.310 ± 0.39^{ab}

NABE 5C	Instant iron fortified product (90:10) + spices	9.782 ± 0.16^{ab}	6.527 ± 0.19^{ab}	3.463 ± 0.62^{b}	24.410 ± 0.18^{bc}	23.347 ± 0.23^{bc}
NABE 5C	Instant iron fortified product (80:20) + spices	8.725 ± 0.21^{b}	6.789 ± 0.14^{ab}	3.669 ± 008^{b}	28.673 ± 0.42^{ab}	23.041 ± 0.60^{bc}
NABE 5C	Instant iron fortified product (70:30) + spices	9.298 ± 0.25^{ab}	8.072 ± 0.01^{a}	4.447 ± 0.23^{ab}	30.300 ± 1.33^{a}	23.206 ± 0.64^{bc}

Values are means of two replicates ± standard deviations of the means. Values in columns with the same superscript letters are not significantly different ($p>0.05$);

Moisture content was close to values reported found by Oliveira, Ribeiro, Jost and Londero (2008) and Ramírez-Cárdenasi, Leonel and Costa (2008), who reported moisture content of beans in the range 9 to 11% on dry basis. Apart from the 10.33% result for moisture content obtained from this study, the rest were found below 10% recommended by specification (Uganda Standard 1852:2019). High moisture content in beans is associated with their degree of hydration during maceration which helps to reduce the anti-nutritional factors in beans (Canniatt-Brazaca and Silva, 2014).

Results of ash content obtained in the present study were higher than those reported by Barampama and Simard (1993) and Brigide *et al*. (2014) which ranged 3.8% to 4.5% dry weight basis of raw beans. Also, recent research yielded 1.41% to 4.22% ash ((Elolu and Ongeng, 2020). The difference could be explained by addition of silver fish, spices, fortification and variations in bean cultivars used in the different studies. NABE 1 had the highest ash content among the three varieties studied. Extrusion alone and extrusion with addition of spices produced the lowest ash content in the composite samples while addition of silver fish and final instant product formulation produced the highest ash content in the samples especially NABE 5C. Section 4.1.1 presents the results of mineral analysis in order to characterize the individual mineral nutrients in the ash of the instant extruded beans composited with silver fish for nutritional recommendation to the target individuals.

The findings of fat content in this study were slightly higher than those reported by Brigide, Canniatti-Brazaca and Silva (2014) for bio-fortified beans which ranged from 1.66% to 2.13% dry weight basis. The variations could be due to addition of silver fish in the current study, and the fact that different studies used different cultivars and sample treatments for experimentation. In this study, fat content did not vary with addition of silver fish and in the final instant iron bio-fortified product since both sets of samples did not significantly differ in fat content ($p > 0.05$). However, extrusion alone and extrusion with the addition of spices significantly produced ($p<0.05$) the lowest fat content of the beans with NABE 1 and NABE 2 having the lowest content while NABE 5C had the highest.

Results of protein content in this study were close to those reported by Esteves (2000) which ranged from 22% to 26% on dry weight basis. Addition of silver fish up to 30% to NABE 5C increased the protein content of the composite sample compared to samples of bean varieties without

silverfish added. In general, extrusion, addition of spices and silver fish did not significantly affect ($p>0.05$) the protein content of the composite bean samples. However, NABE 5C composited with 30% silver fish had the highest protein content among the three bean varieties studied. A study done earlier indicated protein content of plain bean to be 15-30% on dry weight basis (Mutambuka, 2013). The results of carbohydrate content were close to those of Brigide *et al*.
(2014) which ranged from 16.18 to 22.68% on dry weight basis in raw bio-fortified beans.

4.1.3 Folic acid

The results of folic acid content of selected bean varieties are presented in Figure 4.1. The folic acid content ranged from 7.75 mg/kg to 47.47 mg/kg. NABE 1 fortified instant product and extruded NABE 1 beans composited with silver fish (80:20) had the highest folic acid content (47.47 mg/kg and 35.18 mg/kg, respectively) which differed significantly ($p<0.05$) from that of NABE 5C beans composited with silver fish (80:20), and NABE 5C fortified instant product which had the lowest values of 7.75 mg/kg and 19.74 mg/kg, respectively.

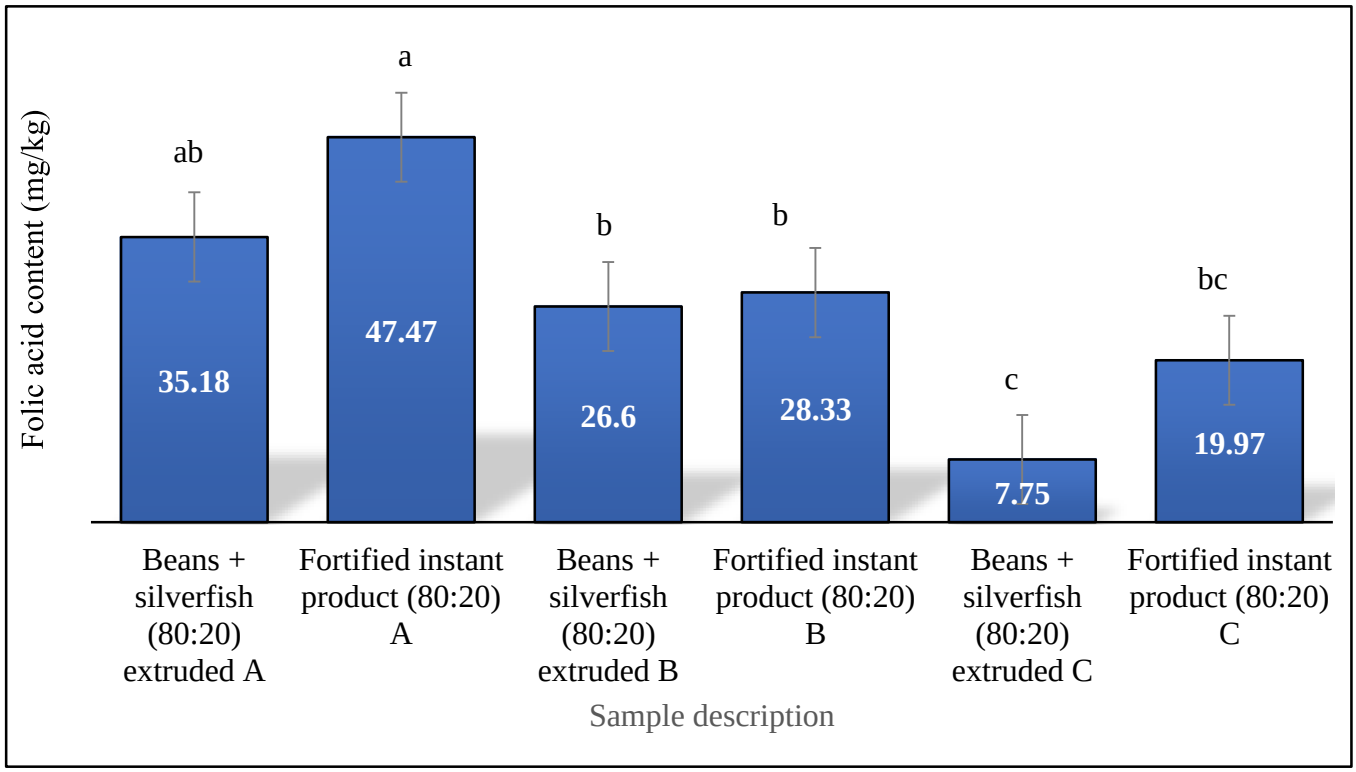


Figure 4. 1Folic acid content (dry weight) of selected bean varieties under different preparation treatments

KEY:
A, NABE 1;
B, NABE 2;
C, NABE 5C

Folic acid is responsible for DNA/RNA synthesis, amino acid transformation, formation of red blood cells and reducing incidences of malformation of embryonic brain and/or spinal cord (neural tube defects) during pre-conception period and first trimester of pregnancy which are common causes of morbidity and mortality among infants and neonates (Coskun and Özdemir, 2009; Çoşar,

Köken, Köken, Şahin, Yeşildağer and Arıöz, 2009). Therefore, consumption of NABE 1 fortified instant product and extruded NABE 1 beans composited with silver fish (80:20) could help pregnant women prevent the effects of folic acid deficiency during pregnancy to meet their RDA of~600 µg/day (de Benoist, 2008).

4.1.4 Phytates

There were siginificant variations in the phytic acid content of the composite samples ($p<0.05$) (Fig. 4. 2) with values ranginged from 12.92 mg/g (extruded NABE 1) to 17.36 mg/g (with raw NABE 1).

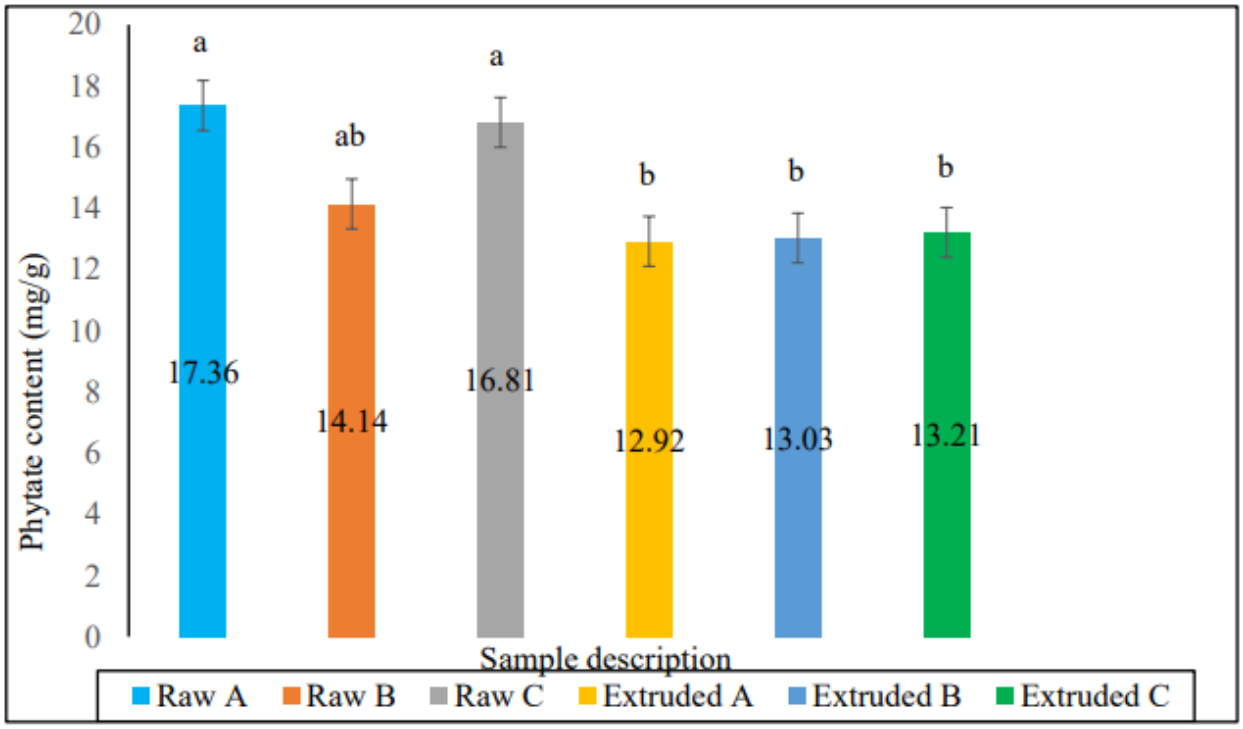


Figure 4. 2 Phytate content (dry weight) of selected bean varieties under different preparation treatments. KEY: A, NABE 1; B, NABE 2; C, NABE 5C

These findings were close to those of Dahiya et al. (2013) and Kumar and Pandey (2020) who reported that the phytate content of beans ranges from 7.34– 8.06 mg/g to 2.93–3.53 mg/g dry weight basis. Phytates bind essential mineral nutrients and proteins due to their amphiphilic nature thereby reducing mineral bioavailability in the body (Lynch, Beard, Dassenko and Cook, 1984; Ganesan and Xu, 2017).

4.1.5 Aflatoxin content of the iron bio-fortified bean - silver fish instant composite sauce

All the final instant iron bio-fortified beans – silverfish (80:20) composite samples in this study had low levels of aflatoxin B2, aflatoxin G1 and G2, total aflatoxins and aflatoxin B1 according to Uganda National Bureau of Standards (<0.25 ppb) indicating they are safe for human consumption (Table 4.3). This could be due to their low moisture content which created unfavourable condition for filamenteous aflatoxin producing mold growth hence absence of aflatoxin in the samples.

Table 4. 3 Aflatoxin content of instant iron bio-fortified beans – silverfish (80:20) composite flour

Sample description	Total (ppb)	B1 (ppb)	B2 (ppb)	G1 (ppb)	G2 (ppb)
NAROBEAN 1	<1 (LOQ)	<0.25 (LOQ)	<0.25 (LOQ)	<0.25 (LOQ)	<0.25 (LOQ)
NAROBEAN 2	<1 (LOQ)	<0.25 (LOQ)	<0.25 (LOQ)	<0.25 (LOQ)	<0.25 (LOQ)
NAROBEAN 5C	<1 (LOQ)	<0.25 (LOQ)	<0.25 (LOQ)	<0.25 (LOQ)	<0.25(LOQ)

LOQ = limit of quantification

Aflatoxins are secondary metabolites of moulds mostly belonging to the *Penicillium*, *Fusarium* and *Aspergillus* genera, which negatively affects pregnancy (Zain, 2011). The total aflatoxin AFs and AFB1 levels in cereal based foods should not exceed 4 µg/kg and 2 µg/kg, respectively according to the European Commission Regulation (European Commission Regulation No 165/2010, 2010). The main aflatoxin producing species are *A. flavus* and *A. parasitica* mainly associated with agricultural food products such as beans, coffee, groundnuts among others (Okun, Khamis, Muluvi, Ngeranwa, Ombura and Yongo, 2015). These toxigenic moulds mainly grow under humid conditions during storage of food products. Aflatoxin B1 (AFB1) and Aflatoxin B2 (AFB2) are among the commonest aflatoxins in the food industry (Lalah, Omwoma and Orony, 2019). Aflatoxin contamination is a serious challenge in Uganda mainly in cereals (maize, sorghum, and millet), legumes (simsim and beans), in sunflower and cassava. Their high prevalence in these foods is mainly due to poor postharvest handling, weak government policies, low level of awareness and education among the stakeholders including farmers and consumers (Omara et al., 2020). Aflatoxins cause low birth weight, and their presence in breast milk can lead to harmfull effects in babies (Ghiasian and Maghsood, 2012). Aflatoxins are also potent mutagens, carcinogens, teratogens, and immuno-suppressants and interfere with protein synthesis and inhibit metabolic systems which leads to damage to various body organs and systems (Zain, 2011; Bbosa, Kitya, Lubega, Ogwal-Okeng, Anokbonggo and Kyegombe, 2013).

4.2 Microbiological safety of the iron bio-fortified bean - silver fish instant composite sauce

All the instant iron bio-fortified beans – silverfish (80:20) composite samples in this study had microbial counts below the maximum recommended safe levels by Uganda National Bureau of Standards (UNBS, Table 4.4) meaning they are safe for consumption by pregnant women.

Table 4. 4 Microbial counts (cfu/g) of instant iron bio-fortified beans – silverfish NABE 5C (80:20) composite flour

Parameter	Sample results	UNBS specification	Status
Escherichia coli	<10	Absent	Pass
Yeast and moulds	<10	100 (maximum)	Pass
Salmonella (25 g)	Not detected	Absent	Pass
Total plate count	<10	10,000 (maximum)	Pass
Staphylococcus aureus	<10	NA	NA
Total coliforms	<10	NA	NA

Values are means of two replicates. NA= Not applicable

Salmonella enterica causes systemic infection and enteritis. The most susceptible populations for these infections include the young, old, pregnant, transplant patients and HIV infected persons (van der Klooster and Roelofs, 1997; Doffinger, Patel and Kumararatne, 2005). Pregnancy can have deleterious effects such as severe *S. typhi* gastroenteritis, infective endocarditis, sepsis and disseminated intravascular coagulation (Krishnan, Guilbert, Russell, Wegmann, Mosmann and Belosevic, 1996; Fievet et al., 2001; Ozer, Sari, Davutoglu and Cebesoy, 2009).

E. coli induces fetal growth retardation, resorptions, malformations, fetal morbidity and mortality (Collins, Smith, Arnold and Offenbacher, 1994). Studies have also reported that *E. coli* endotoxins induce severe physiological disorders, abortion, and organ damage in fetous (Coid, 1976; Ornoy and Altshuler, 1976; Awadalla, Mercer and Brown, 1985; Sharma and Thapa, 2007). *Staphylococcus aureus* intoxications are on a rise in pregnant and postpartum women, as well as in healthy neonates and in infants hospitalized in intensive care units (Fortunov, Hulten, Hammerman, Mason and Kaplan, 2006; Carey, Duchon, Della-Latta and Saiman, 2010). It was therefore favorable that the samples tested in this study were free from these pathogenic organisms making the product safe for consumption by the target population since all samples met the UNBS

safety standards of zero *Salmonella*, <10 cfu/ml for *Escherichia coli*, yeast and moulds, total plate counts, *Staphylococcus aureus*, and total coliforms.

4.3 Sensory acceptability of the iron bio-fortified bean - silver fish instant composite sauce

The indices for each of the various sensory attributes of the iron bio-fortified instant sauce produced from the three bean varieties NABE 1, NABE 2 and NABE 5C did not differ significantly ($p>0.05$) (Table 4.5). NABE 5C (80:20) was preferred the most since it registered the highest score for overall acceptability while the controls for all the three bean varieties had the lowest scores. The high acceptability of NABE 5C (80 beans: 20 silver fish) could be explained by its desirable appearance, texture and colour (Table 4.5). This sample was also averagely preferred for its mouth feel, taste and flavour. Therefore, NABE 5C composited with silver fish in the proportion of 80:20 could be used as a vehicle for iron in pregnant women. However, its taste and flavour attributes should be improved for better acceptance by the target group.

Table 4. 5 Sensory acceptability scores of the instant sauce containing different proportions of iron bio-fortified beans - silver fish composite flour

Attributes	B1-90:10	B1-80:20	B1-70:30	B1-CNTR	B2-90:10	B2-80:20	B2-70:30	B2-CNTR	B5-90:10	B5-80:20	B5-70:30	B5-CNTR
Colour	6.9 ± 0.10^{a}	6.7 ± 0.51^{a}	7.1 ± 0.21^{a}	5.2 ± 0.37^{a}	6.7 ± 0.09^{a}	6.6 ± 0.43^{a}	7.6 ± 0.24^{a}	5.4 ± 0.51^{b}	6.8 ± 0.14^{a}	7.0 ± 0.39^{a}	7.3 ± 0.02^{a}	5.1 ± 1.26^{b}
Appearance	6.9 ± 0.39^{a}	6.6 ± 0.54^{a}	7.2 ± 0.11^{a}	4.7 ± 0.40^{b}	6.8 ± 0.16^{a}	6.6 ± 0.17^{a}	7.4 ± 0.29 ^{a}	5.1 ± 0.41^{b}	6.9 ± 0.11^{a}	7.3 ± 0.19^{a}	7.4 ±0.14 ^{a}	4.6 ± 0.49^{b}
Texture	6.3 ± 0.27^{a}	6.6 ± 0.04^{a}	6.8 ± 0.18^{a}	4.8 ± 0.24^{b}	6.5 ± 0.25^{a}	6.4 ± 0.24^{a}	7.2 ± 0.53^{a}	5.3 ± 0.09^{b}	6.4 ± 0.66^{a}	7.1 ± 0.14^{a}	6.7 ± 0.58^{a}	4.9 ± 0.17^{b}
Taste	6.6 ± 0.35^{a}	6.3 ± 0.19^{a}	6.4 ± 0.21^{a}	5.9 ± 0.23^{b}	6.1 ± 0.52^{a}	6.6 ±0.26 ^{a}	6.4 ± 0.44^{a}	6.2 ± 0.39^{a}	6.3 ± 0.39^{a}	6.7 ± 0.55^{a}	6.3 ± 0.96^{a}	5.7 ± 0.18^{b}
Mouth feel	6.1 ± 0.35^{a}	6.3 ± 0.12^{a}	6.4 ± 1.12^{a}	5.6 ± 0.41^{b}	6.3 ± 0.48^{a}	6.3 ± 0.32^{a}	6.7 ± 0.70^{a}	5.7 ± 0.35^{b}	6.3 ± 0.59^{a}	6.9 ± 0.47^{a}	6.4 ± 0.83^{a}	5.7 ± 0.28^{b}
Flavour	6.4 ± 0.09^{a}	6.4 ± 0.05^{a}	6.1 ±0.43 ^{a}	6.2 ± 0.44 ^{a}	5.9 ± 0.33^{a}	6.2 ± 0.39^{a}	6.2 ± 0.86^{a}	6.2 ± 0.26^{a}	6.3 ± 0.46^{a}	6.6 ± 0.21^{a}	6.3 ± 0.95^{a}	5.6 ± 0.32^{b}
Overall acceptability	6.5 ± 0.15^{a}	6.6 ± 0.33^{a}	6.7 ± 0.56^{a}	5.4 ± 0.09^{b}	6.2 ± 0.64^{a}	6.3 ± 0.12^{a}	6.4 ± 0.13^{a}	5.5 ± 0.04^{b}	6.3 ± 0.19^{a}	**6.9** ± 0.41^{a}	6.6 ± 027^{a}	5.0 ± 0.14^{b}

Values are mean scores from 50 panellists. Values in columns with the same superscript letters are not significantly different ($p>0.05$); B1, NABE 1; B2, NABE 2; B5C, NABE 5C; CNTR, control sample made from 100% bean flour

4.4 Bioavailability of non-fortified bean - silverfish composites formulation and of fortified bean - silverfish composites formulated with three different biofortified bean varieties

For bioavailability of non-fortified bean - silverfish composites formulation, of iron in extruded beans ranged from 0.64 to 21.68 ng ferritin where the lowest bioavailability was in composite flour ratio 90:10 in NABE 1 and the highest in composite flour ratio 70:30 in NABE 5C that is 21.68 ng ferritin/mg cell protein. Generally, bioavailability of iron for non-fortified bean-silverfish composite flour was lower than for fortified samples.

Table 4. 6 Iron concentrations (μg/g) and iron bioavailability (ng ferritin/mg cell protein) of non-fortified bean - silverfish composites formulated with three different of biofortified bean varieties

	Extruded Bean		90:10 Bean: Silverfish		80:20 Bean: Silverfish		70:30 Bean: Silverfish	
Variety)	Fe	Fe Bioavailability	Fe	Fe Bioavailability	Fe	Fe Bioavailability	Fe	Fe Bioavailability
NABE 1	80.6 ± 1.3[b]	0.65 ± 0.05[c]	85.7 ± 0.4[b]	0.64 ± 0.05[c]	94.1 ± 1.1[c]	0.90 ± 0.06[b]	128.8 ± 2.5[c]	2.73 ± 0.34[c]
NABE 2	79.9 ± 0.7[b]	0.83 ± 0.11[b]	94.9 ± 3.2[b]	1.64 ± 0.55[b]	110.8 ± 6.7[b]	2.00 ± 0.40[b]	142.7 ± 3.9[b]	6.31 ± 0.53[b]
NABE 5C	127.9 ± 2.5[a]	8.82 ± 2.62[a]	125.2 ± 0.6[a]	9.37 ± 4.30[a]	130.2 ± 1.3[a]	14.27 ± 1.58[a]	171.2 ± 1.2[a]	21.68 ± 5.13[a]

Values are means ± standard deviations of three replicates for each samples. Means in columns with the same letter are not significantly different ($p \leq 0.05$).

Bioavailability of iron in fortified bean- silver fish composite flour ranged from 23.7 to 163.4 ng ferritin/mg cell protein. The bioavailability of iron was lowest in whole extruded bean NABE 2 23.7 ng ferritin/mg cell protein and the highest in the composite flour in ratio 70:30 NABE 5C: silverfish with the value 163.4 ng ferritin/mg cell protein. Throughout the table, formulations containing NABE 5C had the highest iron bio-availabilty and therefore this variety should be adopted for formulation of the composite sauce. Results from this research clearly showed an increased bioavailability of iron in the fortified bean - silverfish composites formulations.

Table 4. 7 Iron concentrations (µg/g) and iron bioavailability (ng ferritin/mg cell protein) of fortified bean - silverfish composites formulated with three different biofortified bean varieties

	Extruded Whole Bean		90:10 Bean: Silverfish		80:20 Bean: Silverfish		70:30 Bean: Silverfish	
Variety	Fe	Fe Bioavailability	Fe	Fe Bioavailability	Fe	Fe Bioavailability	Fe	Fe Bioavailability
NABE 1	343.7 ± 3.7[b]	39.9 ± 14.6[b]	339.6 ± 8.3[b]	36.8 ± 8.4[c]	342.4 ± 15.9[b]	61.5 ± 14.0[c]	393.6 ± 14.2[c]	138.2 ± 7.5[b]
NABE 2	339.6 ± 17.0[b]	23.7 ± 4.7[b]	363.3 ± 15.7[b]	68.5 ± 19.8[b]	345.9 ± 12.6[b]	118.8 ± 17.1[b]	418.5 ± 8.1[b]	124.2 ± 9.6[b]
NABE 5C	421.6 ± 17.8[a]	93.3 ± 16.4[a]	478.9 ± 13.1[a]	94.9 ± 9.3[a]	426.8 ± 30.6[a]	134.5 ± 12.1[a]	457.5 ± 7.9[a]	163.4 ± 10.9[a]

Values are means ± standard deviations of three replicates for each samples. Means in columns with the same letter are not significantly different ($p \leq 0.05$).

CHAPTER FIVE: CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

The instant composite sauce made from 80:20 extruded NABE beans: silver fish had the highest ash and protein content. Thus, addition of silver fish increased the content of these parameters in the instant sauce but did not affect fat and carbohydrate levels. This implies that compositing NABE beans with silver fish, extrusion cooking and iron fortification of the instant sauce are major contributory treatments for enhancing the proximate composition of the product except for fat and carbohydrate content.

Extrusion cooking and compositing NABE beans with silver fish increased the iron, calcium, zinc, potassium, magnesium, sodium and folic acid content of the instant sauce, but decreased its total phytate content. As these effects were more pronounced in the treatments involving NABE 1 compared with NABE 2 and NABE 5C, consumption of the composite instant product made from NABE 1 could improve the mineral and folic acid nutrition of pregnant mothers. Given that the product also had enhanced iron levels, it could be concluded that iron biofortification of beans positively affected the iron content of the composite sauce. Moreover, the nutritive value of the product could be high due to reduced levels of phytates which normally reduce nutrient bioavailability in the body.

The instant iron bio-fortified composite sauce had low microbial counts, total aflatoxins, aflatoxin B1 and aflatoxin B2 below the maximum recommended levels (<10 cfu/ml for microbial samples except *Salmonella* which should not be present at all and <0.25 ppb for Aflatoxins, respectively) by Uganda National Bureau of Standards implying that the product was safe for human consumption.

NABE 5C (80:20) had the most preferred composite for its appearance, texture, colour, mouth feel, taste and flavour. Therefore, the latter could be used as a vehicle for iron intake in pregnant women.

5.2 Recommendations

(i) Since fortification enhanced the nutrient composition of the final product, about 150 g of the iron bio-fortified beans – silver fish instant sauce (80:20) can be fed to pregnant mothers to meet more than 70% RDA for fat, protein, iron, calcium, zinc, potassium, magnesium and

sodium given the high sensory acceptability, nutritional value and microbial safety of this composite product.

(ii) Extruded iron bio-fortified beans – silver fish instant sauce (80:20) should therefore, be included in pregnant women's diets and in therapeutic nutrition and dietetics for its nutritional components. The formulation is more convenient to prepare since it is an extruded precooked sauce. Therefore, there is a need for society mobilisation to improve social behaviour change communication to ensure enhanced consumption of the sauce in order to prevent iron deficiency anaemia in pregnant mothers.

(iii) Extruded iron bio-fortified beans – silver fish instant sauce with fortificant can provide the required iron bioavailable in the body hence this product can combat iron deficiency anaemia in pregnant mothers.

(iv) Further research should investigate the effect of the investigated treatments on phytate content and bioavailability of iron and folic acid in the product. Further research should look into the effect of bio-fortification of beans on iron overload in humans, and on application of other NABE bean varieties in nutritional enhancement.

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APPENDICES

Appendix 1: The ballot for sensory evaluation

Panelist No._____

You are provided with samples of iron-bio-fortified beans – silver fish instant sauce prepared with varying contents of beans and fish flour. Please observe and record your liking of the samples on the scale of 1 to 9 by placing your score in the box next to the sensory parameter under each sample in the table. Please evaluate the products in the order in which they are presented. Use the water provided to refresh your palate before and between samples.

ANSWER ALL QUESTIONS. We would like to know what you think!!

If you have any questions, please ask the study coordinators.

Score the products using hedonic scale below	
Like extremely	9
Like very much	8
Like moderately	7
Like slightly	6
Neither like nor dislike	5
Dislike slightly	4
Dislike moderately	3
Dislike very much	2
Dislike extremely	1

Quality attributes	Sample No			
Appearance				
Colour				
Flavour				
Texture				
Mouth feel				
General acceptability				

Which sample (only one) would you most prefer and why?

.....……...

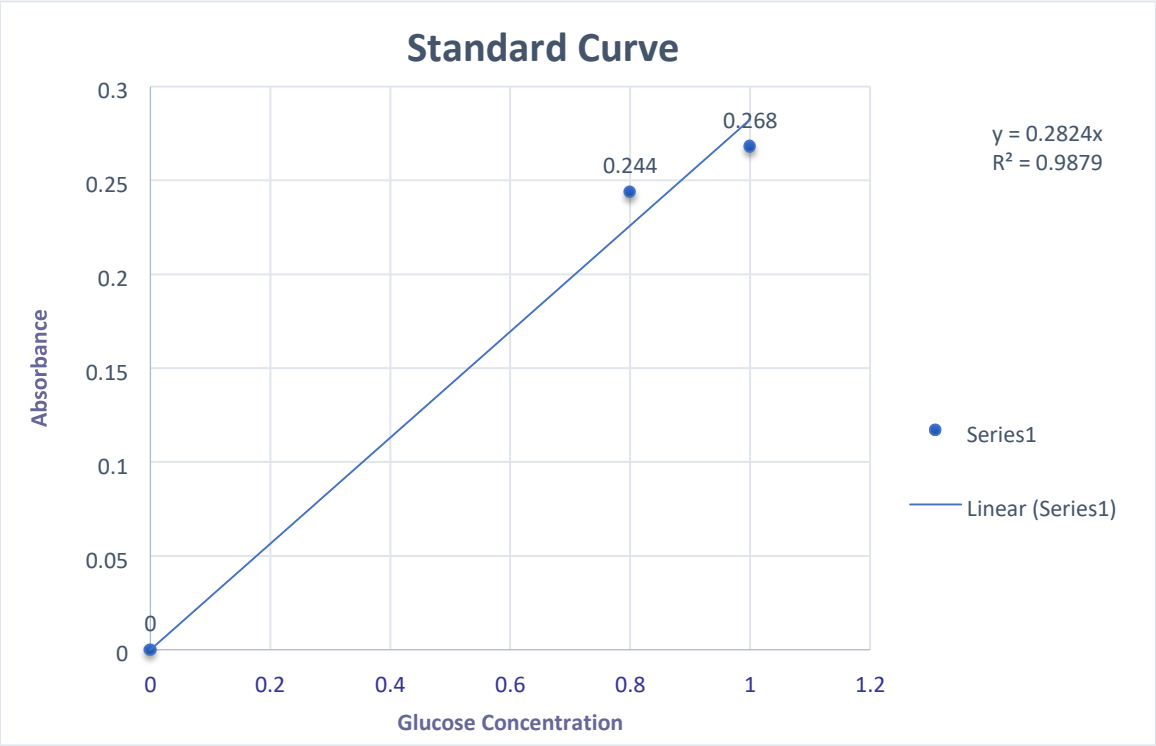
General comments:

……

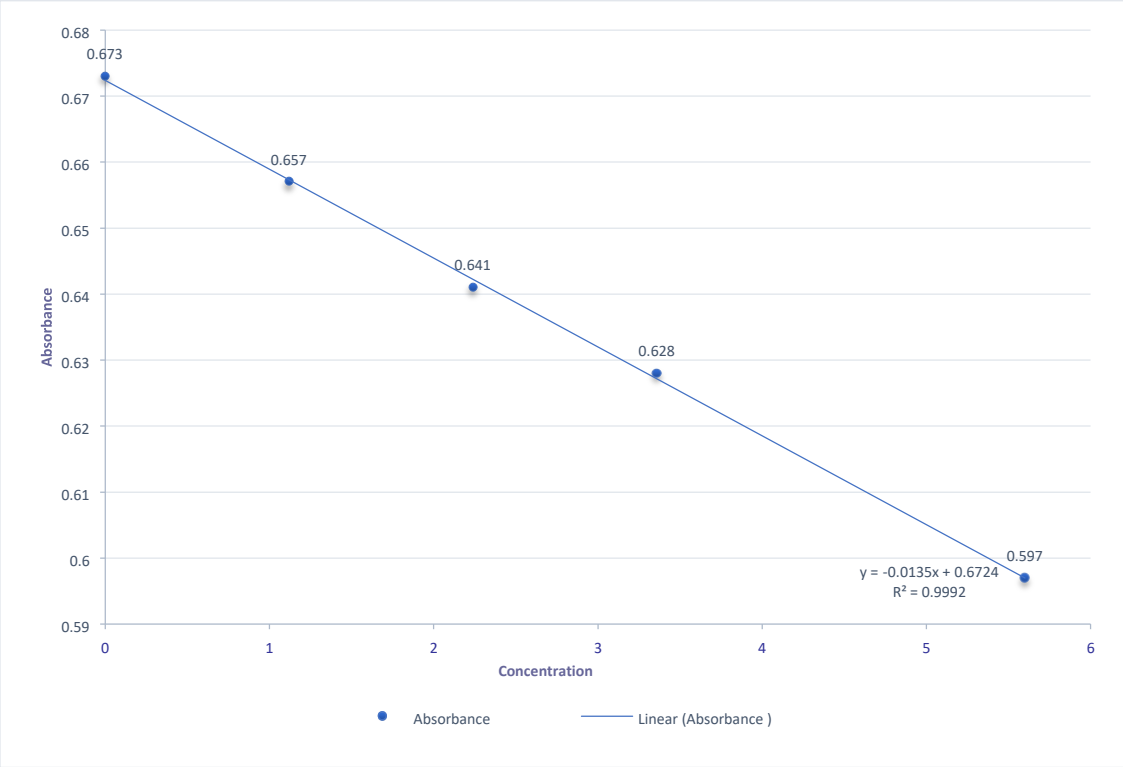
Appendix 2: Extruded product (mixture of grounded dried beans, roasted sliver fish and spices) ready for milling into flour

Appendix 3: Packaged iron bio-fortified bean – silver fish composite flour

Appendix 4: A standard curve of absorbance verses glucose concentration (µg).



Appendix 5: A graph of absorbance verses concentration for phytic acid



Appendix 6: Ethical approval from Makerere University School of Health Sciences

MAKERERE UNIVERSITY
P.O. Box 7072 Kampala, Uganda
E-mail: healthsciences.irb@gmail.com
deanshs@chs.mak.ac.ug
Tel: 256 0200903786
Tel: 256 414 531533
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COLLEGE OF HEALTH SCIENCES
SCHOOL OF HEALTH SCIENCES
OFFICE OF THE DEAN

26/07/2020

To: Peter Rukundo

Kyambogo University
0782425076

Type: Initial Review

Re: MAKSHSREC-2020-8: Next Generation Nutrition in Uganda: Enhancing Dietary Intake of Iron and Folic Acid in a Culturally Acceptable Food Product for Pregnant Women to Improve Maternal and Child Outcomes, Revised July 2020, 2020-07-22

I am pleased to inform you that at the **73rd** convened meeting on **16/06/2020**, the Makerere University School of Health Sciences REC, committee meeting, etc voted to approve the above referenced application.
Approval of the research is for the period of **26/07/2020** to **26/07/2021.**

As Principal Investigator of the research, you are responsible for fulfilling the following requirements of approval:

1. All co-investigators must be kept informed of the status of the research.
2. Changes, amendments, and addenda to the protocol or the consent form must be submitted to the REC for re-review and approval **prior** to the activation of the changes.
3. Reports of unanticipated problems involving risks to participants or any new information which could change the risk benefit: ratio must be submitted to the REC.
4. Only approved consent forms are to be used in the enrollment of participants. All consent forms signed by participants and/or witnesses should be retained on file. The REC may conduct audits of all study records, and consent documentation may be part of such audits.
5. Continuing review application must be submitted to the REC **eight weeks** prior to the expiration date of **26/07/2021** in order to continue the study beyond the approved period. Failure to submit a continuing review application in a timely fashion may result in suspension or termination of the study.
6. The REC application number assigned to the research should be cited in any correspondence with the REC of record.
7. You are required to register the research protocol with the Uganda National Council for Science and Technology (UNCST) for final clearance to undertake the study in Uganda.

The following is the list of all documents approved in this application by Makerere University School of Health Sciences REC:

No.	Document Title	Language	Version Number	Version Date
1	Protocol	English	Revised July 2020	2020-07-22
2	Data collection tools	Luganda	1	2020-05-21
3	Informed Consent forms	Luganda	1	2020-05-21

Yours Sincerely

Kalidi Rajab
For: Makerere University School of Health Sciences REC

MAKERERE UNIVERSITY
SCHOOL OF HEALTH SCIENCES
APPROVED
VALID UNTIL
26 JUL 2021
RESEARCH & ETHICS COMMITTEE
P. O. BOX 7072, KAMPALA

Appendix 7: Ethical approval from Uganda National Council for Science and Technology

Uganda National Council for Science and Technology

(Established by Act of Parliament of the Republic of Uganda)

Our Ref: HS828ES **18 November 2020**

Peter Rukundo
KYAMBOGO UNIVERSITY
Kampala

Re: Research Approval: **Next Generation Nutrition in Uganda: Enhancing Dietary Intake of Iron and Folic Acid in a Culturally Acceptable Food Product for Pregnant Women to Improve Maternal and Child Outcomes**

I am pleased to inform you that on **18/11/2020**, the Uganda National Council for Science and Technology (UNCST) approved the above referenced research project. The Approval of the research project is for the period of **18/11/2020** to **18/11/2021**.

Your research registration number with the UNCST is **HS828ES**. Please, cite this number in all your future correspondences with UNCST in respect of the above research project. As the Principal Investigator of the research project, you are responsible for fulfilling the following requirements of approval:

1. Keeping all co-investigators informed of the status of the research.
2. Submitting all changes, amendments, and addenda to the research protocol or the consent form (where applicable) to the designated Research Ethics Committee (REC) or Lead Agency for re-review and approval **prior** to the activation of the changes. UNCST must be notified of the approved changes within five working days.
3. For clinical trials, all serious adverse events must be reported promptly to the designated local REC for review with copies to the National Drug Authority and a notification to the UNCST.
4. Unanticipated problems involving risks to research participants or other must be reported promptly to the UNCST. New information that becomes available which could change the risk/benefit ratio must be submitted promptly for UNCST notification after review by the REC.
5. Only approved study procedures are to be implemented. The UNCST may conduct impromptu audits of all study records.
6. An annual progress report and approval letter of continuation from the REC must be submitted electronically to UNCST. Failure to do so may result in termination of the research project.

Please note that this approval includes all study related tools submitted as part of the application as shown below:

No.	Document Title	Language	Version Number	Version Date
1	Data collection tools	Luganda	1	21 May 2020
2	Informed Consent forms	Luganda	1	21 May 2020
3	Project Proposal	English	REVISED JULY 2020	
4	Approval Letter	English	REVISED JULY 2020	2020-07-22
5	Administrative Clearance	English	REVISED JULY 2020	2020-07-22
5	Letter from Naguru Hospital to ES UNCST	English	2020	07 February 2020
6	Partnership letter from MSU to VC KyU	English	Letter 2019	15 June 2019
7	Rs 6 updated_Peter Rukundo	English	2020	18 November 2020
8	RS 6 updated Lorraine	English	2020	18 November 2020

Yours Sincerely

Hellen Opolot
For: Executive Secretary
UGANDA NATIONAL COUNCIL FOR SCIENCE AND TECHNOLOGY

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