# EFFECT OF POST-HARVEST HANDLING ON MYCOTOXIN LEVELS IN SOYBEANS FROM RWANDA AND PROCESSING EFFECTS ON NUTRITIONAL VALUE AND ACCEPTABILITY OF SOYMILK

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# Effect of post-harvest handling on mycotoxin levels in soybeans from Rwanda and processing effects on nutritional value and acceptability of soymilk

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## DECLARATION

This Thesis is my original work and has not been presented for a degree in any other University

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## DEDICATION

To my mother Ngendabanka Modesta, my husband Mpumuje Callixte, my sons Mucyo Evrard, Shingiro Ghislain, Sangwa Deus Dedit and my daughters Sugira Bellinda and Sangano Modesta, for their constant support and encouragement.

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Glory to God

## TABLE OF CONTENTS

## LIST OF TABLES

## LIST OF FIGURES

## LIST OF PLATES

## LIST OF APPENDICES

## LIST OF ACRONYMS

| | |
|---|---|
| **AAS** | Atomic absorption spectrophotometer |
| **ADON** | Acetyldeoxynivalenol |
| **AF** | Aflatoxins |
| **ANOVA** | Analysis of Variance |
| **AOAC** | Association of Official Analytical Chemists |
| **AOH** | Alternariol |
| **BCCM-LMG** | Belgian Coordinated Collections of Microorganisms-Laboratory of Microbiology Gent, Belgium |
| **BecA-ILRI** | Biosciences eastern and central Africa-International Livestock Research Institute |
| **BS** | Bovine Serum Albumin |
| **CFU** | Colony Forming Units |
| **CIP** | Crops Intensification Program |
| **CO2** | Carbone dioxide |
| **DAS** | Diacetoxyscirpenol |
| **DOM** | Deepoxy-deoxynivalenol |
| **DON** | Deoxynivalenol |
| **DPPH** | 1, 1-diphenyl-2-picrylhydrazyl |
| **EFSA** | European Food Safety Authority |
| **ELISA** | Enzyme-Linked Immune Sorbent Assay |
| **EOs** | Essential Oils |
| **EC** | European Commission |
| **EU** | European Union |

| | |
|---|---|
| **FAO** | Food and Agriculture Organization |
| **FB** | Fumonisins |
| **FC** | Folin Ciocalteu |
| **FSM** | Fermented soymilk |
| **FUS-X** | Fusarenon X |
| **GAE** | Gallic acid equivalents |
| **GC-MS** | Gas chromatography - Mass spectrometer |
| **GPS** | Global Positioning System |
| **HIV/AIDS** | Human Immunodeficiency Virus/Acquired Immunodeficiency Syndrome |
| **HNO3** | Nitric acid |
| **HPLC** | High Performance Liquid Chromatography |
| **IBM** | International Business Machines |
| **LSD** | Least significant difference |
| **LAB** | lactic acid bacteria |
| **LC-MS** | Liquid chromatography-mass spectrometry |
| **LOD** | Limit of detection |
| **MIC** | Minimum inhibitory concentration |
| **MRS** | De Man, Rogosa and Sharpe |
| **MT** | Metric Tones |
| **NaCl** | Sodium chloride |
| **NaHCO3** | Sodium hydrogen carbonate/ bicarbonate of soda |
| **NaOH** | Sodium hydroxide |
| **NEO** | Neosolaniol |

| | |
|---|---|
| **NGFA** | National Grain and Feed Association |
| **NIST** | National Institute of Standards and Technology |
| **NIV** | Nivalenol |
| **NSRL** | National Soybean Research Laboratory |
| **OCRI** | Oil Crops Research Institute |
| **OD** | Optical Density |
| **OTA** | Ochratoxin |
| **PACA** | Partnership for Aflatoxin Control in Africa |
| **PDA** | Potatoes Dextrose Agar |
| **PH** | Potential of Hydrogen |
| **RAB** | Rwanda Agriculture and Animal Resources Development Board |
| **RCF** | Relative Centrifugal Forces |
| **RDB** | Rwanda Development Board |
| **ROQ-C** | Roquefortin C |
| **SD** | Standard deviation |
| **SF** | Soybean flour |
| **SM** | Soymilk |
| **S/N** | Signal to Noise |
| **SPSS** | Statistical Package for the Social Sciences |
| **TE** | Trolox equivalent |
| **TPC** | Total phenolic compound content |
| **USA** | United States of America |
| **UV-VIS** | Ultraviolet and visible |

| | |
|---|---|
| **WHO** | World Health Organization |
| **ZAN** | Zearalanone |
| **ZEN** | Zearalenone |

**ABSTRACT**

Soybean is considered to be a critical food and nutritional security crop in Rwanda. Soybean is largely consumed in such processed forms as soymilk and soy bean curd. One of the hindrances to increased soymilk consumption is the development of an unpleasant beany flavour due to oxidation of soybean lipids by soybean lipoxygenase. Several methods have been reported for processing soymilk, but their effects on nutritional and phytochemical quality are not well established. Other problems associated with soymilk consumption include the occurrence of flatulence in some individuals, due to the fermentation of soybean oligosaccharides by gut bacteria, and the potential susceptibility of soybeans to mycotoxin contamination. Thus, the objectives of this study were to determine (i) the level of mycotoxin contamination in soybeans from different production regions in Rwanda, and the influence of postharvest handling methods on the mycotoxin levels (ii) the effect of three common processing methods on nutrient and isoflavone content of soymilk (iii) the growth of different probiotic bacteria in soymilk and their effect on antioxidant activity and oligosaccharides content and (iv) compounds of five essential oils from cinnamon, basil, citronella, mint and eucalyptus by GC-MS and their effect on fungal growth and sensory acceptability of soymilk. Soybean samples were collected from farmers (n=300). The farmers also completed questionnaires about their preharvest farm practices, post-harvest farm practices, and aflatoxin awareness. Aflatoxin content in the soybean samples was determined by enzyme-linked immune sorbent assay (ELISA). Detection and quantification of other mycotoxins was done by liquid chromatography-mass spectrometry (LC-MS). Only 7.3 % of the respondents were found to have some knowledge of aflatoxin contamination, indicating a low level of awareness among the farmers. Despite the low levels of awareness on mycotoxins, the farmers were found to observe good postharvest practices including harvesting the crop when the pods were dry. Consistent with this, there was low contamination of the soybeans with aflatoxins and other mycotoxins. Only one sample (0.33%) had an aflatoxin concentration that exceeded the most stringent EU maximum permitted limit of 4 µg/kg. Moreover, apart from one sample contained 13µg/kg of sterigmatocystine, the rest of the samples had very low or undetectable levels of other mycotoxins. From these results, it can be concluded that, based on continued good pre- and postharvest practices, soybean can be promoted as a food product with low risk for mycotoxin contamination in Rwanda. The methods of processing involved (i) soaking soybeans (M1), (ii) blanching soybeans (M2) and (iii) soaking and cooking soybeans prior to soymilk extraction (M3). M1 was found to produce soymilk with significantly higher nutrient and isoflavone extraction than M2 and M3, and can be recommended for soymilk production. Sc. Squire variety has advantage of higher isoflavone content than the other varieties. The growth of seven probiotic lactic acid bacteria (LAB) attained around 8 log CFU/ml, which is sufficient for probiotic effects. However, only *L. reuteri*, *L. brevis* and *L. plantarum* induced a sufficient drop in pH and imparted a viscosity increase characteristic of a good fermented product. Fermentation with these LABS reduced the oligosaccharide content, and may therefore reduce the flatulence associated with unfermented soymilk. The volatile flavor components of the essential oils of five spices, namely mint, basil, cinnamon, eucalyptus and citronella were analyzed by gas chromatography-mass spectrometry(GC-MS).The Minimum inhibitory concentrations (MICs) of the essential oils were tested on the fungus. Cinnamon,

with MIC of ≤0.1 µl/ml was the most effective at inhibiting fungal growth, while eucalyptus, with MIC >>10 µl/ml was the least effective. Sensory evaluation of soymilk flavored with the essential oils of citronella, basil and mint at different concentrations was done. The mint-flavored soymilk had the most preferred flavor, followed by citronella- and basil-flavored soymilk. Thus, essential oils can be used for improving acceptability of soymilk. In conclusion, soybean grown in Rwanda was found to be low in mycotoxin contamination. However, there is a need to educate farmers on mycotoxin contamination in food and feed. Soymilk extraction after soaking but not heating soybeans affords better nutrient and isoflavone extraction than methods involving heating before extraction, and soymilk quality can be improved by fermentation or the use of essential oils such as mint, citronella and basil. Breeders should develop varieties of high yield of soymilk and high nutrients contents for value-added soybean milk both at the household level and industrial level.

# CHAPTER ONE

# INTRODUCTION

## 1.1 Background

Soybean (Glycine max L.), one of the most important food legumes, originated in China before 2500BC (Iruhvwu, 2010). In the Western world, it gained importance in the 19th century, as a source of dietary oil and protein (Iruhvwu, 2010). The USA, which produced 106.93 million metric tons (MT) of soybean in 2016, is the leading producer (Statista, 2018). Soybean production in Africa remains dismal as compared to the US and other leading producers. For example, only 2.1 million metric tons was produced in 2016 in Africa, (FAOSTAT, 2018, May 6). The annual soybean production in Rwanda is estimated at 57,089MT (RDB, 2015) which is cultivated in approximately 42,160 ha of land (RDB, 2015). In terms of area under soybean cultivation, Rwanda ranks sixth in Africa after Zimbabwe, Malawi, Uganda, South Africa and Nigeria (RDB, 2015). The Government of Rwanda has recognized the importance of soybean as a food and nutritional security crop, and earmarked it for increased production under the Crop Intensification Program (Kathiresan, 2012)

Soybean is remarkable in its nutritional and health benefits due to their high content of high quality protein and isoflavones (Jooyandeh, 2011) (Messina & Barnes, 1991). The soybean proteins and isoflavones have been shown to have various health benefits such as reducing the risks for obesity, lowering serum cholesterol, insulin resistance, cardiovascular diseases, osteoporosis and alleviate menopausal symptoms (Alekel, et al., 2000). Soy isoflavones have antioxidant and estrogen-like activity, and high phytoestrogen consumption is associated with low rate of breast cancer in countries like Japan (Lahmann, et al., 2012) (Rassem, Nour, & Yunus, 2016). Reduction of plasma cholesterol is an important intervention against the development of cardiovascular disease, and, from clinical studies, a daily intake of 25 g of soy protein is recommended for adequate effect (Vij, Hati, & Yadav, 2011). Protein-energy malnutrition continues to be a serious nutritional challenge in developing countries. Soybean products were found to effectively improve weight gain, with a monthly weight gain of 0.9 Kg, and to reverse severe malnutrition in

children within three months of intervention (Niyibituronsa, M., Kyallo, Mugo, & Gaidashova, 2014)

Mycotoxin, and especially aflatoxin contamination of cereals and legumes is a serious problem especially in the developing countries (Wild, Miller, Groopman, & D., 2015). For example, deaths due to acute aflatoxicosis occur from time to time due to consumption of contaminated maize or peanuts (Feed the Future, 2012).Lower levels of mycotoxins that may not cause acute effects are also harmful in that they may contribute to the development of chronic health complications, such as eventual induction of liver carcinogenesis by aflatoxins (Wild & Gong, 2010). Thus, although soybean should be promoted due to its nutritional and health promotion potential, such benefits may be negated by mycotoxin contamination.

Reports of the susceptibility of soybean to contamination with mycotoxins have been contradictory. On the one hand, it has been suggested that soybeans are susceptible to growth of molds and contamination with mycotoxins such as ochratoxins, aflatoxins, trichothecenes and cytochalasins (Rodrigues & Naehrer , 2012); (Gallardo, 2008). On the other hand, it has been reported that soybeans are not good substrates for the growth of aflatoxin-producing molds, and this was suggested to be due to the binding of zinc (an important factor for aflatoxin production) by phytates in soybeans (Dharmaputra, 2002). Aflatoxins are produced by molds of the species *Aspergillus flavus* and *Aspergillus parasiticus*, and they have been shown to cause liver damage and cancer (Dohlman, 2003); (Isara-Lyon, 2012). Aflatoxin consumption is also associated with immunity deficiency and malnutrition-related disorders such as stunting (Feed the Future, 2012). Therefore, alongside efforts to increase soybean production and consumption, there should be reliable data generation and documentation on the occurrence and extent of mycotoxin contamination in soybean and soybean products.

Unlike other beans, soybean is not commonly served as cooked whole beans because they are generally hard to cook. Thus, they are processed into products such as soy flour, soymilk, soy beverage powder, or bean curds. Soymilk is considered as an alternative to cow's milk, especially for lactose intolerant individuals or for people trying to reduce the risk for developing non-communicable diseases (Sumarna,

2008). Soybean contains oil rich in the polyunsaturated fatty acid i.e. linoleic and small amounts of linolenic acid. The soybean lipoxygenase-catalyzed oxidation of these fatty acids during processing is associated with the beany flavors responsible for reduced consumer acceptability of soymilk (Zhang, Guo, Liu, & Chang, 2012). Various processing methods have been developed to reduce the soybean beany odor (Zhang, Guo, Liu, & Chang, 2012), but the effects of such processing on the nutritional or phytochemical quality of the products are not well established.

Soymilk fermentation is potentially a good method for improving soymilk quality in terms of flavor, reduction of flatulence-causing oligosaccharides, and improving the antioxidant activity. However, fermentation with different microorganisms is expected to produce different results on different quality aspects. The use of probiotic bacteria may further improve the health benefits of soymilk since such bacteria inhibit growth of pathogens and produce metabolites, such as short chain fatty acids, which reduce the risk for non-communicable diseases (Sumarna, 2008) (Chien, Yang, & C., 2013). However, there is limited information available on the use of probiotic bacteria in soymilk. Essential oils have been used to add flavor and to extend shelf life of various food products (Hyldgaard, Mygind, & Meyer, 2012), and could be useful in improving soymilk flavor. However, not much has been reported on their use in soymilk.

### 1.2 Problem statement

Aflatoxin, which is produced by Aspergillus flavus and Aspergillus parasiticus, is a mycotoxin of great public health concern, particularly in developing countries including Rwanda. These mycotoxins have been found to cause liver damage and liver cancer, recurrent infections due to immunity suppression and malnutrition (Grace, et al., 2015). However, no study has been conducted to determine the levels of aflatoxin contamination of soybeans grown and consumed in Rwanda. Such investigation is needed for preventive measures.

Although soymilk is promoted as a nutritious and health-promoting product, its consumer acceptability is negatively affected by an unpleasant beany flavor due to the enzymatic activity of soybean lipoxygenase, and the presence of flatulence-causing oligosaccharides. Although some processing methods (like cooking

soybeans before extraction of soymilk) that reduce the beany flavor have been reported, these do not achieve complete elimination of the off flavor. Moreover, the effects of such methods on nutritional quality are not well known, thus the study was needed.

### 1.3 Justification

Approximately 5 million people annually are exposed to mycotoxins through contaminated food, and many studies have demonstrated a strong relationship between aflatoxins and adverse health effects such as acute toxicity, liver cancer, immunodeficiency and growth impairment (WHO, 2005); (Krishnamachari, Bhat, Nagarajan, & Tilak, 1975); (Wang, Dou, Macura, Durance, & Nakai, 1997), (Feed the Future, 2012). Mycotoxin contamination of soybean grown in Rwanda and soybean products processed from it has never been determined and documented. This needs to be investigated for awareness of aflatoxin, postharvest handling and food safety. There is need to conduct research on aflatoxins to connect agriculture, health and nutrition outcomes (Grace, et al., 2015)

In Rwanda soybean is one of the crops targeted for increased production in the Crop Intensification Program, (Kathiresan, 2012). Its production increased from 13.922 Metric Tons (MT) in 2000 to 57.100MT in 2010 (RDB, 2015), but decreased to 37.426MT in 2011 and further to 23.934MT in 2017 (Factfish, 2019, February 6). Such decreases in soybean production have been attributed both to the scarcity of high yielding cultivars, and the low sensory acceptability of some soy products, such as soymilk due to beany flavor (Mugabo, Tollens, Chianu, Obi, & Vanlauwe, 2014); (Nsengiyumva, Byamushana, & Rurangwa, 2017); (RDB, 2015), (Niyibituronsa, M., Kyallo, Mugo, & Gaidashova, 2014). Thus the potential use of essential oils from citronella, cinnamon, eucalyptus, mint and basil as a flavoring agent was deemed to be of significance. Moreover, since essential oils have been reported to have some antifungal activity (Hyldgaard, Mygind, & Meyer, 2012), the use of such oils in soybean products may have the double benefit of flavor improvement and safety enhancement. Because of variability in chemical compositions of essential oils based on genetic and environmental factors, the chemical composition of the tested essential oils was determined.

Probiotic bacteria are rapidly gaining acceptance for their health benefits such as suppressing the growth of pathogenic bacteria and thus preventing gastrointestinal infections (Horackova, Muhlhansova, Slukova, Schulzova, & Plockova, 2015).They also produce metabolites such as short chain fatty acids that positively influence physiological processes, such as prevention of certain forms of carcinogenesis, insulin resistance, and diabetes. The use of probiotic bacteria for the fermentation of soybean products has the advantage of not just improving the acceptability and benefits of soybean (Boye & Ribereau, 2011), but also imparting health benefits that are directly due to the probiotic microorganisms. Thus, various probiotic bacteria were tested for their growth in soymilk and their influence on soymilk composition.

## 1.4 Objectives

### 1.4.1 Main objective

The main objective of the study was to determine the level of mycotoxin contamination in soybeans grown in Rwanda and the effects of different processing methods and essential oils on soymilk quality

### 1.4.2 Specific objectives

1. To document the major soybean postharvest handling methods used by the farmers and to determine the level of mycotoxin contamination of soybeans from different production regions in Rwanda
2. To determine the effect of three common processing methods (soaking, blanching and cooking soybeans before soymilk extraction) on nutrient and isoflavone content of soymilk.
3. To determine the growth of different probiotic bacteria in soymilk and their effect on antioxidant activity and oligosaccharides content.
4. To determine the major volatile flavor compounds in five (basil, cinnamon, citronella, eucalyptus and mint) essential oils by GC-MS and their effect on fungal growth and sensory acceptability of soymilk

## 1.5 Research questions

1. Does the production region and postharvest handling practices have any effect on levels of aflatoxin contamination in Rwandese soybeans?

2. Do different processing methods (soaking, blanching and cooking soybeans before soymilk extraction) have any effect on the nutritional composition of soybean milk?
3. Do different probiotic microorganisms grow differently in soymilk?
4. Do soybean fermentation using probiotic bacteria have any effect on antioxidant activity and oligosaccharides content?
5. Do basil, cinnamon, citronella, eucalyptus and mint essential oils compounds have any effect on fungal growth and sensory acceptability of soymilk?

# CHAPTER TWO

# LITERATURE REVIEW

## 2.1 Nutritional importance of soybean

Soybean, (Glycine max (L.) Merrill, which belongs to the family Leguminosae and subfamily Papilionidae, is one of the most important food legumes (Shurtleff & Aoyagi, 2007). It best grows in warm climates, at temperatures close to 25°C and rainfall of 500-900mm, and, depending on variety, can be harvested 120-130 days after sowing (Dugje, et al., 2009). In 2017/2018, a total of 336.7 million metric tons of soybean were produced globally, of which the leading producer, the US, contributed 119.52 million metric tons (USDA, 2008). Compared to this, 2.1 million metric tons was produced in 2016 in Africa (FAOSTAT, 2018, May 6).

Soybean seed is a highly nutritious food that contains approximately 40% protein, 40% carbohydrate (including fiber), 20% fats, and 5% ash (Mateos-Aparico et al, 2018). It is also a good source of minerals like calcium (276mg/100g), magnesium (280 mg/100g), potassium (1797 mg/100g), iron (16mg/100g) and zinc (4.8 mg/100g) (Mateos-Aparicio, Cuenca, Villanueva-Suárez, & Zapata-Revilla, 2008).

Its protein is of high quality, having a balance of essential amino acids that is close to animal proteins, and as such is good for the growth of children and for management of diseases such as HIV/AIDS and tuberculosis (Ghandi, 2009). Soybean is also a rich source of isoflavones which are connected with prevention of various physiological disorders and non-communicable diseases (Jooyandeh, 2011); (Hirose, et al., 2005); (Messina M. J., 1999); (Xu, Harris, Wang, Murphy, & Hendrich, 1995).

Soybean is neither cooked nor eaten like other beans, but is rather processed into different products for improved palatability and nutritional gain. It is mostly processed into soy flour, soymilk and tofu, in Rwanda, (Niyibituronsa, M., Kyallo, Mugo, & Gaidashova, 2014).

## 2.2 Mycotoxins in soybeans

Mycotoxins (from “myco” fungus and toxin) are relatively low-molecular weight, fungal secondary metabolic products that may cause harm to human health once exposed to the toxins (Barros et al., 2011). Mycotoxin contamination is a serious food safety concern for cereal grains and legumes (Wild & Gong, 2010). Since the mycotoxins are formed both pre-harvest and post-harvest under natural environmental conditions, their total eradication is very difficult (Milani, 2013).The common mycotoxins that pose the greatest health concern are aflatoxins, ochratoxin, fumonisins, zearalenone and deoxynivalenol. Aflatoxins are produced by *Aspergillus flavus*, *A. parasiticus* and *A. nomius* (Bhatnagar, Cary, Ehrlich, Yu, & Cleveland, 2006); (Gallardo, 2008); (Wild & Gong, 2010), and contaminate many types of produce including cereals, nuts, dried fruit, legumes, fruits, spices, and oil seeds (Filazi & Sireli, 2013).

There is evidence that aflatoxins and fumonisins cause liver damage, including carcinogenesis (Dohlman, 2003); (Wild, Miller, Groopman, & D., 2015)). The populations with the highest aflatoxin exposures are mainly in Sub-Saharan Africa, Southeast Asia, and China, and the contribution of aflatoxins to liver damage ranges between 4.6% and 28.2% (Feed the Future, 2012). Other health problems associated with the consumption of aflatoxins include acute aflatoxicosis, immune deficiency, and malnutrition-related disorders such as stunting (Wild, Miller, Groopman, & D., 2015) (Feed the Future, 2012). Periodic episodes of acute aflatoxicosis and related deaths have been reported in the literature, in countries such as India and Kenya (Krishnamachari, Bhat, Nagarajan, & Tilak, 1975); (Probst, Njapau, & Cotty, 2007). In Kenya, 317 deaths from aflatoxicosis were reported in 2004 (Feed the Future, 2012) .In Tanzania, 14 deaths from acute aflatoxin poisoning were reported in 2016 (Buguzi, 2016). Aflatoxins readily accumulate in produce stored under conditions that promote post-harvest fungal growth, but the initial contamination can start pre-harvest after crop maturity, under warm and humid conditions (Wild & Gong, 2010); (Milani, 2013); (Pratiwi, et al., 2015). Therefore, awareness creation and capacity building among various stakeholders in agricultural products value chains to

implement good agricultural practices and good manufacturing practices has been proposed as a strategy to lower health risks of mycotoxins in foods (Dohlman, 2003).

The European Commission has set maximum levels for 11 mycotoxins in foods namely, Ochratoxin A (OTA), aflatoxin AFB1, AFB2, AFG1 and AFG2, deoxynivalenol (DON), zearalenone (ZEN), fumonisin (FB1 and FB2), T-2 toxin and HT-2-toxin (EC, 2006) (EC, 2014). However, no such standards have been established for many mycotoxins originating from several fungal genera, and these toxins include fumonisin (FB3), nivalenol (NIV), 3-acetyldeoxynivalenol (3-ADON), 15-acetyldeoxynivalenol (15-ADON), diacetoxyscirpenol (DAS), fusarenone-X (F-X), neosolaniol (NEO), alternariol (AOH), alternariol methyl ether (AME), roquefortine-C (ROQ-C) and sterigmatocystine (STERIG), (Di Mavungu, et al., 2009); (EC, 2006); (Monbaliu, et al., 2009).

Although maximum levels have been set for individual mycotoxins, standards have not been set for mixtures of mycotoxins despite the fact that such mixtures may have additive and/or synergistic effects (Speijers, 2004; Manafi et al., 2012). Therefore, it is important to determine all mycotoxins in any given food sample. Literature on the susceptibility of soybean to contamination with aflatoxin-producing moulds and aflatoxins has been contradictory. For example, (Barros, Ramirez, & Schulze, 2011) reported that soybean is a good medium for the growth of moulds that produce mycotoxins such as aflatoxins and trichothecenes. On the other hand, there are reports that soybean is less susceptible to mycotoxin contamination than other food and feedstuffs (Valenta & Bluthgen, 2002). To-date no data has been published on the level of mycotoxin in soybeans in Rwanda, although the consumption of this crop by adults and as a weaning food by Rwandese is on the increase. Such information is necessary for people to know the overall benefits of soybean, including its safety, rather than just the perception that it is a healthy and nutritious food.

### 2.3 Soymilk processing methods

Soymilk is one of the major soybean products in Rwanda and other countries. It is regarded as a cow's milk substitute for lactose-intolerant people (Kundu, Dhankhar,

& Sharma, 2018). Soymilk production involves the water extraction of soybeans by the following fundamental preparation procedures: soybeans selection, water addition, grinding in wet condition, soymilk separation from fiber (okra), cooking to inactivate lipoxygenase and trypsin inhibitors, (Hosken, 1999). Despite its high nutritional value and health-promoting bioactive compounds, the consumption of soymilk is limited by an unpleasant beany odor, which is mainly caused by soybean lipoxygenase-catalysed oxidation of soybean's polyunsaturated fatty acids, namely linoleic acid and alpha-linolenic acid (Wang, Dou, Macura, Durance, & Nakai, 1997) (Zhang, Guo, Liu, & Chang, 2012). One of the most common methods for soymilk preparation involves soaking soybeans overnight followed by milk extraction without any prior heat treatment (Hosken, 1999); (Niyibituronsa, M., Kyallo, Mugo, & Gaidashova, 2014); (Nyagaya, 2008). However, for odor minimization, and to enhance soymilk palatability, various treatment combinations have been developed for lipoxygenase inactivation, involving pH modification and heating, with varying levels of favourable outcome (Kale, Pandhare, Satwase, & Goswami, 2012); (Krishnan & Darly-Kindelspire, 2013); (Yang, Chen, Zhang, Chen, & Liu, 2012). However, the effects of such treatments on nutrient extraction into the soymilk are not well established.

### 2.4. Soymilk fermentation with lactic acid bacteria

Similarly, to cow's milk, soymilk undergoes fermentation with lactic acid bacteria, which is considered to improve certain aspects of soymilk quality. The effect of fermentation on soymilk flavour differs depending on the bacteria used. For example, some studies reported that the beany odor which reduces soymilk palatability (Kumari, et al., 2015); (Min, Yu, Yoo, & Martin, 2005); (Yu, Liu, Hu, & Xu, 2017)) may be reduced by fermentation (Peng & Guo, 2015)

Reduction in the amounts of soymilk oligosaccharides is another potential benefit of soybean fermentation, depending on the microorganisms used (Kaczmarska, Chandra-Hioe, Zabaras, Frank, & Arcot, 2017).

Some individuals find soybean or soymilk consumption undesirable due to the problem of flatulence; which is at least partly due to the presence of raffinose family

oligosaccharides, which are not digested by human enzymes in the small intestines, but are fermented by microorganisms in the colon, leading to gas production (Battistini et al., 2018; Gote et al., 2004). These oligosaccharides, including verbascose, stachyose and raffinose, can be reduced by fermentation (Battistini, et al., 2018); (Gote, Umalkar, Khan, & Khire, 2004); (Kaczmarska, Chandra-Hioe, Zabaras, Frank, & Arcot, 2017)

It has also been reported that fermented soymilk has better antioxidant properties that are suggested to potentially prevent cancer (Jooyandeh, 2011); (Takagi, Kano, & Kaga, 2015); (Telang, Joshi, Sutar, & Thorat, 2010); (Vij, Hati, & Yadav, 2011); (Ziaei & Halaby, 2017). This may be due to increased β-galactosidase activity during fermentation, resulting in the conversion of isoflavone glycosides to aglycones, the latter being the bioactive forms known for their health benefits (Liu, Yang, & Fang, 2018); (Otieno, Ashton, & Shah, 2006); (Tsangalis, Ashton, Stojanovska, Wilcox, & Shah, 2004); (Villares, Rostagno, García-Lafuente, Guillamón, & Martínez, 2011). A study done previously on enhancement of bioactivity of soymilk beverages by fermentation in Argentina reported stronger antioxidant activity by isoflavones extract from fermented soybean milk than from the unfermented one (Marazza, Nazareno, Savoy, Giori, & Garro, 2012). Fermentation also improves the texture and could have some protective effects against intestinal infections (Chien, Yang, & C., 2013); (Shurtleff & Aoyagi, History of Fermented Soymilk and Its Products, in: History of Soybeans and Soyfoods, 1100 B.C. to the 1980s. Soyinfo Center, Lafayette, California., 2004).

Probiotic microorganisms are those microorganisms that when ingested, impart health benefits to the human host. They do this by inhibiting the growth of pathogens, and by producing metabolites in the gut that improve health through various mechanisms (Amutha & Kokila, 2015); (Fuentes, Lajo, Carrión, & Cuñé, 2013). For example, they may produce short chain fatty acids such as butyric and propionic acids. These acids improve glucose metabolism in the body and thus reduce the risk for insulin resistance, diabetes, cardiovascular diseases and even cancer (Amutha & Kokila, 2015). Butyrate also directly acts on colonic epithelial cells to promote apoptosis and prevent the development of colon cancer. Thus, the

health benefits of soymilk may be further enhanced through fermentation with probiotic microorganisms (Fuentes, Lajo, Carrión, & Cuñé, 2013). Bacterial strains have different optimal growth conditions depending on the characteristics of the organism and the culture substrate (Bansal, Mangal, Sharma, Yadav, & Gupta, 2015); (Breed, Murray, & Hitchens, 1948)

Some of the probiotic strains that have been reported to grow in soymilk include *Lactobacillus casei* Zhang, *Bifidobacterium animalis* ssp. lactis V9, *Lactobacillus acidophilus* NCFM, *Lactobacillus rhamnosus* GG, *Bifidobacterium animalis* Bb12, and *Lactobacillus casei* Shirota at 37°C (Li, Yan, Wang, & Zhang, 2012). A study done by (Mishra & Mishra, 2013) showed that the combination of *L. acidophilus* and *Lactobacillus plantarum* was very good in regard to growth of bacteria, counting more than 9 log CFU/ml (Mishra & Mishra, 2013) s. However, there is need to test the growth of more probiotic strains on soymilk from different soybean varieties.

### 2.5 Potential use of essential oils for improvement of soymilk quality

Soybean oil contains the polyunsaturated fatty acids linoleic acid and alpha-linolenic acid, whose enzymatic oxidation during soymilk processing is associated with the beany flavors responsible for reduced consumer acceptability of the milk (Zhang, Guo, Liu, & Chang, 2012). Apart from attempts at reduction of off flavours from soymilk during extraction, other options have been undertaken to remove or mask the beany odor (Kinney, 2003). For example, (Davles, Nlelsen, & Nielsen, 1987)carried a study to remove the off flavor by genetically reducing lipoxygenase content of soybean. Orange juice, pineapple and banana have also been used to improve the taste of soybean milk (Kale, Pandhare, Satwase, & Goswami, 2012); (Laswai, et al., 2009). However, not much has been done on the use of essential oils for improving soybean flavor.

Essential oils are plant active botanical constituents derived from plant materials such as leaves (lemongrass, ocimum, mint), leaves and stems (geranium, patchouli, cinnamon), flowers (rose, mimosa, lavender), seeds (fennel, coriander, nutmeg), fruits (orange, lemon, bergamot), rhizomes (ginger, curcuma, orris), gums (storax, myrrh, balsam of Peru), bark (cinnamon, cassia, canella), roots (vetiver, valerian,

angelica) and wood (cedar, sandal, pine) (Handa, Khanuja, Longo, & Rakesh, 2008). The essential oils are extracted by different methods, such as water distillation, steam distillation, solvent extraction, CO2 extraction, maceration, effleurage, cold press extraction (Handa, Khanuja, Longo, & Rakesh, 2008). The boiling points of essential oils, which consist of mixtures of monoterpenes, sesquiterpenes and their oxygenated products, range from 150°C to 300°C, which is way higher than the boiling point of water, but during water distillation, these essential oils and the boiling water form a biphasic mixture with a boiling point lower than the boiling point of water (Tandon, 2008).

For better yield and quality of the essential oils the temperature should be maintained as low as possible and plant material packed well in the distillation still (Koul et al, 2004). Fresh oils do not have color but with time oxidation may occurs leading to dark color. Thus, they need to be stored in a cool, dry place in a dark glass container and closed tightly (Rassem, Nour, & Yunus, 2016). The essential oils (EOs) are widely used in the food industry as food preservatives (Hyldgaard, Mygind, & Meyer, 2012). Because of an increasing consumer preference for natural products, and the fact that essential oils have antibacterial and antifungal properties, their use as both flavor compounds and preservatives is increasing (Nazzaro, Fratianni, De Martino, Coppola, & De Feo, 2013). The composition of essential oils of any species varies according to genotypes and environmental factors. For example, three different chemotypes were reported for the essential oil of *Ocimum ciliatum* accessions in Iran. Therefore, in utilizing essential oils for improving the flavor of soymilk, it is necessary to characterize the chemical composition of the essential oils to allow for product standardization.

Basil essential oils are used to flavor foods (Özcan & Chalchat, 2011). For flavoring recipes, 1-3 drops of basil essential oil is better than the use of fresh or dried basil (Sustainable Baby Steps , May, 2019). Mint can also be used as flavoring agent in food (Fatih, Madani, Chibane, & Duez, 2017). Essential oils from *Cymbopogon* (Citronella and Lemon grass), an aromatic tropical plant in the family of Poaceae, which gives flavor to recipes including tea, could potentially be used for improving soymilk flavor. Essential oil of Cymbopogon has been obtained by water steam

distillation (Millet, 2015), (Ranitha, Abdurahman, Sulaiman, Nour, & Thana, 2014). Cymbopogon is relatively cheap and available (Laswai, et al., 2009); however, they have not been tested for quantity of extract to be used in soybean milk.

# CHAPTER THREE

# THE METHODOLOGY

## 3.1 Determination of the effect of production region and postharvest handling practices on the level of selected mycotoxins contamination in Rwandan soybeans

### 3.1.1 Sampling

From among the soybean growing regions in Rwanda, two districts were randomly selected from each of the different agro-ecological zones. The selected districts were Kirehe (average altitude 1521 m) and Kayonza (1431 m) in East-Rwanda, Huye (1704 m) and Kamonyi (1662 m) in South-Rwanda, and Nyamasheke (1677 m) and Rusizi (1501 m) in South-West-Rwanda (Figure 1). Within these districts sample collection was done from three types of actors in the soybean value chain, namely households, markets and the Rwanda Agriculture Board stores (RAB), who were paid for the samples. Sampling was done in August 2015 according to Whitaker's guidelines of food sampling for mycotoxin analysis (Whitaker, 2006). Briefly, one kilogram of soybeans was scooped with a cup from the upper, middle and lower parts of the storage bag, and poured into a paper bag (Kaki No14). The sample bags were then transferred to the RAB Rubona cold room (4˚C). When samples were obtained from farmers, a questionnaire was also administered to them regarding pre- and post-harvest farm practices, and aflatoxin awareness (Appendix I).

Sample size calculation was based on the following formula (Dhulkhed, Dhorigol, Mane, Gogate, & Dhulkhed, 2008)

$$n = \frac{(Z_{1/2\alpha} + Z_{(1-1/2\beta)})^2\, p(1-p)}{d^2} = \frac{(1.96+1.282)^2\,(0.4)(0.6)}{0.1^2} = 252 \quad \text{(Equation 1)}$$

The soybean-growing households were estimated to be 40% based on a recent report by (Tukamuhabwa, Kwikiriza, & Ariong, 2016). A type I error probability (alpha 0.05 giving 1.96) for estimating proportions with probability (power) of 0.90 (1.282)

was taken (Dhulkhed, Dhorigol, Mane, Gogate, & Dhulkhed, 2008); (Niyibituronsa, Kyallo, Mugo, & Gaidashova, 2015). The number of samples was adjusted to 300 to cover for possible losses. Thus 300 samples were collected from six districts.

Each bag was labelled with the following information: collection date, source, variety, planting and harvesting time, drying, storage duration, and equipment used for storage, type of pesticides used, and global positioning system (GPS) coordinates. The survey on awareness concerning aflatoxin contamination was conducted in the local language (Kinyarwanda) starting by the knowledge of moulds ("uruhumbu") that produce toxins ("uburozi").

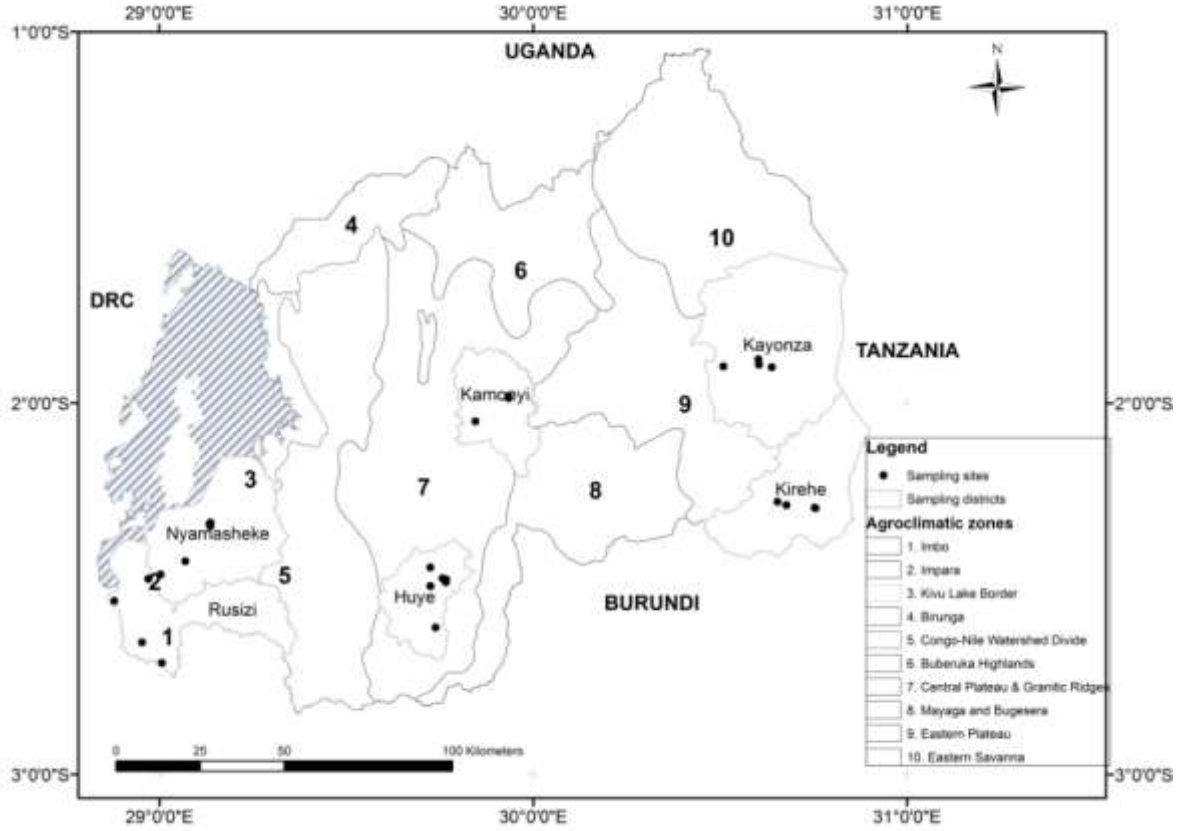


**Figure 3.1: Sampling sites in agro-ecological zones favorable for soybean growth in Rwanda**

One hundred grams from each of the 300 soybean samples was put in a zip-lock bag. The zip-lock bags were packed in a box which was then transferred to the Biosciences Eastern and Central Africa-International Livestock Research Institute (BecA-ILRI) Hub (Nairobi, Kenya) for aflatoxins analysis by enzyme-linked immunosorbent assay (ELISA). The remainder of the 300 soybean samples were mixed per district, and 10 subsamples from three randomly selected districts in the agro-ecological zones (East: Kirehe 2 subsamples, West: Nyamasheke 2, and South: Kamonyi 1 and 5 RAB varieties subsamples) were transported to Bioanalysis Laboratory, Ghent University, Belgium for analysis of multiple mycotoxins using LC-MS. At both BECA Hub and the University of Ghent, samples were stored under refrigeration at 4˚C until analysis.

### 3.1.2 Sample preparation for aflatoxin and multiple mycotoxin analyses

Upon removal from cold storage, samples were kept at room temperature for one hour. A 50 g sub-sample was taken from each sample and ground with a coffee grinder to obtain a fine powder (< 5mm sieve) to facilitate the extraction of mycotoxins.

For multi-mycotoxins analysis, a 200 g of subsample was ground with an IKA® M20 universal mill (Staufen, Germany). To avoid cross-contamination, the grinder was cleaned before milling each sample. The ground sample was transferred to a zip-lock bag, and stored in the cold room at 4˚C.

### 3.1.3 Determination of aflatoxin levels in soybean flour samples by ELISA

Total aflatoxin assay enzyme-linked immunosorbent assay (ELISA, Cat. No. 981AFL01LM-96, Helica Biosystems Inc, Santa Ana, CA 92704, USA) were used for aflatoxins analysis in soybean flour. Five grams of ground soybean (5.0 g) were weighed into 50 mL Falcon tubes. To each Falcon tube was added 25 mL of acetonitrile/water (80/20, v/v) for aflatoxin extraction. After capping, each tube was placed into an orbital shaker set at 6 relative centrifugal forces (RCF), and the shaking was continued for 5 minutes at 25°C. The samples were subsequently left to stand for 15 minutes to allow settling of the solids. An aliquot of 100 µL was drawn

from the liquid phase, diluted with 900 µL of reconstituted wash buffer in 2.0 mL tubes, and subsequently vortexed for 5 seconds. The ELISA kit consisted of a 96-wells micro titer plate coated with antibody that would bind to aflatoxins, if present. The analysis was done following the Assay kit manufacturer's instructions. A random sample was analysed twice for each plate to measure the accuracy of the data generated. The optical density of samples and standards were read on a microtiter plate reader set at a wavelength of 450nm. The optical density (OD) reading of each microwell was recorded by computer. Aflatoxin concentrations were calculated as µg/kg from the logit regression equation generated in the standard ODs, with r2>0.98. The limit of detection (LOD) was calculated for each plate in the matrix of standards at 0.02, 0.05, 0.1, 0.2 and 0.4µg/kg. The recovery of 5µg/kg spiked soybean samples (n=3) extracted with acetonitrile/water (80/20, v/v) was 4.6µg/kg (92%) in four independent experiments (Helica, 2007).

### 3.1.4 Determination of multi-mycotoxins levels in soybean flour samples by Liquid Chromatography-Mass Spectrometry

All reagents used were of analytical grade. Acetic acid was obtained from Merck. Methanol and HPLC-R were from and Biosolve. n-Hexane Hipersolv Chromanoform for HPLC was obtained from VWR International. Mycotoxin standards were from VWR International.

A quantitative LC-MS method used to determine the levels of multiple mycotoxins in ground soybean samples was performed according to the method of (Monbaliu, et al., 2009). Five grams (5g) of sample was weighed with an analytical balance (Sartorius, Goettingen, Germany). The sample was placed into a 50 mL extraction tube. To the extraction tube containing soybean sample was added 20 mL of acetonitrile/water/acetic acid (79/20/1, v/v/v) as the extraction solvent. After wrapping the extraction tubes with aluminium foil, they were agitated in a shaker for one hour. Then they were centrifuged for 15 minutes at 3300 relative centrifugal force (RCF). Next, the supernatants were purified on a C18 column (500 mg/6 mL, Grace, Alltech, Columbia, USA) installed on a vacuum elution manifold. The eluent was collected in volumetric flasks. The eluent was defatted according to the

procedure of (Monbaliu, et al., 2009). From the defatted extract, a 12.5ml was drawn, mixed with 27.5ml of acetonitrile/acetic acid (99/1, v/v), and cleaned up through a MultiSep®226-column (Romer Labs, Tulln, Austria). Another 5ml portion of the remaining defatted extract was filtered through a folded glass filter on a plastic tube of 10ml. Two milliliters was added to the cleaned eluent (to avoid loss of some compounds like fumonisins that could remain in the column). This was followed by evaporation until complete dryness at 40°C under a gentle nitrogen flow. The mobile phase solution (150 µL) was added to dissolve the residue. The filtrate was transferred into a vial for subsequent analysis by LC-MS.

Soybean samples spiked with different concentrations of mycotoxin standards were also prepared prior to extraction as already described for the unspiked sample. Spiking was done at 0.5 X µg/kg, X µg/kg, 1.5 X µg/kg, 2 X µg/kg), where X was equal to the cut-off level of the mycotoxin. This was used for drawing a calibration curve. Internal standards were added, namely 100 µL of zearalenone (ZAN) and 25 µL of deoxynivalenol (DOM).

The HPLC-MS analysis was done on an Acquity HPLC-Quattro Premier (Waters, Milford, USA). Four identification criteria were used to confirm mycotoxin identity by LC-MS, namely (i) appearance of two selected fragment ions (ii) a signal to noise ratio >3 (iii) a relative retention time of ±2.5% and (iv) a relative peak area of ±25%. More details on the used LC-MS are reported in (Monbaliu, et al., 2009) and other studies, which emphasize the importance of clean-up and defatting for elimination of interfering compounds (Monbaliu, et al., 2009); (Gilbert & Anklam, 2002); (Rahmani, Jinap, & Soleimany, 2009).

### 3.1.5 Quality control and data validation

At the end of a series of LC-MS analyses, a reinjection of the cut-off (middle standard of calibration curve) was done to confirm check recovery of the LC-MS. Moreover, the extraction, purification and analysis of a blank sample spiked at cut-off level were done to check the recovery of the complete analysis. To evaluate data precision, two wheat control samples were used during analysis. In-house validation data are detailed in (Chilaka, 2017).

### 3.1.6 Data analysis

SPSS 16.0 and Excel software's were used for data analysis. Findings could not be tested for statistical significance by ANOVA due to the low percentage of positive samples.

## 3.2 Determination of the effect of different processing methods on nutrient and isoflavone content of soymilk

### 3.2.1 Selection of soybean varieties

The widely cultivated variety of soybean, Peka 6 and unknown locally grown variety in Western province-Rwanda were among the six varieties (Figure 2) selected for this study in addition to four varieties that has been promoted by the Rwanda Agriculture Board (RAB). SB 24, Sc. Saga, Sc. Sequel and Sc. Squire are the four varieties which are considered to be better than Peka 6 in terms of yield per unit area, early maturity and resistance against diseases such a rust, Read leaf blotch and frog eye leaf spot (RDB, 2015). According to RAB-N2Africa report (2013), Peka 6 originated from India, SB24 from Nigeria, Sc. Saga, Sc. Squire and Sc. Sequel from Zimbabwe and the grain yield (kg/ha) in 2013 was 2406, 2700, 3264, 3278 and 2128, respectively (N2Africa, 2014).

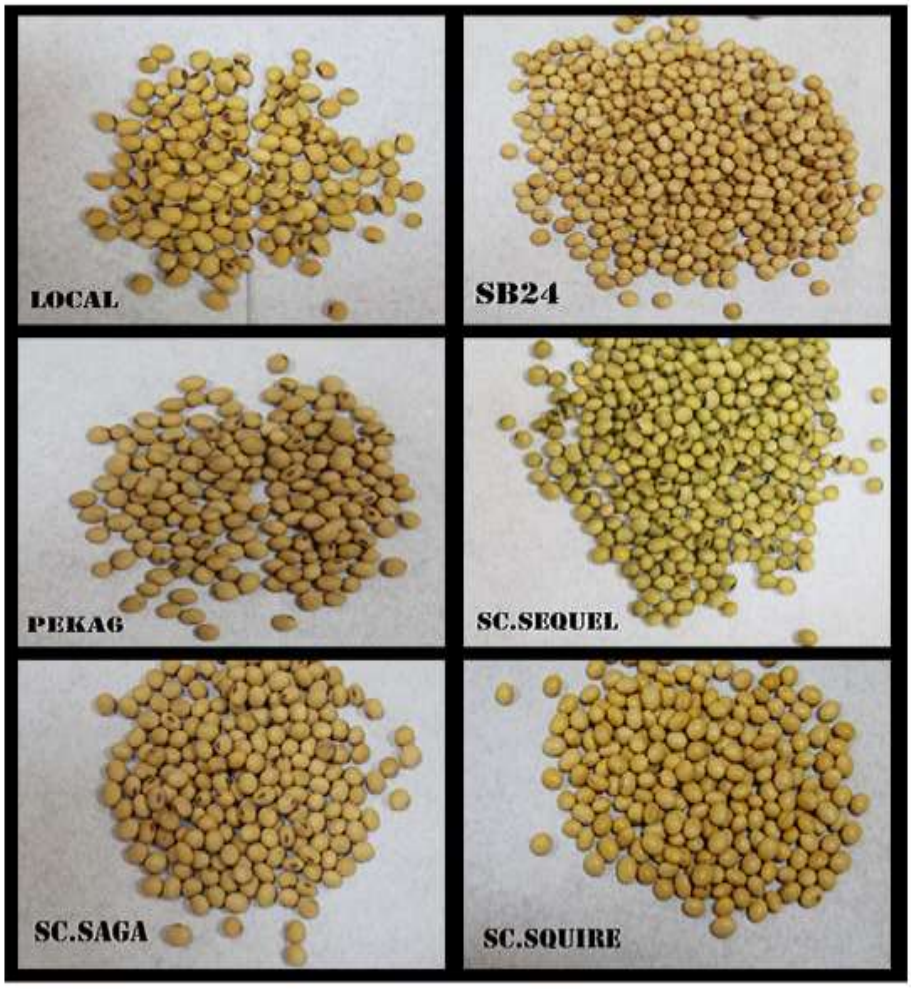


**Figure 3.2: Soybean varieties used for nutrients analysis and soybean milk extraction**

### 3.2.2 Sampling

Peka 6, SB 24, Sc. Saga, Sc. Sequel and Sc. Squire samples were obtained from RAB stores in Southern and Eastern provinces while the unknown local variety was obtained from farmers in the Western province where there is no RAB soybean program. Samples, 1kg of each variety, were collected in triplicates and stored in a cold room at 4˚C.

### 3.2.3 Soymilk preparation

Three different methods were used to prepare soymilk. The first method (M1) involved soaking the soybeans prior to extraction (M1), the second method (M2)

involved blanching in alkaline solution prior to extraction (M2), while the third method (M3) involved soaking in alkaline solution followed by cooking prior to extraction. In M1, 25g of soybeans were soaked in 100 ml of tap water for 12 hours at room temperature (mean temperature 25°C) (Hosken, 1999); (Nyagaya, 2008). After draining the soaking water and rinsing with cold water, the soybeans were ground in 200 ml of water by a warring laboratory electric blender (HGBTWTG4, USA). Subsequently, the mixture was filtered through a muslin cloth to obtain the soymilk as the filtrate. The soymilk was then boiled for 10 minutes in a beaker (to make it safe for consumption).

In M2, 25g of soybeans and 0.05g of NaHCO3 in 100 ml of water was blanched at 100˚C in a beaker for 5 minutes. This was followed by draining of the alkaline water and rinsing twice with hot water. Next, the beans were ground for 3 minutes in a warring blender with 200 ml of water at 80˚C temperature, according to the method of the National Soybean Research Laboratory at Illinois University (2015). The mixture was filtered with a muslin cloth and then the filtrate was boiled in a beaker for 10 minutes.

In M3, of 25g of soybeans was soaked in 5% NaHCO3 at 25˚C for a duration of 16 hours. After draining off the alkaline water, the beans were cooked at 100°C for a duration of 20 minutes. Subsequently, the beans were ground with 200ml of boiled water, which was followed by further cooking of the mixture at a temperature of 90°C for 10 minutes, homogenization, and separation of insoluble and soluble portion using muslin cloth (Kale, Pandhare, Satwase, & Goswami, 2012). The soymilk was stored in a cold room 4°C.

### 3.2.4 Determination of nutrient content in soybean and soymilk

Soybean grains and soymilk were evaluated for protein, fat, minerals and isoflavones. Analysis of Crude protein was done by the Folin-Lowry method (Lowry, Rosebrough, Farr, & Randall, 1951). Briefly, extraction was done using 5% sodium dodecylsuphate (SDS). The measurement of protein was done following a calibration curve from Bovine Serum Albumin (BSA) used at λnm= 750 as standard.

Official Method 983.23 of AOAC was used to determine crude fat. This method involved enzymatic incubation of samples with Clarase to break down starch and subsequent extraction, by blending with chloroform/methanol mixture. The extracts aliquoted were evaporated until dry and the total fat content was determined gravimetrically.

Mineral content of the samples was determined using AOAC Official Methods 9.1.09 and 50.1.14 (Horwitz, 2000). A block digester (Velp Scientifica DK20/26 230V, Europe) was used in extraction with concentrated nitric acid (HNO3) and H2O2. The extracts were diluted, and the determination of the target analytes calcium (Ca), magnesium (Mg) and potassium (K) was done using flame atomic absorption spectrophotometer (AAS) (GBC Savant AA 01-1006-03, Australia) set at wavelengths 422.70 nm, 285.20 nm and 766.50 nm, respectively. Determination of Phosphorus was done using UV-VIS (Agilent G6860A, Australia) set at 400 nm. BCR-708 (Institute for reference materials and measurements-JRC European commission) was analysed for quality control purposes, alongside the samples in determination of crude protein, magnesium, oil, phosphorous and calcium.

Determination of Isoflavones was done using AOAC Official Method 2001.10 (Latimer, 2012) (Collison, et al., 2008): Extraction was done at 65˚C for two hours in 80 % methanol and the extracts saponified at ambient temperature with NaOH 2M solution. The extracts were acidified, filtered and diluted with 50% methanol. Thereafter, the extracts were centrifuged to clarify them and analysed by liquid chromatography on a reverse-phase C-18 column using High Performance Liquid Chromatography (Shimadzu LC-30A, Japan) coupled to a UV detector (SPD-M20A, Japan) set at λnm= 260. The target analytes were Genistein, Genistin, Daidzin, and Daidzein. All analysis was performed in triplicates.

### 3.2.5 Data analysis

Data were analysed using IBM statistics, SPSS 20 software and the analysis of variance was used to test the significance of difference between variables at 95% level of confidence. Means were separated using least significance difference (LSD) post hoc tests.

## 3.3 Determination of the growth of different probiotic bacteria in soymilk and their effects on anti-oxidant activity and oligosaccharide content

### 3.3.1 Soybeans

Eight soybean samples, consisting of five known and three unknown local varieties were used. The five known varieties, namely Peka 6, Sc. Sequel, Sc. Squire, SB24 and Sc. Saga were obtained from the Rwanda Agriculture Board stores. The 3 unknown local varieties were those grown by farmers in Eastern, southern and Western provinces and were designated as LocE, LocS and LocW, respectively.

### 3.3.2 Soymilk preparation

The Hosken method (Hosken, 1999), M1 in the Materials and Methods subsection 3.2.3, which was found to give better nutrient retention was used to prepare the soymilk.

### 3.3.3 Inocula preparation

Seven probiotic lactic acid bacteria stock culture: *Lactobacillus plantarum*(93), *Lactobacillus acidophilus* (88), *Lactobacillus brevis*(89), *Lactobacillus reuteri*(94), *Lactobacillus rhamnosus*, *Lactococcus cremoris* SK11 and *Lactobacillus casei* (shirota), were obtained from the Belgian Coordinated Collections of Microorganisms-Laboratory of Microbiology: BCCM-LMG (Ghent, Belgium), this is a bacterial culture collection containing over 25000 well-characterized strains.

A mother culture was prepared by adding 0.1ml stock culture (stored in glycerol at -18˚C) to 10 ml sterile medium De Man, Rogosa and Sharpe (MRS) for each bacterium. From the mother culture, inocula were prepared by adding 0.1 ml mother culture into fresh MRS medium. Tubes were incubated for 18h at 30˚C. The CFU/ml of the inocula was measured by plating the appropriate dilutions (made in 0.9% NaCl) on MRS agar plates. Plates were incubated at 30˚C for 24 h.

### 3.3.4 Fermentation of soymilk: screening experiment

Soymilk (20ml) prepared from soybeans (kindly received from Alpro, Wevelgem, Belgium) was inoculated with about 5.106 CFU of the 7 different lactic acid bacteria. The flasks were then incubated for 24h at 30˚C. At several time points during 24h, pH was measured to follow the acidification profile during fermentation. Subjective inspection of the change in viscosity was done by scoring it as 1, 2 and 3 for high viscosity, medium viscosity and low viscosity, respectively. After 24h incubation, the appropriate dilutions of the fermented soymilk were plated out on MRS agar plates for counting the CFU/ml. Plates were incubated at 30˚C for 24 h.

### 3.3.5 Production of fermented soymilk from different soybean varieties using three selected lactic acid bacteria

Based on the results of the screening experiment, 3 strains, namely *L. plantarum, L. brevis* and *L. reuteri* were selected to ferment soymilk from 8 different soybean varieties of Rwanda. A total of 3ml inoculum (about 106 CFU/ml) was added to 60 ml of soymilk. Incubation was done at 30˚C for 24h. Subjective inspection of the change in viscosity was done by observation and shaking the bottle used for fermentation. The pH was measured at t = 0 and t = 24h. Titratable acidity was determined after 24h and expressed as g lactic acid/l (g LA/l). Total count numbers were determined by plating. Soymilk samples (0 and 24h fermentation) were also stored at -20˚C for further analysis of total phenolic compounds, antioxidant activity and oligosaccharide content.

### 3.3.6 Total phenolic compounds extraction

From 2.5g of soymilk or from 0.5g of soybean flour samples, phenolic compounds were extracted using 15 ml of methanol (100%). After homogenization for 45 s at 1000 rpm using an Ultraturax centrifuge, tubes were kept for 15 minutes on ice, and then centrifuged for another 15 minutes at 4000 rpm at 4˚C. The supernatant was filtered into a 25ml volumetric flask using Whatman No.2 filter paper. Methanol 80% (10 ml) was added to the residues, centrifuged for 20 s at 1000 rpm, and filtered in the flask as previously (Shumoy, Gabaza, Vandevelde, & Raes, 2017); (Singleton,

Orthofer, & Lamuela-Raventós, 1999). The flask was topped up to 25ml with the extraction solvent (Methanol 80%). The extract was kept in the cold room at 4˚C for further analysis of antioxidant activity and total phenolic compounds.

### 3.3.7 Total phenolic compound content (TPC) using Folin Ciocalteu phenol reagent

Some of the reagents used were 20% Na2CO3, Folin Ciocalteu reagent (FC), 90% methanol. A gallic acid stock solution (400 mg/l) was prepared, from which serial dilutions were made between 0 and 50 mg/l. Both stock solution and dilutions were made in 90% methanol. As a blank, a 1 ml 90% methanol solution was used. One ml of standard or 1 ml of sample extract was added to each test tube. FC (0.5 ml) was added, vortexed, and incubated for 6 min. After this, 1.5 mL Na2CO3-solution and 1 mL bi distilled water was added to each test tube. After vortexing, test tubes were kept in the dark for 2 hours. A spectrometer (Thermo Spectronic, GENESYS 20 Cambridge, England) was used to measure the absorbance at 760nm. The quantification was done against a standard curve of gallic acid. Results were expressed in terms of mg gallic acid equivalents per 100g (mg GAE/100g).

### 3.3.8 Determination of antioxidant (free radical scavenging) activity using 1, 1-diphenyl-2-picrylhydrazyl (DPPH)

Determination of the scavenging activity of samples was done by adding 4ml DPPH (0.1mM) into 200µL methanolic extracts or into 200µL trolox standards (0.01, 0.02, 0.04, 0.08 and 0.1mg/L) and a blank (MeOH 90%) (Kumaran & Karunakaran, 2006); (Shumoy, Gabaza, Vandevelde, & Raes, 2017). The test tubes were kept in the dark for 30 min, after vortexing. Absorbance was read using a spectrometer at 517nm, and results were expressed in terms of mg trolox equivalents per 100g.

### 3.3.9 Determination of oligosaccharides

Raffinose, stachyose and verbascose were measured by the raffinose/D-galactose kit from Megazyme (Megazyme International Ireland, 2014). All of the reagents and filters were purchased from VWR chemicals, Leuven, Belgium. Prior to analysis,

samples were clarified using carrez solutions. Therefore, 5ml of fermented and non-fermented soybean milk or 1g of soybean flour were pipetted in a 100ml volumetric flask containing 60ml of water and mixed thoroughly. Then, 5ml of Carrez I, 5ml of Carrez II, and 10ml of NaOH were added, mixed and the volumetric flask was filled up with distilled water to the mark. The filtration was done with a Whatman filter No2 to get a clear solution for use in the Megazyme assay. Each sample was measured using a UV-VIS called UV1 (from Thermo Spectronic Cambridge, England) for the combined raffinose and the free D-galactose, which was subtracted at the end of the analysis.

### 3.3.10 Data analysis

Data were analysed using IBM statistics, SPSS 20 software and the analysis of variance was used to test the significance of difference between variables at 95% level of confidence. Means were separated using least significance difference (LSD) post hoc tests.

## 3.4 Determination of the compounds of five essential oils by gas chromatography-mass spectrometry and their effect on fungal growth inhibition and sensory acceptability of soymilk

### 3.4.1 Essential Oils compounds analysis by GC-MS

Soybeans and essential oils (EOs) (*Cymbopogon nardus, Ocimum basilicum, Cinnamomum verum, Eucalyptus globulus* and *Mentha)* were purchased at Hengcheng natural perfume oil co., Ltd, JiangXi province, China. Analysis of EOs compounds by Gas Chromatography- Mass Spectrometer (GC-MS) was done using GC-MS Agilent at OCRI; Mass spectra were obtained on Agilent 5973 MSD mass spectrometer, coupled directly to 7890A gas chromatograph fitted with a J & W DB-5ms, 0.25 mm i.d. x 30 m, 0.25 micron coating thickness, fused silica capillary column. The GC/MSD was operated under the following conditions: injector temperature 240˚C; transfer line 300˚C; the column temperature was initially held at 50˚C for 3 min, increased to 240˚C at a rate of 3˚C/min, and then held at this higher temperature for 2 min; injection 0.1 µl (10% soln.), split 1:20, and helium with a

flow rate of 1.2 ml/min was used as the carrier gas; Electron energy of 70 eV in the electron ionization mode, and an ion source temperature of 200˚C; Scan Range 41 - 415, 1 scan/ sec., Solvent delay 2.00 min (Adams, 2017). A comparison of retention time of compounds with the standards was done. In addition, a comparison of mass spectra of the components with the mass spectra stored in the National Institute of Standards and Technology (NIST) reference library was done, and the calculation of percentages of compounds in the EOs.

### 3.4.2 Test of Essential Oils Minimum inhibitory concentration on fungal growth

Strains of fungi species Aspergillus flavus 3.4408 were obtained from the culture collection of Oil Crops Research Institute of the Chinese Academy of Agricultural Science, Department of Mycotoxins Research, Wuhan in China. The fungi were cultured in petri dishes on Potato dextrose agar (PDA) for 7 days at 28˚C. Extraction of the spores was done by washing the colonies in petri dishes with Tween 80 0.1% v/v distilled water. Using a pipette tip, the extract/spores suspension were collected in a tube (Moosavi-Nasab, Jamalian, Heshmati, & Haghighi-Manesh, 2018)). The suspension was tested for contamination by adding 0.5 ml in 50ml of Liquid Sabourand Medium and incubating at 28°C in an incubator shaker for 48 hrs. Hyphens growth was visible in small ball in the medium if the spore's suspension was not contaminated. The spore concentration was determined using a hemocytometer slide by an optical microscope (Nikon eclipse E100, Japan) and the Aspergillus flavus spore suspension was diluted in 0.1 % Tween 80 to bring the final inoculum to 5.105 CFU/ml (Clinical and Laboratory Standards Institute (CLSI), 2012)

The EOs and Medium PDA were prepared using agar dilution method (Hammer et al., 1999; Davari and Ezazi, 2017). The concentrations of 1, 3, 5, 10, 20, 50 and 100µl/10mL of PDA were used. Inoculation was done with 10µl of fungal suspension in triplicate and sample control. Petri dishes were sealed and incubated at 28°C. The growth was observed on 2 days basis and each fungal colony was measured in two perpendicular directions, and the colony mean diameter was obtained after 7 days. The minimum inhibition concentration (MIC) was determined

using the formula: Inhibition % = (C - T/C)*100; Where C: Diameter of fungal colony in control plates and T: Diameter of fungal colony in treated plates (Davari & Ezazi, 2017); (Li, et al., 2016).

### 3.4.3 Sensory evaluation of soymilk flavored with Essential Oils

Different levels of EOs flavor 1 drop, 2 drops and 3 drops were added in 1L of soymilk except the control. The method 1 of soymilk preparation that has highest nutrient retention was used (Hosken, 1999). A ten-member taste panel determined the acceptability of different soymilk flavored with Basil, Citronella and Mint by using a 9-point hedonic scale from like extremely = 9 to dislike extremely = 1 (Hashmi, 2007). The products were evaluated for the taste, color, appearance, odor using an evaluation form (Appendix 2).

### 3.4.4 Data analysis

Data was analyzed using SPSS 22.0 and MS Excel. Analysis of variance (ANOVA) was conducted and significance of differences were declared significant at $P< 0.05$ probability levels.

# CHAPTER FOUR

# RESEARCH RESULTS AND DISCUSSION

## 4.1 The effect of postharvest handling practices and production region on the level of selected mycotoxins contamination in Rwandan soybeans

### 4.1.1 Soybean pre- and post-harvest handling

Table 1 shows information on aspects of pre- and postharvest history of the soybean samples used in the study. The planting date was mainly in February 2015 and the harvesting time in June 2015 for 81.6% of farmers. The drying duration of soybean samples by farmers was between one and eight days. Most of the farmers (58%) dried their soybeans for 5 days and others dried for 1 to 4 days (38%) and 6 to 8 days (4%). The storage duration was between 1 and 4 months (99.3%), where only 2 samples (0.7%) were stored for 12 months. The storage methods were polyethylene bags (99.3%, n=298) and plastic containers (0.7%, n=2) (Table 1). Only 7.7% of the farmers (n=23) used pesticides, normally cypermethrin purchased in Agro-vet shops.

Mycotoxin formation is difficult to prevent as they are formed both pre- and post-harvest under natural environmental conditions (wild and Gong, 2010). Pre- and post-harvest conditions and agricultural practices play a critical role in modulating the risk of mycotoxigenic fungal colonization and growth, as well as mycotoxin contamination (Mutiga, et al., 2014); (Milicevic, Nastasijevic, & Petrovic, 2016). Farmers were able to dry soybeans and store in proper equipment, one of the strategies proposed to lower health risks associated with mycotoxins in foods (Dohlman, 2003). According to Harner (1997), soybeans are harvested when the moisture content is 16-18%, further dried until the moisture content is ≤14% before storage. Post-harvest handling that assures dry seed and integrity of the seed coat has been shown to reduce colonization of soybeans by *Apergillus flavus* (Barros, Ramirez, & Schulze, 2011).

**Table 4.1: Soybeans pre and post-harvest handling by farmers**

| Variables | Number of samples /Respondents (n) | Percentage (%) |
|---|---|---|
| Source of samples: | | |
| Household | 138 | 46.0 |
| Market | 140 | 46.7 |
| Rwanda Agriculture Board | 22 | 7.3 |
| Planting time and harvesting time: | | |
| 03/2014-07/2014 | 2 | 0.7 |
| 09/2014-03/2015 | 25 | 8.3 |
| 10/2014-03/2015 | 20 | 6.7 |
| 02/2015-06/2015 | 245 | 81.7 |
| 04/2015-07/2015 | 8 | 2.7 |
| Drying duration (days): | | |
| 1 | 33 | 11.0 |
| 2 | 24 | 8.0 |
| 3 | 16 | 5.3 |
| 4 | 41 | 13.7 |
| 5 | 174 | 58.0 |
| 6 | 1 | 0.3 |
| 7 | 10 | 3.3 |
| 8 | 1 | 0.3 |
| Storage duration (in months): | | |
| 1 | 251 | 83.7 |
| 2 | 1 | 0.3 |
| 3 | 1 | 0.3 |
| 4 | 45 | 15.0 |
| 12 | 2 | 0.7 |
| Storage equipment: | | |
| Polyethylene bags | 298 | 99.3 |
| Gerican/plastic containers | 2 | 0.7 |
| Pesticides use: | | |
| Cypermethrin | 23 | 7.7 |
| No pesticides use | 277 | 92.3 |

### 4.1.2 Aflatoxin awareness

Only 7.3% (n=22) of the respondents were aware of aflatoxins and the related human health impact. These persons were educated staff members of RAB stores. More than 92.7% of the respondents did not know what aflatoxin was and the health implications associated with it. These findings clearly have shown that there is a need to create awareness on aflatoxins among the Rwandese by training to prevent food colonization by mycotoxigenic fungi, as was suggested by (Nishimwe, Wanjuki, Karangwa, Darnell, & Harvey, 2016). Ignorance on the understanding of aflatoxins and the conditions in which the fungal species producing the toxin grow, could lead to acute and chronic toxicity (EFSA, 2013), and subsequent aflatoxins outbreaks ( (Feed the Future, 2012); (Krishnamachari, Bhat, Nagarajan, & Tilak, 1975). Thus, it is important to train farmers on mycotoxins contamination in food and feed to ensure better standards are adhered to safeguard the health of the consumers in regard to these fungal secondary metabolites.

### 4.1.3 Total aflatoxins in soybeans

The quantification of aflatoxins was obtained from a logit-log standard curve and the concentrations were adjusted according to a dilution factor of 50. The Limit of Detection (LOD) was 1 µg/kg, the correlation factor ($R^2$) was 0.98. The aflatoxins incidence found in soybean in this study was low (3%) as shown in Table 2.

**Table 4.2: Total aflatoxin content by region/Province**

| Categorized soybean aflatoxin measurements | Number of samples | District | Agro-ecological zone/Province |
|---|---|---|---|
| Below LOD (undetectable) | | | |
| <1µg/kg | 291 | 6 Districts | 3 AEZ/Province |
| Positive, below maximum allowable limit (µg/kg) | | | |
| 1.00 | 1 | Kamonyi | South |
| 1.00 | 1 | Kirehe | East |
| 1.00 | 1 | Kayonza | East |
| 1.10 | 1 | Kayonza | East |
| 1.10 | 1 | Nyamasheke | West |
| 1.20 | 1 | Kirehe | East |
| 1.40 | 1 | Kayonza | East |
| 1.60 | 1 | Kirehe | East |
| Positive, above maximum limit (µg/kg) | | | |
| 11.2 | 1 | Kirehe | East |
| **Total** | **300** | | |

Most of the positive samples were collected in the Eastern zone of Rwanda known to have a hot and dry climate, which is favourable conditions for A. flavus (Milani, 2013); (Pratiwi, et al., 2015). This may explain the effect of production region on the aflatoxin contamination where the Eastern Rwanda, which is hotter and more dry than the other soybean growing areas, may be confirmed by the previous reports that hot and dry weather conditions favour A. flavus infection and aflatoxin production (Cotty & Ramon, 2007). For the same reason, aflatoxins are a more serious problem in tropical and sub-tropical regions (Milicevic, Nastasijevic, & Petrovic, 2016); (Pratiwi, et al., 2015). The least contaminated sample above the LOD contained 1 µg/kg aflatoxins, and the highest contaminated had 11.2µg/kg from the Eastern region. In the EU, current maximum levels for total aflatoxins in ground nuts, cereals and dried fruits (AFB1, AFB2, AFG1 and AFG2) are 4 µg/kg (EC, 2006). In the US, the maximum limit for grain and grain products is 20 µg/kg (NGFA, 2011)

The low levels of aflatoxins in the samples are consistent with previous reports that soybeans are not a good substrate for the production of aflatoxins even when they are infected with A. flavus (Dharmaputra, 2002). Nevertheless, good post-harvest handling practices by the farmers, including proper drying on polyethylene sheets and grain storage in polyethylene sacks could contribute to the good quality. This is because pre-, peri- and post-harvest conditions and agricultural practices modulate the risk of product colonization and growth of mycotoxigenic fungi, as well as the associated contamination with mycotoxins (Milicevic, Nastasijevic, & Petrovic, 2016); (Mutiga, et al., 2014)). For example, in Uganda where drying was done on bare ground, (Kaaya, 1992)) reported higher levels of contamination (up-to 40 µg/kg) than were obtained in the present study. Similarly, while a study done by (Dharmaputra, 2002) in Indonesian soybean meal revealed no aflatoxin contamination in soybean. (Lutfullah & Hussain, 2012) found that 15% of soybean samples from Pakistan were contaminated.

In the present study, Rwandan farmers were found to harvest soybeans only when pods were dry, and they dried the bean further for an average of 5 days. Such drying facilitates removal of the beans from the pods without breaking, which is advantageous in that broken grains are more susceptible to A. flavus infestation (Focus, 2013). The reduction in moisture content as a result of drying for an average of 5 days also serves to inhibit growth and aflatoxin production by A. flavus (Dohlman, 2003); (Harner, 1997).

### 4.1.4 Multi mycotoxins in soybean

The four parameters required to confirm a mycotoxin presence were not fulfilled for most mycotoxins analysed: OTA, AFB1, AFB2, AFG1, AFG2, DON, ZEN, FB1, FB2, T-2, HT-2, ROQ-C, NIV, NEO, FB3, AOH, AME, 3-ADON, 15-ADON, DAS and FUS-X. The signal to noise ratio was <3 for all "legislation" mycotoxins in all samples. One subsample had a S/N ratio >3 for STERIG. Sterigmatocystine was at a level of 13 µg/kg (Kirehe subsample 2 in the Eastern agro ecological zone). Sterigmatocystine is among mycotoxins, which do not have maximum levels set by EU (Di Mavungu, et al., 2009); (Monbaliu, et al., 2009). According to the scientific

opinion of the European Food Safety Agency (EFSA), the acute oral toxicity of STERIG in food is relatively low (EFSA, 2013).

As is the case with aflatoxins, many studies have found generally low rates of soybean contamination with other mycotoxins. However, cases of contamination still do occur and are of concern. For example, in Brazil, 0.5 µg/kg, 30 µg/kg and 57 µg/kg of AF, ZEN and FB in soybean were reported in one study (Daga et al., 2015). Moreover, 24 % of soy products marketed in Germany were found to be contaminated with Fusarium toxins, especially DON and ZEN (Schollenberger et al., 2007). Although some OTA contamination of Korean fermented soybean was reported, the concentration of the mycotoxin was low (0.14 µg/kg) (Ahn, Lee, Lee, & Kim, 2016). In a four-year surveillance for OTA and FB in retail foods in Japan, 65% of soybeans were contaminated with FB1 at a mean concentration of 4.5 µg/kg, 15% were contaminated with FB2 at a mean concentration of 4.3 µg/kg) while OTA was not detected (Aoyama, et al., 2010).

There is no literature on the levels of soybean contamination with mycotoxins in Rwanda. On the other hand, (Nishimwe, Wanjuki, Karangwa, Darnell, & Harvey, 2016) reported high levels of contamination of Rwandan maize with aflatoxins, where aflatoxin concentrations were as high as 26 µg/kg. In the latter study, up to 35% of the samples exceeded the maximum accepted limit for total aflatoxins according to US standards, while up to 100% of the samples exceed the stricter EU limits (Nishimwe, Wanjuki, Karangwa, Darnell, & Harvey, 2016). A comprehensive survey conducted in America, Europe, Asia and Oceania reported that soybean was mostly contaminated by FB (range, 12 µg/kg – 2,966 µg/kg) (Rodrigues & Naehrer , 2012). From these studies, it can be concluded that, with proper postharvest handling, soybean is not very susceptible to mycotoxin, and especially aflatoxin contamination, which further enhances the value of soybean in combating malnutrition and contributing to the prevention of non-communicable diseases.

## 4.2 The effect of different processing methods on nutrient and isoflavone content of soymilk

### 4.2.1 Nutrient and isoflavone content of the six soybean varieties

Protein, fat, minerals and isoflavones content analysed in the six varieties of soybean grown in Rwanda are presented in Table 3. Protein content ranged between 34.7 and 36.7 %, but these differences were not significant ($P>0.05$). There were significant differences ($P<0.05$) in fat content where the lowest (SB24 and Sc. Saga) had 11.1% while the highest had 16.6%. All these values were within the range found in previous studies (Penalvo et al., 2004). There were no significant differences in Potassium (K) content ($P>0.05$) which ranged between 1451.2 and 1857.5 mg/100g. These are in the range of values found by Mateos-Aparicio et al., 2008. The Magnesium (Mg) content of Sc. Sequel (141.9 mg/100g) was significantly lower ($P<0.05$) than the other five varieties.

**Table 4.3: Nutrients and isoflavone content of flour from six soybean varieties grown in Rwanda**

| Varieties | Protein (g/100g) | Fat (g/100g) | Minerals (mg/100g) | | | | Isoflavones total (µg/g) |
|---|---|---|---|---|---|---|---|
| | | | Ca (mg/100g) | Mg (mg/100g) | K (mg/100g) | P (mg/100g) | |
| Peka 6 | 36.7 ±4.4 | 12.4 ±0.9 | 131.7 ±12.2 | 164.9 ±12.8* | 1453.5 ±299.1 | 325.5 ±60.9 | 2193.5 ±196.6 |
| Sc. Saga | 34.7 ±2.3 | 16.6 ±2.3* | 148.2 ±24.8* | 166.4 ±15.7* | 1701.9 ±300.9 | 318.5 ±72.9 | 1942.6 ±591.4 |
| Sc. Sequel | 36.1 ±5.4 | 14.9 ±1.9 | 170.5±47.5* | 141.9 ±11.2 | 1451.2 ±315.5 | 360.7 ±108.7 | 1666.6 ±346.5 |
| Sc. Squire | 36.0 ±3.5 | 15.6 ±1.2* | 120.7 ±8.5 | 150.1 ±10.9* | 1857.5 ±371.3 | 426.1 ±51.2 | 4839.9 ±375.3* |
| SB24 | 36.4 ±1.3 | 11.1 ±0.7 | 100.5±11.8 | 167.2 ±31.9* | 1625.4 ±343.9 | 430.9 ±50.3* | 2272.3 ±341.3 |
| Local | 36.7 ±2.0 | 12.7 ±0.7 | 144.4 ±3.3* | 154.0 ±9.5* | 1669.8 ±6.1 | 414.9 ±3.5 | 2528.6 ±444.9 |
| P value | >0.05 | <0.05 | <0.05 | <0.05 | >0.05 | <0.05 | <0.05 |

There were significant differences (P<0.05) in calcium, which ranged between 100.5 and 170mg/kg, and phosphorus, which ranged between 318.5 and 430.9 mg/100g. The Phosphorous (P) content of SB24 (430.9 mg/100 g) was significantly higher (P<0.05) than the other five varieties whose concentrations did not significantly differ from one another (P>0.05).

The isoflavones content was significantly different between varieties (P<0.05). Sc. Squire had more than double the content of isoflavones (4839.9 µg/g) as compared to other varieties, while Sc. Sequel had the lowest (1666.6 µg/g). The values are higher than those reported for Korean varieties, where the values ranged between 1248 to 1528 mg/Kg (Yong-Soon, Bung-Hoon, Jong-Hwa, & Nam-Soo, 2000). Thus, varieties may contribute to lower or higher isoflavones content.

### 4.2.2 Nutrient content of soymilk prepared from six soybean varieties by the three processing methods

The method M1 (soaking soybeans before extraction) was found in this study to give better nutrient extraction than M2 (blanching) and M3 (cooking). The two methods (M2 and M3) were developed for reducing beany flavor (Zhang, Guo, Liu, & Chang, 2012). Moreover, at least in one study, it has been demonstrated that the efficiency of extraction of nutrients from soybeans is dependent on the variety (Zhang, Chang & Liu, 2015). Thus, it was important to determine whether M2 and M3 might have better nutrient extraction for some of the six varieties. M1 produced soymilk with higher protein content in all the varieties as shown in Figure 3. However, M2 gave protein content comparable to M1 for Sc. Squire and the local variety and this might be related to the texture of the grain as observed during the preparation of soybean milk where the two varieties were relatively softer and tender. The difference between varieties was not significantly different (P >0.05).

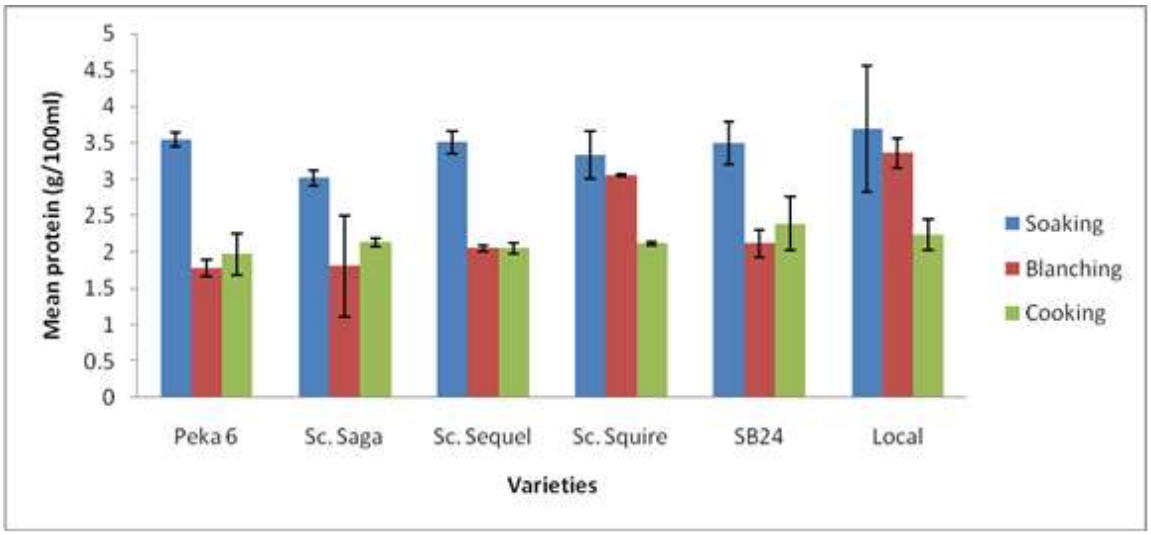


**Figure 4.3: Mean protein content of soymilk from six selected soybean varieties as influenced by three extraction methods**

Figure 4 shows that M2 gave the lowest fat content in all the varieties and M3 gave fat contents comparable to M1 for Sc. Squire and the Local variety. The difference between varieties was not significantly different (P >0.05).

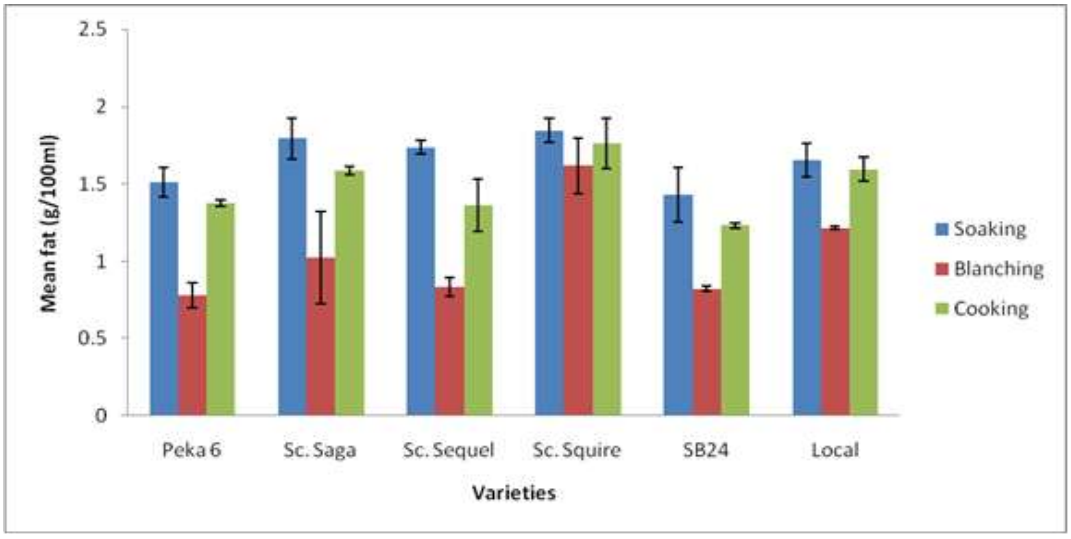


**Figure 4.4: Mean fat content of soymilk from six selected soybean varieties as influenced by three extraction methods**

The lower fat extraction by M2 was consistent with previous finding that showed that increasing temperature in the range of 100 -150˚C led to a reduction in fat extraction (Adejumo, Alakowe, & Obi, 2013); (Saldaña & Martínez –Monteagudo, 2013).

In Table 4, the means minerals content in soymilk from six selected varieties the three processing methods were highlighted.

**Table 4.4: Means minerals content in soymilk from six selected varieties as influenced by three extraction methods**

| Varieties | Method | Ca (mg/100g) | Mg (mg/100g) | K (mg/100g) | P (mg/100g) |
|---|---|---|---|---|---|
| Peka6 | M1 | 8.33±0.52 | 14.86±1.06 | 119.44±4.58 | 32.59±1.25 |
| | M2 | 5.36±1.16 | 10.38±0.71 | 79.86±4.12 | 14.71±0.43 |
| | M3 | 4.64±0.08 | 8.32±0.55 | 77.77±4.96 | 16.66±0.64 |
| Sc. Saga | M1 | 8.85±0.32 | 13.91±1.09 | 179.19±5.25 | 20.28±0.72 |
| | M2 | 5.92±0.25 | 9.5±0.49 | 130.61±6.39 | 13.53±0.54 |
| | M3 | 6.55±0.29 | 8.94±0.34 | 120.18±4.69 | 13.47±0.6 |
| Sc. Sequel | M1 | 12.27±0.29 | 11.41±0.11 | 138.95±6.2 | 17.29±1.82 |
| | M2 | 8.38±0.25 | 9.47±0.8 | 116.98±7.52 | 15.23±0.15 |
| | M3 | 6.22±0.28 | 7.59±0.38 | 97.66±9.77 | 15.57±1.06 |
| Sc. Squire | M1 | 6.78±0.47 | 12.57±0.76 | 151.47±7.1 | 34.08±1.41* |
| | M2 | 7.4±0.36 | 12.45±1.06 | 141.75±11.53 | 25.81±1.08* |
| | M3 | 4.9±0.26 | 7.28±0.47 | 100.42±6.44 | 21.26±0.92* |
| SB24 | M1 | 6.95±0.20 | 15.50±0.65 | 134.43±5.43 | 26.00±1.07* |
| | M2 | 5.99±0.38 | 11.74±0.44 | 101.27±9.96 | 24.82±1.15* |
| | M3 | 4.63±0.30 | 9.28±0.34 | 90.15±9.56 | 25.25±1.16* |
| Local | M1 | 10.75±0.85 | 14.87±0.24 | 156.71±12.32 | 29.18±1.44* |
| | M2 | 9.49±0.15 | 14.42±0.65 | 121.06±7.58 | 30.94±2.91* |
| | M3 | 4.27±0.28 | 7.69±0.61 | 103.75±7.46 | 23.26±0.42* |
| P value | | >0.05 | >0.05 | >0.05 | <0.05 |

The difference between varieties was not significant for Calcium, Magnesium and Potatium (P value >0.05). Soymilk from Sc. quire, SB24 and the Local varieties had higher amount of Phosphorus (P value <0.05).

As shown in Figure 5, isoflavones were also better extracted by M1 than M2 and M3 in all the varieties. This is consistent with the study done previously showing that soymilk produced at strong heat had lower total isoflavones (Toda, Sakamoto, Takayanagi, & Yokotsuka, 2000).

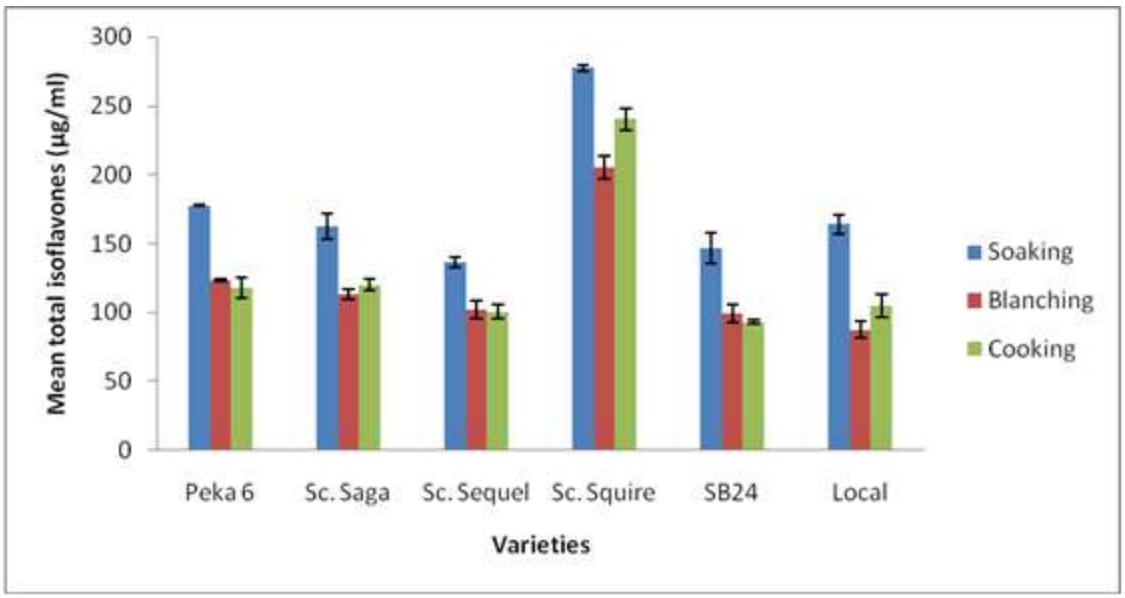


**Figure 4.5: Mean total isoflavones content of soymilk from six selected soybean Varieties as influenced by three extraction methods**

Neither M2 nor M3 afforded comparable isoflavone extraction as M1 in any of the varieties. Soymilk from variety Sc. Squire had significantly higher isoflavones content than the rest of the varieties (P>0.05). Sc. Squire Soymilk obtained by M2 had higher isoflavone content than soymilks obtained from the other varieties, by even M1. This indicates that the nutrients were extracted in proportion to the quantity available in the soybean variety.

### 4.2.3 Nutrient and isoflavone content of soymilk processed by different methods

Table 5 shows the means of nutrient and isoflavone content of soymilk obtained by the three processing methods from the six varieties. The nutrients (proteins, fat and minerals) and isoflavones (diadzin, genistin, genistein and daidzein) were better extracted by M1 than M2 and M3. Remarkably, the amount of the isoflavones daidzein and genistein extracted by M1 (9.8 and 9.4µg/ml, respectively), were between 4 and 10 times higher than M2 and M3 (Table 4). This is consistent with a previous report that soaking or heating led to a 12% or 49% loss, respectively, in soy isoflavones during Tempeh processing, and that alkaline extraction caused a 53% loss during soy protein isolate production (Wang and Murphy, 1996). The more the bioactive aglycones isoflavones (daidzein and genistein) in the diet, the more the health benefits in prevention/treatment of hormonal disorder diseases (Setchel & Cassidy, 1999).

**Table 4.5: Effect of different methods on nutrient content of soymilk prepared from six soybeans varieties**

| Nutrients means | | Method1 | Method2 | Method3 | P-Value |
|---|---|---|---|---|---|
| Protein (g/100g) | | 3.4±0.3* | 2.3±0.7 | 2.1±0.2 | <0.05 |
| Fat (g/100g) | | 1.7±0.2* | 1.1±0.4 | 1.5±0.2* | <0.05 |
| Minerals | Ca (mg/100g) | 9.0±2.3* | 6.9±1.7 | 5.4±1.4 | <0.05 |
| | Mg (mg/100g) | 13.6±1.7* | 10.9±1.9 | 8.1±0.8 | <0.05 |
| | K (mg/100g) | 146.9±30.9* | 116.1±27.4 | 98.6±15.9 | <0.05 |
| | P (mg/100g) | 26.4±8.6* | 18.9±7.3 | 18.3±5.8 | <0.05 |
| Isoflavones | Daidzin (µg/ml) | 71.6±24.1 | 61.1±19.7 | 58.8±25.4 | >0.05 |
| | Genistin (µg/ml) | 80.6±25.8* | 55.7±19.3 | 62.1±12.4 | <0.05 |
| | Daidzein (µg/ml) | 9.8±3.4* | 1.6±0.5 | 2.1±1.2 | <0.05 |
| | Genistein (µg/ml) | 9.4±4.2* | 0.6±0.4 | 1.0±0.3 | <0.05 |
| Total isoflavones (µg/ml) | | 171.3±53.2* | 119.0±38.1 | 124.1±48.4 | <0.05 |

Data are expressed as means ±SD. *Means within the row are significantly different from others. (Method 1 = soaking grains for 12 h; Method 2 = blanching grains; Method 3 = cooking grains)

The extraction of low nutrients and isoflavones by M2 and M3 may be attributable to a common factor. This is because, unlike M1, these two methods involved heating prior to extraction. Thermal treatment denatures proteins and could reduce their extraction due to decreased solubility (Nufer, B., & Hayes, 2009); (Zhang, Guo, Liu, & Chang, 2012). Isoflavone thermal stability is also affected by protein denaturation (Malaypally & Ismail, 2010); (Zhang, Chang & Liu, 2015). Due to hydrophobic residues and S-S linkages,

many plant proteins are not readily soluble in water and alkaline solutions are often used to increase their solubility through breakage of hydrogen bonds (Cui, et al., 2017). M3, however, involving soaking in NaHCO3 solution for a duration of three hours followed by heating had lower protein extraction than M1. This indicates that the effect of heating on reducing protein extraction is stronger than the potential alkaline treatment-mediated increase in protein extraction. Between M2 and M3 for protein, Calcium and Phosphorous extraction, no differences were observed. The method M2 showed more extraction of magnesium than M3 on the other hand and there is no clear reason for this difference.

### 4.3 The growth of different probiotic bacteria in soymilk and their effects on anti-oxidant activity and oligosaccharide content

#### 4.3.1 Screening experiment

The first screening of seven probiotic lactic acid bacteria used to ferment soybean milk from model soybean at 30˚C showed that most of the strains could grow well in soymilk (Table 6).

**Table 4.6: Screening of the growth (log CFU/ml soymilk) of seven lactic acid bacteria in soymilk**

| *Lactobacillus* | Mean log CFU/ml FSM at 0h | Mean log CFU/ml FSM at 24h | Real growth log CFU/ml |
|---|---|---|---|
| *L. brevis* | 6.24±0.10 | 8.88±0.06 | 2.64±0.03 |
| *L. plantarum* | 6.33±0.04 | 9.02±0.07 | 2.69±0.11 |
| *L. reuteri* | 5.90±0.06 | 8.92±0.00 | 3.02±0.06* |
| *L. cremoris* | 5.88±0.00 | 8.59±0.12 | 2.71±0.11 |
| *L. casei* | 5.84±0.06 | 8.28±0.44 | 2.44±0.50 |
| *L. acidophilus* | 6.38±0.04 | 7.94±0.07 | 1.56±0.11 |
| *L. rhamnosus* | 6.10±0.11 | 8.38±0.19 | 2.28±0.30 |

*Significant real growth in soymilk at 0.05 significance level; CFU = colony forming unit, FSM = Fermented soymilk

The mean log CFU/ml in soymilk after 24 hours of fermentation ranged from 7.94 *(L. acidophilus*) to 9.02 (*L. plantarum*), showing that *L. acidophilus* had the lowest growth in soymilk. For most of these bacteria, the logs CFU/ml at 24 hours were higher than the 8 log CFU/ml recommended for health benefit (Champagne, Raymond, Guertin, Martoni, & Jones, 2016); (Donkor, Henriksson, Vasiljevic, & Shah, 2007). The real growth of lactic acid bacteria in fermented soymilk FSM (log CFU/ml at 24h- log CFU/ml at 0 h) was significantly higher for *L. reuteri* ($P<0.05$) followed by *L. cremoris, L. plantarum* and *L. brevis.* Despite the high CFU levels attained by all the seven bacteria studied, only 3 strains gave good results for pH and viscosity after 24 hours of incubation. For the screening experiment, the soymilk fermented with *L. plantarum, L. brevis* and *L. reuteri* had a pH of 4.7, 4.8 and 4.7, respectively, after 24 h incubation, and all showed high viscosity. This is consistent with previous studies where a pH of 5 was reported after 24 h of soymilk fermentation with mixed cultures (Garro, de Valdez, & de Giori, 2004). The viscosity was high, thus creamy fermented soymilk was made. For other strains i.e. *L. cremoris*, *L. casei*, *L. acidophilus* and *L. rhamnosus* the pH did not change significantly ($P>0.05$) from time 0 (pH = 6.6) to time 24h (pH = 6.2), and the viscosity remained low. Molina et al. (2012) reported that soymilk fermented with *L. reuteri* for 6 hours attained a fairly high population of 1.6 x 107 CFU/ml but without much acidification (pH of 6.8). In a previous study, *L acidophilus* at log 8.73 CFU/ml in soymilk gave a pH of 4.8, log 8.98 CFU/ml in soymilk-apple juice blend (85:15) gave a pH of 3.83, while log 9.08 in soymilk-apple juice blend (75:25) gave a pH of 4.18 (Icier et al., 2015) showing that a higher CFU/ml does not directly translate into lower pH.

### 4.3.2 Soymilk fermentation with *L. plantarum, L. reuteri* and *L. brevis*

#### 4.3.2.1. Growth of the lactic acid bacteria in soymilk from the eight varieties of soybean

The three selected lactic acid bacteria *L. plantarum, L. reuteri* and *L. brevis* were used to ferment soybean milk from the eight soybean varieties grown in Rwanda. Table 7 summarizes the growth of the three strains in soymilk from the eight soybean varieties in a period of 24 hours. The final population ranged between 8.85 and 9.08 log CFU/ml. The mean final population after 24h was 9.03, 8.99 and 8.98 log CFU/mL for *L. plantarum, L. reuteri* and *L. brevis*, respectively. The real growth of *L. reuteri* in all varieties of fermented soybean milk was significantly higher than *L. plantarum* and *L. brevis* ($P<0.05$).

**Table 4.7: The growth (log CFU/ml) of the three strains of *lactobacillus* in soymilk obtained from eight soybean varieties**

| *Lactobacillus* | Varieties | Mean log CFU/ml 0h | Mean log CFU/ml 24h | Real growth log CFU/ml |
|---|---|---|---|---|
| *L. plantarum* | Peka 6 | 6.41±0.11 | 9.01±0.13 | 2.61±0.14 |
| *L. plantarum* | Sc. Saga | 6.33±0.17 | 9.00±0.02 | 2.67±0.18 |
| *L. plantarum* | LocS | 6.45±0.06 | 9.05±0.00 | 2.60±0.06 |
| *L. plantarum* | Sc. Squire | 6.34±0.14 | 9.00±0.03 | 2.67±0.11 |
| *L. plantarum* | SB24 | 6.40±0.10 | 9.10±0.10 | 2.70±0.06 |
| *L. plantarum* | Sc. Sequel | 6.44±0.05 | 9.10±0.06 | 2.66±0.03 |
| *L. plantarum* | LocE | 6.32±0.16 | 9.08±0.09 | 2.76±0.08 |
| *L. plantarum* | LocW | 6.36±0.16 | 8.94±0.06 | 2.58±0.13 |
| *L. reuteri* | Peka 6 | 5.96±0.19 | 8.85±0.07 | 2.89±0.25* |
| *L. reuteri* | Sc. Saga | 5.93±0.21 | 8.89±0.07 | 2.95±0.28* |
| *L. reuteri* | LocS | 6.24±0.28 | 9.24±0.52 | 3.00±0.24* |
| *L. reuteri* | Sc. Squire | 5.99±0.20 | 8.91±0.05 | 2.92±0.15* |
| *L. reuteri* | SB24 | 5.89±0.07 | 8.99±0.12 | 3.10±0.05* |
| *L. reuteri* | Sc. Sequel | 5.94±0.09 | 9.13±0.08 | 3.19±0.14* |
| *L. reuteri* | LocE | 5.83±0.05 | 8.98±0.02 | 3.15±0.04* |
| *L. reuteri* | LocW | 5.92±0.13 | 8.90±0.02 | 2.98±0.15* |
| *L. brevis* | Peka 6 | 6.18±0.34 | 8.99±0.10 | 2.81±0.24* |
| *L. brevis* | Sc. Saga | 6.44±0.04 | 8.98±0.02 | 2.54±0.01 |
| *L. brevis* | LocS | 6.28±0.26 | 9.00±0.16 | 2.72±0.12 |
| *L. brevis* | Sc. Squire | 6.41±0.01 | 8.88±0.06 | 2.48±0.04 |
| *L. brevis* | SB24 | 6.14±0.28 | 9.04±0.04 | 2.90±0.32 |
| *L. brevis* | Sc. Sequel | 6.13±0.26 | 9.14±0.09 | 3.02±0.19 |
| *L. brevis* | LocE | 6.40±0.00 | 8.98±0.15 | 2.58±0.15 |
| *L. brevis* | LocW | 6.41±0.01 | 8.86±0.10 | 2.44±0.11 |

*The real growth using *Lactobacillus reuteri* is significantly different from *Lactobacillus plantarum* and *Lactobacillus brevis* at 0.05 significance level.

*L. reuteri* showed better real growth log CFU/ml in soymilk than *L. plantarum* and *L. brevis. L. reuteri* can be promoted not only for fermentation of soybean milk but also for its health benefit as vitamin B-12 producer in the body (Molina, Medici, Font, & Taranto, 2012). Among other benefits, *L. plantarum* metabolizes cholesterol and may reduce the risk of cardiovascular disease (Amutha & Kokila, 2015); (Fuentes, Lajo, Carrión, &

Cuñé, 2013). One strain of *L. brevis* has been reported to reduce the incidence of influenza in school children (Waki, Matsumoto, Fukui, & Suganuma, 2014).

The soymilk from different varieties displayed differences in their promotion of the growth of the lactic acid bacteria. For example, growth of *L. reuteri* was significantly higher ($P<0.05$) in the variety LocS than in Peka 6, Saga and LocW. This may be due to the solid content of soymilk as reported in previous study that the higher the solid level the higher the growth of lactic acid bacteria (Estévez, Mejía, Figuerola, & Escobar, 2010).

#### 4.3.2.2 pH during soymilk fermentation

The pH at time 0 was 6.62 ± 0.02. At time 24h the mean pH was 4.5, 4.6 and 4.9 for *L. plantarum, L. reuteri* and *L. brevis*, respectively. The pH between the strains was significantly different ($P<0.05$), Figure 6. The difference between varieties was not significant ($P<0.05$)

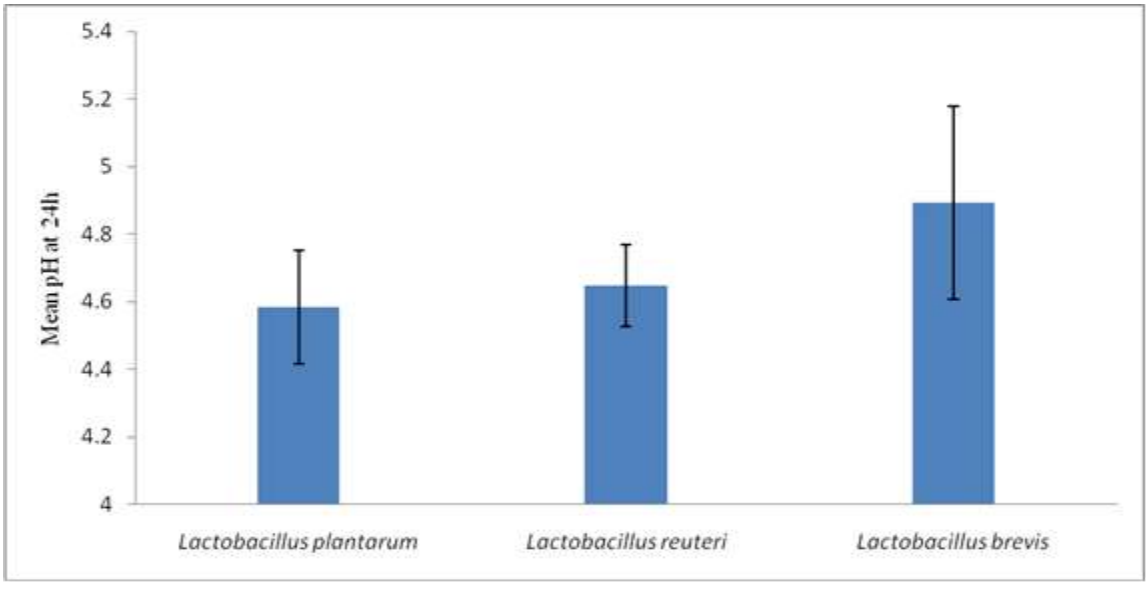


**Figure 4.6: Mean pH after 24 hours of soybean milk fermentation by three lactic acid bacteria**

Fermentation with *L. plantarum* resulted in the most acidic fermented soymilk followed by *L. reuteri* and lastly *L. brevis*. These results were similar to the pH found using the soybean model for the screening of 7 lactic acid bacteria and previous findings by (Garro, de Valdez, & de Giori, 2004) while investigating the growth of pure and mixed culture in soymilk, pH between 4.5 and 5 (Garro, de Valdez, & de Giori, 2004).

#### 4.3.2.3. Titratable Acidity (TA) of fermented soymilk

The TA of fermented soymilk varied between 2.17 g LA/L (0.2%TA) and 4.20 g LA/L (0.42%) (Figure 4.7). This is in the range with values found by (Obadina, Akinola, Shittu, & Bakare, 2013) of 0.42%. Although it was expected that the soymilk fermented with *L. brevis* would have a lower acidity than the other two strains because of its relatively higher pH (Fig 6), the product acidity difference between strains as well as between varieties was not significant (P>0.05). This might be due to the production of different profiles of other organic acids with different strengths besides lactic acid.

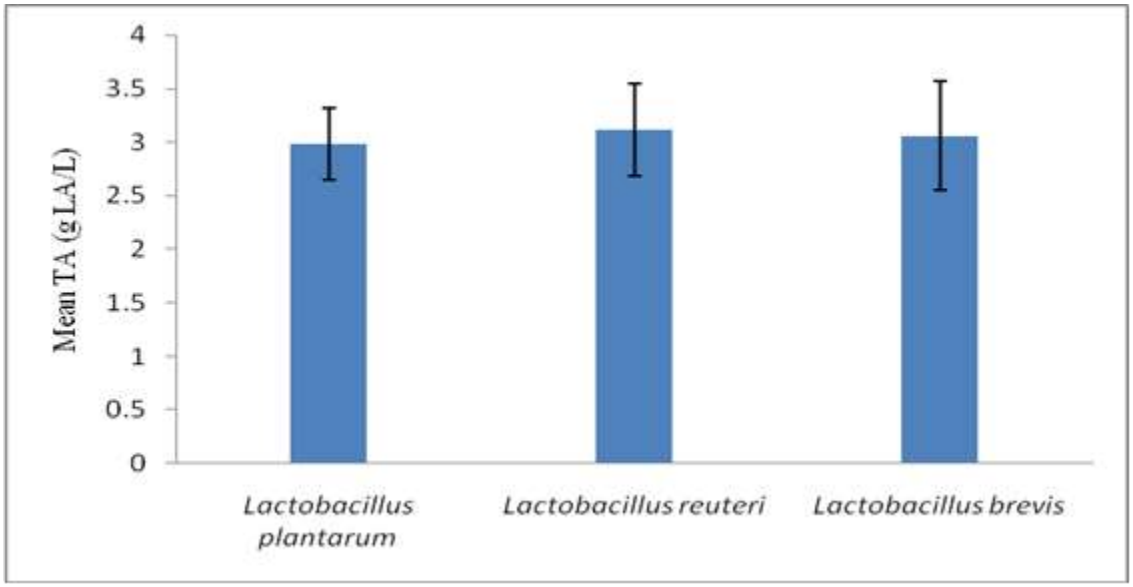


**Figure 4.7: Titratable acidity of fermented soymilk after 24hours**

The development of acidity during fermentation is higher in cow's milk than in soymilk, which takes a long time to ferment as reported in previous study (Ismail, 2016). This was

observed in this study as well as where the soybean milk started to coagulate only after 6 hours.

#### 4.3.2.4 Effect of fermentation on Total Phenolic Compound content (TPC) and antioxidant activity

Soybean flour had a mean TPC of 82.65mg GAE/100g. LocE variety had the highest TPC content as compared to other varieties for soymilk, fermented soymilk and soybean flour. Soymilk had a mean TPC of 10.16mg GAE/100g. The mean TPC for fermented soybean milk was 7.72mg GAE/100g (Table 8). There were significant differences in TPC of soymilk made from the different varieties of soybeans ($P<0.05$) but, there were no significant differences ($P>0.05$) between bacterial strains.

**Table 4.8: Total Phenolic Compound content in soybean flour, soymilk and fermented soymilk**

| Varieties | Mean TPC mg GAE/100g in soy flour | Mean TPC mg GAE/100g in soymilk | Mean TPC mg GAE/100g in fermented SM |
|---|---|---|---|
| Sc. Saga | 78.58±9.56 | 8.39±0.63 | 6.74±1.40 |
| SB24 | 85.97±4.03 | 10.38±0.24 | 7.28±0.65 |
| LocW | 79.20±3.52 | 10.23±0.17 | 7.59±0.43 |
| Sc. Squire | 80.33±4.12 | 9.96±0.92 | 7.39±0.20 |
| Peka6 | 85.92±0.49 | 10.18±0.74 | 7.97±0.54 |
| LocS | 78.80±3.64 | 9.38±1.15 | 7.22±0.48 |
| LocE | 87.75±3.19* | 12.65±0.68* | 9.50±1.34* |
| Sc. Sequel | 84.66±1.85 | 10.18±0.96 | 8.11±0.99* |
| P value | <0.05 | <0.05 | <0.05 |

*Means significantly different from others in the same column

Fermented soymilk had a slightly higher DPPH scavenging activity (3.20±0.12 mg TE/100g) than non-fermented soymilk (3.08±0.07) even though the latter had higher TPC than the former. This is consistent with previous reports that fermented soybean products have higher antioxidant activity than unfermented ones (Di Cagno, et al., 2010); (Riciputi, et al., 2016); (Yang, Chen, Zhang, Chen, & Liu, 2012); (Yao, Xiao-Nan, & Dong, 2010) (Chien, Yang, & C., 2013) reported that fermented soymilk had lower total isoflavones than non-fermented soymilk but had a high antioxidant activity due to the increase in aglycones during fermentation. β-Galactosidase transforms glycosides into aglycones (daidzein and genistein) (Malashree, et al., 2016).

The Trolox equivalent (TE) of soybean flour was 17.20±0.40 mg TE/100g. The difference in antioxidant activity was not significant ($P>0.05$) between the three bacteria. However, in Figure 8, Saga variety showed a significantly higher scavenging activity than SB24 and Peka 6 ($P<0.05$).

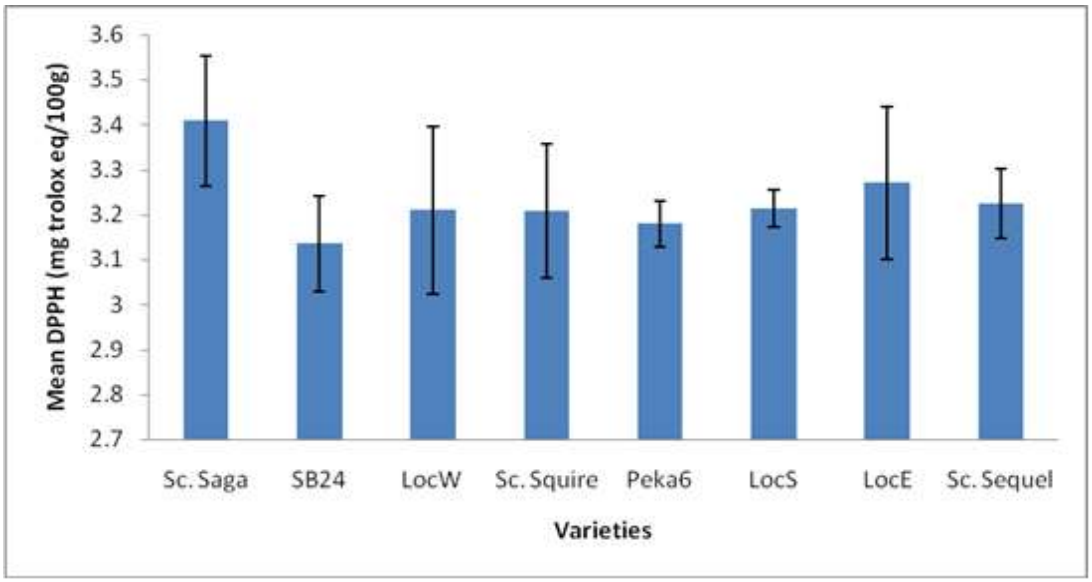


**Figure 4.8: Antioxidant activity of soymilk in eight varieties, fermented by *L. reuteri***

#### 4.3.2.5. Effect of fermentation on oligosaccharides

Concentrations of oligosaccharides in term of g/100g raffinose in soy flour (SF), soymilk (SM) and fermented soymilk (FSM) obtained from different soybean varieties are presented in Table 9. Varieties like Saga, SB24 and Squire have significantly less oligosaccharides ($P<0.05$) than Peka 6, Sequel and the three local varieties (LocE, LocS and LocW).

**Table 4.9: Concentrations of oligosaccharides in soybean flour (SF), soymilk (SM) and fermented soymilk (FSM)**

| Soybean varieties | Concentration raffinose SF (g/100g) | Concentration raffinose SM (g/100g) | Concentration raffinose FSM (g/100g) |
|---|---|---|---|
| Sc. Saga | 6.67±0.24 | 0.47±0.01 | 0.41±0.02 |
| SB24 | 6.90±0.18 | 0.52±0.01 | 0.45±0.04 |
| LocW | 6.72±0.08 | 0.64±0.03* | 0.53±0.01* |
| Sc. Squire | 6.34±0.10 | 0.52±0.02 | 0.47±0.01 |
| Peka6 | 7.55±0.12* | 0.68±0.05* | 0.58±0.02* |
| LocS | 7.73±0.16* | 0.67±0.02* | 0.54±0.08* |
| LocE | 7.11±0.18 | 0.67±0.02* | 0.56±0.04* |
| Sc. Sequel | 7.95±0.08* | 0.60±0.02 | 0.53±0.02* |

*Significantly different from others in the same column

Upon fermentation of soymilk by *L. reuteri*, *L. brevis* and *L. plantarum*, the concentration of oligosaccharides expressed in term of raffinose (g/100g) was significantly different by strains and by varieties $P<0.05$. *L. reuteri* reduced the most of

the oligosaccharides followed by *L. brevis* (Figure 9). This is consistent with the results that *L. reuteri* was the fastest growing, followed by *L. brevis*.

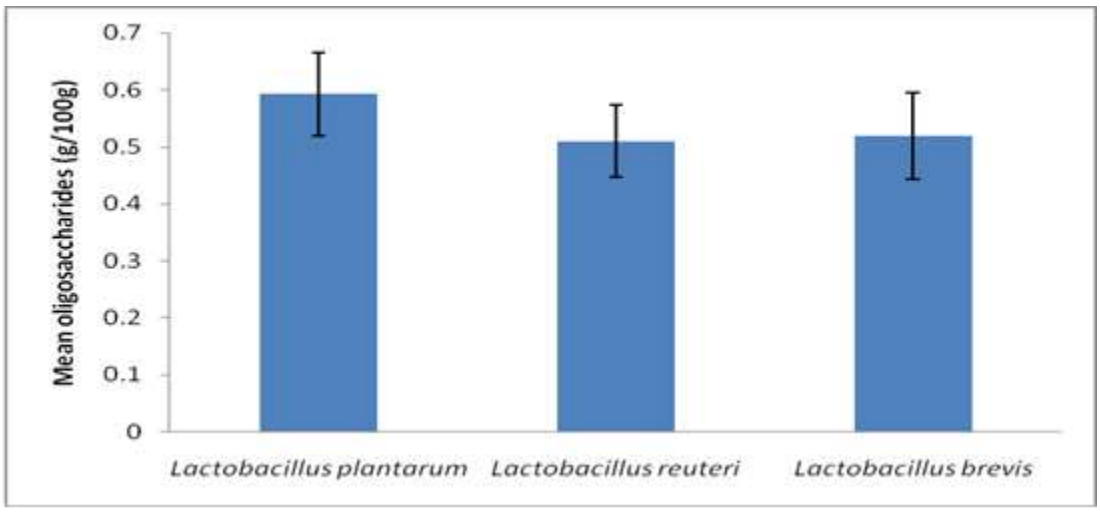


**Figure 4.9: Oligosaccharides content in fermented soymilk by *L. plantarum, L. reuteri* and *L. brevis***

Fermentation reduces sucrose, raffinose and stachyose (Length, 2011) due to the α-galactosidase activity of lactic acid bacteria on oligosaccharides (Donkor, Henriksson, Vasiljevic, & Shah, 2007). The presence of α-D-galactosyl oligosaccharides stimulates the activity of this enzyme (Scalabrini, Rossi, Spettoli, & Matteuzzi, 1998); (Tsangalis, Ashton, Stojanovska, Wilcox, & Shah, 2004). As shown in Table 9, fermentation led to a reduction in oligosaccharide content of the soymilk from different varieties. A paired samples test showed a significant difference between fermented soymilk and non-fermented soymilk in terms of oligosaccharides content ($P<0.05$).

## 4.4 Determination of compounds of five essential oils by gas chromatography and their effect on fungal growth and sensory acceptability of soymilk

### 4.4.1 Analysis of essential oils compounds by GC-MS

The five essential oils (basil, cinnamon, citronella, Eucalyptus and Mint) were analysed for compounds by GC-MS.

The main compound for Cinnamon (Cinnamomum verum) was Cinnamaldehyde (97.26%) at 18.99 RT. The composition depends on the growth stage and the segment of the plant (Vangalapati, Sree, Surya, & Avanigadda, 2012). Cinnamon leaves essential oil contain cinnamaldehyde (1.00 to 5.00%) and eugenol: (70.00 to 95.00%) while the bark contain cinnamaldehyde (65.00 to 80.00%) and eugenol (5.00 to 10.00%) (Jayaprakasha, Rao, & Sakariah, 2002); (Rao & Gan, 2014).

The compounds in Citronella (Cymbopogon nardus) are Limonene (38.51%) at 8.23 RT, Citronellal (30.29%) at 8.17 RT, Citronillol (14.32%) at 12.84 RT, Geraniol/citral (7.9%) at 14.61 RT and Cuparene (9%) at 17.02 RT. A study by Regnault-Roger (1997) reported Citronellal (33.8%), geraniol (21.6%), citronellol (9.2%) (Regnault-Roger, 1997). Compounds differ from one species of *Cymbopogon* to another; *C. martini* has Geraniol (64.0%-92.6%), *C. flexuosus* Citral (80.6%-84.4%), *C. pendulus* (Lemongrass) Citral (75.9%), limonene (5.5%), *C. winterianus* of Kashmir Citronellal (31.1-35.4%), Geraniol (22.4-30.2%), Citronellol (7.4- 11.0%) while *C. winterianus* of Hymalayan region of India contain Geraniol (50.1%), Citral (21.8%), Citronellal (11.8%) (Wany, Jha, Nigam, & Pandey, 2013).

Figure 10 shows a chromatogram of the compounds in Basil (*Ocimum basilicum*) essential oil. The main compounds for Basil were eugenol 83.26% at 22.06 Retention time (RT) and Caryophyllene (10.36%) at 23.98 RT.

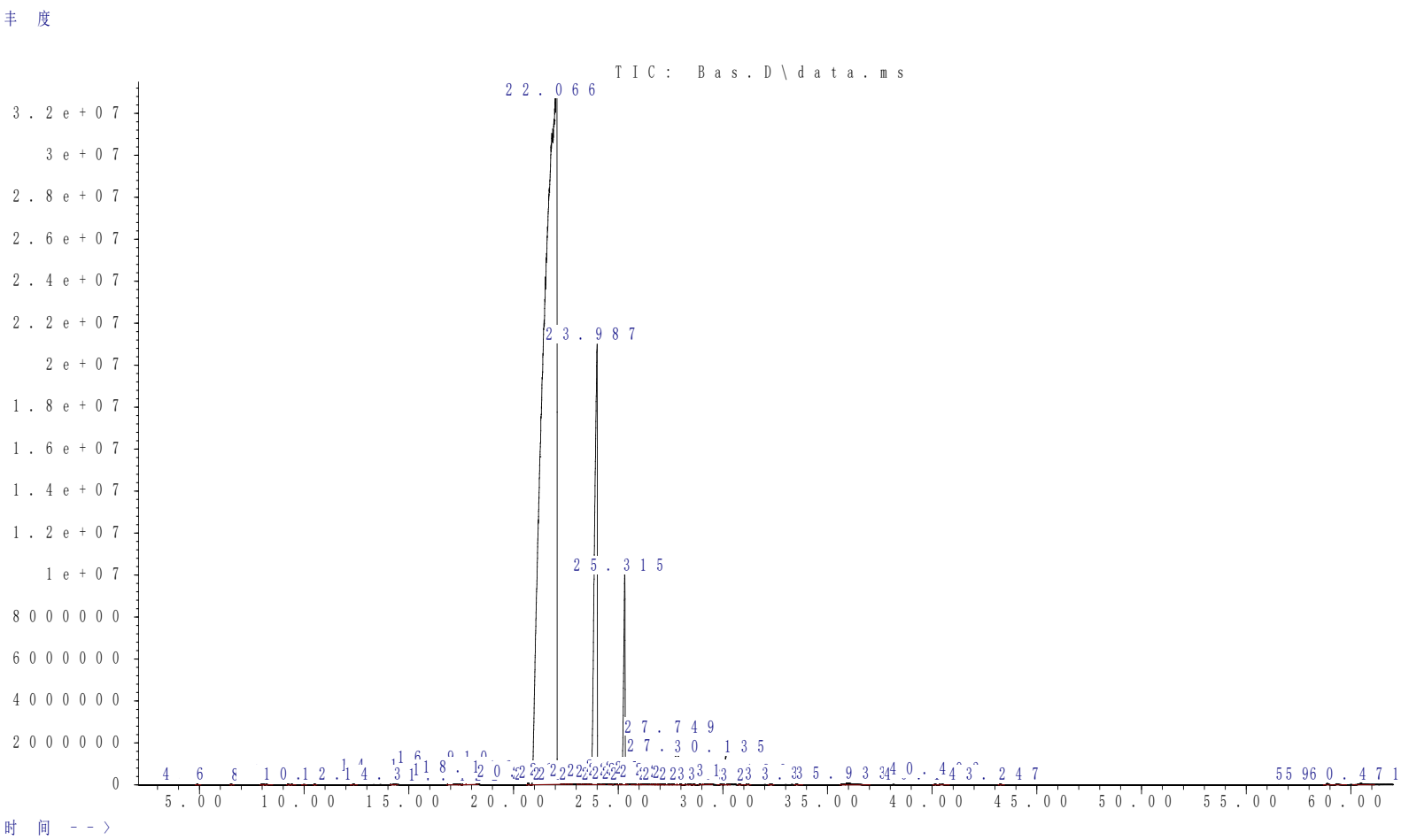


**Figure 4.10: Basil essential oil compounds**

The compounds in Basil vary depending on the species, growth stage, season and the geographic area where the plants are found, (Chamorro, et al., 2012); (Poonkodi, 2016). Some have eugenol as the major compound up to 87%, especially for species of eugenol chemotype, similar to our findings (Koutsos, Chatzopoulou, & Katsiotis, 2009); (Murárikovᡠet al., 2017). Others have linalool and chavicol as major compounds (Chamorro, et al., 2012); (Murárikovᡠet al., 2017); (Poonkodi, 2016)). According to (De Martino, De Feo, & Nazzaro, 2009) study, the main constituents of basil oil were iso-pinocamphone (35.10%) and carvone (39.70%), while eugenol was in trace, only 1% (De Martino, De Feo, & Nazzaro, 2009)

The main compounds in Eucalyptus (Eucalyptus globulus) are Eucayptol/Cineole 76.70% at 8.48 RT, Carene 9.70% at 7.61 RT, Terpinene 4.16% at 6.71 RT and Phellandrene 3% at 7.26 RT. Previous study reported cineole content of eucalyptus essential oil was 86%, pinene 3.9% and cymene 2.4% (Regnault-Roger, 1997). Some study found 44.08% of Cineole, 1.51% of Terpinene and many traces of compounds (Davari & Ezazi, 2017). Chemical compounds may vary within the same species due to the development stage of the plant used to extract the essential oils or the plant adaptation to the environment (Chamorro et al., 2012).

In mint essential oil, the main compounds are Menthol (42.72%) at 14.25 RT, Menthone (25.72%) at 13.04 RT, Limonene (4.92%) at 8.05 RT and Pinene (3.18%) at 5.25 RT. Previous studies found menthol 85.89%, menthone 2.99% depending on the development stage of the plant (Lopez-Reyes et al., 2010). Pino et al. (1999) found the major constituents as menthol (51.68%), menthone (26.08%) and menthyl acetate (10.55%), (Pino et al., 1996) and this was consistent to our study.

### 4.4.2 Essential oils on fungal growth

The Minimum Inhibitory Concentrations (MICs) were determined as the lowest concentration of essential oil inhibiting the visible growth of Aspergillus flavus 3.4408 on the PDA plate, (Table 10). The MIC is estimated at one fold the concentration > 80%

of inhibition (Clinical and Laboratory Standards Institute (CLSI), 2012); (Carson, Hammer, & Riley, 1995).

**Table 4.10: The minimum inhibitory concentration of EOs on fungal growth**

| Essential oil | Scientific name | $MIC_{>80\%}$ (µl/ml) |
|---|---|---|
| Cinnamon | *Cinnamomum verum* | ≤0.1 |
| Basil | *Ocimum basilicum* | 0.5-1 |
| Citronella | *Cymbopogon nardus* | 5-10 |
| Mint | *Mentha arvensis* | 10-20 |
| Eucalyptus | *Eucalyptus globules* | >>10 |

Cinnamon was the most effective in fungal growth at the concentration less than 0.1 µl/ml followed by Basil at 0.5 µl/ml and Citronella 5 µl/ml. Mint showed fungal inhibition growth at 10 µl/ml, the limit concentration set for the study and Eucalyptus didn't inhibit the fungal growth, it required higher concentration than 10 µl/ml.

In Figure 11 the concentration of EOs from citronella were plotted in accordance with the diameter of the colonies measured after 7 days of incubation. As the concentration increased the diameter of the colony decreased. The diameter of 3.8mm was measured for the concentration of 5µl/ml and the control sample had 80mm of diameter, thus the MIC was equal to 95.2%: ((80-3.8)/80)*100 (Davari and Ezazi, 2017; (Li et al., 2016)

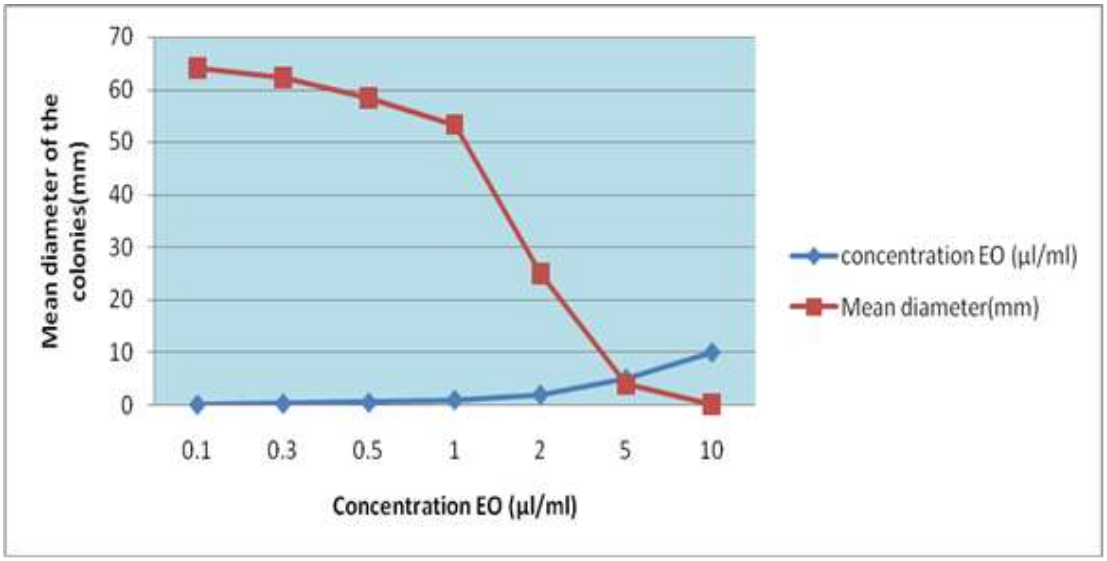


**Figure 4.11: Fungal growth per concentration of Citronella**

The inhibition is apparent in Plate 1 from the concentration of 30µl/10ml of medium up to the MIC of 50µl/10ml or 5µl/ml and no visible growth at the concentration of 100µl/10ml (10µl/ml). This may be due to the damage of cell wall of the fungi by the essential oil leading to the cytoplasm retraction in the hyphae and the death of mycelium (Sharma & Tripathi, 2008), or mitochondrial dysfunction (Bakkali, Averbeck, Averbeck, & I, 2008)

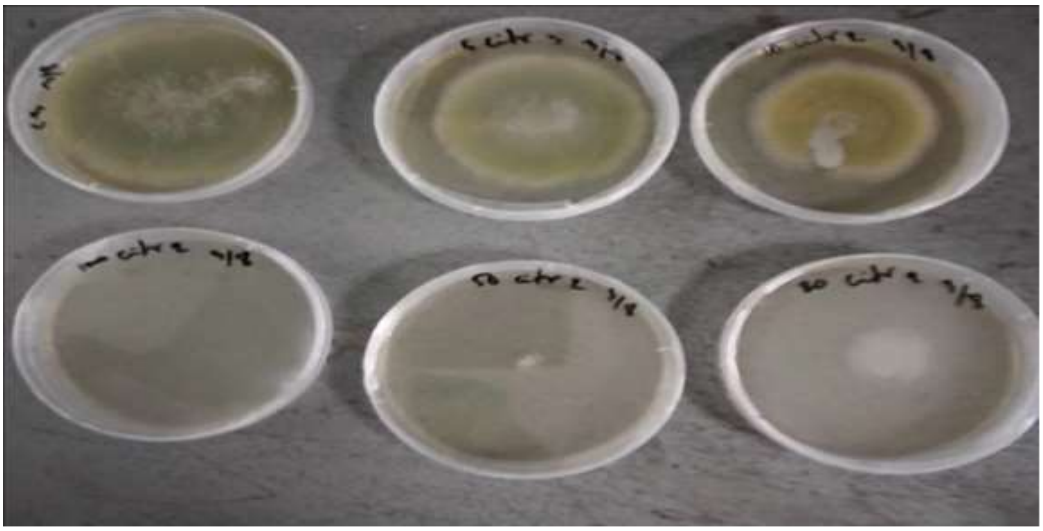

**Plate 4.1: Citronella essential oil inhibiting fungal vs control**

The essential oils showed the capacity as food preservative (Hyldgaard, Mygind, & Meyer, 2012); (Harich, Maherani, Salmieri, & Lacroix, 2018) and can increase the shelf life and acceptability of soymilk.

Table 4.11 shows the percentages of fungal growth inhibition per concentration and per EOs namely Cinnamon, Basil, Citronella, Mint and Eucalyptus.

**Table 4.11: The EOs concentrations vs Minimum inhibitory % of fungal growth**

| Essential oils source | Scientific name | concentrations (µl/ml) / %inhibition | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | 10 | 5 | 2 | 1 | 0.5 | 0.3 | 0.1 |
| Cinnamon | *Cinnamomum verum* | 100 | 100 | 100 | 100 | 100 | 100 | 100* |
| Basil | *Ocimum basilicum* | 100 | 100 | 100 | 100 | 86.9* | 74.8 | 41.3 |
| Citronella | *Cymbopogon nardus* | 100 | 95.2* | 68.8 | 33.3 | 27.1 | 22.1 | 19.8 |
| Mint | *Mentha arvensis* | 84.0* | 32.3 | 20.8 | 14.6 | 12.9 | 10.8 | 10.6 |
| Eucalyptus | *Eucalyptus globulus* | 29.4 | 23.1 | 18.8 | 17.3 | 16.3 | 12.7 | 12.1 |

*% MIC > 80% inhibition

Cinnamon, Basil, Citronella and Mint EOs inhibit growth of fungal microorganisms. However, some EOs compounds were more efficient in fungal inhibition. This was consistent to previous studies reporting EOs as natural fumigant and playing a significant role to eliminate storage fungi and increase the shelf life in food (Kohiyama, et al., 2015); (Prakash, Singh, Kedia, & Dubey, 2012); (Pratiwi, et al., 2015). A study done by (Tian, et al., 2012) showed that cinnamon is a powerful inhibitor of spore germination and synthesis of Aflatoxins by A. flavus (Tian, et al., 2012). The Clinical and Laboratory Standards Institute (CLSI) estimate that an antimicrobial agent, which can inhibit bacterial and fungal growth at 4µl/ml to be effective (Clinical and Laboratory Standards Institute (CLSI), 2012). Thus, Cinnamon, Basil and Citronella can be recommended as preservatives. Eucalyptus was less effective, only 29.4% inhibition at the highest

concentration studied of 10µl/ml. This has been reported in previous study done by (Davari & Ezazi, 2017) where 1-8 cineole, the main compound of eucalyptus essential oil was not effective for fungal growth inhibition (Davari & Ezazi, 2017).

### 4.4.3 Sensory test of soymilk flavored with three selected essential oils

Citronella, Basil and mint were randomly selected for sensory evaluation of soymilk as shown in Table 12. A scale of 6 to 9 score means that the product is liked (Hashmi, 2007). All the soymilk samples were accepted (score above 6) for aroma, color, clarity, taste and overall acceptability except the control for aroma only. The more preferred was mint with a score of 8 corresponding to 'Like very much' followed by Citronella with a score of 7 (Like moderately) and Basil with a mean score of 6.70. The difference between flavors was significant for aroma, taste and overall acceptability ($P<0.05$).

**Table 4.12: Sensory evaluation of soymilk flavored with 3 EOs at 3 drops per liter**

| Essential oils | Aroma | Color | Clarity | Taste | Overall acceptability |
|---|---|---|---|---|---|
| Citronella | 6.20 ± 1.61 | 8.00 ± 0.94 | 7.10 ± 1.28 | 7.10 ± 0.99 | 7.00± 0.81 |
| Mint | 8.10 ± 1.28 | 7.90 ± 0.87 | 7.70 ± 1.25 | 7.70 ± 1.05 | 8.00 ± 0.66 |
| Basil | 6.30 ± 1.63 | 7.80 ± 1.13 | 7.30 ± 1.49 | 6.30 ± 1.41 | 6.70 ± 1.25 |
| Control | 5.80 ± 1.03 | 8.00 ± 0.94 | 7.70 ± 1.16 | 6.10 ± 1.52 | 6.20 ± 1.31 |
| P value | 0.004 | 0.962 | 0.665 | 0.028 | 0.004 |

Table 4.13 reports the aroma, color, clarity, taste and overall acceptability of soymilk for 0, 1, 2, 3 drops of EO used to flavor one liter of soymilk.

**Table 4.13: Sensory evaluation of soymilk using 3 concentrations of Citronella**

| Concentrations (drops) Cymbopogon | Aroma | Color | Clarity | Taste | Overall acceptability |
|---|---|---|---|---|---|
| 0 | 5.80 ± 1.31 | 7.60 ± 1.26 | 7.80 ± 1.03 | 6.00 ± 0.94 | 6.60 ± 0.96 |
| 1 | 6.50 ± 0.97 | 7.70 ± 1.25 | 7.80 ± 1.03 | 6.80 ± 0.91 | 6.90 ± 0.73 |
| 2 | 6.60 ± 0.84 | 7.50 ± 1.50 | 7.80 ± 1.03 | 6.80 ± 1.03 | 7.00 ± 0.81 |
| 3 | 7.90 ± 0.73 | 7.50 ± 1.65 | 7.60 ± 1.17 | 7.90 ± 0.87 | 7.80 ± 0.91 |
| P value | 0.000 | 0.987 | 0.967 | 0.001 | 0.025 |

The three concentrations of Citronella EOs used to flavor soymilk had a high acceptability (>6) on a scale of 1 to 9 except for the control for aroma, which had a mean score of 5.8. This indicates that flavoring soymilk with essential oils camouflages the beany odor and enhances the organoleptic attributes of soymilk. Three drops of EOs concentrations were liked very much in soymilk flavoring and the difference between concentrations was significant for aroma, taste and overall acceptability ($P<0.05$). The soymilk flavored with essential oils could be used as potential to promote the method of processing that the preserve nutrients such as protein and isoflavones required for the beneficial health effect (Boye & Ribereau, 2011).

# CHAPTER FIVE

# CONCLUSIONS AND RECOMMENDATIONS

## 5.1 Conclusions

Soybean from Rwanda was found to have low rates of mycotoxin contamination while farmer's awareness on mycotoxins contamination and harm to the human health was low. The effect of post-harvest handling, especially drying on polyethylene sheets and drying duration of 5 days might have contributed to maintaining soybean safe for human consumption as it had low mycotoxin levels. The production region of high temperature may contribute to levels of contamination. The nutritional value of soymilk was affected by the processing methods used. Various methods used in soybean milk preparation intend to reduce the lipoxygenase activity; however, cooking the soybean before soymilk extraction reduces nutrients content. Soymilk processing by a method involving soaking the soybeans for 12 hours prior to extraction and subsequent boiling exhibited better extraction of nutrients and isoflavones. Sc. Squire and the Local varieties exhibited higher extraction of proteins and fat than the other varieties during soymilk processing by the methods involving heating prior to extraction. Fermentation by different lactic acid bacteria decreased flatulence-causing oligosaccharides and increased antioxidant activity in fermented soymilk, which translates into health benefits of fermented soybean products. Three probiotic bacteria, namely *Lactobacillus reuteri*, *Lactobacillus brevis* and *Lactobacillus plantarum* were found to be suitable for soymilk fermentation. Finally, there was effect of cinnamon, basil, citronella and mint Essential oils (EOs) on fungal growth and sensory acceptability of soybean milk. Essential oils are good substances for flavoring soymilk to improve organoleptic attributes and inhibit fungal growth, thus can contribute to increase the shelf life of soymilk.

## 5.2 Recommendations and scope for further work

There should be a deliberate effort to educate farmers and other actors in the Rwanda value chain on mycotoxins and practices for preventing their contamination of food.

Given the relatively low levels of soybean contamination with mycotoxins, soybean consumptions should be promoted not only as a nutritious product but also as a safe product. However, there should be closer surveillance of mycotoxin contamination, especially in the Eastern region, not only for soybean but also for other susceptible produce such maize. Two varieties Sc. Squire and Local, especially, Sc. Squire have high isoflavone content may be recommended for soymilk preparation. *Lactobacillus reuteri*, *Lactobacillus brevis* and *Lactobacillus plantarum* can be recommended to ferment soy yoghurt for its increased nutritional value/health benefit and consumption in Rwanda. For fermented soymilk production, the milk extraction can be done by a method involving soaking for 12 hours followed by extraction prior to boiling. Although this method does not eliminate beany flavor, it gives better extraction of nutrients and isoflavone. The beany flavor of the fermented soymilk can then be masked by essential oils such as from mint, citronella and basil. Future comprehensive studies on sensory acceptability of soybean products made from different varieties by different methods among different consumer categories will be necessary.

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## APPENDICES

### Appendix I: Label samples/Questionnaire pre and post-harvest handling, and aflatoxins awareness

CODE:

Date:

District:

Sector:

Village:

Variety:

Planting time:

Harvesting time:

Drying duration:

Drying equipment:

Storage duration:

Storage equipment:

Pesticides used:

Soy Products known:

**Appendix II: Sensory evaluation of soymilk**

**Date:…….....**

Put the sign × in the case where your age and gender belong

**Age** **20 – 30** ☐ **31 – 40** ☐ **>41** ☐

**Gender** **Male** ☐ **Female** ☐

**Instructions:** You are provided with different numbered samples of soymilk and you are request to carry out the sensory evaluation. Please rinse your mouth after testing each sample using provided water. Do not seek another person's opinion. Please indicate your degree of liking or disliking on provided parameters by scoring the given samples using the scale below.

Evaluate the samples of soymilk for the attributes given against the following scale:

Like extremely…….9
Like very much…..……8
Like moderately……7
Like slightly……..6
Neither likes nor dislikes…… 5
Dislike slightly……..……4
Dislike moderately……..……..3
Dislike very much……...……..2
Dislike extremely……1

| Sample | Aroma | Colour | Clarity | Taste | Overall acceptability |
|---|---|---|---|---|---|
| **222** | | | | | |
| **223** | | | | | |
| **221** | | | | | |
| **224** | | | | | |

**Thank you**

**Appendix III: Data, papers and abstracts published**

Niyibituronsa M, Onyango N. A., Gaidashova S., Imathiu S., Ming Z., Ruinan Y., Weiqi Z., XiuPin W., Qi Z., Zhaowei Z. and Peiwu L. (2020) Evaluation of five essential oils by Gas Chromatography-Mass Spectrometry and their effect on fungal growth inhibition and sensory acceptability of Soymilk. Journal of Food Research; 9 (2): 36-47. https://doi:10.5539/jfr.v9n2p36

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