NUTRITIONAL, ANTIOXIDANT AND METAGENOMIC ANALYSIS OF 'SUPERFOODS' PRODUCED WITH SPROUTED SOYBEANS

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Declaration

I hereby declare that the work reported in this thesis and submitted to the Department of Biotechnology and Food Technology at the Durban University of Technology for a Doctoral Degree is my original work. I confirm that it has not been previously submitted for a degree at any other University or Higher Education Learning Institution.

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Preface

This thesis is organized into nine chapters and presented in the format submitted for publication. Chapter one is a general introduction to the thesis. Chapter two presents a critical review of relevant literature and the areas covered in the literature review include the utilization of soybeans for the production of fermented and unfermented foods, methods of microbial population determination in fermented soy-based foods, health benefits of soy consumption, effect of sprouting on soybeans and its products and optimization of processing conditions in soy-based food production. Chapter three presents the optimization of sprouting parameters of soybeans. Chapters four, five and six present the nutritional and functional attributes of soy-based foods produced from optimized sprouted soybeans in Chapter three. Chapter seven describes the microbial community of fermented soy-based foods using molecular and metagenomic approaches. Chapter eight is the general discussion of the entire findings, with conclusions and recommendations for possible future works. Chapter nine is for the references.

Publications and Conference Outputs

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Abstract

This research was aimed at developing different 'superfoods' from sprouted soybeans and determining their nutritional, antioxidant and microbial characteristics. The specific objectives were to optimize the sprouting conditions (soaking and germination) of soybeans for the production of soymilk using response surface methodology, determine the effect of these optimized sprouting conditions of soybeans on the nutritional and quality attributes of resulting superfoods, determine their antioxidant activities, identify and evaluate the potentials of optimum sprouting conditions in reducing soy allergens and to determine the microbial community of fermented soy-based superfoods using next generation sequencing approach. In all cases, soy-based foods from unsprouted soybeans served as control samples. For sprouted soymilk production, soaking (12-24 h) and germination times (48-96 h) were optimized with response surface methodology, at a constant temperature of 25°C using central composite rotatable design. The optimum sprouting conditions of soybeans obtained were 12 h soaking and 52 h germination using desirability concept. Soymilk made from optimized conditions had 17% increase in total proteins, 50% reduction in phytic acid, 1.7% increase in total phenolics and a colour change of 4.89 compared to the control produced from unsprouted soybeans. There was a significant reduction in trypsin inhibitor activity (0.03 mg/g TI), with increase in total amino acids and similar rheological properties in optimized soymilk to the control.

Soy-based foods, including tofu, naturally fermented soymilk and soymilk-kefir, were produced from soymilk obtained at optimized conditions of soybean sprouting and their nutritional and colour attributes were investigated. Total protein contents of sprouted products were higher than their controls and there were no significant differences in the ash contents ($p \leq 0.05$). *In vitro* protein digestibility ranged between (85.70-99.82%) for sprouted and (81.33-99.50%) for unsprouted ones, with fermented products having the highest digestibility. Generally, no significant difference in amino acid contents ($p \leq 0.05$) was recorded for all sprouted soy foods and their controls. Total flavonoids were higher in sprouted tofu coagulated with $CaCl_2$ (60.41mg/g) and sprouted fermented soymilk (48 h) (8.93 mg/g) and there was a general reduction in total condensed tannins for all sprouted products.

Colour deviation of sprouted products from their controls were minimally perceivable, as the highest ΔE value was 5.30. Soy products obtained from sprouted soybeans at optimized conditions had nutritional advantage over unsprouted ones, with negligible colour deviations.

Antioxidant activities of soy-based foods produced at optimized conditions of soybean sprouting were investigated using different assays. Total phenolic content (TPC) was determined using Folin Ciocalteau method and samples (0.5-5 mg/ml), in appropriate buffers were tested for their abilities to scavenge free radicals. Sprouted soy-based food products had significantly higher ($p \leq 0.05$) TPC (13.21-14.21 mgGAE/g) when compared to their controls (13.01-14.07 mgGAE/g). Sprouted soy-based foods scavenged free radicals and demonstrated higher diphenyl-1-picrylhydrazyl (DPPH), superoxide and hydroxyl radical scavenging activities. Over 50% increase in metal ion chelation was observed in all sprouted soy-based food products, however, ferric reducing antioxidant power (FRAP) in both sprouted and unsprouted products were not significantly different ($p \leq 0.05$). Production and consumption of soy foods from soybeans sprouted at these optimized conditions as functional foods could be a promising means of preventing the excessive production of reactive oxygen species.

The allergenic proteins in optimized sprouted soy-based foods and their respective controls were identified and their levels were evaluated. Protein was extracted from each soy-based food and their concentrations were determined using Bradford assay. Aliquots of proteins previously dissolved in appropriate buffers were separated according to their molecular weight and ionic strengths using 2-dimensional electrophoresis. Protein spots were then excised from the gels and made to undergo complete tryptic digestion to produce peptides. Identities of peptides were determined using MALDI-TOF mass spectrometry and the volumes of protein spots in each gel was determined using PDQuest image analyzer. Protein concentration significantly increased in sprouted products (up to 149% in SSK). Eight differentially expressed protein spots were selected and identified by MALDI- TOF/TOF mass spectrometry as glycinin subunit G2, β-conglycinin (α, α' and β subunits), trypsin inhibitor, 34 kDa soy seed protein and sucrose-binding proteins.

Higher extent of breakdown of soy storage protein was obtained in sprouted soy products, as shown by higher protein concentration and spot volumes. Lower spot volumes were obtained in all fermented products. This study suggests higher potentials for soybean-storage-protein breakdown and reduced allergen contents in sprouted soy-based foods.

The microbial community of fermented soy-based foods from sprouted soy foods and their respective controls was determined using rep-PCR and high throughput amplicon sequencing (HTS). Samples of spontaneously fermented soymilk and soymilk-kefir were collected at 6 h interval from 0-48 h and subjected to analyses. From rep-PCR, identified LAB species include *Weissela cibaria, Lactococcus lactis, Leuconostoc lactis, Leuconostoc messenteroides,* and *Lecleria adecarboxylata* while yeast species are *Saccharomyces cerevisae, Pichia fermentans* and *Torulaspora delbrueckii*. In addition to the genera revealed in rep-PCR, HTS showed the presence of *Bacillaceae* and other bacteria involved in spontaneous and kefir fermentation of soymilk. It could be concluded from the findings of this research that sprouting of soybeans at the suggested optimized conditions has potential for extension of use of soybeans in the production of soy-based foods with enhanced nutritional and functional properties.

Chapter 1: Introduction

Soybean (*Glycine max*) is a popular plant food source due to its various nutritive components (Messina, 1999). The major proximate composition of soybean seeds, on dry basis, includes approximately 40% protein, 30% carbohydrates and 20% oil, which makes it a major plant based food source of nutrients for human consumption (Yu *et al*., 2016). Soybeans have also been found to contain isoflavones, saponins, phytosterols, phytic acid, lectin and trypsin inhibitors, whose metabolites offer potential health benefits such as the prevention of diabetes and cancer (Jooyandeh, 2011). In addition to being consumed as roasted or boiled beans, soybeans have also been processed into many products such as soymilk, tofu (cheese-like product), soy flour, sauce, among many others, providing a significant food source of protein, oil, carbohydrates and phytochemicals (Yoshikawa *et al*., 2014). Soy proteins are also known to contain balanced amino acids which can compete with proteins from animal sources such as eggs, beef and fish from a nutritional perspective (Setchell and Radd, 2000). Soybeans have a high lysine content, and as such, soymilk can be used in place of animal milk to complement cereal foods (Jooyandeh, 2011). Many products have been made from soybeans to serve nutritional, functional and therapeutic purposes and isolates such as soy protein and oil are prepared from soybeans. Food products traditionally prepared from soybeans are either non-fermented; soymilk, tofu, whole dry beans, soy sprouts, soy nuts (Golbitz, 1995, Jooyandeh, 2011) or fermented such as tempeh, *natto*, soy sauce and *sufu* (Murooka and Yamshita, 2008). Fermented soy products have been found to contain higher levels of antioxidants and bioavailable nutrients than the unfermented ones (Tsangalis *et al*., 2002).

Soybeans have been traditionally consumed in Asia and is gaining wide popularity in other parts of the world due to the health benefits linked to its consumption (Golbitz, 1995). Consistent consumption of soy products has been found to reduce the risk of chronic diseases, and provide the body with minerals, vitamins and other nutritive metabolites. Isoflavones such as genistein, diadzein and glycitein, abundantly available in soy products, have been found to reduce the risk of cancers (He and Chen, 2013, Aresta *et al*., 2016).

Other components such as saponins, phytosterols, phytic acid and lectins have been linked with various beneficial effects, including anti-carcinogenic activities, anti-oxidation and inhibition of platelet aggregation (Jooyandeh, 2011).

'Superfoods' is a general term given to functional foods which have the characteristics of improved beneficial physiological effects and potentials for reduction of risk of disease development when consumed, as compared with regular foods (van den Driessche *et al*., 2018). Sprouting and fermentation have been used to achieve these purposes in foods. Sprouting of seeds prior to use triggers the enzymatic activity, leading to the breakdown of proteins, carbohydrates and lipids into their simpler, bioavailable forms (Nout and Ngoddy, 1997). Almost all the commercially available products of soybeans have been derived from their dry, unsprouted forms. The sprouting process is aimed at increasing the versatility and extensive utilization of soybeans, and to break down the complex constituents, leading to the release of the nutrients in simpler forms (Paucar-Menacho *et al*., 2010b). Germination of legumes had been used to improve the nutritional values, reduce the anti-nutritional factors and also improve protein digestibility (Rumiyati *et al*., 2012). Factors to be closely monitored while sprouting seeds for food processing include soaking time, germination temperature, relative humidity, variety of seeds and germination time, as their effective combination determines the characteristics of the resulting sprouted seeds (Fernandez-Orozco *et al*., 2008, Jiang *et al*., 2013, Murugkar, 2014).

Fermentation is a food process technology which brings about the improvement of the nutritional, functional and sensory qualities of foods, as well as enhancement of their shelf lives (Ijabadeniyi, 2007). The biochemical changes occurring during fermentation lead to alterations in structure and nutritional composition of foods, bringing about digestibility and bioavailability of food constituents and as such, it has been applied in the production and extraction of bioactive compounds from foods (Martins *et al*., 2011, Zhang *et al*., 2012). In legumes, fermentation results in increase in the bioactive phenolic compounds and this has enhanced the antioxidant activity of fermented legume products, hence, improving their health-linked functionalities (Torino *et al*., 2013). For example, the development of angiotensin converting enzyme (ACE)-inhibitory peptides and alpha-aminobutyric acid as a result of lactic acid bacteria fermentation has helped in the prevention and treatment of hypertension (Kono and Himeno, 2000, Torino *et al*., 2013).

Also, exposure of microorganisms to oxidative stress during fermentation may bring about the production of protective mechanisms, such as the production of enzymatic antioxidants which may in-turn contribute to the anti-oxidation ability of the food being processed (Kim *et al*., 2005).

Optimization of food and other biochemical processes is a technology aimed at obtaining the best processing conditions, resulting in products of acceptable quality attributes (Sobukola *et al*., 2010). Optimization could either be achieved through traditional methods (one variable) or response surface methodology (RSM) (multi-variate) (Baş and Boyacı, 2007). RSM has been suggested and used as the preferred tool of optimization because many biological, chemical and biochemical processes often require the interrelationships of independent variables to determine the nature of the resulting responses (dependent variables) (Baş and Boyacı, 2007, Bezerra *et al*., 2008). Also, RSM helps to reduce the number of experimental runs necessary to conduct a research, through the formation of adequate combination of factors, thereby increasing the reliability of results and reducing the cost and time of research (Bezerra *et al*., 2008). Therefore, optimization of sprouting conditions, using RSM, can be employed to determine the best levels for the combination of factors of sprouting, in order to obtain optimum levels of desired attributes (responses) in the resulting soy products (Paucar-Menacho *et al*., 2010a). Many approaches to the optimization of sprouting parameters of soybeans, using different optimization tools, have been suggested in literature for optimal production of nutrients, bioactive compounds and antioxidant capacities (Paucar-Menacho *et al*., 2010b, Guo *et al*., 2011, Jiang *et al*., 2013, Huang *et al*., 2014) with differing suggestions of results of optimal sprouting conditions.

The effect of soaking time, as an important factor of sprouting, is yet to be extensively studied singly or in combination with other factors. Information about the use of sprouted seeds for the production of soy-based foods and their resulting quality attributes, with respect to nutrition, antinutritional factors, allergens and antioxidant activity is yet to be sufficiently documented. Also, the effect of pre-process sprouting of seeds on the microbial community of fermented soy-based food, especially for spontaneous fermentation, is yet to be adequately explained. Therefore, it is imperative to investigate the effects of optimization of soaking and germination times of sprouted soybean seeds on the quality attributes of soy-based 'superfoods' that would be produced from these sprouted soybeans.

This study aims to investigate the improvement of nutritional attributes of fermented and unfermented soy-based foods as a result of optimization of sprouting conditions of soybeans, relative to previous studies. Antioxidant (oxidative radicals-scavenging) potentials of sprouted soy-based foods will also be compared with those of unsprouted ones, using different antioxidant assays.

The potential for enhanced breakdown of soybean storage proteins will be considered, in order to investigate the potentials of optimized sprouting on the allergenic tendencies of sprouted soy-based foods. Also, the microbial community of spontaneously fermented products from sprouted and unsprouted soybeans will be determined using culture independent and metagenomic approaches, to adequately understand their microbial diversity and how sprouting, as a pre-processing operation, affects the community of microorganisms.

Chapter 2: Literature Review

2.1. Soybeans as a food source

Soybean (*Glycine max*) is a very important leguminous crop that has been used as a good source of plant protein for human diet, and also constitutes a large percentage of the protein content in the feed formulations for livestock and aquatic animals (Masuda and Goldsmith, 2009). Apart from proteins, soybeans are excellent sources of phytochemicals, flavonoids, fibre, polyunsaturated fats and are low in calories. Soybean is estimated to contain 36.9% protein, 6.1% carbohydrates, 20.9% fibre, 18.1% lipid and 4.7% ash (Muzquiz *et al.*, 2012). The components of soybean, often referred to as phytochemicals, include bioactive proteins and peptides, phytosterols (including phenolic acids and isoflavones), phytic acid and saponins. Although, some of these phytochemicals have been considered as antinutrients in some literature, they have recently been found to exert beneficial health and therapeutic effects. The beneficial physiological impact of these substances is as presented in Table 1.

Table 1: Physiological importance of soybean constituents

Components in soybeans	Physiological functions
Soybean proteins	Reduction of serum cholesterol, prevention of cardiovascular diseases, reduction of body fat and promotion of serum insulin
Peptide from proteins	Antioxidant activities, inhibition of angiotensin-converting enzymes and promoting action of phagocytosis
Isoflavones	Anti-carcinogenetic activities, prevention of cardiovascular diseases, prevention of osteoporosis, antioxidant activities and alleviation of menopausal symptoms
Saponins	Anticarcinogenic activities, hypocholesterolemic effects, inhibition of platelet aggregation, HIV preventing effects and antioxidant activities
Phytosterol	Anti-carcinogenic activities
Phytic acid	Anti-carcinogenic activities
Lectin (Hemagglutinin)	Activation of lymphocytes (T cell) and aggregating action of tumor cells
Nicotianamine	Inhibitor of angiotensin-converting enzyme
Protease inhibitors	Anti-carcinogenic activities

Adapted from (Jooyandeh, 2011)

2.2. Soy-based food products

Food products from soybeans have been broadly classified into two: fermented and non-fermented soy foods.

2.2.1. Fermented soy foods

Most Asian fermented foods are produced with soybeans. Fermentation was the traditional solution found by the Asian soy food producers, to the objections of consumers, to the intrinsic beany or grassy flavour of soybeans, and the phenomenon of flatulence associated with the consumption of unfermented soy products. Soybean is found to contain alpha-galactosides including raffinose (0.5-1.3%), stachyose (2.2-4.3%) and verbascose (0-0.3%) (Guillon and Champ, 2002, Hood-Niefer *et al*., 2012). Fermentation was used to achieve the reduction in beany flavour, flatulence and the reduction in antinutritional factors, such as phytates and oxalates, which have been implicated in the blockage of the uptake of essential minerals such as Calcium, magnesium, iron, copper and zinc. Fermentation of soy foods are either carried out using single cultures, mixed cultures or spontaneously by allowing the products to ferment and cure naturally. With the proximate composition of soybean, it is considered a good substrate for the production of functional foods. Therefore, subjecting soybeans to fermentation will help to improve phytochemical content, such as free isoflavones, breakdown complex proteins and carbohydrates into simpler, absorbable forms by the actions of hydrolytic enzymes produced by microbial activities before consumption and also produce probiotics that may be beneficial to the gastrointestinal tract of consumers. (Jooyandeh, 2011).

2.2.1.1. Fermented soybean-barley paste: *Miso*

The first reference to *Miso* was in ancient Chinese text around 700BC. *Miso* is a thick paste which is rich in protein, vitamins and minerals and has played a very important role in Japanese nutrition as it is consumed by over 90 percent of Japanese population (Murooka and Yamshita, 2008). It is a Japanese food (or sometimes seasoning) produced by the fermentation of soybeans with *Actinomucor oryzae* mould cultivated on steamed rice or barley and mixed with salt (*koji*).

The most common types of *Miso* are made from soybeans, while others are obtained from barley and rice and they differ in the types of ingredients, temperature and duration of fermentation, salt content, variety of starter culture used and the variety of fermentation vessel used. Consumption of *Miso* may help in reducing the risk of gastric cancer (Hirayama, 1981), due to the presence of oleic and linoleic acids and their esters which help to slow down the proliferation of cancer cells (Ueoka and Yamauchi, 2004). Consistent consumption of *Miso* has also been suggested to have anti-aging effects, prevent arteriosclerosis (Kawano) and also lower cholesterol levels (HORII *et al*., 1990) due to the presence of vitamins B2, B12 and E, as well as lecithin, choline, dietary fibre and isoflavones in it.

2.2.1.2. Soy sauce: *Shoyu*

Soy sauce or *Shoyu* is made from almost equal quantity (by weight) of cooked soybeans and roasted wheat. Traditionally, to produce soy sauce, moistened soybeans are first cooked at high temperature and pressure and mixed with almost the same quantity of grounded roasted wheat, whole wheat. Thereafter, a mash is made from the mixture by the addition of a small amount of koji mold (*Actinomucor oryzae* or *Aspergillus kojae*) (Kobayashi, 2005), NaCl (15-17%) and water and allowed to ferment in wooden vessels for 1-2 years. Currently, commercial production of soy sauce has been automated from raw material mixing to packaging with reduction in the duration of fermentation (6-8 months). After fermentation, the aged mash is pressed to release soy sauce, which is further pasteurized before being made available commercially. More than being a sauce, soy sauce could be regarded as a functional food. Soy sauce has demonstrated antioxidant potentials (Ando *et al*., 2003, Long *et al*., 2000), antimicrobial activity against pathogenic microorganisms and potentials for angiotensin I converting enzyme inhibition (Kinoshita *et al*., 1993). The active compounds of soy sauce are formed during enzymatic digestion of soybeans and roasted wheat due to the metabolic activities of fermentative microorganisms. Due to prolonged aging, there are prolonged activities of proteolytic enzymes, leading to the production of short chain amino acids and peptides and complete elimination of allergens present in the raw materials (Tsuji *et al*., 1995, Ogawa *et al*., 2000).

2.2.1.3. *Natto*

Natto is a fermented soy product obtained from soybeans cultured with *Bacillus subtilis*. It is prepared by steaming soybeans and then inoculating with *B. subtilis*. *B. subtilis* is traditionally obtained from rice straw and hence, *Natto* could be produced by covering steamed soybeans with rice straw and allowing to ferment for at least 20 h (Murooka and Yamshita, 2008). Activities of *B. subtilis* brings about the degradation of soybean starch and protein into simpler forms of sugars and amino acids. Also, vitamins, isoflavones, saponins and fibrinolytic enzymes are released during the fermentation process. About 124 fold increase in vitamin K was found during natto fermentation (Yanagisawa and Sumi, 2005). The sticky nature of *Natto* could be traced to the formation of polyglutamic acid network. Beneficial effects of *Natto* consumption include antimicrobial activity against *E. coli* and *Helicobacter pylori*, prevention of osteoporosis, breakdown of blood clots through the action of nattokinase and also the breakdown of fibrin buildup, thereby enhancing the functionality of the heart (Fujita *et al*., 1995, Sumi *et al*., 1990).

2.2.1.4. *Tempeh*

Tempeh is traditionally produced by dehulling and boiling soybeans and then fermenting with *Rhizopus sp*. On achieving the desired colour and texture preferences, usually after 1-2 days of fermentation, Tempeh is pressed into blocks or cubes, with different colours depending on the soybean and mold used. Tempeh is rich in aminobutyric acid and isoflavones and the recently available methods of tempeh production are such that improves their levels in the final products (Nakajima *et al*., 2005, Aoki *et al*., 2003). The richness in aminobutyric acid and isoflavones makes the consumption of Tempeh a good nutritional supplement for the elderly (Tsuchida *et al*., 1999), and a possible way of alleviating the symptoms of osteoporosis (Brzezinski *et al*., 1997, Murooka and Yamshita, 2008) and suppress the onset of arteriosclerosis due to improved lipid and cholesterol metabolism.

2.2.1.5. Fermented soymilk

Soymilk is a water extract of soybeans and it is obtained by soaking raw soybeans in water for a prolonged time (usually overnight), grinding the soaked beans in a blender, sieving through muslin cloth and boiling the water extract at temperatures over 100°C for a short time. (Wang *et al*., 2006). Fermentation of soymilk has been extensively carried out by the addition of pure culture of fermentative bacteria, including lactic acid bacteria such as *Lactobacillus acidophilus*, *Streptococcus thermophilus* (Hsieh and Chou, 2006), *Lactobacillus rhamnosus* (Marazza *et al*., 2012) and bifidobacteria such as *Bifidobacterium infantis* and *B. longum* (Wang *et al*., 2006) and *B. bifidum* (Zhao and Shah, 2014b). These authors reported antimutagenic effects, improved antioxidant activity, and increase availability of bioactive components in fermented soymilk. Spontaneous fermentation of soymilk had been attempted (Obadina *et al*., 2013) with increase in the macro nutrients after fermentation for 72 h. The viability of natural fermentation of soymilk, with respect to antioxidant activity, bioavailability of nutrients and isolation and purification of possible starter cultures of fermentative microorganisms, is yet to be exhaustively explored.

2.2.2. Non-fermented soy products

2.2.2.1. Soymilk

As earlier described, soymilk is the water extract of whole soybeans, with some similarities to cow milk in resemblance and some chemical contents (Cruz *et al*., 2007). The nutritional contents of soymilk (Table 2) are as presented by Giri and Mangaraj (2012). Apart from the common method of production which involves soaking, wet milling, filtering (to remove okara) and cooking, soymilk can also be made by reconstituting full soy flour in water to the desired consistency. Soymilk could either be consumed directly or serve as a raw material for the production of other soy-based foods including tofu, soy yoghurt, ice cream and other soy cheeses (Golbitz, 1995). For traditional method of soymilk production, sound kernels are manually separated from a soybean batch, after which water will be added to soak for 4-12 h. Soaked beans are manually dehulled to separate the shafts and naked seeds. Water is added to dehulled seeds, up to ratio 10:1 (water/seeds) and then ground. Some methods involve steaming the mixture before filtering or filtering the water extracts from the residue before cooking the extracts (Murugkar, 2014).

Steaming and cooking are usually carried out to inactivate antinutrients such as lipoxygenase and trypsin inhibitor. Industrial approach to soymilk production involves ultra-high temperature processing and aseptic packaging, to enhance the shelf life of soymilk (Giri and Mangaraj, 2012). The methods of soymilk processing with the resultant physical characteristics of the resulting products are presented in Table 3. Different factors that can affect the quality of soymilk include extraction methods (Prabhakaran and Perera, 2006), heat treatment method used (Poliseli-Scopel *et al*., 2012), soybean seed composition (Poysa and Woodrow, 2002), pre-process sprouting (Murugkar, 2014) among many others.

Table 2: Nutritional content of soymilk

Component	Amount	Component	Amount
Water	93 (g)	Sodium, Na	12 (mg)
Energy	138 (kJ)	Zinc, Zn	0.23 (mg)
Protein	2.8 (g)	Copper, Cu	0.12 (mg)
Fat (total lipid)	2 (g)	Manganese, Mn	0.17 (mg)
Fatty acids, saturated	0.214 (g)	Selenium, Se	1.3 (lg)
Fatty acids, mono-unsaturated	0.326 (g)	Vitamin C (ascorbic acid)	0 (mg)
Fatty acids, poly-unsaturated	0.833 (g)	Thiamin (vitamin B1)	0.161 (mg)
Carbohydrates	1.8 (g)	Riboflavin (vitamin B2)	0.07 (mg)
Fiber	1.3 (g)	Niacin (vitamin B3)	0.147 (mg)
Ash	0.27 (g)	Panthotenic acid (vitamin B5)	0.048 (mg)
Isoflavones	8.8 (mg)	Vitamin B6	0.041 (mg)
Calcium, Ca	4.0 (mg)	Folic acid	1.5 (lg)
Iron, Fe	0.58 (mg)	Vitamin B12	0 (lg)
Magnesium, Mg	19 (mg)	Vitamin A	3 (lg)
Phosphorus, Mg	49 (mg)	Vitamin E	0.01 (mg)
Potassium, K	141 (mg)	-	-

Source: (Giri and Mangaraj, 2012)

Table 3: Comparison of basic soymilk manufacturing processes

Comparison of constituents	Traditional	Hot grind	Hot blanch	Canadian
Beans	Whole, any type	Whole or dehulled, high quality	Whole or dehulled, good quality	Whole or dehulled, any type
Processing chemicals	None	NaHCO$_3$/HCl, etc.	NaHCO$_3$/HCl, etc.	None
Soaking	Yes	Optional	Hot Blanch	Preferred
Grinding	Cold grind/ filter/cook	Hot grind/filter	Hot grind and high-pressure homogenization	Airless/cold grind/ filter or filter/cook
Soymilk	Dissolved solids	Mostly dissolved solids	Mostly suspended solids	Dissolved solids
Odor	Rancid	Less rancid	Roasted nut	Cereal
Mouthfeel	Smooth	Chalky	Very chalky	Smooth
Protein yield	70–90 %	60–80 %	98%	70–90 %
Basic plant cost	Low	Medium to very high	High	Low to medium

Source: (Giri and Mangaraj, 2012)

2.2.2.2. Tofu

Tofu is a curd-like product formed from soymilk using chemical and natural coagulants. Chemical coagulants used include calcium and magnesium salts while natural coagulant that have been explored include rossell leaf extract, lemon juice, alum and top water of fermented maize (Obatolu, 2008). Coagulation refers to the precipitation of soy protein from soymilk, with the aid of coagulants and the type and concentration of different coagulants determine the chemical composition of the resulting tofu. Curds produced are similar to fresh cottage cheese from cow milk and can be further pressed into various hardness, depending on the preference of consumers and the desired quality leading to the variations in the protein contents of the resulting tofu products (Kada *et al*., 2008). Obatolu (2008) reported protein (58.2g/100g), fat (13.7%), ash (7.9%) and calcium (312.7mg/100g) contents in tofu coagulated with calcium sulphate. These values are higher than those obtained for other coagulants. Prabhakaran *et al*. (2006) reported differences in the physical properties (texture, colour and yield) and isoflavone levels when varying concentrations (0.4 and 0.5% w/w) of different chemical coagulants (chloride, sulphate and lactate salts of magnesium and calcium) were used.

In other studies by Sun and Breene (1991) it was reported that differences in the yield, protein quality, total solids and textural characteristics at calcium sulphate concentrations of 0.02-0.06 N. The quality of tofu produced from sprouted soybeans at various levels has also been found to be different from those made from unsprouted seeds. Murugkar (2014) reported increase in protein content but reduction in fat, trypsin inhibitor and phytic acid contents in tofu produced from soybean seeds subjected to 4 h soaking and 72 h germination at 25°C and 90% humidity. Limiting information exists on the characteristics of tofu produced from sprouted soybeans, despite many sprouting conditions for soybeans having been documented.

2.2.2.3 Other soy-based foods

Due to changes in consumer demands, traditional foods are being increasingly modified to meet market needs. Many soy-based food materials have been recently developed to effectively substitute traditional foods in diets (Alezandro *et al*., 2011). Commercial products containing soy-based products such as soy protein isolates, concentrates and textured soy protein, as major starting materials and representing a newer generation of soy foods have been developed. Meat analogues, including soy steak, soy hamburger and soy sausage, have been developed from soy textured proteins through extrusion cooking of soy flour. During this process, high extrusion temperature brings about the opening of protein quaternary structure, leading to protein polymerization, reorientation and development of strong inter-molecular bonds, which gives the product a final texture similar to meat (Hutabarat *et al*., 2001). This process also destroys inhibitory enzymes such as urease, lipoxygenase and trypsin inhibitor due to high process temperature. Soy protein extracts have been used in the production of condensed milk and chocolates as excellent substitutes for cowmilk and as ingredients in the production of bread and cereal bars, thereby improving their nutritional attributes (Alezandro *et al*., 2011). Also, because of their functional properties such as high water and oil absorption capacities, gel formation and emulsification, soy ingredients have been used as base starting materials in the production of beverages, soups and sauces (Genovese and Lajolo, 2002). Table 4 summarizes some food products that have been derived from the incorporation of soy ingredients into their matrices.

Table 4. Some soy-based food products and their ingredients

Soy-based foods	Ingredients
Soy condensed milk	soybean extract
Soy bread	soy flour
Soy cereal bar	soy protein isolates, soy flakes
Soy chocolate	soybean extracts
Soy cookies	soy protein isolates, soy flour
Soy steak	textured soy protein
Soy hamburger	textured soy protein, soy protein isolates and concentrates
Soy lasagna	textured soy protein
Soy kibe	textured soy protein
Soy nuggets	textured soy protein, soy protein isolates
Soy pasta	textured soy protein
Soy sausage	textured soy protein
Soy stroganoff	textured soy protein

Source: (Alezandro *et al.*, 2011)

2.3. Methods of microbial population determination

Fermenting microorganisms (including lactic acid bacteria, yeast and other microorganisms) are heterogenous in nature, consist of different species and strains and produce lactic acid and ethanol as their major metabolites (Temmerman *et al*., 2004). They survive in various substrates, including foods from plant and animal origin. They are generally regarded as safe (GRAS) and have been used for many industrial applications due to their metabolic activities, and also imparting desirable characteristics on fermented foods (Holzapfel *et al*., 2001). They are used as probiotics, due to their potential health benefits and in biotechnological techniques for the reduction of biopolymers and enzymes, or as delivery aids for bioactive substances (Klaenhammer *et al*., 2002). Extensive applications of fermenting organisms had necessitated their wide characterization to understand their identity, growth, metabolism, performance, resistance and sustainability, as well as their quality and safety controls (Saarela *et al*., 2000, Temmerman *et al*., 2004). Over the past two decades, attention has been placed on the identification of fermenting microorganisms, especially those meant to be consumed in fermented foods. The different methods of identification differ in time of analysis, accuracy of discrimination, reproducibility and work load (Temmerman *et al*., 2004). The choice of method to be used therefore depends on the intended use.

Methods that have been explored for fermenting microorganisms' identification and characterization could be broadly divided into culture dependent, culture independent and metagenomics. A balance for the desired characteristic of fermenting microorganisms, such as the desired taxonomic identity, workload, speed, cost and expertise, must be struck to be able to adequately determine the suitable method of identification and characterization (Temmerman *et al*., 2004).

2.3.1 Culture-dependent phenotypic identification

This method of fermenting bacteria or yeast identification is based primarily on the phenotypic characteristics of these microorganisms. It involves the inoculation of appropriate dilution of fermented foods into suitable growth media (agar or broth) and the microorganisms are allowed to thrive at their specific optimal growth conditions including temperature, oxygen availability and salt concentration (Adimpong *et al*., 2012). Pure cultures of these microorganisms are usually obtained by re-streaking cultures exhibiting their preliminary phenotypic characteristics, including shape, orientation and colour, into specialized growth media designed for their growths, to obtain pure cultures (Nielsen *et al*., 2007). The phenotypic characteristics of these pure cultures are then determined to identify the organism. Phenotypic methods of identification and characterization of fermenting organisms are cheaper than genotypic methods and has been proven to be effective for the identification of certain LAB and yeast (Akabanda *et al*., 2010). In many industrial microbiology units, especially food industries, routine phenotypic tests which involves the morphological and physiological characterization, protein and carbohydrate tests are still being used.

The popularity of phenotypic determination is because it is cheap and no specialized equipment is needed. However, phenotypic methods of microbial identification are grossly inadequate in providing robust classification and differentiation, and consequently, there have been shifts by researchers towards genotypic characterization methods (McCartney, 2002). Other weaknesses of phenotypic characterization include poor reproducibility, ambiguity in identities due to poor discriminatory powers, inability to characterize minor population of microorganisms which

require selective enrichment, inadequacy of detection of non-culturable cells, time consumption and labour demands (Justé *et al*., 2008).

According to Temmerman *et al*. (2004), previous works on the phenotypical identification of LAB in 317 presumptive isolates was only 38% accurate (Corsetti *et al*., 2001), while the identities of 14 LAB isolates to their specie level could not be determined in the study by Wijtzes *et al*. (1997), despite the use of two carbohydrate fermentation kits.

2.3.2 Culture independent methods

The use of polymerase chain reaction (PCR) techniques in the identification of microbial strains has been developed as advancement over the culture-dependent phenotypic methods. It involves the identification of amplified nucleic acid components of these organisms (Yang *et al*., 2001). Microbial growth and metabolism in fermented foods brings about changes in their physical, sensory and storage qualities, as observed in wine, cheese, fermented milk and sour dough. Therefore, it is important to understand the ecology of fermenting microorganisms and their metabolic activities per time (Willems *et al*., 2003, Giraffa, 2004, Justé *et al*., 2008). This is done by closely monitoring the changes in microbial community as enhanced by the various stages of production. Therefore, accurate detection and identification of fermenting organisms to species and strain levels, and the study of microbial community ecology in fermented foods support the management of quality and safety of such foods and a better understanding of their processing (Delfini and Formica, 2001, Justé *et al*., 2008). Since its introduction, PCR techniques has been widely used in microbial molecular ecology and many modified methods have been developed to adapt to the desired outcomes. Deoxyribonucleic acid (DNA) is the substrate used for PCR amplification, predicated on the target of genetic materials of desired microorganisms through the use of primers which amplify the target sequences within a given population. Therefore, in PCR-based molecular determination of microbial diversity of fermented food samples, selection of gene markers or primers that will be able to differentiate the wide variety of microorganisms present is very crucial, as ubiquitously conserved genes and functional genes are the major target sites for PCR markers (Justé *et al*., 2008).

Bacterial ribosomal regions such as 16S rRNA, 23S rRNA and D1/D2 regions of 26S rRNA of yeasts are ubiquitous conserved gene regions targeted by PCR primers and this is because of their universal abundance, phylogenetic properties as depicted by variable and highly conserved sequence domains, high potentials for discrimination of organisms, sensitivity and the availability of their sequences in databases that are widely available, which enhances the identification and description of microbial community (Benson *et al*., 2004, Chakravorty *et al*., 2007). Functional genes could also be targeted when a microbial process specific to a strain, rather than a group of microorganisms is of interest. The process involves assessing the diversity of the functional genes by targeting the key enzymes in the process of interest, followed by the identification of the predominant gene polymorphisms (Justé *et al*., 2008). This has been employed in soil and environmental microbial diversity characterizations, as well as study of food-borne human pathogens (Wu *et al*., 2001, Chiu *et al*., 2005, Geets *et al*., 2006)

Coupled with PCR, molecular fingerprint identification techniques have been adopted for genotypic analysis of microbial communities, because they provide comprehensive information about the differences between the community of microorganisms and between the different treatments and environments, subsequently, giving a basis for comparison of these situations. These methods include denaturation and temperature gradient gel electrophoresis (DGGE and TGGE), amplified ribosomal restriction analysis (ARDRA), terminal restriction fragment length polymorphism (T-RFLP) and singe-strand conformation polymorphism (SSLP) (Justé *et al*., 2008). Repetitive element based PCR (rep-PCR) products could also be run directly on agarose gels to obtain the fingerprints of the diversities of microbial communities (Adimpong *et al*., 2012). To identify the fingerprints obtained by profiling PCR products with these different gel-based identification approaches, bands from the gels may either be excised, cloned and sequenced (Nielsen *et al*., 2007) or the PCR products already amplified with target primers may be directly cloned and sequenced, to allow the identification of individual communities (Adimpong *et al*., 2012). Sequences obtained are then processed by trimming and filtering and then compared with public databases to obtain the identity and characteristics of microorganism. More recently, pyrosequencing and whole-genome shotgun sequencing techniques have been developed because

of their higher accuracy in the determination of microbial diversity of foods, soil and environmental samples.

Pyrosequencing removes the need for cloning, as obtainable in many molecular-based methods, thereby reducing the potential for production of aberrant recombinants and cloning related errors (Speksnijder *et al*., 2001). Also, whole-genome shotgun sequencing (metagenomics), which involves the extraction, lysis and fractionation of whole genomic DNA from food samples, as against pure microbial cultures, followed by amplification of target genomic region and sequencing of fragments is currently, widely employed in the characterization of microbial community.

2.3.3 High throughput sequencing

The most recent approach of analyzing the microbial community of foods, especially fermented foods are mainly based on high-throughput DNA sequencing, amplicon analysis and metagenomic sequencing (Bokulich *et al*., 2014, Franzosa *et al*., 2015, Chen *et al*., 2017). This approach may depend on the genomic DNA extraction from single organism or the mixed genome DNA obtained directly from fermented food samples. For amplicon analysis, conserved 16S rRNA and ITS regions (bacteria and yeast respectively) from single or mixed genomic DNA are amplified with universal PCR primers, followed by thermocycling at pre-defined conditions. Appropriate gel electrophoresis methods for these products are then carried out to display their fingerprints, followed by direct sequencing of the PCR products. Thereafter, the compositions of the microbial communities are identified by alignment of DNA sequences against public databases (Chen *et al*., 2017). This method has been extensively utilized in determining the microbial community of fermented foods (Tamang *et al*., 2016). Amplicon sequencing helps to understand the normal and novel successions of changes in microbial community, especially for spontaneous fermentation and multiple stages of complex microbiota, to explain product quality and consistency. Advantages of amplicon sequencing include higher throughput (compared to molecular methods) and low cost, which makes it suitable for the display of large scale microbial diversity across time and space while the drawbacks include low resolution of taxonomy assignment at species level, bias in PCR amplification and that it is unable to detect organisms without gene markers (Franzosa *et al*., 2015).

These drawbacks could be addressed through the use of metagenomic sequencing. Metagenomics, other than the different genomic approaches that are based on the DNA sequences of individual organisms, involves the sequencing of mixed genomic DNA extracted directly from fermented food, soil or environmental samples after fragmentation and library construction. Therefore, entire information about the target organism could be obtained, by reconstruction and assignment to accurate taxonomies at species level (Chen *et al*., 2017). Metagenomic approach is most suitable for the determination of the identity of novel species and unculturable microbial strains, especially those of low abundance, due to its higher resolution and accuracy in the determination of microbial diversity (Nalbantoglu *et al*., 2014, Krych *et al*., 2018).

Through metagenomics, alien microorganism including viruses, could be detected as part of the microbial community of fermented foods, a detection which could not be possible by other microbial analysis methods (Jung *et al*., 2011). Metagenomics can also be used for comparative analysis of the abundance of genes in metagenome samples, to provide information about their diversity and functionality (Chen *et al*., 2017)

2.4. Health benefits of soy-based food consumption

2.4.1. Prevention of Oxidative Stress

Normal metabolic reactions of the body, especially during respiration and cellular activities, bring about the production of free radicals, in humans and vertebrates (Cross *et al*., 2004, Liwanpo and Hershman, 2009). Oxidants can also be externally introduced into the body by the consumption of foods that have undergone oxidation during processing and storage, worsening their quality, nutritive values and safety (Hubert *et al*., 2005). Example of such is the consumption of rancid lipids. More prone to production of free radicals are consumers of alcohol, tobacco and other potentially toxic food materials, as they can be absorbed directly into the blood stream to cause adverse health effects (Mbithi *et al*., 2001). Adverse effects of unacceptable levels of free radicals in the body include break down of disease defense mechanisms in the body (Chavarro *et al*., 2008, Bair *et al*., 2008), through cell damage, which in turn predisposes the body to disease conditions

such as atherosclerosis, arthritis, diabetes and cancer (Hamilton-Reeves *et al*., 2010, Gilani *et al*., 2005).

Consumption of antioxidative peptides from foods such as soybean, have been found to have advantages over synthetic antioxidants, as they have simpler structures, are more stable, do not have hazardous tendencies and present nutritional and functional advantages to the consumers (Gibson *et al*., 1998). Peptides serve as inhibitors of lipid peroxidation (Liwanpo and Hershman, 2009), free radical scavengers (Gee *et al*., 1997) and transition metal chelators (Hubert *et al*., 2005). Soy protein fractions and peptides of <10kDa molecular weights have been found to demonstrate radical scavenging abilities, reducing powers and metal chelation abilities (Adeoya-Osiguwa *et al*., 2003). Also, Lee *et al*. (2004) found general free radical and superoxide anion scavenging abilities in aliquots of extracts of 17 varieties of Ohio soybeans in appropriate buffers.

2.4.2. Potential protective effects against cancer

Although still controversial, consumption of soy foods have been reported to have anti-cancer effects. The consumption of soy-foods increases isoflavones concentrations in breast tissues to levels that can have favourable health effects, such as prevention of breast cancer (Bolca *et al*., 2010). Also, there have been studies relating the lower incidences of breast cancer, especially pre-menopausal breast cancer, to lifelong consumption of soy-based foods (He and Chen, 2013). Much as this could be traced to the beneficial effects of soy isoflavones, the effect of soy saponins with respect to breast cancer prevention is also gaining research interests (Jooyandeh, 2011). From animal studies, it has been demonstrated that soy isoflavones play important roles in the prevention of prostate cancer cell proliferation (Lakshman *et al*., 2008, Pollard and Suckow, 2006). The higher protective tendencies to prostate cancer recorded in Asian populations (combined risk/odd ratio of 0.52) compared with Western populations (combined risk/odd ratio of 0.85) may be due to the abundance of soy foods in Asian diets. A study by Xu *et al*. (2009) reported that the exposure of prostate cancer cells of patients to genistein of soybeans *in vitro* suggests an inhibition of cancer cells, with a decrease in the level of matrix metalloproteinase-2 (MMP-2) decreased the levels of vitamin D.

2.4.3. Protection against heart disease

The Food and Drugs Administration of the United States consented to labelling foods rich in soy protein with claims that they could reduce the risk of coronary heart diseases. Dietary soy isoflavones have also been suggested to activate signal pathways that may increase the production of nitrous oxide, whose vasodilatory effects have been reported, thereby preventing the incidences of cardiovascular diseases (Siow and Mann, 2010).

Also, the risk of heart diseases and mortality have been found to reduce by 2-5% for every 1% decrease in low density lipoprotein (LDL) cholesterol and consumption of adequate levels of soy isoflavones has been reported to regulate the levels of LDL receptors in the liver (Law *et al*., 1994, Friedman and Brandon, 2001), thereby preventing buildup of LDL cholesterol in the heart. Therefore, it could be suggested that the consumption of soy rich foods, especially those of improved isoflavone levels, may have preventive effects against coronary heart diseases, since studies have suggested a reduction in LDL, without increase in high density lipoproteins and total cholesterol.

2.5. Antinutritional components of soybeans

Much as the health and nutritional benefits of soy-based foods have been widely reported in literature, some of its constituents such as lectins, trypsin inhibitors, saponins and phytic acids have been reported as anti-nutritional and toxic factors. As a legume, soy contains substantial amounts of lectins, although, they are exhaustively destroyed by prolonged heat treatment (Friedman and Brandon, 2001). Lectins have been found to be allergenic, leading to food sensitivity in consumers (Friedman and Brandon, 2001, Breiteneder and Mills, 2005). Saponin contents at the levels that can pose health risks have also been reported in soy. At very high levels, soy saponins can act as immunological adjuvants and also increase *in vitro* intestinal permeability, causing undue sensitization of the immune system, leading to immunity dysfunctionalities (Gee *et al*., 1997). Soy saponins are heat stable and may not considerably reduce during processing (Hubert *et al*., 2005). However, soy saponins have been suggested to have anticarcinogenic effects (Jooyandeh, 2011, Messina and Barnes, 1991, Hasler, 1998) and hypocholesterolemic effects with animal studies (Thompson, 1993).

Other effects of soy antinutrients are as presented in Table 4. It is important to note that adverse health risks posed by these anti-nutrients could only be initiated when very large quantities are ingested (Sahin, 2014). Sprouting and fermentation are some of the processing operations that have been suggested to reduce the anti-nutritional components of soy-based food to acceptable levels.

Sprouting of legumes, which include soaking and germination, brings about physiological activities that could result in many changes, including leaching and breakdown of phytic acid, disintegration of lectins and reduction or inactivation of enzyme inhibitors, leading to improved bioavailability of minerals and vitamins. (Mahgoub and Elhag, 1998, Mbithi *et al*., 2001). Also, the lack of difficulties in digesting fermented soy products, which has motivated their traditional consumption, suggests fermentation as a highly likely way of reducing soy antinutrients (Sahin, 2014).

Table 4. Antinutrients present in Soy and Potentially Harmful By-Products of Soy Processing

Antinutrients	Study	Study Design	Properties
Lectin	Breiteneder and Mills (2005)	Review of laboratory analyses	Linked to food sensitivities, highly allergenic hemagglutinin proteins are present in soy in high concentrations
	Friedman and Brandon (2001)	Review of laboratory analyses	They are destroyed by heat.
Saponin	Gee *et al.* (1997)	Animal trial	Shown to increase intestinal permeability *in vitro*, which may contribute to auto-immune disease, they have the potential to increase sensitization to dietary antigens.
	Hubert *et al.* (2005)	Laboratory analysis	Not removed by heating or other standard food-processing methods,
Enzyme Inhibitor	Koiwa *et al.* (1997)	Review of laboratory analyses	They bind digestive enzymes chymotrypsin and trypsin.
	Hathcock (1991)	Narrative review	Inhibiting protein digestion and increasing pancreatic function, result in increased pancreatic protein synthesis.
	Friedman and Brandon (2001)	Review of laboratory analyses	Reported to be the cause of pancreatic hyperplasia and adenomas in animal models that using high soy diets, retain 25% of their activity even after heating.
Phytic acid	Liener (1994)	Narrative review	Reducing bioavailability of vitamins A, B, D, and E, it can create a potential for multiple nutritional deficiencies.
	Martino *et al.* (2007)	Animal trial	It decreases the bioavailability of zinc and iron
	Rekha and Vijayalakshmi (2010a)	Laboratory analysis	Destroyed by fermentation but not heating, resulting in increased mineral bioavailability.

Source: (Sahin, 2014)

2.6. Soybean sprouting

Sprouting is a biological process aimed at bringing viable seeds out of their latent states, to produce plantlets, once the environmental conditions required for growth and development have been adequately met (Laitila *et al*., 2007). For seeds to germinate, the environmental factors such as humidity of substrates, oxygen availability for aerobic respiration and the required temperature for metabolic and enzymatic processes required for the development of plantlets must be adequately considered. Germination has been used as traditional methods of treating some of the disadvantages inherent in grains and legumes, such as presence of antinutrients, undesirable tastes and flavours (Suberbie *et al*., 1981), resulting in quality changes in the seeds. Sprouting is an inexpensive method of seed pretreatment aimed at the improvement of availability, palatability and digestibility of nutrients (Kumar *et al*., 2006a) and the resulting effect of germination depends on factors such as the soaking time, germination time, seed type or variety and temperature (Savelkoul *et al*., 1992). Therefore, after germination, legumes and pulses generally demonstrate improved nutritional quality and increased levels of bioactive components (Tarzi *et al*., 2012, Lin and Lai, 2006).

2.6.1. Effect of sprouting of soybeans on the resulting soy-based foods

2.6.1.1. Proteins

Metabolic activities and enzymatic hydrolysis occurring during sprouting bring about the breakdown of some complex protein molecules and the synthesis of new proteins (Zhang *et al.*, 2015, Moongngarm and Saetung, 2010). During the development of seeds of leguminous plants, complex proteins molecules form the larger portion of its nutrient reserve, on weight basis. (Duranti *et al.*, 2008). Although, Savelkoul *et al.* (1992) reported no significant change in the total crude protein after 7 days germination after the seeds were soaked for 12 h and sprouted in the light at 20°C, Sathe *et al.* (1987) observed, from SDS-PAGE, a remarkable breakdown of native proteins of soybeans. They observed the disappearance of glycinin and beta-conglycinin units of stored proteins after 1-2 days germination. They are thought to have broken down into 31500 and 97400 molecular weight proteins. Gao *et al.* (2007) therefore postulated that since germination brings about the breakdown and synthesis of protein moieties, there could have been a turnover between protein and non-protein nitrogen, bringing about equilibrium between the degradation and synthesis of proteins.

2.6.1.2. Amino acids

Soybeans contain balanced essential amino acids but are limiting in cysteine and methionine. Proteins from soybeans, when adequately processed, can be a major or sole protein source, and compete in quantity with proteins of animal origin. Soybean proteins are high in lysine and makes them a useful complementary protein food with cereals (Singh *et al.*, 2008). Sprouting conditions used in the treatment of soybeans determine the resultant effect on their essential and non-essential amino acids. As such, conditions of sprouting play important roles in the quality of resulting amino acids in sprouted soybeans. Increase in the essential and non-essential amino acid contents of soybean was observed by Mostafa *et al.* (1987) after 3 days (8.9% and 17.6%) and 6 days of germination (22.4% and 17.6%). (Gulewicz *et al.*, 2008) reported significant increase in sulphur amino acids in lupin sprouts, as compared to whole seeds while Bau *et al.* (1994) reported a 54.9% decrease in lysine content of soybeans after 5 days of germination.

2.6.1.3. Alpha-Galactoside carbohydrates

The major alpha-galactosides of soybeans include raffinose and stachyose, and they are important sources of energy for seeds during germination and early growth of plants. Oligosaccharides such as sucrose, raffinose and stachyose are all present in germinating seeds, together with monosaccharides. (Shimelis and Rakshit, 2007), reported a progressive degradation of these oligosaccharides to over 50% of their initial contents after 48 h germination, They also found an increased level of monosaccharides, which could be as a result of collective degradation of sucrose, raffinose and stachyose. Kuo *et al.* (1990) studied sugar metabolism of germinating soybean seeds and reported the conversion of oil soluble oligosaccharides into sucrose, which is in turn metabolized into simple sugars which are readily used up during seed expansion and growth of plantlets.

2.6.1.4. Trypsin inhibitory activity (TIA)

Trypsin inhibitors are present in leguminous seeds, and those of soybeans have been identified as Kunitz inhibitor and Bowman-Birk inhibitor (Wati *et al.*, 2010). They are long chain polypeptides which form stable enzyme-inhibitor complexes with trypsin in, making them unavailable for metabolic activities (Macedo *et al.*, 2007).

Freed and Ryan (1978) found 12.13mg/g Kunitz trypsin inhibitor in Steele and Harosoy variety of soybeans and is responsible for most of the trypsin inhibitor activity found in this variety. Germination has been widely reported to reduce the trypsin inhibitor contents of sprouted beans. Mostafa *et al.* (1987) found about 32% degradation of trypsin inhibitor activity (TIA) after overnight soaking and 6 days of germination in Calland soybean variety. Steaming, together with germination for 5 days was found to reduce the TIA of soybean by up to 92%. They suggested that heat treatment may have aided the degradation, as it increased the accessibility of seed proteins (Bau *et al.*, 1997). Papastoitsis and Wilson (1991) attributed the degradation of Kunitz trypsin inhibitors in soybean seeds during germination to the activities of Proteinase K1, which is produced during germination and increases with the increase in the duration of germination, up to 4 days. Proteinase K1 is reportedly absent in dry soybean beans, but is produced during germination and is active against Bownam-Birk trypsin inhibitor, and also the subunits of beta-conglycinin (Kumar *et al.*, 2006b). Savelkoul *et al.* (1992) reported an improved *in vitro* protein digestibility of germinated soybeans and Bau *et al.* (1997) attributed this to the decrease in TIA and lectin content, and the availability of protein moieties in simpler and absorbable forms due to their susceptibility to more efficient enzymatic activities during germination.

2.6.1.5. Phytic acid

Phytic acid is widespread in many leguminous and oil seeds. Kirby and Nelson (1988) reported that 1g of soybeans contain about 4mg phytates, which is 57% of its total organics phosphorus content. Phytic acids bind with major and trace minerals, such as zinc, magnesium, calcium, manganese, copper and molybdenum, preventing their bioavailability (Beleia *et al*., 1993). Phytic acids also binds with proteins, thereby decreasing its solubility and functionality. (Bau *et al*., 1997). Sprouting conditions, especially soaking and germination, bring about the reduction of phytic acid content in soybeans. The reduction of phytic acid in leguminous seeds has been attributed to the activation and increase in the functions of enzyme phytase. Bau *et al*. (1994) reported a 17% decrease in phytic acid in soybean seeds after 5 days germination while a rapid reduction of over 50% was recorded after 9 days by (Chandrasiri *et al*., 1987). Gibson *et al*. (1998) were able to purify enzyme phytase from the cotyledons of soybean samples that have germinated for 10 days and the phytase was reported to have a very strong affinity for phytic acid. Also, soaking of soybean seeds before germination have been found to reduce the phytic acid contents of soybeans.

Phytic acid is water soluble and leaches into the soaking water at extended soaking time (Liang *et al*., 2009). Soaking of soybeans and other leguminous seeds, for 12 h and beyond have been found to drastically reduce the phytic acid contents and improve mineral and vitamin availability (Mbithi *et al*., 2001).

2.6.1.6. Phenolics and antioxidant properties

Phenolic acid

Phenolic compounds have been widely documented to have beneficial roles in reducing the risk of chronic diseases and many oxidative stress disorders due to its antioxidant potentials. As such, they have been sought after in functional food and nutraceuticals development (Jiang *et al*., 2013). Phenolic compounds of leguminous foods include its phenol acids, lignins, condensed tannins and flavonoids, including isoflavones. Sprouting is a potent way of increasing the phenolic acids in leguminous seeds, as phenolics are biosynthesized and bioaccumulated in germinating seeds, as response and defense mechanisms of seeds to the adverse environmental stress created by sprouting conditions López-Martínez *et al*. (2017). Soaking time was also demonstrated to play an important role in the synthesis of phenolics in soybeans. Cevallos-Casals and Cisneros-Zevallos (2010) evaluated the phenolic contents of 13 edible seeds and found about 30% synthesis of phenolic compounds in soybeans, which is an increase over the dormant phenolic content (about 10%). However, they found over 60% synthesis after 7 days of germination and an overall 271% increase in phenolic contents from dormant seeds to 7 days germination.

Isoflavones

Flavonoids, as an important phenolic compound, is found in considerably high quantities in legumes (Aguilera *et al*., 2010). They are of low molecular weight, having two aromatic rings joined by a 3-carbon bridge (Singh *et al*., 2017). They could exist as flavones, flavonols, isoflavones and they are mostly present in leguminous seeds (Amarowicz and Pegg, 2008). Zhu *et al*. (2005) considered the isoflavone contents of two varieties of soybeans (Hutcheson and Caviness) commonly grown in Arkansas, United States, soaked for 12 h and germinated for 0-24 h. They reported an increase in the isoflavone contents of the two varieties after 12 h soaking.

Maximum isoflavone content was reached when the hypocotyl was 0.5mm long (2.491 mg/g flour) and 2.55mm long (2.78 mg/g flour) for Hutcheson and Caviness varieties respectively. They attributed the increase in the isoflavone contents to the induction of metabolic pathways of the precursors of isoflavonoids at these stages of germination and possible bio-conversion of other phenolic compounds to isoflavones during the process of germination. On the evaluation of isoflavone contents of yellow and black soybeans, Huang *et al*. (2017) reported a 700% increase in the aglycone content of yellow soybeans (105.5-917.3 mg/g) and 850% increase in black soybeans (124.8-1186.4 mg/g) from raw to germinated seeds. The seeds were subjected to 10 h soaking and 3 days germination. They also reported the bio-conversion of isoflavone glucosides into aglycones during germination with 111% and 81% decrease in diadzin, 130 and 77% decrease in genistin and 300 and 88% decrease in glycitin of yellow and black soybeans respectively throughout the germination.

Antioxidant activity

Sprouting of leguminous seeds have been linked to the increase in the levels of their functional substances, with various nutritional and health benefits to humans, compared to unsprouted seeds. Also, increase in the phenolic compounds such as phenolic acids and isoflavones in germinated soybeans has been linked to the increase in their antioxidant capacities (Paucar-Menacho *et al*., 2010a, Cevallos-Casals and Cisneros-Zevallos, 2010). Therefore, there is a positive interaction between the increase in the phenolic compounds in germinating legumes and their antioxidant capacities. Huang *et al*. (2014), while studying the kinetics of changes in the antioxidant activity of germinated soybeans and mung beans, reported an increase in the DPPH free radical scavenging abilities of germinated soybeans, starting from 2 days of germination. They reported about 143% increase in DPPH radical scavenging activity in germinating soybeans after 2 days germination, as compared to ungerminated soybeans. Also, as the germination days increased, they reported increase in the antioxidant activity. For example, they found 220%, 233% and 201% increase in antioxidant capacities after 3,4 and 5 days of germination respectively and suggested 4 days of germination for peak antioxidant activity. The increase was directly attributed to the increase in the level of phenolic compounds due to germination, as they also reported a successive increase in the total phenolic compound as the number of days of germination increased, peaking at 330% after 4 days.

The total phenolic content of soybeans was also correlated with its anti-radical potentials by Mamilla and Mishra (2017). They attributed the increase in the antioxidant activity of soybeans to its increase in total phenolic contents. Among the legume seeds studied, soybean exhibited the highest increase in total phenolic contents (2.35g/kg) and DPPH scavenging activity (35%) after 5 days of germination in a dark chamber. They attributed this behaviour in soybeans to higher activities in its hydrolases and polyphenol oxidases during germination and also the differences in the matrices of seeds, which in turn affects the release of metabolites as germination progressed.

2.7. Soy proteins as allergens

Food allergies have been described as the hypersensitive reaction of human immune system to some proteins contained in foods. These proteins are generally referred to as allergens and the eight major foods that have been reported to have allergenic reaction in consumers include egg, fish, milk, peanut, shellfish, tree nuts, wheat and soy (Koeberl *et al*., 2014). Various soybean allergenic proteins have been reported, including glycinin and β-conglycinin being the most researched (Wang *et al*., 2014). Also, soybean agglutinin and protease inhibitors are sometimes referred to as soy allergens. Soy agglutinins are glycoproteins with isoelectric point pH of 6.0 and molecular weight of 120kDa. It preferentially binds with oligosaccharides with α or β-linked N-acetylglucosamine, leading to poor availability of these nutrients for digestion, thereby impairing their utilization. Soybean protease inhibitors including Kunitz trypsin inhibitors and Bowman-Birk trypsin inhibitors reduces the availability of trypsin and chymotrypsin enzymes for protein digestion (Wang *et al*., 2014).

As earlier stated, glycinin and β-conglycinin are the two major allergenic proteins in soybeans. They account for 70-80% of its storage proteins and are referred to as 11s and 7s globulins according to their sedimentation coefficients (Krishnan *et al*., 2009). Glycinin is the most predominant, accounting for about 50% of storage proteins in many soy varieties (Staswick *et al*., 1984). Its molecular weight ranges between 320-360kDa (Lallès *et al*., 1999) and contains five subunits A1aB1b, A1bB2, A2B1a, A3B4 and A5A4B3 (sometimes written in their short form such as B1, B2 etc.), with considerable variations (Natarajan *et al*., 2007).

Denaturation of native glycinin (through heat treatment or enzymatic activities) brings about the loss of activity of anti-glycinin antibodies to react with them, suggesting considerable structural rearrangement or disintegration during these denaturation processes (Moreira *et al*., 1981). β-conglycinin has a molecular weight of 180-220kDa and contains about 5% carbohydrates, which may explain its immunoreactivity (Amigo-Benavent *et al*., 2009). It has 3 subunits namely α, α' and β subunits with theoretical molecular weights of 76, 72 and 53 kDa. The differences in the subunits is in their amino acid composition (Wang *et al*., 2014).

2.7.1. Allergen detection and quantification methods

2.7.1.1. Enzyme linked immunosorbent assay (ELISA)

ELISA has been widely used to determine the allergenic proteins in food products, especially soy-based foods (Wang *et al*., 2014). ELISA can be used for large number of samples simultaneously (Koppelman *et al*., 2004). To perform ELISA, there is a need for the use of at least one antibody, and the mostly used antibody is serum obtained from animals, including rabbits or mice (Wang *et al*., 2014). Types of assays used include Sandwich ELISA, competitive ELISA and indirect ELISA. Despite that these assays have been widely used and can be used for a large number of samples, some of its major drawbacks include time consumption, especially in the preparation of assays and antibodies. Antibodies should be highly specific and sensitive to detect cross-reaction of other sources of allergens with the target allergen, which is facilitated by their affinity for the target allergen. Again, antibodies, even if they are properly prepared, may not be entirely suitable for all food samples, depending on the nature of the target proteins (either native or denatured). This has led to the development of commercial kits, with high precision and are ready for immediate use (Sakai *et al*., 2010).

2.7.1.2. Immunoblotting

This is a method of allergen detection which is capable of indicating the molecular weight and immunoreactivity of allergenic proteins, with sensitivity of detection up to nanogram level (Meisel, 1993, Ogawa *et al*., 1993, Wang *et al*., 2014). Immunoblotting can also be used to study the digestion pattern of glycinin and β-conglycinin, as was used in the study using calves and rats (Lallès *et al*., 1999, Perez *et al*., 2000) as reported by Wang *et al*. (2014). Authors found accelerated digestibility of glycinin and β-conglycinin in rats compared to calves.

Immunoblotting could be favourably used for soybean allergen studies, however, it is necessary to choose the correct membranes, find appropriate antibodies and their concentration and also use the correct reagents.

2.7.1.3. Mass spectrometry

Recent advance in allergen determination involves the use of mass spectrometry in determining the identity of food allergens. Mass spectrometry is currently used in proteomics in determining the identity of soluble proteins that have been separated through in-solution digestion or through electrophoresis in 2-dimesnsional gels (2-DE), which separate proteins into their subunits according to their molecular weight and isoelectric points (Koeberl *et al*., 2014, Seo and Cho, 2016). The flow of process (Fig. 1) in the proteomic analysis of food allergens using assisted mass spectrometry, coupled with 2-DE or in-solution protein separation gives an overview of the entire procedure (Koeberl *et al*., 2014).

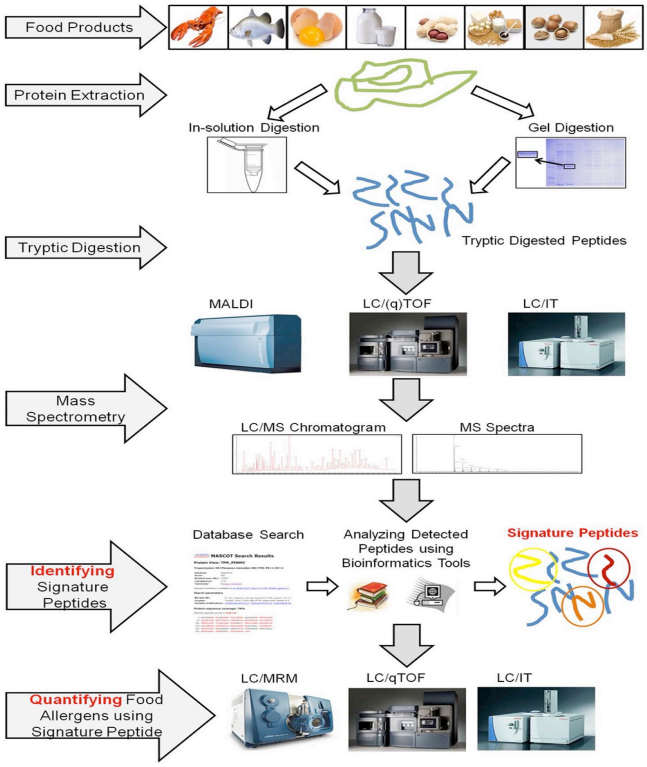

Figure 1. Procedure for food allergen peptide identity and characterization and quantification of food allergens. Adapted from Koeberl *et al.* (2014).

Separated proteins are further digested into peptides using appropriate enzymes such as trypsin, for identification and characterization using mass spectrometry. A mass spectrometer consists of three important parts; ion source, detector and mass analyzer. For ion source, many technologies including matrix-assisted laser desorption/ionization, electrospray ionization (ESI), nanoelectron spray ionization (nanoESI), atmospheric pressure chemical ionization (APCI), desorption/ionization on silicon (DIOS), fast atom/ion bombardment (FAB), electron ionization (EI) and chemical ionization (CI) (Siuzdak, 2004). For proteomics, these ion sources are combined with suitable mass detection mechanisms, including time of flight (TOF) and ion trap (IT) to form hybrid systems such as MALDI-TOF, ESI-qTOF, ESI-IT for optimal performance and accurate detection of mass peaks.

2.8. Optimization of sprouting conditions

Optimization of process parameters involve the improvement of performance of systems to produce increase in yield, without increasing costs and time (Baş and Boyacı, 2007). Optimization has been traditionally carried out by determining the influence of one factor at a time on the responses of the experiment, while other factors are kept constant. The disadvantages of this optimization approach include the omission of the interactive approach to studying the relationship between variables within the experiment, thereby neglecting the complete effect of the parameter on the resulting response. Again, large number of experimental runs would also be required to conduct researches, leading to increase in time, expenses and consumption of research materials (Bezerra *et al*., 2008). Response surface methodology (RSM) has since been developed to overcome the problems of optimization of experimental process parameters. With the combination of at least two experimental factors (multivariate technique), RSM involves the collection of mathematical and statistical procedures important in the development, improvement and optimization of responses of interest, through the various combination of variables, with the aim of obtaining the optimum characteristics in the desired response (Baş and Boyacı, 2007). RSM analyzes the effect of independent variables and generates a model to describe the biochemical processes involved (Anjum *et al*., 1997).

To efficiently apply RSM, it is necessary to choose an experimental design that adequately fits the characteristics of factors and describes the experiments to be carried out within the region being studied. For example, experimental designs leading to the generation of first order models, such as factorial designs, can be used when the factors do not present the possibility of curvature. On the other hand, designs such as Box-Behnken and central composite would be appropriate for experimental data which cannot be described by linear functions (Hanrahan and Lu, 2006). Definite terms used in the application of RSM include experimental domain, which describes the region to be investigated. The domain is defined by setting the minimum and maximum levels of experimental factors (or variables) to be studied. Experimental design refers to the combination of different levels of independent variables or factors in a matrix, which will be applied to obtain the responses. Factors or independent variables are experimental variables which could be altered within the combinations, independent of one another, to obtain responses. Levels of factors are the different values of variables within which the experiment falls.

It defines the maximum and minimum values within which the experiment must be carried out. Responses or dependent variables are the results obtained from the combinations of factors or independent variables within the levels preset in the experimental design. Residual is the difference between the experimented and calculated values, which determines the fitness of the experimental design or model used. The effectiveness of a mathematical model used for an experiment or generated by the experimental design is determined by the residual values it presents: Low residuals show good mathematical models and are a good fit. (Bezerra *et al*., 2008).

There had been attempts to optimize the sprouting parameters in order to produce soybeans and soy-products with improved nutritional characteristics, antioxidant activities and bioactive compounds of soybeans, using response surface methodology (multi-variate approach) or traditional optimization approaches using one factor. Fernandez-Orozco *et al*. (2008), attempted to optimize the germination time of soybeans after subjecting soybean seeds, initially soaked in distilled water for 5h 30 min, to germinate for 2-6 days.

Rather than response surface methodology, authors used kinetics of changes in the attributes studied to determine the optimum sprouting conditions. Four (4) days of germination was suggested as the optimized germination time, to achieve increased synthesis of antioxidant compounds and improvement in antioxidant activity. Huang *et al*. (2014) also attempted to optimize the germination time of soybeans by studying the kinetics and dynamics of changes in the nutrient and antioxidant activities of soybeans. Seeds were soaked for 10 h and subjected to germination for 24-120 h (1-5 days) in the dark. The study suggested 4 days of germination as the optimum germination time for enhanced nutrient content and antioxidant activity. Jiang *et al*. (2013) determined the optimal germination time of sprouted soybeans soaked for approximately 12 h and sprouted for 28, 50 and 72 h. Response surface methodology was not employed by authors in the optimization of sprouting conditions. Their results suggested 28 h as the optimum germination time for improved nutritional, physicochemical and quality attributes. On the other hand, Paucar-Menacho *et al*. (2010a) employed RSM techniques to optimize the contents of bioactive compounds of germinated Brazillian soybean (cultivar BRS 258). A two-factor central composite rotational design (CCRD) and the two factors studied were germination temperature (18, 20, 25, 30 and 32°C) and germination time (12, 21, 42, 63, and 72 h).

Authors reported different optimal gemination temperatures and times for each bioactive compound studied. For example, 12 h germination at 25°C was suggested for optimal lunasin production while 63 h germination at 30°C was suggested for optimal aglycones and saponin production. Again, Paucar-Menacho *et al*. (2010b) attempted to optimize the germination temperature and time for increased production of bioactive compounds in another cultivar of Brazillian soybeans (BRS 133), using response surface methodology. They suggested 42 h germination at 25°C to be the optimal condition for lunasin production and 63 h at 30°C for optimal isoflavone and saponin production. Differences in the optimum conditions for each bioactive compound within the same cultivar may be a challenge, especially since the bioactive compounds do not exist in isolation within the seed framework.

Guo *et al*. (2011) optimized the effect of temperature, pH of germination cultivating solution and air-flow rate on the production of Gamma-aminobutyric acid (GABA) content of sprouted soybeans using response surface methodology.

They suggested a germination temperature of 30°C, pH of 4.1 and air-flow rate of 0.9L/min as the optimum conditions for the highest production of GABA (2.60 mg/g) in the study. Also, an optimum soaking temperature and time of 30°C and 4 h was also suggested for satisfactory GABA production, however, these optimum soaking conditions were reached without employing RSM techniques.

Sprouting of soybeans and fermentation of soy-based foods are processing operations with vast potential of producing food products with high levels of nutrients and increased levels of nutrient bio-availability. Subjecting soybeans to sprouting, prior to use, is a mechanism capable of triggering many enzymatic reactions which in turn releases potent antioxidants, reduces antinutrients and triggers the production of other bioactive compounds. Sprouting can alter the microbial community of soy-food fermentation, due to the change in substrates available to fermentative microorganisms. Therefore, it is necessary to determine the microbiome of products obtained from sprouted soybeans. Many optimized sprouting conditions have been suggested in literature, with inconsistent effects on sprouted soybean and their products. The use of response surface methodology in optimizing the sprouting conditions of soybeans, that would be used as raw materials for the production of soy-based foods, could therefore be a favourable approach towards ensuring the presence of desired nutritional, antioxidant and other quality attributes in them.

2.9. Aim, Hypotheses and Objectives

Aim

The aim of this research is to determine the nutritional characteristics, antioxidant activities and microbial community of soy-based foods produced from sprouted soybeans.

Objectives

- To optimize the sprouting conditions of soybeans using response surface methodology and to determine the effect of optimized sprouting conditions on the nutritional and quality attributes of the resulting soy-based foods (superfoods).
- To determine the antioxidant activities of superfoods.

- To identify and determine the level of allergens in the resulting superfoods using 2D proteomic analysis approach.
- To establish the microbial community of superfoods made from optimized conditions of soybean sprouting, using next generation sequencing techniques.

Hypotheses

1. Optimization of sprouting conditions using response surface methodology will bring about the best levels of the processing conditions for production of sprouted soybeans, with improved quality attributes, for the production of soy-based foods. Sprouting of seeds has been found to increase the nutritive value and therapeutic properties of foods in a natural way (Jiang *et al*., 2013, Plaza *et al*., 2003).
2. Soy foods produced from optimized sprouted soybeans will have improved nutritional properties, antioxidant activities and enhanced levels of bioactive compounds compared to those from unsprouted seeds. Soybean upon germination exhibited marked increase in nutrients, bioactive compounds antioxidant activity (Huang *et al*., 2014, Paucar-Menacho *et al*., 2010a)
3. There will be reduction in the levels of allergens in fermented and unfermented soy-based foods produced from soybeans sprouted at optimized conditions. Seo and Cho (2016) reported a significant decrease in the volumes of soy allergenic proteins in soybean meal subjected to solid state fermentation with *Bacillus subtilis* for 12-24h.
4. Sprouting of soybeans, as a pre-treatment method, will affect the microbial community of the resulting soymilk. During sprouting, diverse microbial community occurs in the sprouting bed of barley and this has an effect on the quality of malt and as such, characterization of the microbiome has a great potential in ensuring the quality and safety of the resulting beer (Laitila *et al*., 2007, Justé *et al*., 2011).

Chapter 3: Improvement of quality attributes of soymilk through optimization of selected soybean sprouting parameters using response surface methodology

Abstract

This chapter was aimed at producing functional soymilk through optimization of the sprouting conditions of soybeans using response surface methodology. Soaking (12-24 h) and germination times (48-96 h) were optimized using a central composite rotatable design. Responses obtained from experimental runs were fitted into second order polynomial regression model and the significance of model parameters tested using ANOVA and R^2 values. The optimum sprouting conditions of soybeans were 12 h soaking and 52 h germination using the desirability concept. Soymilk made from optimized conditions had a 17% increase in total proteins, 50% reduction in phytic acid, 1.7% increase in total phenolics and a colour change of 4.89 when compared with the control. There was a significant reduction in trypsin inhibitor activity (0.03 mg/g TI), with an increase in total amino acids and similar rheological properties in optimized soymilk. Optimized production conditions of soymilk are shown to have improved both nutritional and quality attributes.

Keywords: Soymilk, Soaking, Germination, Optimization, Response surface methodology

3.1. Introduction

Soybean (*Glycine max*) is an important plant source of major nutrients and phytochemicals such as proteins, fatty acids, phytosterols and phenolic acids. The consumption of soybean helps in preventing and combating some diseases such as cancer, osteoporosis and cardiovascular diseases (Kim *et al.*, 2016). Soybean is cheap, accessible and easy to process into many fermented and non-fermented products such as soymilk made by grinding and expression of the milk component. Soymilk has been found to be a suitable alternative for milk from animal sources, especially for vegetarians and those that are lactose intolerant (Jiang *et al.*, 2013). It can be consumed directly as a food or used as an intermediate product in the production of other fermented and non-fermented soy products such as tofu and soy yoghurt.

Sprouting of soybeans before production of soy-based products, as a means of improving the functionality of the resulting foods has been explored in previous studies (Jiang *et al.*, 2013, Murugkar, 2014, Paucar-Menacho *et al.*, 2010b). Subjecting soybeans to sprouting, prior to processing into a desired product, is a cheap and effective pretreatment aimed at stimulating metabolic activities. It helps in the breakdown of complex components in the seeds into simpler, absorbable forms, hence improving nutritional quality, digestibility and overall functionality (Fernandez-Orozco *et al.*, 2008). The quality of sprouted grain may be influenced by many factors including the soaking time, germination time, temperature and variety of the grain (Jiang *et al.*, 2013, Huang *et al.*, 2014, Murugkar, 2014, Paucar-Menacho *et al.*, 2010b). Jiang *et al.* (2013) studied the effect of varying germination times (28-72 h) of soybeans on the quality of soymilk and reported that 28 h germination resulted in the highest increase in protein and total phenolic contents. These authors also suggested that soymilk prepared from sprouted soybean had comparable physicochemical properties to traditional soymilk. Huang *et al.* (2014) studied the kinetics of changes in the nutrient and antioxidant capacities of soybeans at extended germination times (24-120 h) and found that approximately 72 to 96 h germination time resulted in an increase in the nutrient contents of the soybean. Other optimization studies on the bioactive components in soybean reported that 45 h germination time was optimum for the production of lunasin, and reduction of lectin and lipoxygenase activities (Paucar-Menacho *et al.*, 2010b).

Soaking, as a factor of germination, enhances the softening of seed-coats, emergence of sprouts and reduction of antinutritional factors such as phytates (Lestienne *et al.*, 2005). However, there are limited studies on the soaking conditions required for sprouting of legumes, especially in combination with other factors. Since different germination times for desirable results were suggested by previous authors, there is a need to optimize the soaking and germination times on the resulting quality attributes of soymilk. The aim of this study was therefore to employ response surface methodology in optimizing the sprouting conditions (soaking and germination time) in the production of functional soymilk from South African soybeans, with improved nutritional and quality attributes, as an intermediate product for the production of other soy-based foods.

3.2. Materials and methods

3.2.1. Experimental materials

Soybeans grown in South Africa (variety: DM 5.1i RR) were obtained from Agricol, KwaZulu Natal branch. Seeds were manually sorted to remove defective ones. All chemicals and solvents used were laboratory grade.

3.2.2. Experimental design for sprouting conditions.

The factors of sprouting studied were sprouting time and germination time. The effect of variations in these factors on some quality attributes of the resulting soymilk from each experimental run was determined using a two-factor central composite rotatable design. The centres of rotation were chosen to be 5, thereby making $\alpha=\pm1.414$. Thirteen experimental runs were generated from the combination of factors, with experimental conditions presented in Table 1.

Table 1. Experimental conditions for optimization of sprouting conditions (soaking and germination times)

Factors	Codes	Levels				
		-α	**-1**	**0**	**1**	**α**
Soaking time (h)	X_1	9.5	12	18	24	26.5
Germination time (h)	X_2	38	48	72	96	106

3.2.3. Sprouting of soybean seeds

For each experimental run, 100 g of seeds were thoroughly washed with distilled water and rinsed thereafter. Sprouting of soybean seeds were carried out in the dark in a growth chamber (Hotpack 352643, Warminster PA.) with the temperature within the chamber maintained at 25±2°C. Samples were rinsed and kept humid by manual wetting at 3-4 h interval throughout the germination period (24 - 96 h) for each experimental run.

3.2.4. Preparation of soymilk from sprouted seeds

Sprouted soybeans were manually dehulled to obtain dehulled seeds and sprouts. Distilled water (1000 ml) was added to the mixture (1:10 w/v dehulled soybean and sprouts/water ratio) and blended using a laboratory blender (Kenwood BL 380, China) until a fine consistency was obtained. Raw soymilk was obtained by sieving through muslin cloth and the residue discarded. Thereafter, the raw milk was cooked at 100°C for approximately 10 mins and allowed to cool at ambient temperature before storing in the refrigerator at 4°C for further analyses. Soymilk produced from unsprouted seeds soaked for 12 h using the same procedure described above served as the control.

3.2.5. Analyses

3.2.5.1. Total protein and total solids

Total protein content of soymilk samples was determined using Kjeldahl method with the conversion factor of 6.25. Also, total solids were obtained by placing 5 g of sample into pre-weighed crucibles and then dried in a hot air oven at 105°C for 3 h. The dry matter was weighed and used to calculate the percentage total solids (Liu and Chang, 2013).

3.2.5.2. Determination of colour parameters

The lightness, redness (or greenness) and yellowness colour attributes (L*, a* and b* respectively) of each soymilk sample obtained from sprouted soybeans and control were determined using the ColourFlex EZ benchtop spectrophotometer (HunterLab, USA). The equipment was calibrated with tiles fitted with black and white standard colours. Liquid samples were poured into the sample cup to fill the entire base and was covered with the lid, before the parameters were measured. The change in colour (ΔE) between the optimized sprouted soymilk and the control was also determined using the equation:

$$\Delta E = \sqrt{(\Delta L)^2 + (\Delta a)^2 + (\Delta b)^2} \quad \text{.... 1}$$

3.2.5.3. Total phenolic content

The method described by Xu and Chang (2007) was used to determine total phenolic content of the samples. Soymilk samples were frozen at -80°C for 24h and then lyophilized in an Alpha 2-4 LDplus freeze drier (Christ, Germany) set at 1 atm and -80°C. Phenolic compounds were extracted from 0.3 g of freeze-dried samples of soymilk with a mixture of acetone, acetic acid and water, referred to as acidic acetone (Jiang *et al.*, 2013) in the ratio 70:0.5:29.5. Gallic acid calibration curve was used to obtain the total phenolic content and the results were expressed in mg GAE/g of sample.

3.2.5.4. Total phytic acid content

Wade reagent was used in the determination of phytic acid from freeze dried soymilk samples. The reagent was prepared by the addition of 0.03% $FeCl_3 \cdot 6H_2O$ and 0.3% sulfocalicyclic acid. 2.4% HCl solution was mixed with 0.3 g freeze dried samples and was shaken in an orbital shaker at 220 rpm for 6 h. Thereafter, 0.1 ml of clear supernatant was mixed with 3 ml water and 0.2 ml Wade reagent before centrifugation at 5500 rpm for 10 mins. The absorbance was measured with distilled water as blank at 500nm and the results were expressed as mg/g phytic acid/freeze-dried soymilk (Gao *et al.*, 2007)

3.2.6. Analyses of optimized soymilk and the control

3.2.6.1. Trypsin inhibitor activity

The method of Hamerstrand *et al.* (1981) as illustrated by Poliseli-Scopel *et al.* (2012) was used to determine the trypsin inhibitor activity of optimized soymilk and the control. Tris buffer, N_α-Benzoyl-L-arginine 4-nitroanilide hydrochloride (L-BAPA) and trypsin solutions were prepared in line with the methods described and the samples were extracted with 0.01 N HCl solution by shaking in an orbital shaker. Absorbance was measured at 410 nm against sample blanks and trypsin inhibitor content was determined.

3.2.6.2. Rheology

Viscosity and other rheological properties of soymilk were measured according to the modified method of Oyeyinka *et al.* (2015). Briefly, samples were allowed to equilibrate at 25°C for about 10 mins in the sample cup of a Rheometer (Rheolab 80732808, Anton Paar, Austria). Data generated at shear rates between 750 and 1500 s^{-1} were fitted into the Power law equation and the rheological properties were determined as follows:

$$\boldsymbol{\tau = k\gamma^{n}} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\mathbf{2}$$

where τ, k, γ and n are shear stress (Pa), consistency coefficient $(Pa.s)^n$ shear rate (s^{-1}) and flow behavior index respectively.

3.2.6.3. Amino acid content determination

Amino acid content was determined using the method described by Grobbelaar et al. (2014) with minor modifications. Amino acid separation and detection was performed using a Waters Acquity Ultra Performance Liquid Chromatograph (UPLC) fitted with a photodiode array (PDA) detector. Derivatization was performed using Waters AccQ Tag Ultra Derivatization kit, according to the manufacturer's guide. Sample/standard solution (1 µl) was injected into the mobile phase [AccQ-Tag Ultra Eluent A and B (Waters)] which conveys the derivatized amino acids onto a Waters UltraTag C_{18} column (2.1 x 50 mm x 1.7 µm) held at 60°C.

3.2.7. Optimization of responses

As explained by Akinpelu *et al.* (2014), optimization of the processing parameters were achieved using the numerical optimization technique, which was aimed at simultaneous combination of parameters to find a point that maximizes the desirability functions. These desirable qualities in the resulting soymilk include the highest possible total solid, total protein, phenolic acid and lightness and the lowest redness, yellowness and phytic acid contents. Optimized sprouting conditions (soaking and germination times) were derived from the values of responses obtained from each experimental run.

Since the characteristics of each desired goal can be altered by adjusting the importance of each of the responses, maximization (for total solids, total proteins, total phenolic acid and lightness) and minimization (for redness, yellowness and phytic acid content) were preset to obtain the desired outcomes. 3-dimensional response surface plots were also generated to show the effects of processing parameters on the responses, with respect to the preset optimization conditions.

3.2.8. Statistical analyses

Design expert 10.0 software (StatEase Inc., USA) was used to generate the experimental design, with the two-independent variables; soaking time (X_1) and germination time (X_2). Data obtained from responses were fitted into a second order polynomial equation as shown below:

$$\boldsymbol{Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{12} X_1 X_2} \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \mathbf{3}$$

where Y is the response variable, X_1 and X_2 are process variables and $\beta_0, \beta_1, \ldots, \beta_{22}$ are coefficients of regression.

Response surface methodology was used to determine the optimum sprouting conditions using numerical optimization and choosing the solution that best turns in the desirable levels, after the responses have either been minimized (redness, yellowness and phytic acid) or maximized (total solids, total protein, lightness, and total phenolic content). Analyses of variance (ANOVA) was used to check the significant differences in responses determined in triplicates and to examine the statistical significance of the regression equation used.

3.3. Results and discussion

3.3.1. Total solids and protein contents

The total solids contents of soymilk samples obtained from sprouted beans ranged between 2.88 to 4.43 g/100 g (Table 2). Response surface plot showed that the total solid content decreased as soaking and germination time increased (Fig. 1A). The reduction during germination could be attributed to loss of nutrient reserves, especially at extended germination times.

This seems plausible since sprouting as a metabolic process involves the breakdown of nutrients, which is accompanied with the outgrowth of cotyledons and the release of energy (Murugkar, 2014). Retention of adequate nutrient reserves in the form of total solids in soymilk is of critical importance to the quality of soy-based foods produced (Denkova and Murgov, 2005).

An increase in the total protein contents (1.24-2.44 g/100g) (Table 2) on a wet basis was recorded with an increase in soaking and germination time (Fig. 1B). Sprouting triggers the production of diverse enzymes, which could either be responsible for the biosynthesis of proteins, or breakdown of complex proteins. Previous studies similarly reported slight increases in the protein content of sprouted soybeans (Jiang *et al.*, 2013) and buckwheat for up to 72 h germination (Zhang *et al.*, 2015). However, studies by other authors have shown that soybean germinated for 48 h resulted in a decrease in protein content of the sprouted grains (Radzi *et al.*, 2012). Hence, the protein content of sprouted grains may be influenced by the grain variety and germination conditions. The types of enzymes produced during germination and their effect on the residual proteins of the legume may also influence changes in protein content of sprouted grains (Zhang *et al.*, 2015).

Table 2: Responses of sprouted soymilk for different experimental runs*

Experimental run	Soaking time (h)	Germination time (h)	Total Solid (g/100g)	Total Protein (g/100g)	Lightness	Redness	Yellowness	Total Phenolics (mg GAE/g)	Phytic acid (mg/g)
1	18	106	3.06	1.61	81.42	-1.83	11.95	12.93	0.22
2	26	72	3.63	2.26	81.59	-1.95	12.13	12.97	0.21
3	24	96	4.19	1.83	81.27	-2.11	11.46	14.08	0.22
4	24	48	4.43	2.44	83.59	-2.14	12.96	13.34	0.21
5	18	72	2.89	1.79	79.81	-2.52	9.03	13.09	0.22
6	12	48	3.83	2.24	78.82	-2.83	7.78	13.03	0.20
7	18	38	3.96	2.03	83.21	-2.17	12.61	13.16	0.23
8	9.5	72	3.82	2.21	81.47	-2.37	11.09	13.26	0.19
9	18	72	3.21	1.85	80.35	-2.27	10.86	13.11	0.24
10	18	72	3.30	1.35	50.46	-2.37	10.87	13.24	0.21
11	18	72	4.07	2.35	82.58	-1.92	12.70	13.28	0.19
12	12	96	3.83	1.30	78.90	-2.34	9.71	13.04	0.19
13	18	72	4.13	1.24	82.95	-1.79	12.77	12.96	0.19

*Total solids, total protein and colour attribute values calculated on wet basis

3.3.2. Colour

The colour of soymilk as measured by lightness, redness and yellowness ranged between 79.81 to 83.58, -1.83 to -2.82 and 9.03 to 12.76, respectively (Table 2), with their response plots presented in Fig. 1 C, D and E, respectively. The least change in colour attributes, with respect to the control were observed at lower soaking and germination times. This could be attributed to lower metabolic stress during the breakdown of carbohydrate components of sprouting seeds into reducing sugars. Earlier studies indicated that these sugars may result in off-colour development especially at extended sprouting times (Charoenthaikij *et al.*, 2009). The inclusion of cotyledons and sprouted seeds in the production of soymilk may also be implicated for colour loss. However, minimal colour change (ΔE) was observed when the optimized soymilk was compared with the control, suggesting similar appearance of optimized soymilk to the control. Similar minimal ΔE values between soymilk and tofu from sprouted and unsprouted sources have been reported in earlier other studies (Murugkar, 2014).

3.3.3. Total phenolic and phytic acid content

Total phenolic content of soymilk samples varied between 12.93 and 14.07 mg GAE/g (Table 2). The phenolic content of the samples increased with soaking and germination times (Fig. 1F) and may be due to the response of the sprouted grains to stress induced during germination. A similar trend was seen in a study which found that phenolic compounds are synthesized and accumulated in sprouting legumes in response to the stress induced during germination, especially in the dark (Randhir *et al.*, 2004). The increase observed in soymilk produced from sprouted soybeans, as compared with the control could be seen as an advantage. Phenolic compounds have been found to exhibit bioactive properties, which may help in reducing the incidence of chronic ailments such as cancer and cardiovascular diseases (Jooyandeh, 2011).

Phytic acid content of soymilk produced from sprouted soybeans for all experimental runs (0.19-0.23 mg/g) (Table 2 and Fig. 1G) were low when compared to the control (0.41 mg/g). Soaking has been found to reduce the phytic acid content of legumes due to leaching into water (Liang *et al.*, 2009).

The reduction in phytic acid could also be attributed to the activity of phytase released during sprouting (Murugkar, 2014). According to a study by Dave *et al.* (2008), phytase activity may be up to 5-fold in sprouted soybean compared with the unsprouted ones. Furthermore, the cooking process involved in the production of soymilk could also contribute to the overall reduction in its phytic acid content. For instance, the phytic acid content of kidney beans reportedly reduced by 61% after cooking (Shimelis and Rakshit, 2007, Fabbri and Crosby, 2016). Phytic acid binds with essential minerals such as zinc, calcium and iron to form complexes, making them biologically unavailable from foods. Although phytic acid had recently been found to be a potent anti-cancer agent, they should be consumed at very low doses (Jooyandeh, 2011, Jiang *et al.*, 2013).

3.3.4. Model description

The responses from different experimental runs and the mean values of total solids, total protein, colour, total phenolics and phytic acid contents of soymilk varied significantly (Table 2). The estimated values of parameters obtained for the second order polynomial model are presented in Table 3. Optimization of the dependent responses was carried out to obtain the best conditions for sprouting soybeans which will be used to produce functional soymilk that will serve as suitable intermediate for other soy-based products. Response plots are used to show the effect of processing parameters on responses (Chakravorty *et al.*, 2007). The effect of soaking and germination time on the responses investigated are shown in the individual 3-dimensional plots (Fig 1). The significance of the model parameters were tested using ANOVA and the results are as stated in Table 3. Most parameters are significant ($p \leq 0.05$) for all the responses evaluated. The coefficient of determination (R^2), which is a measure of the reduction in response variability using repressor variables in the model equation ranged between 0.59 and 0.91 (Akinpelu *et al.*, 2014, Esan *et al.*, 2015). This suggests a considerable fit of responses into the second order polynomial equation used.

Table 3: Significance of model parameters with corresponding coefficients of determination

Coefficients	Y_1	Y_2	Y_3	Y_4	Y_5	Y_6	Y_7
β	3.5219	1.7143	81.2313	-2.1733	11.2453	13.1386	0.2107
X_1	**0.0861**	0.0996	0.9126	0.1894	1.0516	0.1177	0.006
X_2	-0.1889	-0.267	-0.5958	0.1244	**-0.0625**	**0.0515**	-0.003
X_1^2	0.2134	0.2413	**-0.1705**	**-0.0615**	**-0.1856**	**0.0618**	**-0.006**
X_2^2	**0.1069**	**0.0353**	**0.222**	**0.0168**	**0.151**	**0.0284**	**0.0061**
X_1X_2	**-0.0622**	**0.0799**	-0.6	-0.1142	-0.8567	0.1832	0.005
R^2	0.82	0.8	0.78	0.87	0.91	0.82	0.59

β: Intercept X_1: Soaking time X_2: Germination time; Responses Y_1: Total solids, Y_2: Total proteins, Y_3: Lightness, Y_4: Redness, Y_5: Yellowness, Y_6: Total phenolics, Y_7: Total phytic acid. Values in bold font are significant ($p \leq 0.05$)

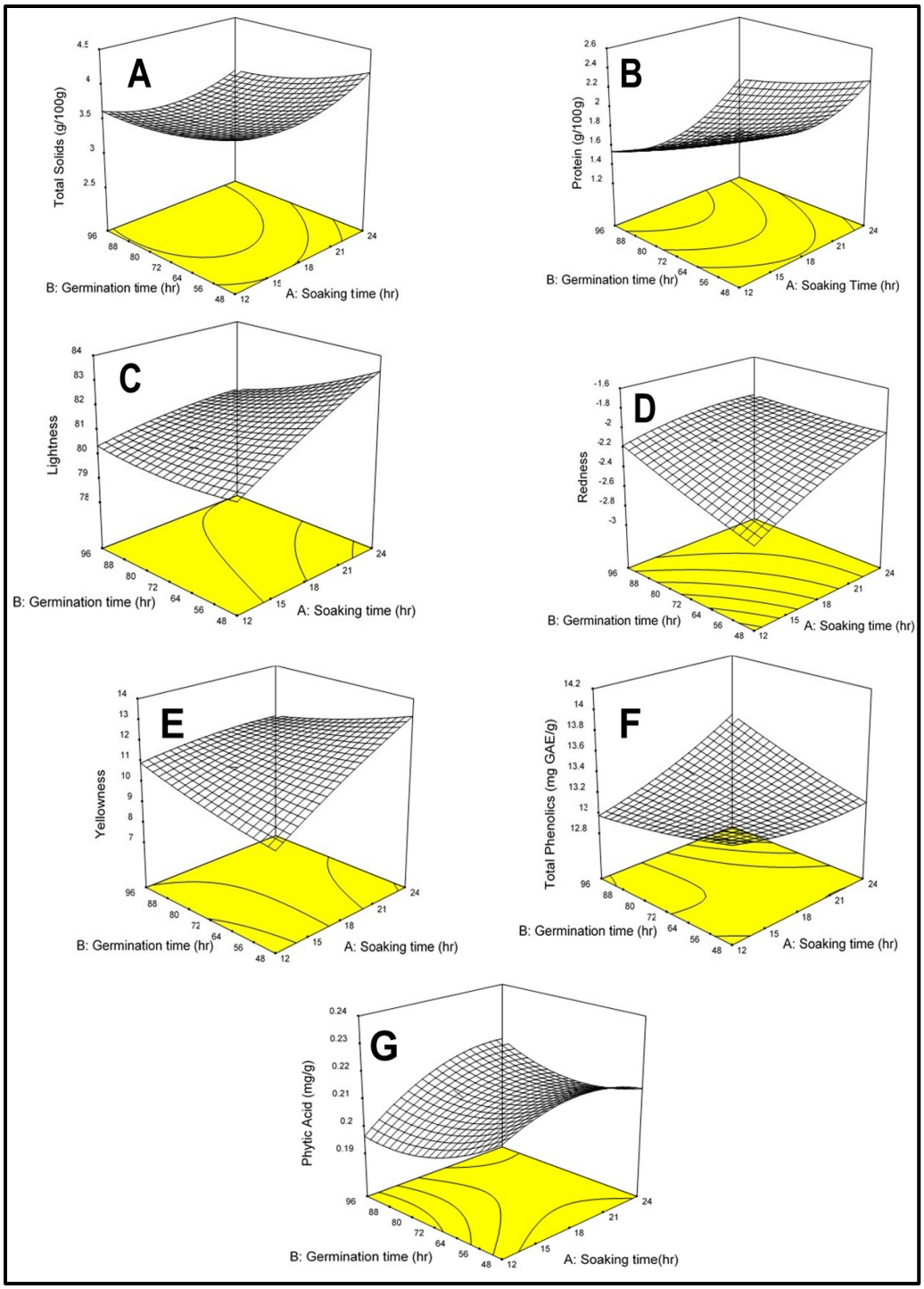

Figure 1: Response surface plots for sprouting conditions of soybeans (A) Total solids (B) Total Protein (C) Lightness (D) Rightness (E) Yellowness (F) Total phenolics (G) Total phytic acid

3.3.5. Optimized conditions for soymilk production

Numerical optimization of sprouting conditions with respect to the preset conditions of responses produced 7 results and the optimum solution of responses from the result with the highest value for desirability function was selected. The selected optimized sprouting conditions were 12 h of soaking and 52 h of germination (in the dark). Optimum responses of the measured quality attributes for soymilk prepared from sprouted soybean gave total solids of 3.83 g/100 g (wet basis), total protein of 2.40 g/100 g (wet basis), 80.30 lightness, 2.61 redness, 9.4 yellowness, 13.21 mg GAE/g total phenolic content and 0.21 mg/g phytic acid. The values obtained for soymilk prepared from unsprouted beans were 5.07 g/100 g, total solids 2.06 g/100 g total protein (wet basis), 84.05 lightness, -1.78 redness, 12.48 yellowness, 13.01 mg GAE/g total phenolic content and 0.41mg/g phytic acid. The overall change in colour (ΔE) of optimized sprouted soymilk, as compared to the control was 4.89. To adequately understand the effect of optimization on the sprouting parameters, soymilk from optimized sprouting conditions of seeds and the control were compared by determining their trypsin inhibitor activities, rheological properties and amino acid contents.

3.3.6. Comparison of soymilk from sprouted and unsprouted soybeans

3.3.6.1. Rheology

The apparent viscosity of the optimized and control increased as the shear rate increased, suggesting a shear-thickening behavior with $n > 1$ (Fig. 2; appendix). Our result contradicts earlier reports where the viscosity of soymilk prepared from sprouted soybean (at 1:6 soybean-water ratio) decreased with increase in shear rate (Zhang *et al.*, 2015). This difference in rheological behavior could be attributed to the soybean-water ratio used in this study (1:10). To characterize the flow properties of the soymilk samples, the power law model was used. Experimental data showed a good fit to the model with determination coefficients (R^2) = 0.99 (Table 4). The power law model constants were similar for sprouted sample and the control suggesting that sprouting did not significantly affect the viscosity (Fig. 2; appendix) of optimized soymilk.

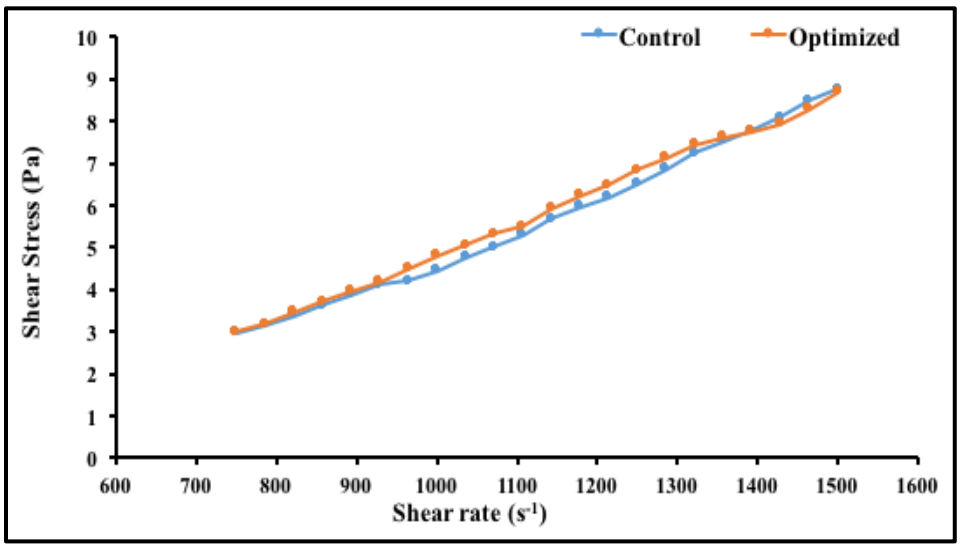

Figure 2: Flow behaviour curves for soymilk samples. Data represents mean (n=3).

Table 4: Power law coefficients for control and optimized soymilk

Treatments	n	k(Pa.s)^n	R^2
Control	1.5832	0.0000815	0.99
Optimized	1.5485	0.000107	0.99

3.3.6.2. Trypsin inhibitor activity

The trypsin inhibitor activity of sprouted soymilk was significantly lower (0.03 mg/g TI) than the control (0.08mg/g TI). The massive reduction in sprouted soymilk samples could be as a result of the presence of higher amounts of Kunitz trypsin inhibitors. These inhibitors are unstable and incorporated into protein aggregates at cooking temperatures compared to the Bowman-Birk trypsin inhibitors which are more heat stable (Xu *et al.*, 2012). Furthermore, the decrease in trypsin inhibition upon germination has been attributed to the synthesis of amino acids from the hydrolytic break-down of trysin inhibitors, to support the growth of sprouts (Sugawara *et al.*, 2007). This may account for the lower values obtained for soymilk made from optimized sprouted soybeans. Jiang *et al.* (2013) also observed that up to 91% reduction in trypsin inhibitor activity occurred between 28-72 h of germination.

3.3.6.3. Amino acid content

Soymilk prepared from sprouted soybeans had a higher total sum of amino acids than soymilk made from the control. Eighteen amino acids were detected, with lower values for sulphur-containing amino acids when compared to others (Table 5). Yang *et al.* (2012) similarly reported low values for sulphur-containing amino acids in yoghurts made from sprouted soybeans. Although there was slight reduction in the individual contents of serine, threonine, lysine, phenylalanine and methionine after sprouting, the reduction was not statistically significant. Reduction in amino acid contents after sprouting had previously been reported (Kuo *et al.*, 2004, Sulieman *et al.*, 2008, Yang *et al.*, 2012, Fouad and Rehab, 2015). The changes observed in the amino acid contents of sprouted legumes during the first 72 h of sprouting had been attributed to hydrolysis, synthesis and rearrangement of protein moieties (Taraseviciene *et al.*, 2009). There is competition for the synthesized amino acids as energy source for germinating seeds, and this may explain the reduction in some of the amino acids (Taraseviciene *et al.*, 2009, Fouad and Rehab, 2015). Thus, extended germination times may lead to depletion of amino acids due to extended sprout growth. The optimized sprouting conditions obtained could ensure a considerable retention of amino acids in soymilk, which makes it a suitable intermediate for other non-fermented soy-based products. Furthermore, the observed increase in some individual amino acid may be advantageous for improved microbial activities during the production of fermented products such as fermented soymilk and *sufu*.

Table 5: Amino acid composition of control (unsprouted) and optimized (sprouted) soymilk (µg/g)

Amino acids	Control (unsprouted)	Optimized (sprouted)
Histidine	1691.0[a]	1874.5[a]
Serine	2138.3[a]	2093.6[a]
Arginine	4450.6[b]	5078.5[a]
Glycine	2873.2[a]	2970.2[a]
Aspartine	5200.7[a]	5448.0[a]
Glutamine	8104.1[a]	8550.0[a]
Threonine	1232.4[a]	1113.5[a]
Alanine	1954.9[a]	2191.7[a]
Proline	2882.8[a]	3209.6[a]
Lysine	1757[a]	1241.5[a]
Tyrosine	1971.4[a]	2462.8[a]
Valine	2301.1[a]	2615.6[a]
Isoleucine	2259.7[a]	2579.7[a]
Leucine	3325.6[a]	3836.7[a]
Phenylalanine	9731.9[a]	7847.1[a]
Asparagine	931.8[a]	1188.8[a]
Cystine	160.8[a]	171.8[a]
Methionine	303.5[a]	121.7[b]
Total Amino acids	53271.0[a]	54595.5[a]

Means with the same superscripts are not significantly different (across the rows)

3.4. Conclusion

Process optimization of soybeans sprouting using response surface methodology showed that the optimum soaking and germination time was 12h and 52h. Soymilk produced from sprouted soybeans at these optimized conditions displayed improved nutritional attributes (protein, phenolic acid, total amino acids), reduced anti-nutrient activity (trypsin inhibitors and phytic acid) and compared favourably in quality attributes (colour and rheology) with that which is made from unsprouted beans (control). Soymilk made from the optimized sprouting conditions of beans could be a nutritious alternative milk from plant source and an important intermediate raw material in the production of other fermented or non-fermented soy-based products.

Chapter 4: Nutritional and colour attributes of soy-based foods produced from soybeans at optimized sprouting condition

Abstract

In this chapter, the nutritional and colour properties of soy-based foods produced from optimized conditions of soybean sprouting were investigated. Five soy-based foods were produced with sprouted soybeans (12 h. soaking and 52 h germination) while controls were made from un-sprouted soybeans, with all samples subjected to nutritional and colour analyses. Total protein contents of sprouted products were higher than their controls with no significant difference in the ash contents ($p > 0.05$). *In vitro* protein digestibility ranged between (85.70-99.82%) for sprouted and (81.33-99.50%) for unsprouted samples, with fermented products having the highest digestibility. No significant difference in amino acid contents ($p > 0.05$) were recorded for all sprouted soy foods and controls, apart from proline and phenylalanine in sprouted fermented soymilk (24 h) and lysine in sprouted soymilk-kefir. Total flavonoids were higher in sprouted tofu ($CaCl_2$) (60.41 mg/g) and sprouted fermented soymilk (48 h) (8.93 mg/g) and there was a general reduction in total condensed tannins for all sprouted products. Colour deviation of sprouted products from their controls was minimally perceivable as the highest ΔE value was 5.30. This study suggests that soy products obtained from sprouted soybeans at optimized conditions have a nutritional advantage over unsprouted ones, with negligible colour deviations.

Keywords: Soy-based foods, nutritional properties, colour, optimized conditions, soaking, germination.

4.1. Introduction

Awareness for the consumption of highly nutritious foods is fast rising with the nutritional condition of foods directly linked to health and general well-being. These dietary approaches have been successfully employed to prevent and control the incidence of life-threatening diseases such as cancer, heart diseases and osteoporosis (Rodríguez-Roque *et al*., 2013b, Sęczyk *et al*., 2017).

Soybean is among the cheap and abundantly available food sources with the potential of providing the body with rich nutritional requirements, either as a singular food source or composite of other foods. Soy-based foods are widely consumed in Asia and are gaining popularity in other parts of the world due to their abundant nutrients and functional compounds such as proteins, phytochemicals and vitamins (Huang *et al*., 2014). In recent times, there have been established links of consumption of soy-based foods and health-promoting effects (Jiang *et al*., 2013). It is also important to note the presence of anti-nutrients and a beany flavour associated with soybeans, which have been argued to be of detrimental nutritional and consumer appeal effects.

Sprouting of soybeans is one of the methods used to improve the nutritional composition of soy foods and reduce their antinutrient levels (Paucar-Menacho *et al.*, 2010b). Fernandez-Orozco *et al.* (2008) and Yang *et al.* (2012) reported the production of L-ascorbic acid in germinated soybeans and mung beans, which was not present in the un-germinated samples. McCue *et al.* (2005) reported the antidiabetic and antihypertensive potentials of sprouted soybeans while Murugkar (2014) reported quality improvement and antinutrient reduction in soymilk and tofu produced from sprouted soybeans. Soymilk is the major intermediate in the production of soy-based foods, including fermented soymilk, tofu, tempeh and sufu. Soymilk produced with sprouted soybeans are regarded as functional soymilk because they contain increased levels of bioactive compounds and reduced antinutrients (Jiang *et al.*, 2013). Factors affecting soybean sprouting include germination temperature, time, relative humidity, variety of soybeans and soaking time. These factors have been previously studied in isolation or in combination with other factors.

In the present study, soymilk produced from soybeans sprouted at the optimized soaking and germination times were used to produce soy-based foods. Optimization of soaking and germination periods, as factors of sprouting, is necessary to improve the functionality of soymilk. In Chapter 3, the soaking and germination times of soybeans (12 h and 15 h respectively) were optimized to obtain soymilk with high protein and reduced antinutrient levels. Since sprouted soymilk is an intermediate product, it is imperative to study the nutritional characteristics and appearance (colour) of foods produced from it. It is also necessary to investigate the effects of processing activities involved in the production of these foods on the retention of desired characteristics obtained at optimized soybean sprouting conditions.

Comparing these foods with those produced from unsprouted soybeans will also provide information on their nutritional advantage and colour deviations. This study was carried out to determine selected nutritional and colour properties of five different soy-based foods obtained from soymilk produced at optimized conditions of soybean sprouting (soaking and germination times) and to compare these with those obtained from unsprouted soybeans.

4.2. Materials and methods

4.2.1. Soybean Material

Soybean seeds (variety: DM 5.1i RR) were obtained from Agricol, KwaZulu Natal, South Africa. The soybean samples were carefully inspected and any damaged or infected sample discarded. Using the method of Murugkar (2014), soymilk was produced from sprouted soybeans at optimized conditions of soaking time (12 h) and germination time (52 h) obtained from previous studies. Soy based foods namely; tofu (soy-*wara*), naturally fermented soymilk (soy-*nono*) and soymilk-kefir were then produced from sprouted soymilk and subjected to nutritional and colour analyses. Control samples for each of the soy-based foods were also produced from un-sprouted soybeans.

4.2.2. Production of soy-based foods

Five food products were made from optimized sprouted soymilk: STC (tofu from sprouted soybeans coagulated by $CaCl_2$); STH (tofu from sprouted soybeans coagulated with *Hibiscus sabdariffa* flower extracts); SFS24 (sprouted fermented soymilk - 24 h); SFS48 (sprouted fermented soymilk – 48 h); SSK (sprouted soymilk-kefir). UTC, UTH, UFS24, UFS48 and USK were the respective products from un-sprouted soymilk used as controls in each case.

4.2.2.1. Tofu (*soy wara*)

Two separate tofu (cheese-like) products were made from soymilk samples using natural and chemical coagulants. As described by Rekha and Vijayalakshmi (2010b), 10 ml of 5% water extract of dried *Hibiscus sabdariffa* flowers was added to 100 ml of soymilk from sprouted and unsprouted beans at 80°C. The mixture was mildly swirled to ensure proper incorporation of *H. sabdariffa* and allowed to stand for approximately 30 min to coagulate.

Thereafter, tofu (from natural coagulant) was obtained by allowing the coagulant to pass through muslin cloth, any remaining liquid was further removed by pressing with weights, to separate the whey. For tofu from a synthetic coagulant, a similar approach was carried out using 0.4% $CaCl_2$ as the coagulant (Prabhakaran *et al.*, 2006).

4.2.2.2. Naturally fermented soymilk (*soy nono*)

The method of Obadina *et al.* (2013) was modified to obtain these products. Briefly, sucrose (5%) was added to 100 ml soymilk samples in 250 ml conical flasks. The mixture was loosely covered with aluminum foil and fermented at 25±2°C. Samples were drawn after 24 (pH 4.6) and 48 h (pH 4.0) of fermentation for nutritional analyses. Sucrose was added to trigger spontaneous fermentation and the quantity added was determined through preliminary fermentations with 1%, 2.5%, 4% and 5% sucrose.

4.2.2.3. Soymilk-kefir

The method of Liu *et al.* (2005) was used to produce soymilk-kefir. Dried water-kefir grains (5 g), which are potential repositories for fermentation microorganisms, were added to 100 ml soymilk samples and incubated at 25°C for 24 h. Wet kefir grains were later separated from the fermented milk and kept for further use, while the fermented soymilk-kefir samples were subjected to analyses.

4.2.3. Analyses

4.2.3.1. Total solid, moisture, protein and ash contents

Total solid and moisture contents of samples were obtained by drying approximately 5 g of each in a convection oven for 3 h at 103±2°C. Dry matters obtained were regarded as total solid content and mass losses as moisture content in all cases. Results were expressed as percentage of the original mass of samples (Liu and Chang, 2013). Total protein (Kjeldahl) and ash contents were determined using the methods described by Murugkar (2014)

4.2.3.2. *In vitro* protein digestibility (IVPD)

The method described by Osman *et al.* (2014) with minor modifications were used to determine the *in vitro* protein digestibility of each product. To enhance digestion, 15 ml of 0.1M HCl was added to 0.2 g of each freeze-dried sample (containing up to 16 mg nitrogen), and thereafter, 0.1 mg pepsin per ml of HCl was added.

The mixture was digested for 3 h in a shaking incubator at 37°C. The reaction was stopped by adding 15 ml of trichloroacetic acid, and the mixture filtered through Whatman filter paper (No. 1). The protein content of the residue on the filter paper was determined using the Kjeldahl method and percentage IVPD calculated as:

$$Protein\ digestibility\ (\%) = 100 - \left(\frac{undigestible\ protein}{total\ protein} \times 100\right)$$

4.2.3.3. Amino acid analyses

Amino acid analysis was carried out using Waters Acquity Ultra-Performance Liquid Chromatography fitted with a photodiode array (PDA) detector, with reference to the method described by Grobbelaar *et al.* (2014). Sample and standard solutions of 1 µl were injected into the mobile phase (Waters AccQ-Tag Ultra Eluents A and B) conveying the derivatized amino acids into a C_{18} Column (2.1 x 50 mm x 1.7 µm) held at 60°C. Derivitization was performed with a Waters AccQ Tag kit in line with manufacturer's instructions and results were expressed in mg amino acids/g freeze-dried samples.

4.2.3.4. Total flavonoid and condensed tannin contents determination

Colorimetric methods described by Heimler *et al*. (2005) and Xu and Chang (2007) were used to determine the total flavonoids and condensed tannins respectively. Catechin solution was used as a standard in both cases. Freeze-dried samples (0.3 g) were extracted with acidic acetone (70% acetone, 25% distilled water and 5% acetic acid) and aliquots of these extracts used. For total flavonoids, 0.25 ml of extracts was mixed with appropriate volumes and concentrations of $NaNO_2$, $AlCl_3 \cdot 6H_2O$ and NaOH. Absorbance was measured at 510 nm using UV visible spectrophotometer against the blank. To determine total condensed tannins, 50 µl of sample extracts were mixed with 3 ml of 4% methanol Vanillin and 1.5 ml concentrated HCl. After 15 mins the absorbance was taken against methanol at 500 nm. Standard curves were constructed with appropriate concentrations of (+)-catechin in both cases and concentrations of total flavonoids and tannins in all samples expressed in mg catechin equivalents (CAE)/g freeze-dried samples.

4.2.3.5. Colour

Colour parameters (L, a and b) of freeze-dried samples of sprouted soy-based foods and their respective controls were determined using table top ColourFlex EZ spectrophotometer (Hunterlab,

USA). The base of sample cup was sufficiently filled with portions of dried samples and then covered with the lid to properly measure the colour attributes.

4.2.4. Statistical analysis

All data was statistically analyzed using analysis of variance (ANOVA) at 95% confidence level and means compared using Duncan and Least Significant Difference (LSD) tests with SPSS version 24 (IBM, USA).

4.3. Results and discussion

Total solids, moisture and ash contents

The advantages associated with the production of soymilk at optimized conditions of soybean sprouting (12 h of soaking and 52 h of germination) has been outlined in Chapter 3. Total solids obtained for tofu products ranged between 28.12-35.02 mg/100 g with UTH having the highest solid contents (Table 1). STC had higher total solids than UTC while UTH had a higher content than STH ($p \leq 0.05$). The reduction obtained for STH could be due to the breaking down of food reserves in soybean seeds through various enzymatic activities during germination, leading to the oxidation of starch, sugars and proteins, to enhance respiration and energy production (Mbithi *et al.*, 2001). It could also be because of the loss of soluble food components with the whey during tofu pressing. Higher recovery of solids was recorded in UTH compared to UTC. Obatolu (2008) reported a higher retention of solids with the use of lemon over other chemical coagulants. Solid content retention in SFS 24 and SFS 48 was lower than their respective controls. This is expected because of germination and fermentation since both processes involve metabolic activities leading to depletion of nutrient reserves. These results are also supported by the moisture contents obtained for each of the food products. Ash contents were significantly higher ($p \leq 0.05$) in SFS48 and SSK compared with the control, while the amount is lower in sprouted tofu products.

Sangronis and Machado (2007) explained that the increase in mineral components in *Phaseolus vulgaris* and pigeon beans, up to 17%, was a result of reduction in phytic acid during germination, leading to the release of minerals previously bound to phytates. Reduction in ash contents recorded for tofu from sprouted soymilk could be due to diffusion of minerals already released from the phytic acid complex into whey as a result of pre-coagulation cooking of soymilk. This trend is

supported by the findings of Mubarak (2005) who reported the leaching of minerals of processed mung beans into cooking water.

Table 1: Proximate composition of soy-based foods from sprouted soymilk and their control samples

Sample	Total Solid (g/100g)	Total Protein (g/100g)	Moisture content (g/100g)	Ash Content (g/100g)
STC	30.84 ± 1.47^{b}	15.42 ± 0.51^{a}	69.15 ± 1.47^{c}	2.55 ± 0.15^{a}
UTC	28.49 ± 0.47^{b}	13.73 ± 0.19^{b}	71.50 ± 0.47^{c}	2.63 ± 0.22^{a}
STH	28.12 ± 1.98^{b}	15.55 ± 0.71^{b}	71.88 ± 1.98^{c}	2.05 ± 0.06^{b}
UTH	35.02 ± 4.21^{a}	15.41 ± 0.03^{b}	64.98 ± 4.21^{d}	2.18 ± 0.02^{b}
SFT24	9.36 ± 0.66^{c}	3.53 ± 0.30^{c}	90.63 ± 0.66^{b}	1.47 ± 0.16^{c}
UFT24	9.40 ± 0.25^{c}	2.05 ± 0.59^{d}	90.59 ± 0.25^{b}	1.52 ± 0.03^{c}
SFT48	8.88 ± 0.56^{c}	1.98 ± 0.23^{d}	91.11 ± 0.56^{b}	1.56 ± 0.09^{c}
UFT48	8.91 ± 1.08^{c}	1.74 ± 0.19^{d}	91.08 ± 1.08^{b}	0.28 ± 0.08^{e}
SSK	5.28 ± 0.21^{d}	15.51 ± 0.51^{a}	94.71 ± 0.21^{a}	0.53 ± 0.11^{d}
USK	4.77 ± 0.13^{d}	13.56 ± 0.49^{a}	95.22 ± 0.13^{a}	0.34 ± 0.12^{de}

Data represents means (n = 3) with different superscripts are significantly different across the columns ($p \leq 0.05$)
STC: tofu from sprouted soybeans coagulated by $CaCl_2$ STH: tofu from sprouted soybeans coagulated with *Hibiscus sabdariffa* flower extracts, SFS24: sprouted fermented soymilk (24h), SFS48: sprouted fermented soymilk (48h) SSK: sprouted soymilk-kefir. UTC, UTH, UFS24, UFS48 and USK are the respective control samples.

4.3.1. Total protein and *in vitro* protein digestibility

Generally, total protein contents of sprouted products were higher than their respective controls ($p \leq 0.05$) for all samples (Table 1). Increase in total protein in legumes and their products as a result of sprouting (Murugkar, 2014, Jiang *et al.*, 2013) and fermentation (Sanjukta and Rai, 2016) had been reported.

This increase in total proteins has been attributed to the activity of proteolytic enzymes in breaking down complex proteins and synthesis of new ones during these two processes. Soybean seeds sprouted at the optimized conditions (12 h soaking and 52 h germination) had increased protein content in soymilk, which was used as the major raw material in the production of sprouted soy products in this study. All sprouted soy-products had increased protein contents with 12% in STC, 0.97% in STH, 72% in SFS24, 13.7% in SFS48 and 14.8% in SSK relative to their controls.

The minimal increase in the protein content for sprouted tofu coagulated with *H. sabdariffa* could be due to the protein introduced by the extracts of *H. sabdariffa* externally. The high protein content of SFS24 resulted from the activities of fermentative microorganisms and the lower percentage increase observed for SFS48 could be because of the extended fermentation time, making proteins more available for microbial fermentative activities. Increase in protein content in soy-yoghurt with added inulin was reported by Rinaldoni *et al.* (2012). Protein content of the two soymilk-kefir products are very high and comparable to that of coagulated products. This suggests abundant synthesis of proteins by the activities of microorganisms present in kefir grains. However, the higher protein content recorded for SSK could be attributed to the availability of nutrients in their reduced forms to fermentation microorganisms, as a result of germination at the optimized condition. *In vitro* protein digestibility (Fig. 1) was generally higher for sprouted soy-products, reaching 99.8% in sprouted fermented soymilk at 48 h (SFS48). The high IVPD could be due to initial break down of complex protein molecules in soybean seeds by proteolytic enzymatic activities at the optimized germination conditions and further breakdown achieved during fermentation by the activities of microorganisms. Dikshit and Ghadle (2003) reported an *in vitro* protein digestibility of 56.52% for MACS-13 soybeans germinated for 48 h while Shimelis and Rakshit (2007) reported the *in vitro* protein digestibility of 88.74% at 24 h germination for *Phaseolus vulgaris* as the highest value for the three varieties studied. Also, Osman *et al.* (2014) attributed increased *in vitro* protein digestibility to the reduction of antinutrients such as phytates and tannins and this was generally observed in soy-products from sprouted soybeans (results reported in Fig. 2 and 3). Fermented products from sprouted soybeans (SFS24 and SFS48) had the highest digestibility values. This suggests that production of fermented soy foods from sprouted beans, especially at the optimized conditions of sprouting used in this study, assures the availability of protein moieties present in these foods in simple, absorbable forms.

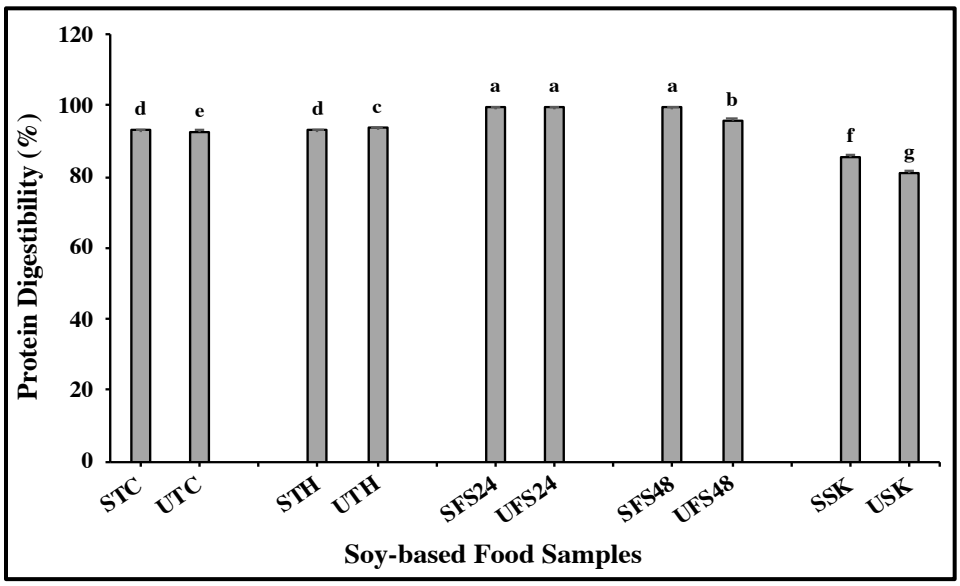

Figure 1: *In vitro* protein digestibility of soy-based foods samples [STC: tofu from sprouted soybeans coagulated by $CaCl_2$ STH: tofu from sprouted soybeans coagulated with *Hibiscus sabdariffa* flower extracts, SFS24: sprouted fermented soymilk (24h), SFS48: sprouted fermented soymilk (48h) SSK: sprouted soymilk-kefir. UTC, UTH, UFS24, UFS48 and USK are the respective control samples]. Data represents mean ± standard deviation (n=3). Means with the same superscripts are not significantly different ($p \leq 0.05$).

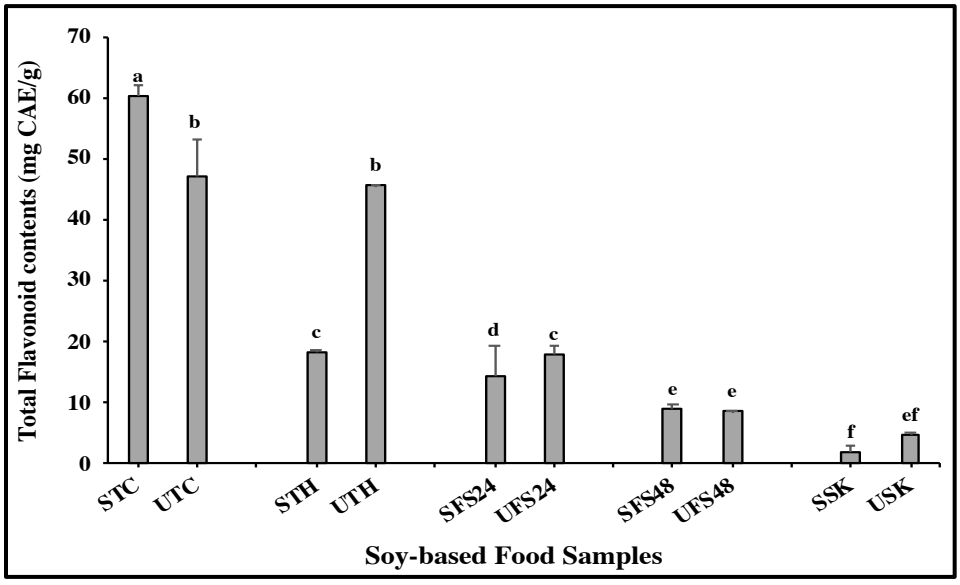

Figure 2: Total flavonoid contents of soy-based food samples [STC: tofu from sprouted soybeans coagulated by $CaCl_2$ STH: tofu from sprouted soybeans coagulated with *Hibiscus sabdariffa* flower extracts, SFS24: sprouted fermented soymilk (24h), SFS48: sprouted fermented soymilk (48h) SSK: sprouted soymilk-kefir. UTC, UTH, UFS24, UFS48 and USK are the respective control samples]. Data represents mean ± standard deviation (n=3). Means with the same superscripts are not significantly different ($p \leq 0.05$).

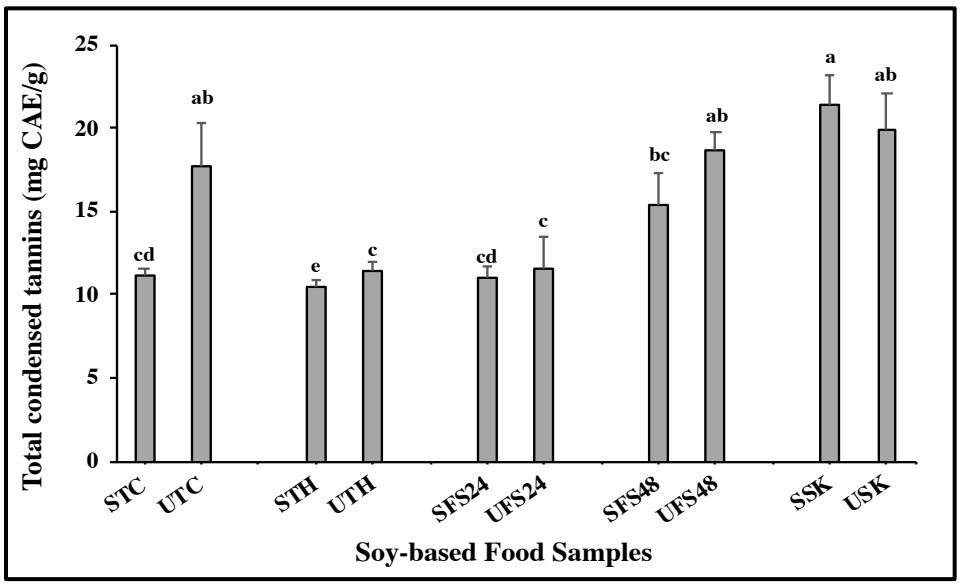

Figure 3: Total condensed tannin contents of soy-based food samples [STC: tofu from sprouted soybeans coagulated by $CaCl_2$ STH: tofu from sprouted soybeans coagulated with *Hibiscus sabdariffa* flower extracts, SFS24: sprouted fermented soymilk (24h), SFS48: sprouted fermented soymilk (48h) SSK: sprouted soymilk-kefir. UTC, UTH, UFS24, UFS48 and USK are the respective control samples]. Data represents mean ± standard deviation (n=3). Means with the same superscripts are not significantly different ($p \leq 0.05$).

4.3.2. Amino acid contents

The quantities of individual amino acids obtained for tofu products was greater than those of other foods (Fig. 4). This could be because they are coagulated products, with higher amounts of proteins pressed together per unit weight. A trend in aspartic and glutamic acids contents was observed for sprouted soy foods and their control; where there is an increase in aspartic acid for sprouted foods, there is a corresponding increase in glutamic acid, and vice-versa. This trend could be due to the extent of breakdown of complex proteins into simpler forms to accumulate as amides which then form aspartic and glutamic acid, as reported by Murugkar (2014). There were no significant differences in the amino acid contents ($p > 0.05$) for all sprouted soy foods and their controls, except for proline and phenylalanine in SFS24 and lysine in SSK. Mbithi-Mwikya *et al.* (2000) reported a 5.1% lysine reduction after 48 h germination of kidney beans. The slight reduction of individual amino acids for sprouted foods may also reflect their availability, in simpler forms, to be used up rapidly during germination for the growth of sprouts.

Higher germination times, beyond the optimized condition, suggested (52 h) may lead to loss of more amino acids, especially during fermentation (Yang *et al.*, 2012).

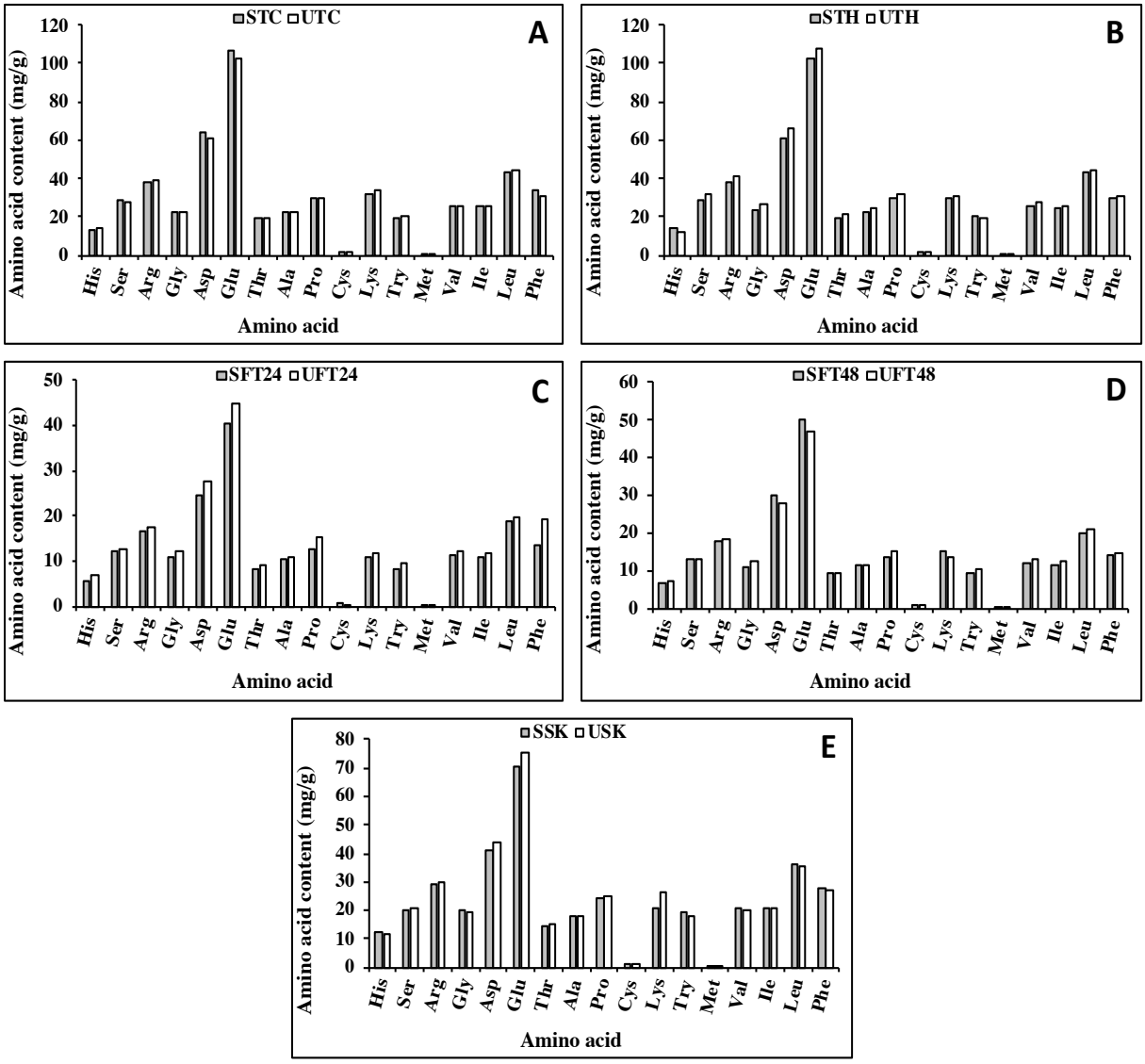

Figure 4: Amino acid content of soy-based samples from sprouted soybeans and their respective controls [A-STC: tofu from sprouted soybeans coagulated by $CaCl_2$; B-STH: tofu from sprouted soybeans coagulated with *Hibiscus sabdariffa* flower extracts; C-SFS24: sprouted fermented soymilk (24h); D-SFS48: sprouted fermented soymilk (48h); E-SSK: sprouted soymilk-kefir. UTC, UTH, UFS24, UFS48 and USK are the respective control samples].

4.3.3. Total flavonoids and condensed tannins

Isoflavones are the major flavonoids found in soybeans (Lee *et al.*, 2011). Soybeans also contain flavanols and condensed tannins (Xu and Chang, 2007). There are conflicting perspectives of researchers to flavonoids, as either an antinutritional factor (Isanga and Zhang, 2008) or a radical scavenging agent protecting against oxidative stress related diseases (Xu and Chang, 2007). Total flavonoid content was higher for STC and SFS48, with STC having 60.14 mg/g freeze-dried product (Fig. 2). The increase in total flavonoids could be viewed as an increase in isoflavones and flavonols during germination, which has been attributed to the production of precursors of isoflavonoids of soybeans (Liu *et al.*, 2002, Zhu *et al.*, 2005). The decrease observed in STH and SSK may be attributed to the interconversion of flavonoid constituents from one form to another during germination and this could be justified by the condensed tannin content of these food products (Fig. 3). Therefore, the increase in flavonoids for sprouted soy products obtained in this study could be an advantage for a possible increase in their antioxidant potentials.

Tannins are water soluble and heat labile (Rakić *et al.*, 2007). Soymilk used as raw material to produce soy-based foods were obtained from soybeans (sprouted or unsprouted) that had been initially subjected to soaking and boiling. There was significant reduction of total condensed tannins in all sprouted soy foods ($p \leq 0.05$), except for sprouted soymilk kefir (Fig. 4). The highest percentage reduction was observed in SFS48 (above 17%). Tannins are released into water during soaking of soybeans. Also, the enzymatic activities of polyphenolase bring about the loss of tannins during germination (Sangronis and Machado, 2007). The further reduction of tannins in legumes during fermentation has been attributed to the activities of polyphenol oxidase and fermentation microflora (Khattab and Arntfield, 2009).

4.3.4. Colour attributes

Processing operations aimed at improving the quality of foods should not have extreme adverse effects on their consumer appeals, especially colour. Colour of food materials is known to influence the perception and preference of consumers (Oyedeji *et al.*, 2017) and could affect the decision to accept or reject a specific food product.

Colour attributes (L,a,b) were significantly different for all food samples, except for L (lightness) for STC and UTC (Table 2). The L values obtained for the two naturally fermented soymilk samples from sprouted soymilk (SFS24 and SFS 48) were higher ($p \leq 0.05$) than their respective controls, while their a and b values were lower. However, lower lightness values were obtained for STC, STH and SSK. Reduction in lightness has been reported for soymilk and tofu produced from sprouted beans (Murugkar, 2014) and was attributed to the inclusion of sprouts while milling soybeans to produce soymilk. Colour change (ΔE) values ranged between 1.02 to 6.88 in all samples, with STC having the least colour change. The minimal colour change observed suggests that products from sprouted soybeans have a close resemblance to their controls and so, their acceptability to consumers who may already be familiar with the unsprouted products would likely not be adversely affected.

Table 2: Colour attributes of soy-based foods from sprouted soymilk and their control samples

Sample	Lightness*	Redness*	Yellowness*	Colour change (ΔE)
STC	78.19 ± 0.16^{e}	3.14 ± 0.01^{a}	28.15 ± 0.02^{b}	5.31
UTC	78.22 ± 0.16^{e}	2.69 ± 0.08^{bc}	29.11 ± 0.10^{a}	
STH	71.14 ± 0.03^{h}	2.53 ± 0.05^{c}	22.65 ± 0.11^{g}	4.76
UTH	76.02 ± 0.02^{g}	1.51 ± 0.17^{d}	25.41 ± 0.01^{d}	
SFT24	86.47 ± 0.23^{a}	0.62 ± 0.02^{d}	20.91 ± 0.06^{h}	4.57
UFT24	83.28 ± 0.02^{a}	1.47 ± 0.02^{f}	23.06 ± 0.13^{f}	
SFT48	85.87 ± 0.24^{b}	0.71 ± 0.04^{f}	19.61 ± 0.12^{i}	4.43
UFT48	82.56 ± 0.11^{d}	1.22 ± 0.03^{e}	24.34 ± 0.32^{e}	
SSK	77.63 ± 0.11^{f}	2.80 ± 0.40^{b}	27.49 ± 0.30^{c}	5.24
USK	82.17 ± 0.73^{d}	1.02 ± 0.14^{e}	22.72 ± 0.16^{g}	

Means with different superscripts are significantly different across the columns ($p \leq 0.05$) [STC: tofu from sprouted soybeans coagulated by $CaCl_2$ STH: tofu from sprouted soybeans coagulated with *Hibiscus sabdariffa* flower extracts, SFS24: sprouted fermented soymilk (24hrs), SFS48: sprouted fermented soymilk (48hrs) SSK: sprouted soymilk-kefir. UTC, UTH, UFS24, UFS48 and USK are the respective control samples].

4.4. Conclusion

Soy-based foods were successfully derived from sprouted soybeans using previously established optimized conditions of sprouting (12 h soaking and 52 h germination). Protein content and *in vitro* protein digestibility was higher in all sprouted soy-based foods, and much more for sprouted fermented soymilk samples, compared with their respective controls. Lower solid content obtained for sprouted fermented products was because of loss of food reserves during sprouting and fermentation. Germination times beyond the one suggested in this study may lead to a greater loss of amino acid components, especially for fermented soy foods. Increase in total flavonoids obtained for sprouted foods in this study may be an advantage as they have been proposed as potent antioxidants. Reduction in condensed tannins observed in all soy foods was due to the extended soaking time (12 h) and further reduction in sprouted and fermented ones stemmed from myriad enzymatic activities during these processes. Colour change for sprouted products was such that their consumer perception may not be affected, relative to the unsprouted ones. Generally, products obtained from sprouted soybeans at the suggested optimized sprouting conditions had better nutritional attributes.

Chapter 5: Antioxidant activities of soy-based foods produced with soybeans sprouted at optimized conditions

Abstract

The antioxidant activities of soy-based foods produced at optimized conditions of soybean sprouting (12 h soaking and 52 h germination) were investigated using different assays. These products included soymilk (unfermented), tofu (coagulated with natural and chemical coagulants), naturally fermented soymilk and soymilk-kefir. The control sample of each food was produced at similar conditions from unsprouted soybeans. Total phenolic content (TPC) was determined using Folin Ciocalteau method and samples (0.5-5 mg/ml), in appropriate buffers were tested for their abilities to scavenge free radicals. Sprouted soy-based food products had significantly higher ($p \leq 0.05$) TPC (13.21-14.21 mg GAE/g) when compared to their controls (13.01-14.07 mg GAE/g). Sprouted soy-based foods were able to scavenge free radicals and demonstrated higher diphenyl-1-picrylhydrazyl (DPPH), superoxide and hydroxyl radical scavenging activities. Over 50% increase in metal ion chelation was observed in all sprouted soy-based food products, however ferric reducing antioxidant power (FRAP) in both sprouted and unsprouted products were not significantly different ($p>0.05$). Production and consumption of soy foods from soybeans sprouted at these optimized conditions as functional foods could be a promising means of preventing accumulation of toxic free radicals.

Keywords: Soybean, Soy milk, Tofu, Antioxidant properties, Free radicals, Sprouting

5.1. Introduction

The incidence of various diseases and physiological breakdowns in humans has been linked to the presence of free radicals and oxygen reactive species produced as a result of regular body cell activities (Udenigwe *et al*., 2009). These radicals are known to promote oxidative disintegration of biological macromolecules, resulting in disease conditions such as cancer, arthritis, and atherosclerosis (Juan and Chou, 2010). Despite this the body of a healthy mammal has defense mechanisms against oxidative damage if the system is overwhelmed with severe oxidative stress (Fasakin *et al*., 2011).

Free radicals, including superoxide moieties, promote the formation of many reactive species such as hydroxyl radicals and singlet oxygen, which bring about the damage of body cell and tissue structures (Lee *et al*., 2004). Chemical antioxidants including butylated hydroxyl anisole (BHA), butylated hydroxyl toluene (BHT) and propyl gallate (PG) has been previously added to foods to reduce these health issues, however their safety concerns have necessitated the use of natural antioxidants present in food.

Consuming antioxidant-rich foods, such as legumes, pulses and peas could support the inherent defense systems of the body against adverse physiological effects of free radical production. Hence, their consumption has been suggested and is fast rising. Soybeans are one of the most commonly consumed legumes worldwide (Sęczyk *et al*., 2017). The consumption of soy-based foods is gaining rapid popularity, especially in countries outside Asia. Soybeans contain high amounts of bioactive compounds such as polyphenols and flavonoids and ingestion of these compounds could help prevent the occurrence of chronic diseases (Rodríguez-Roque *et al*., 2013a, Morales-de La Peña *et al*., 2010, Sęczyk *et al*., 2017). The bioactive components of soy-based foods are potent antioxidants and function as radical scavenging agents, thereby preventing the adverse activities of free radicals (Lee *et al*., 2004).

Sprouting increases the nutritive value of edible seeds and sprouted seeds have been found to be rich sources of phenolic and flavonoid compounds with high antioxidant activities (Pająk *et al*., 2014). This increase has been attributed to stress-induced biochemical activities during sprouting, leading to the enzymatic breakdown of complex nutrients, with increased release of phenolic and non-phenolic antioxidants (Duenas *et al*., 2009). Other food processes, especially fermentation, has also been reported to enhance the antioxidant potential of leguminous foods (Xiao *et al*., 2015). Fermentation of soymilk and other soy-based foods with single or mixed cultures of *Bacillus subtilis*, *Lactobacillus rhamnosus*, and other strains of *Lactobacillus sp*., *Acetobacter sp*., *Coccharomyces sp*. and *Streptomyces sp*. have been reported to improve antioxidant activities (Xiao *et al*., 2015, Marazza *et al*., 2012, Juan and Chou, 2010).

Different food sample treatment approaches have been used in other studies in determining the antioxidant activities of soybeans and their food derivatives. These approaches include the use of aliquots of phenolic extracts (Xu and Chang, 2007), isoflavone extracts (Marazza *et al*., 2012, Pyo *et al*., 2005), hydrophilic and lipophilic phenolic extracts (Zhao and Shah, 2014a, Rodríguez-Roque *et al*., 2013a) and aliquots samples extracted in appropriate buffer solutions (Liu *et al*., 2005, Wang *et al*., 2006, Morales-de La Peña *et al*., 2010). Together with phenolics, other components of soy-based foods including peptides, isoflavones, and other flavonoids could be responsible for their antioxidant activities. There is available literature on the improvement of nutritional composition (Murugkar, 2014) increased levels of bioactive antioxidative peptides (lunasin and Bowman-Birk Inhibitor) (Paucar-Menacho *et al*., 2010b) and reduced antinutrient compounds and antihypertensive potentials (McCue *et al*., 2005) of sprouted soybeans and their products. These studies suggest different sprouting conditions (temperature, soaking and germination times) for soybeans in their studies. This necessitated the optimization of sprouting conditions of soybeans, which was done in Chapter 3, to facilitate optimal production of phenols and antioxidants. Therefore, it is important to study the antioxidant activities of the various soy-based foods produced from soybeans sprouted at the optimized conditions (12 h of soaking and 52 h germination). Little information exists on the antioxidant capacities of natural and kefir fermented soymilk produced from sprouted soybeans, especially regarding specific oxidative radicals. Therefore, the aim of this study was to determine the free radical scavenging capabilities of different soy-based foods produced at optimized sprouting conditions, relative to unsprouted samples.

5.2. Materials and methods

In Chapter 3, the optimum sprouting conditions (soaking and germination) of soybeans obtained were 12 h of soaking and 52 h of germination. Soybeans (variety DM 5.1i RR), obtained from Agricol, KwaZulu Natal, South Africa were sprouted based on the suggested optimized conditions and used to produce six soy-based foods. All reagents and solvents used to produce antioxidant assays were purchased from Sigma Aldrich (Sigma Chemicals St. Louis, MO).

5.2.1. Production of soy-based foods

Six food products were made from optimized sprouted soybean seeds. They include soymilk; tofu from sprouted soybeans coagulated by $CaCl_2$; tofu from sprouted soybeans coagulated with *Hibiscus sabdariffa* flower extracts; sprouted fermented soymilk (24 h); sprouted fermented soymilk (48 h); sprouted soymilk-kefir following the methods described in Chapters 3 and 4. Their respective products from un-sprouted soymilk were used as control in each case.

5.2.2. Sample preparation

All samples were frozen at -80°C for 24 h and freeze-dried at 1 atm using an Alpha 2-4 LDplus freeze-drier (Christ, Germany). Samples were ground into very fine consistencies before use. Sample concentrations of 0.5-5 mg/ml of extraction solvents or buffer were tested for all antioxidant assays and the observed activities of each concentration stated were reported. In cases where no reaction was observed for a concentration, no result was stated.

5.2.3. Total phenolic content determination

Total phenolic content was determined using the Folin-Ciocalteu colorimetric method described by Xu and Chang (2007). Absorbance readings obtained were converted into total phenolic contents using a gallic acid standard curve and results expressed in gallic acid equivalent per gram of freeze-dried samples (mg GAE/g)

5.2.4. Antioxidant analysis

5.2.4.1. Determination of DPPH radical-scavenging activity

The method of Alashi *et al*. (2014) with minor modifications for 96 well clear bottom plates, was used to determine the radical scavenging activity of samples against DPPH. Methanolic DPPH was made to a final concentration of 100 μM, each sample concentration was dissolved in 0.1 M sodium phosphate buffer (pH 7.0) containing 1% (w/v) Triton X-100. Aliquots from dissolved samples (100 μl) were mixed with 100 μl of methanolic DPPH solution in a 96-well plate to a final assay concentration of 0.0625 - 1.0 mg/ml and incubated in the dark for 30 mins at room temperature.

Thereafter, the absorbance of the blank (A_c) and samples (A_s) were measured at 517 nm. The blank consisted of buffer alone, without the samples. The percent DPPH radical scavenging activity of the samples was determined using the following equation:

$$DPPH\ Radical\ Scavenging\ Activity\ (\%) = \frac{A_c - A_s}{A_c} \times 100 \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \mathbf{1}$$

5.2.4.2. Determination of superoxide radical scavenging activity (SRSA)

The method described by Alashi *et al*. (2014) was used to determine the SRSA. Appropriate concentrations of sample aliquots were obtained by dissolving in 50 mM Tris-HCl buffer (pH 8.3) containing 1 mM EDTA. Then, 80 μl of these aliquots were transferred into a 96 well microplate in each case while 80 μl of buffer was added to the blank well. Thereafter, 40 μl 1.5 mM pyrogallol (dissolved in 10 mM HCl) was added into each well in the dark and the change in the reaction rate was immediately measured at room temperature over a period of 4 min at an absorbance of 420 nm. The superoxide radical scavenging activity was calculated using the following equation:

$$\text{SRSA}\ (\%) = \frac{\Delta A/min_b - \Delta A/min_s}{\Delta A/min_b} \times 100 \ \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \mathbf{2}$$

5.2.4.3. Determination of hydroxyl radical scavenging assay

The hydroxyl radical scavenging assay was modified based on a method described by Girgih *et al*. (2013). Samples or standard and 1,10-phenanthroline (3 mM) were each dissolved in 0.1 M phosphate buffer (pH 7.4) while $FeSO_4$ (3 mM) and 0.01% hydrogen peroxide were dissolved in distilled water. Aliquots (50 µl) of sample and buffer (control) was first added to a clear, flat bottom 96-well plate followed by additions of 50 μl of 1,10-phenanthroline and 50 μl of $FeSO_4$ to each well. For the reaction to commence, 50 μl of hydrogen peroxide (H_2O_2) solution was added to the mixture in each well before covering the plate. Thereafter, the absorbance of the mixtures was measured at 536 nm every 10 min for a period of 1 h at 37°C while shaking. The equation below was used to calculate the hydroxyl radical scavenging activity based on change in absorbance (ΔA):

$$Hydroxyl\ radical\ scavenging\ activity\ (\%) = \frac{\Delta A/min_{control} - \Delta A/min_{sample}}{\Delta A/min_{\ control}} \ \ldots\ldots\ldots\ldots \mathbf{3}$$

5.2.4.4. Determination of metal ion chelating activity

The metal ion chelating activity was measured using a modified method of Girgih *et al*. (2011). Five hundred microlitre (500 μl) sample solution or standard (final assay concentration of 1 mg/ml) was combined with 25 µl of 2 mM $FeCl_2$ and 925 µl double distilled water in a reaction tube. Fifty microlitre (50 μl) ferrozine solution (5 mM) was added and mixed thoroughly, allowed to stand at room temperature for 10 min after which an aliquot of 200 μl was pipetted into a clear 96-well microplate. A control was also conducted by replacing the sample with 500 µl of double distilled water. The absorbance values of control (A_c) and sample (A_s) were measured at 562 nm and the percentage chelating effect (%) calculated using the following equation:

$$Metal\ chelating\ activity\ (\%) = \frac{A_c - A_s}{A_c} \times 100 \quad \text{... ... ...} \mathbf{4}$$

5.2.4.5. Determination of ferric reducing antioxidant power (FRAP)

The ferric reducing antioxidant power of samples was measured according to the method described by Benzie and Strain (1996) with minor modifications for a microplate reader. Briefly, the FRAP reagent was freshly prepared by mixing 300 mM acetate buffer (sodium acetate buffer, pH 3.6), 10 mM 4,6-tripryridyls-triazine (TPTZ) in 40 mM HCl and 20 mM ferric chloride in the ratio 5:1:1 (v/v). Thereafter, 200 μl of FRAP reagent preheated to 37°C was added to 40 μl of sample aliquot in a 96 well microplate. Absorbance was measured at 593 nm against an assay blank. Ferrous sulphate (0.0625-1 mM) was used to prepare a standard curve and the results of the samples expressed as mmol Fe^{3+}/g of samples.

5.2.5. Statistical analysis

All experiments were performed in triplicate and means compared using analysis of variance (ANOVA) at 95% confidence level, Duncan and LSD tests were used to test the significant differences.

5.3. Results and discussion

5.3.1. Total phenolic content

The total phenolic contents of different soy-based foods generally increased with sprouting (Fig. 1), apart from sprouted fermented soymilk. Natural fermentation also brought about an increase in phenolic contents for both sprouted and unsprouted soymilk, however, unsprouted fermented soymilk samples had a higher phenolic content. The combined effect of sprouting and fermentation in increasing total phenolic contents of soy foods were observed in sprouted soymilk kefir (14.21 mg GAE/g) relative to its control (14.07 mg GAE/g). Legumes respond to stress induced during sprouting by producing phenolics, especially if germination was carried out in the dark (Randhir *et al*., 2004). The total phenolic contents estimated by Folin's reagent may also include contributions from other compounds such as reducing sugars, soluble proteins, ascorbic acids and other compounds (Prior *et al*., 2005, Morales-de La Peña *et al*., 2010). This also suggests why it is necessary to study the antioxidant potentials of all these compounds together in soy-based foods and not only phenolic extracts.

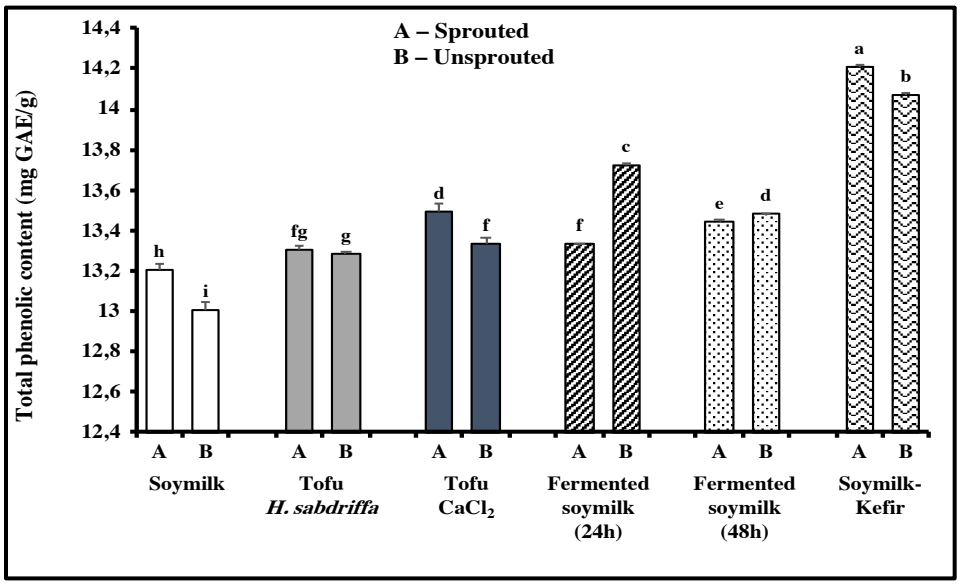

Figure 1: Total phenolic content (Gallic equivalent mg/g) of soy-based food samples. Data represents mean ± standard deviation (n=3). Means with different superscripts are significantly different ($p \leq 0.05$).

5.3.2. DPPH radical scavenging activity

The ability of antioxidant compounds is exhibited by the donation of electrons to DPPH free radicals, often accompanied by a change in colour of DPPH solution (Schaich *et al*., 2015). The DPPH assay had been widely used to measure the antioxidant activities of many natural compounds, including foods and their extracts (Arise *et al*., 2016). All soy-based foods produced from sprouted and unsprouted soy exhibited radical scavenging potentials against DPPH free radicals, except for naturally fermented soymilk samples which showed no reaction (Table 1). Food samples were only reactive to DPPH radicals at a concentration of 1 mg/ml. Generally, sprouted soy-based foods exhibited higher radical scavenging activities when compared with control samples, with sprouted tofu coagulated with *H. sabdariffa* extracts having the highest activity. Alvarez-Jubete *et al*. (2010) attributed the increase in DPPH radical scavenging activity of sprouted seeds to the metabolic changes occurring during seed sprouting due to the activities of hydrolytic enzymes, with other benefits such as increased nutrient digestion and decreased levels of phytase and protease inhibitors. The increased level of compounds other than polyphenols such as vitamins and bioactive peptides in sprouted soy-based foods may also contribute to the higher anti-radical activities observed. The absence of reaction in fermented foods may be due to the inability of their antioxidant moieties, including phenol, to access the hindered DPPH radical active site, thereby impeding the hydrogen atom transfer reaction (Schaich *et al*., 2015).

Table 1: Percentage DPPH radical scavenging activities of soy-based food samples

Samples	Soymilk	Tofu ($CaCl_2$)	Tofu (*H. sabdariffa*)	Fermented soymilk (24 h)	Fermented soymilk (48 h)	Soymilk-kefir
Control	14.87 ± 2.21^{c}	5.72 ± 0.96^{a}	8.02 ± 0.48^{a}	NR	NR	16.24 ± 1.19^{d}
Sprouted	11.29 ± 0.48^{b}	11.18 ± 0.36^{b}	30.27 ± 1.56^{f}	NR	NR	22.15 ± 1.45^{e}

[Data represents means ± standard deviation (n=3). Different letters in the same column are significantly different ($p \leq 0.05$) NR: No reaction]

5.3.3. Superoxide radical scavenging activity (SRSA)

Superoxides are free oxygen radicals which have been implicated in lipid oxidation by their precursory activities for singlet oxygen and hydroxyl radicals (Wang *et al*., 2006). In this study, soy-based foods from sprouted soybeans exhibited higher superoxide radical scavenging potentials as seen in Figure 2. Superoxide antiradical activities were not detected beyond two concentrations of sample extracts (0.5 and 1 mg/ml), with higher activities observed at 1 mg/ml. Higher scavenging potentials were observed in sprouted soymilk (21.73% at 1 mg/ml) and sprouted soymilk-kefir (24.34% at 1 mg/ml). Soybeans naturally contain superoxide dismutase and isoflavones which are potent superoxide scavenger (Marazza *et al*., 2012, Chien *et al*., 2006). This explains the superoxide radical scavenging activities observed in all soy food samples. The higher SRSA activities observed in sprouted foods may be as a result of the improvement of the activities of hydrolytic enzymes and isoflavones due to the stress-induced conditions of sprouting. Fernandez-Orozco *et al*. (2008) reported an increase in SRSA activity of *Vigna radiata* and *Glycine max cv. jutro* sprouted for up to 4 days. The scavenging activity observed in sprouted soymilk kefir may be attributed to the combined effects of sprouting (Fernandez-Orozco *et al*., 2008) and fermentation (Marazza *et al*., 2012).

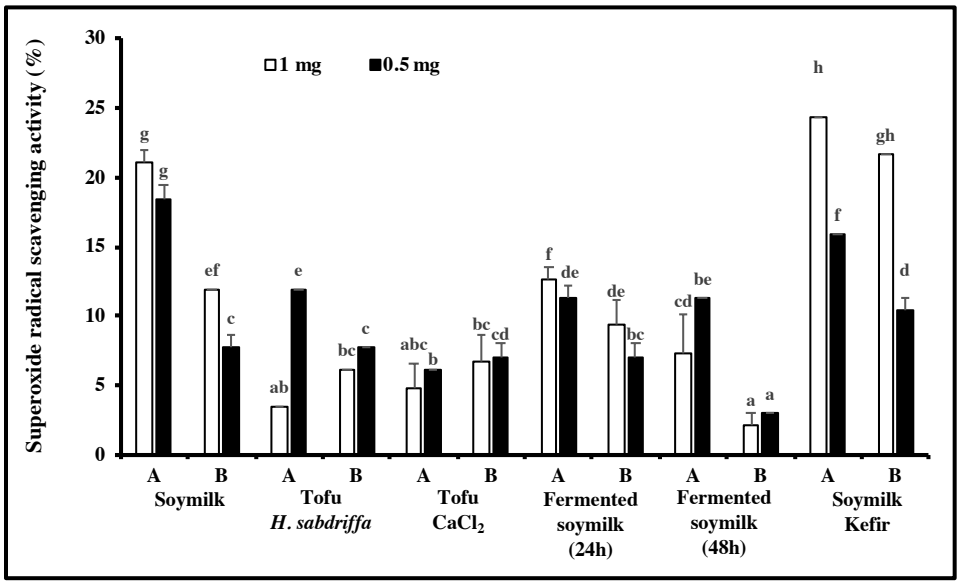

Figure 2: Percentage superoxide radical scavenging activity of soy-based food samples [A – sprouted; B – unsprouted]. Data represents means ± standard deviation (n=3). Different letters in the same concentration group are significantly different (p ≤ 0.05).

5.3.4. Hydroxyl radical scavenging activity

Generally, sprouted soy foods had higher hydroxyl radical scavenging activities as compared to unsprouted, with the highest activity observed in sprouted soymilk kefir at the 0.5 mg/ml (Fig. 3). Oxygen radicals are highly reactive and react with other substances in the body to cause cell damage in humans (Zhu *et al*., 2005). Hydroxyl radicals are formed from the conversion of hydrogen peroxide or superoxide anions in the presence of metal ions and are among the most reactive species of oxygen radicals which function by damaging all forms of macromolecules, including, nucleic acids and proteins (Girgih *et al*., 2011, Arise *et al*., 2016). Above two-fold hydroxyl radical scavenging activity was observed for all sprouted soy foods in both concentrations (0.5 and 1 mg/ml) as compared to unsprouted. This behaviour could be linked to the influence of sprouting at the optimized conditions previously suggested (Chapter 3). High hydroxyl radical activity has been reported at reduced germination times in seeds. Vale *et al*. (2014) found enhanced hydroxyl radical scavenging power in *Brassica oleracea*, with reductions at extended times. Therefore, observed activity in sprouted foods may be due to the optimized germination time (52 h) for sprouted soy foods in this study.

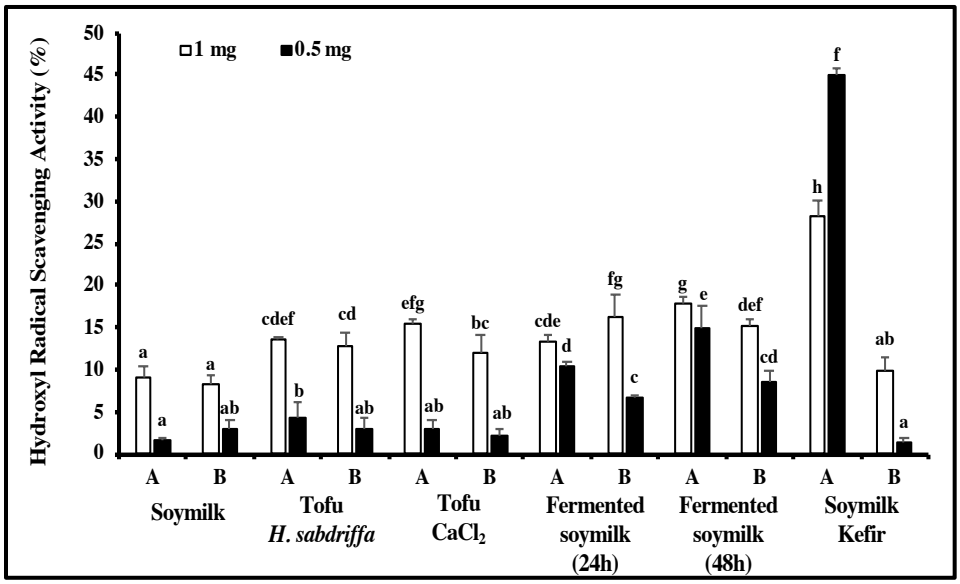

Figure 3: Percentage hydroxyl radical scavenging activity of soy-based food samples [A – sprouted; B – unsprouted]. Data represents means ± standard deviation (n=3). Different letters in the same concentration group are significantly different ($p \leq 0.05$).

5.3.5. Metal ion chelation

Chelation of metal ions involves the formation of ligands with metal ions, making them unavailable for reaction with hydrogen peroxides and superoxide ions by Fenton and Haber-Weiss reactions to form highly reactive hydroxyl radicals (Pownall *et al*., 2010). Therefore, natural foods that could successfully chelate metal ions can serve as secondary antioxidants since they would have prevented the formation of hydroxyl radicals. The mechanism here is more of prevention of the availability of metal ions than scavenging the free oxidative radicals. All the five concentrations of samples (1-5 mg/ml) tested had metal ion chelating activities (Fig. 4) with higher activities in sprouted soy foods, especially sprouted soymilk. Also, the greatest reactivity was obtained at 3 mg/ml in all cases for sprouted soy foods Amino acids and peptides are potent metal chelators (Pownall *et al*., 2010, Megías *et al*., 2008). The higher metal chelation activity observed for sprouted foods may be attributed to the breakdown of complex protein molecules into simpler amino acids and peptides during germination, making them more available for chelation, as compared with the unsprouted ones.

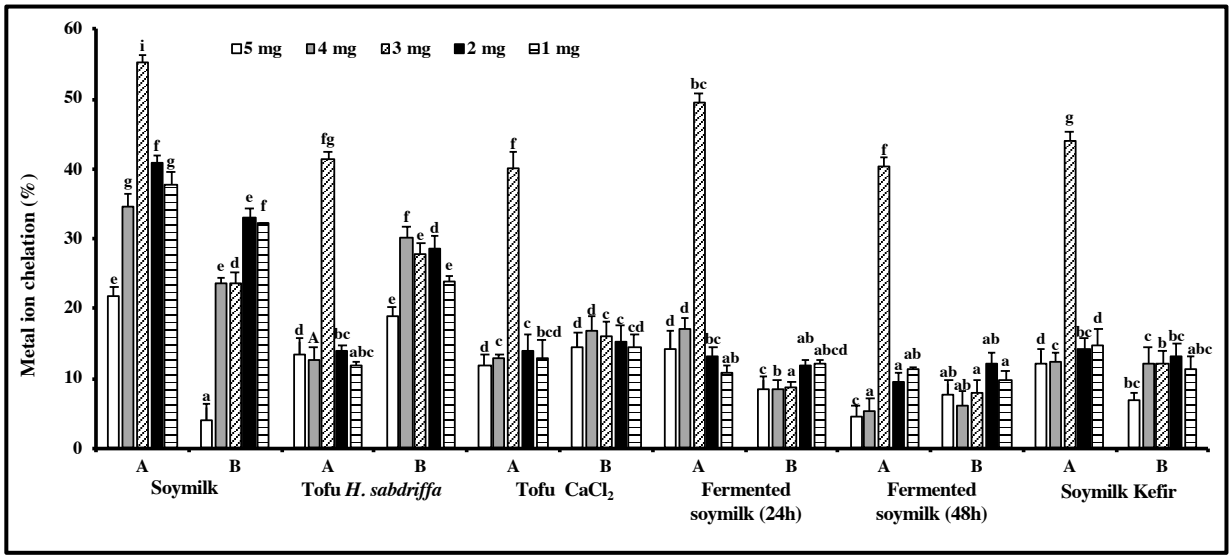

Figure 4: Percentage metal ion chelation activity of soybased food samples [A – sprouted; B – unsprouted]. Data represents means ± standard deviation (n=3). Different letters in the same concentration group are significantly different ($p \leq 0.05$).

5.3.6. Ferric reducing antioxidant power (FRAP)

Iron is the most abundant transition element in the body, with the capacity of catalyzing the generation of reactive oxygen species (Liu *et al*., 2005). Ferric reducing power of antioxidants of food samples or extracts refers to their ability to convert ferric ions from ferricyanide complex to more stable ferrous ions (Girgih *et al*., 2013), and could be used as a measure of antioxidant potentials of such foods. Samples were active against FRAP assays up to a concentration of 2 mg/ml (Fig. 5) with the greatest reactivity observed at this concentration. The reducing power of soymilk samples (sprouted and unsprouted) were highest for all samples at 0.95 and 0.93 Fe^{3+}mmol/g. There were generally no observable significant differences in the reactivity of sprouted and unsprouted samples to ferric ions. The only exception was in sprouted soymilk-kefir, which had significantly higher ferric reducing power (0.80 Fe^{3+}mmol/g at 2 mg/ml) as compared with the unsprouted sample (0.62 Fe^{3+}mmol/g at 2 mg/ml). Ferric reducing antioxidant activity of natural foods has been attributed to their intracellular antioxidants, peptides and hydrogen donating abilities (Wang *et al*., 2006). Again, reductones are produced from the activities of fermenting organisms in soy-based foods, and they have the ability of breaking free-radical chain reactions (Yang *et al*., 2000). With metal chelating potentials and FRAP capacities observed in sprouted soy-based foods in this study, they have the potential of preventing the availability of ferrous radicals, thereby preventing the formation of reactive oxygen moieties.

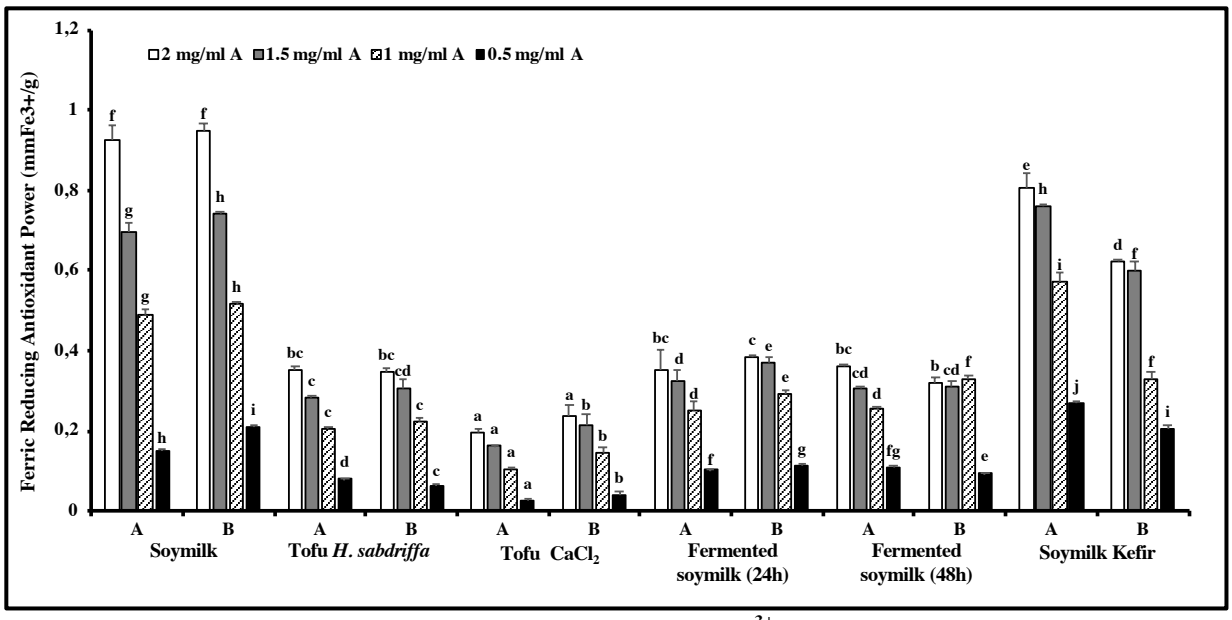

Figure 5: Ferric Reducing Antioxidant Power (mmol Fe^{3+}/g) of soy-based food samples [A – sprouted; B – unsprouted]. Data represents means ± standard deviation (n=3). Different letters in the same concentration group are significantly different ($p \leq 0.05$).

5.4. Conclusion

Sprouting of soybeans resulted in the improvement of antioxidant activities of soy-based foods. The antioxidant capacities of sprouted soy-based foods could be further enhanced through natural and kefir fermentation. High metal ion chelating properties of sprouted soy-based foods suggests that their consumption could be a preventive measure against the production of free oxidative radicals in the body. Different concentrations of samples in appropriate buffers have varying effects on antioxidant assays and this should be taken into consideration while determining their antioxidant potentials.

Chapter 6: Potentials for enhanced soy storage protein breakdown and allergen reduction in soy-based foods produced with optimized sprouted soybeans

Abstract

In this study, potentials for improved soy storage protein breakdown and allergen reduction in soy-based foods produced with soybeans, sprouted at optimized conditions were investigated using proteomic approach. The changes in protein concentration and expression levels of six sprouted soy-based foods, namely SSM: sprouted soymilk, STC: tofu from sprouted soybeans coagulated by $CaCl_2$, STH: tofu from sprouted soybeans coagulated with *Hibiscus sabdariffa* flower extracts, SFS24: sprouted fermented soymilk (24 h), SFS48: sprouted fermented soymilk (48 h) and SSK: sprouted soymilk-kefir and their respective control samples (USM, UTC, UTH, UFS24, UFS48 and USK) were analyzed using 2-dimensional gel electrophoresis and PDQuest image analyses. Protein concentration significantly increased in sprouted products (up to 149% in SSK). Eight differentially expressed protein spots were selected and identified by MALDI- TOF/TOF mass spectrometry as glycinin subunit G2, β-conglycinin (α, α' and β subunits), trypsin inhibitor, 34 kDa soy seed protein and sucrose-binding proteins. Higher extent of breakdown of soy storage protein was obtained in sprouted soy products, as shown by higher protein concentration and spot volumes. Lower spot volumes were obtained in all fermented products. This study suggests higher potentials for soy storage protein breakdown and reduced allergen contents in sprouted soy-based foods.

Keywords: Sprouted soy-based foods, glycinin, β-conglycinin, optimized conditions, allergen, storage protein.

6.1. Introduction

Soybeans is regarded a major crop in human nutrition as it serves as a good source of high quality protein, in addition to other beneficial nutritional contents (Wang *et al.*, 2014). Due to the high protein content and relatively well-balanced amino acids, soybean is extensively used by food and feed processing industries as the primary source of protein in food matrices (Seo and Cho, 2016).

Consumption of soybeans is traditional to Asian countries; however, the high nutritive values and beneficial effects of soybeans has popularized its consumptions in other parts of the world (Krishnan *et al.*, 2000). The major threat to soybean consumption has been the presence of antinutritional factors and allergic reactions by consumers and these can be adequately removed or sufficiently minimized by proper processing (Cervantes-Pahm and Stein, 2010). The major proteins of soy products are glycinin and β-conglycinin, accounting for over 50-90% of its storage proteins, depending on the concentration of proteins after processing, from soy flours (50%) to soy protein isolates (90%) (Plumb *et al.*, 1994, Lallès *et al.*, 1999).

Glycinin, also referred to as 11S globulin, is a hexameric pure protein formed by polypeptide subunits covalently bonded (Wilson *et al.*, 2005). Glycinin is a high molecular weight protein (approximately 360kDa) and the molecular weights of each of its six subunits theoretically being approximately 60kDa. The subunits contain acidic and basic polypeptides, jointed by disulphide bonds. Also, β-conglycinin (7S globulin) is a soybean storage protein having a molecular weight of approximately 220kDa, with three trimers namely α, α' and β subunits of β-conglycinin. (Lallès *et al.*, 1999, Wilson *et al.*, 2005). These proteins, in their native state, are allergenic (Montowska and Fornal, 2018) due to their very high molecular weights. Soy proteins are precursors of bioactive peptides (Yu *et al.*, 2008) and so, it is necessary to break the complex forms of soybean storage proteins down to simpler forms during processing, since proteins are absorbed in the body primarily as dipeptides and tripeptides (Ma *et al.*, 2011, Vernaza *et al.*, 2012). Commercial enzymes have been used in the hydrolysis of soy storage proteins to produce bioactive peptides, but this may not be employable on a cottage scale due to the high cost of enzymes, and on a large scale because of the cost of production, especially in developing countries (Yu *et al.*, 2008).

Fermentation of soy foods has been extensively used in the improvement of the contents of its bioactive substances and reduction of antinutrients (Cho *et al.*, 2011, Egounlety and Aworh, 2003). Complex organic compounds are broken down by the activities of fermentative microorganisms, imparting various functional characteristics to foods, beyond their primary nutritional functions. (Sanjukta and Rai, 2016).

Previous studies have suggested the reduction of soy allergenic and antinutritional components, especially proteins, by the actions of proteolytic enzymes produced by microorganisms during soy-food fermentation (Chi and Cho, 2016, Frias *et al.*, 2007, Aguirre *et al.*, 2014). Hence, fermentation of soy-based foods is helpful in breaking down complex proteins into simpler forms, towards the production of bioactive peptides which are inactive within the sequence of their parent proteins (Sanjukta and Rai, 2016, Sanjukta *et al.*, 2015).

Like fermentation, food processing operations, such as sprouting, have been used as natural, inexpensive methods of reducing the complex components of soy storage proteins into simpler forms. Recent findings have suggested the presence of higher contents of functional substances, with various benefits for human consumption, in sprouted soybeans, as compared to unsprouted ones (Huang *et al.*, 2014). Sprouting is capable of triggering biochemical processes in germinating seeds, leading to changes in the characteristics of storage proteins, including the increase in nutritional value and improved digestibility, due to breakdown of complex forms, bringing about enhanced absorptivity and release of bioactive protein constituents (Bau *et al.*, 2000, Paucar-Menacho *et al.*, 2010a, Vernaza *et al.*, 2012). Also, optimization factors of sprouting, including germination temperature and time, seed variety and other factors, has been explored towards improving the nutritional and biochemical characteristics of sprouted seeds, especially legumes. Optimization of sprouting parameters of soybeans, using either traditional methods or response surface methodology, has also been reported (Jiang *et al.*, 2013, Huang *et al.*, 2014, Guo *et al.*, 2011).

Although previous studies have reported the potentials of fermentation (Kuba *et al.*, 2003, Seo and Cho, 2016, Yu *et al.*, 2008) and germination (Vernaza *et al.*, 2012) in the improvement of the storage protein quality and reduction of allergenic characteristics of soy-based foods, there has been limited information on the allergenic properties of fermented soy-based foods produced with sprouted soybeans, especially after optimization of sprouting parameters. In this study the effect of optimization of soybean sprouting conditions (soaking time and germination time) on protein composition and bioactivity in fermented and unfermented soy-based foods were investigated to evaluate their potentials for reduced allergenic tendencies.

6.2. Materials and methods

6.2.1 Sample preparation

Soybean seeds (variety: DM 5.1i RR) were sprouted at optimized conditions for 12 h soaking and 52 h germination, in line with Chapter 3. Thereafter, experimental methods previously described were used to produce soy-based foods namely soymilk (Murugkar, 2014), tofu (*soy wara*) (Rekha and Vijayalakshmi, 2010b), spontaneously fermented soymilk (*soy nono*) (modified method of Obadina *et al.* (2013)) and soymilk-kefir (Liu *et al.*, 2005) with the optimized sprouted soybeans. For this study, products were named SSM: sprouted soymilk, STC: tofu from sprouted soybeans coagulated by $CaCl_2$, STH: tofu from sprouted soybeans coagulated with *Hibiscus sabdariffa* flower extracts, SFS24: sprouted fermented soymilk (24 h), SFS48: sprouted fermented soymilk (48 h) and SSK: sprouted soymilk-kefir. USM, UTC, UTH, UFS24, UFS48 and USK were the respective control samples produced with un-sprouted soybeans. All samples were freeze-dried at 1 atm using an Alpha 2-4 LD plus freeze-drier (Christ, Germany) after they have been frozen at -80°C for at least 24 h.

6.2.2 Soluble protein extraction

The method of Seo and Cho (2016) was used with slight modifications. Protein extraction was performed by suspending 100 mg of each freeze-dried sample in 1.5 ml of extraction buffer (7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 20 mM DTT and 40 mM Tris) followed by sonication and DNase treatment for 30 min at 4 °C. Thereafter, samples were centrifuged at 13,000 rpm for 30 min and the supernatant mixed with 10% (w/v) trichloroacetic acid/acetone. The samples were incubated at -20 °C overnight to allow protein precipitation, followed by centrifugation at 13,000 rpm for 30 min. Protein pellets obtained were washed 3 times with cold acetone, dried and reconstituted in sample buffer (7 M urea, 2 M thiourea, 4% CHAPS, 20 mM DTT). Bradford assay (Bradford, 1976) was used to determine the protein concentration at 595 nm while one hundred microgram of protein from each sample was used for two-dimensional gel electrophoresis analyses.

6.2.3 2D Electrophoresis (2DE) and spot analysis

Proteins (100 µg) from each sample were mixed with IPG rehydration buffer (7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 20 mM DTT, 0.4% ampholyte) and applied onto pH 3-10 immobilized non-linear gradient strips (BioRad). The strips were rehydrated overnight followed by isoelectric focusing (IEF). IEF was performed at 12,000 Vh on a Biorad Protein IEF cell and the strips equilibrated. For the second dimension, strips were placed on 12% polyacrylamide resolving gels and run at a constant voltage of 80 V for 2.5 h as described previously (Park *et al.*, 2002, Cho *et al.*, 2005). Gels were stained with Coomassie Brilliant Blue G-250 until the spots were visible and scanned using a Bio-Rad Pharos FX plus Molecular imager. Further PDQuest analysis was done on spots using the PDQuest 2-D analysis software (Bio-Rad).

6.2.4 Identification of proteins

For the identification, gel spots of interest were excised and de-stained with 200 µl 100 mM ammonium bicarbonate in 50% (v/v) acetonitrile until clear. Gel pieces were then dehydrated and desiccated with 100 µl acetonitrile (ACN) followed by reduction and alkylation with 2 mM tris (2-carboxyethyl) phosphine (TCEP) and 20 mM iodoacetamide (IAA), respectively. Thereafter, the gel pieces were washed with 25 mM ammonium bicarbonate, treated with 0.4 µg of trypsin (Promega) and incubated on ice for 1 h. Following incubation, excess trypsin solution was discarded and 15 µl of 50 mM ammonium bicarbonate added to the gel pieces. The samples were then allowed to incubate at 37 °C for 16 h. Following complete tryptic digestion, the peptides were extracted from gel networks by adding 15 µl extraction solution (0.1 % TFA in 30 % acetonitrile) and agitation for 45 min at room temperature (Seo and Cho, 2016). The supernatant was transferred to a clean Eppendorf tube and stored until further analysis.

Matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry (MS) and LIFT MS/MS was performed using a UltrafleXtreme MALDI-TOF/TOF system (Bruker Daltonics, Bremen, Germany) with instrument control through Flex control 3.4. The digested samples were spot in a 1:1 ratio with 1.4 mg/ml CHCA matrix [85% acetonitrile, 0.1% TFA, 100 mM $NH_4H_2PO_4$] onto a 800 µm MALDI Anchor chip target plate for peptide mass fingerprinting.

Peptides were ionized with a 337 nm laser and spectra acquired in reflector positive mode at 28 kV using 500 laser shots per spectrum with a scan range of m/z =700 Da - 4000 Da. Spectra were internally calibrated using peptide calibration standard II (Bruker Daltonics, Bremen, Germany). This calibration method provided a mass accuracy of 50 ppm across the mass range 700 Da to 4000 Da. Peptide spectra of accumulated 3,000 shots were automatically processed using ProteinScape software (Bruker Daltonics, Bremen, Germany).

6.2.5 Data analysis

Database interrogation was performed with the Mascot algorithm using the Swissprot and *Glycine max* database. The search parameters used were: taxonomy-Viridiplantae, enzyme-trypsin; missed cleavages:1; fixed modification: carbamidomethyl (C); variable modification: oxidation (M); precursor tolerance: 50 ppm; fragment tolerance: 0.7 Da. Candidate protein matches with individual ion scores greater than 31 and 29 were considered as identified proteins when performing the '*Glycine max*' and Viridiplantae search respectively.

6.3. Results and discussions

6.3.1 Soluble protein concentration of samples

The concentration of soluble proteins extracted from each of the samples, using Bradford assay, are as presented (Table 1). Protein concentration ranged from 4.954 to 12.351 μg/μl for all samples. Sprouted soy-based food samples had higher protein concentrations in all cases, apart from fermented soymilk (48 h) where the protein content was higher in the control sample (9.498 μg/μl). The least percentage increase in protein concentration (9%) in sprouted products, compared to their controls, was observed in tofu products coagulated with *Hibiscus sabdariffa* leaf extracts, while there was up to 149% increase reported for sprouted soymilk kefir. The increase in protein concentration of sprouted products could be as a result of possible synthesis of new protein moieties and the loss of other soybean seed components during sprouting.

Vernaza *et al*. (2012) reported an increase in the soluble protein concentration (52.9-66.8%) and attributed it to the breakdown of higher molecular weight proteins in Brazilian soybean cultivar 133 from 0-72 h germination, while Zhang *et al*. (2015) linked the increase in protein contents of sprouted buckwheat from 144.68 mg/g (0 h) to 155.16 mg/g (72 h) to synthesis of proteins due to biochemical reactions occurring during sprouting. The reduction in protein content observed in sprouted fermented soymilk (48 h) may explain the joint effects of breakdown during sprouting and extended fermentation on storage and synthesized proteins (Zhang *et al*., 2015, Seo and Cho, 2016). The major constituents of proteins in UFS 48 may be of higher molecular weight peptides, while SFS 48 could contain more of peptides with lower molecular weight (Fig.1). The changes observed in protein concentration sprouted samples and their respective controls could therefore be majorly linked to the effects sprouting at the optimized conditions (Chapter 3) for unfermented products, and joint effects of optimized sprouting and activities of fermentative microorganisms in fermented soy-based products.

Table 1: Soluble protein concentration of soy-based food samples

Soy-based foods	Soluble protein concentration ($\mu g/\mu l$)
USM	7.72^{bcd}
SSM	9.10^{bc}
UTH	9.23^{abc}
STH	10.47^{ab}
UTC	9.16^{bc}
STC	10.02^{ab}
UFS24	9.11^{bc}
SFS24	10.06^{ab}
UFS48	9.49^{ab}
SFS48	6.15^{cd}
USK	4.95^{d}
SSK	12.35^{a}

Different letters in the same column indicate significantly difference ($p \leq 0.05$). SSM: sprouted soymilk, STC: tofu from sprouted soybeans coagulated by $CaCl_2$, STH: tofu from sprouted soybeans coagulated with *Hibiscus sabdariffa* flower extracts, SFS24: sprouted fermented soymilk (24h), SFS48: sprouted fermented soymilk (48h) and SSK: sprouted soymilk-kefir. USM, UTC, UTH, UFS24, UFS48 and USK were the respective control samples produced with un-sprouted soybeans

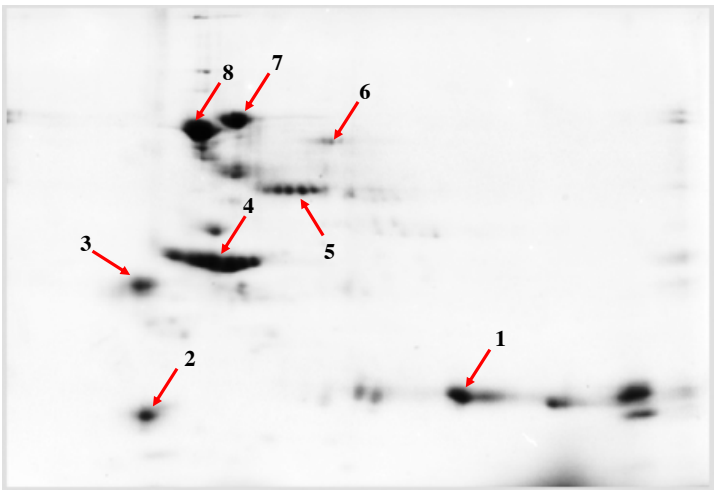

Figure 1: 2-dimensional electrophoresis gel showing the positions of selected protein spots and numbers assigned to them.

6.3.2 Effect of sprouting and fermentation on proteomic profiles of soy-based foods

To evaluate the effect of sprouting and sprouting with fermentation on the proteomic profile and allergen-reduction potentials in soy-based food samples, proteomics approach using 2-D electrophoresis and mass spectrometry was employed. This approach involves the separation of proteins based on their molecular weights and isoelectric points, identification of protein constituents of these foods and determination of spot volumes of separated proteins. The use of thiourea/urea and tricholoroacetic acid (TCA)/acetone solvents in the extraction of pure protein pellets from samples have been reported to be reliable approaches, especially for efficient separation in the 2D gels and identification by mass spectrometry (Natarajan *et al*., 2005). Seo and Cho (2016) also reported this method as most useful for desalting and terminating proteolysis, with clear protein resolutions in all the separated spots of fermented soybean meal samples.

Protein spots formed on 2D gels for all samples were localized within the relative molecular weights of 11-80 kDa (Fig. 2). Over 26 major protein spots were observed on the gels of each sample. To evaluate the allergen reduction potentials of optimized sprouting conditions, as well as fermentation processes used in this study, 8 spots corresponding to soybean's major proteins, with different expression levels for sprouted and unsprouted samples (Fig. 1), were selected for volume determination and identification, based on the soybean protein maps presented by Pedersen *et al*. (2008) and Seo and Cho (2016).

It was observed that the intensities of these spots generally increased in fermented and unfermented sprouted food samples compared to their respective controls. The increase in spot intensities was further depicted by the higher volumes of these spots in sprouted samples, compared to the unsprouted control samples (Table 2). Further processing of peptides obtained after tryptic digestion and MALDI-TOF mass spectrometry using ProteinScape software revealed information such as the ascension identity, protein name, sequence coverage, theoretical molecular weights and isoelectric points, score of each spot as well as the databases from where this information was obtained (Table 3).

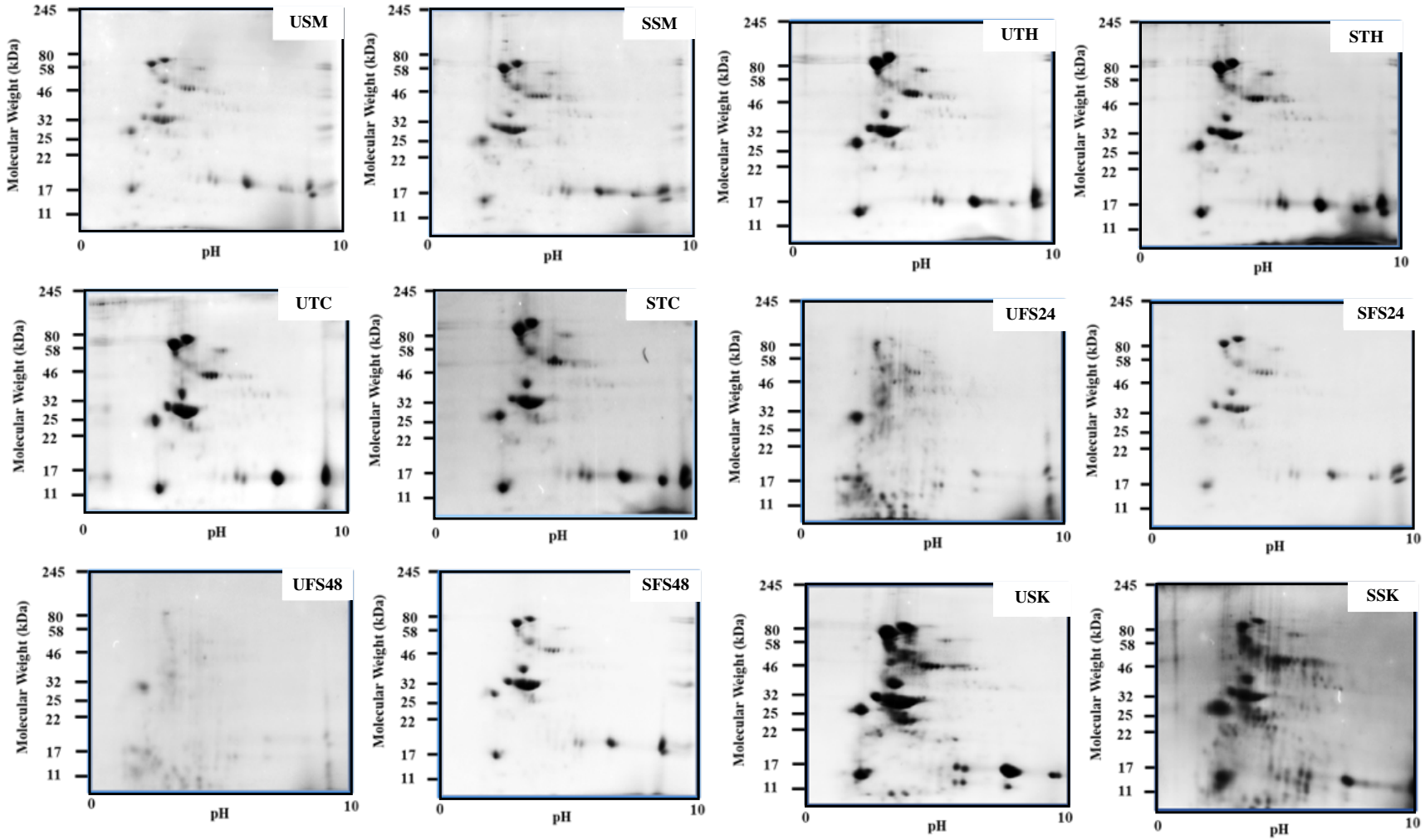

Figure 1: Two-dimensional (2D) gel images of sprouted soy-based foods and their respective controls [SSM: sprouted soymilk, STC: tofu from sprouted soybeans coagulated by $CaCl_2$, STH: tofu from sprouted soybeans coagulated with *Hibiscus sabdariffa* flower extracts, SFS24: sprouted fermented soymilk (24h), SFS48: sprouted fermented soymilk (48h) and SSK: sprouted soymilk-kefir. USM, UTC, UTH, UFS24, UFS48 and USK were the respective control samples produced with un-sprouted soybeans].

Table 2: Comparison of protein spot intensity using PDQuest image analyzer

	Relative Volume											
Spot	**USM**	**SSM**	**UTH**	**STH**	**UTC**	**STC**	**UFS24**	**SFS24**	**UFS48**	**SFS48**	**USK**	**SSK**
1	225349.1	122695.8	74011.1	62063	370574.2	613001	37204.8	141886.9	2635.3	79688.3	33.9	6469.9
2	968558.6	1589589	79986.4	66451.9	24437.5	133126.9	87902	53348	4449.1	41829.5	1357.8	1540.3
3	218224.5	123504.5	36844.9	67665.6	42027.8	136942.4	103889.4	6653	21977.2	111096.2	1236.9	1304.6
4	311043.6	517668.9	81215.5	92788.9	277493.6	144474.9	794.8	254278	5154.2	21951.7	39785.8	8341.2
5	155331.5	140984	62484	71946.1	126719.9	84027.9	30913.9	77272.6	2954	66114.6	16401.2	6232.9
6	177229	184244.2	48435.6	54732.5	3880.4	45829.8	5797.1	27105.3	1166.9	42191.5	9089.1	5409.8
7	845567.4	1235963	99857.5	66451.9	27603.1	82079.6	25604.2	587759.5	2468.3	1881.45	22338.9	10713.7
8	892312.3	1963260	14755.8	56370.8	10279.3	15717.9	48541.2	251133.6	23363.8	114518.6	21501.4	7842.5

(1): Glycinin G2 (2): trypsin inhibitor (3): 3kDa seed protein (4) Glycinin G2 (5): β subunit of β-conglycinin (6) sucrose-binding protein precursor (7): α' subunit of β-conglycinin (8) α subunit of β-conglycinin [SSM: sprouted soymilk, STC: tofu from sprouted soybeans coagulated by $CaCl_2$, STH: tofu from sprouted soybeans coagulated with *Hibiscus sabdariffa* flower extracts, SFS24: sprouted fermented soymilk (24h), SFS48: sprouted fermented soymilk (48h) and SSK: sprouted soymilk-kefir. USM, UTC, UTH, UFS24, UFS48 and USK were the respective control samples produced with un-sprouted soybeans].

Table 3: Identity of protein spots on 2D gels by MALDI LIFT TOF/TOF

			Theoretical				
Spot	**Ascension**	**Protein name**	**Score**	**Sequence coverage**	**Molecular Weight (kDa)**	**pi**	**Database**
1	GLYG2_SOYBN	Glycinin G2	331.45	14.6	54.4	5.46	SwissProt
2	ITRA_SOYBN	Trypsin inhibitor	221.51	23.1	24	4.95	SwissProt
3	tr|O64458|O64458_SOYBN	34 kDa maturing seed protein	54.33	4.2	42.7	5.63	Glycine max
4	GLYG2_SOYBN	Glycinin G2	347.31	9.7	54.4	5.46	SwissProt
5	GLCB_SOYBN	Beta-conglycinin, beta chain	255.76	16.4	50.5	5.88	SwissProt
6	548900	Sucrose-binding protein precursor	113.00	31	60.8	6.42	SoyproDB
7	GLCAP_SOYBN	Beta-conglycinin, alpha' chain	417.56	20	74.3	5.47	SwissProt
8	tr|Q94LX2|Q94LX2_SOYBN	Beta-conglycinin alpha subunit	511.84	26.9	70.13	5.12	Glycine max

(1): Glycinin G2 (2): trypsin inhibitor (3): 3kDa seed protein (4) Glycinin G2 (5): β subunit of β-conglycinin (6) sucrose-binding protein precursor (7): α' subunit of β-conglycinin (8) α subunit of β-conglycinin

Soy-based foods investigated in this study are either unfermented (SSM, STC, STC and their respective controls) and fermented (SFS 24, SFS 48, SSK and their respective controls). Sprouting of soybean seeds at optimized conditions of soaking (12 h) and germination (52 h) was the major experimental difference between sprouted and unsprouted soy-based products in this study. The higher protein concentration of the identical quantity of pure protein in sprouted samples (Table 1), resulting in the higher volumes of identical protein spots in the separate 2D gels of sprouted, relative to the unsprouted ones (Table 2 and Fig. 2) could be directly linked to sprouting. The major storage proteins of soybeans are glycinin and β-conglycinin, and they determine the nutritional, functional and physiological properties of soybeans (Maruyama *et al.*, 2001). Complex biochemical reactions involved in seed sprouting may bring about breakdown of higher molecular weight proteins into simpler forms, buildup of lower molecular weight proteins and synthesis of entirely new proteins (Zhang *et al.*, 2015, Yang *et al.*, 2007). Although, different responses were observed in the behaviour of identified spots to sprouting at the optimized condition, breakdown of soy storage proteins into their simpler subunits was largely noticed in all samples.

Sprouting of seeds brings about structural changes in storage proteins and activates proteinases maintained in inactive forms around seeds storage proteins, to facilitate storage protein mobilization for enhanced enzymatic activities. The identities of the selected protein spots are glycinin subunit G2 (spot 1 and 4), α, α' and β subunits of β-conglycinin (spots 5, 7 and 8), trypsin inhibitor (spot 2) and 34 kDa maturing seed protein (spot 3) and their properties are as stated in Table 3. The identity of spot 6 was not revealed through the SwissProt and Glycine max databases, but was determined using the protein map available through the SoyProDB bioinformatics database (Tavakolan *et al.*, 2013). The increase in volume of these spots in all sprouted seeds, as revealed by PDQuest analysis, could be attributed to higher rate of breakdown of high molecular weight glycinin and β-conglycinin into their subunits, in sprouted seeds, leading to their increased concentration per unit quantity of soluble proteins, as compared with unsprouted seeds. This is as depicted in the 2D gels and PDQuest analysis of sprouted soymilk and tofu samples, and their respective controls (Fig. 2 and Table 2).

Interestingly, fermentation drastically reduced the volumes of protein spots in all fermented samples. Highest reduction was observed in soymilk kefir samples (Table 2). Studies on soluble protein concentration of fermented foods had reported the reduction in spot volumes (Phromraksa *et al.*, 2008, Seo and Cho, 2016). Highest spot volumes were observed in sprouted unfermented products, such as sprouted soymilk (spot 8: 1963260.4) and sprouted tofu coagulated with $CaCl_2$ (spot 1: 613001). As the days of fermentation increased, there was a progressive decrease in the volume of protein spots. The highest decrease was observed in control soymilk-kefir samples. Proteolytic enzymes produced by the microbial community of fermenting foods hydrolyze proteins into peptides and free amino acids (Sanjukta and Rai, 2016). It follows that microbial and enzymatic activities during the fermentation of soy-based foods led to the breakdown of proteins into very low molecular weight peptides (<11 kDa), bringing about the reduction in spot volumes. Also, extent of breakdown of subunits of glycinin, β-conglycinin and other identified proteins by fermentation, as well as the functionality of hydrolyzed products depend on the cultures involved in the fermentation process (Ibe *et al.*, 2006, Sanjukta and Rai, 2016, Rai *et al.*, 2011). This may explain the difference in the extent of fermentation in naturally fermented products compared to those of soymilk-kefir samples, shown by a higher volume reduction in soymilk-kefir fermentation. The higher volumes for protein spots in sprouted samples could be attributed to prior sprouting of seeds, as earlier discussed.

Higher concentration and volume of subunits of glycinin and β-conglycinin per unit quantity of soluble proteins in sprouted soy-foods in this study could be viewed as potentially advantageous in the reduction of their allergenic tendencies. Glycinin (MW approx. 360 kDa) and β-conglycinin (MW approx. 220 kDa) have been reported as potent allergens (Krishnan *et al.*, 2009, Pedersen *et al.*, 2008). Therefore, enhanced and extensive breakdown of these high molecular weight storage proteins into their subunits reduces the allergenic tendencies of their native forms. Also, lower molecular weight proteins (subunits of glycinin and β-conglycinin) are more accessible to digestive enzymes such as pepsin and pancreatin *in-vivo*, leading to a breakdown of these subunits even further into peptides as low as 7 kDa (Lallès *et al.*, 1999, Kella *et al.*, 1986). Hence, sprouting of soybean seeds at the optimized conditions suggested, prior to their use in the production of soy-based foods, would enhance the availability of soy-based proteins in the form of reduced polypeptides, that would be better digestible.

The reduction of quantities of these polypeptides in fermented soy-based products (sprouted and controls) could therefore be viewed as further enhancement of their reduced allergenic tendencies, as further degradation of glycinin and β-conglycinin subunits could be achieved *in vitro*, through microbial activity-induced enzymatic and biochemical processes.

6.4. Conclusion

Sprouting of soybean seeds at optimized conditions of soaking and germination suggested in this study has the capacity for reduction of the large molecular weight of its storage proteins into their subunits, through the activities of enzymes inherent in seeds and activated during germination. The enhanced reduction of soy storage proteins to their lower molecular weight subunits, facilitated by sprouting, suggests increased chances of peptide bioavailability and possible increase in the quantity of bioactive peptides that would be available after *in-vivo* pepsin and pancreatic digestion. Drastic reduction in quantity of soybean storage protein subunits by fermentation implies further breakdown of storage protein subunits, with possible implications of improved bioavailability of lower molecular weight peptides (below 11 kDa). Sprouting of soybean seeds at the suggested optimized conditions, as well as natural and kefir fermentation of soy-based foods produced with these sprouted seeds, have potential for increased bioactive peptides availability and reduced allergenic effects.

Chapter 7: Microbial community of spontaneously fermented and kefir-fermented soymilk as revealed by rep-PCR and high throughput amplicon sequencing

Abstract

In this study, the microbial community of spontaneously fermented soymilk and soymilk-kefir was investigated using two distinct approaches. Soymilk produced with soybean seeds sprouted at optimized conditions of soaking (12 h) and germination (52 h) were subjected to natural fermentation (for 48 h) and kefir fermentation (for 24 h), with samples drawn at 6 h intervals. Fermented soymilk from unsprouted beans served as the control. For the first approach, culture independent rep-PCR, coupled with sequencing of 16S rRNA and D1/D2 regions of 26S rRNA was used to characterize the lactic acid bacteria and yeast community of samples from their pure cultures obtained from appropriate growth media. Cluster analysis of DNA fingerprints of isolates was performed using Bionumerics 7.0. The second approach involved the extraction of metagenomic DNA from samples and determination of their bacterial community using tag-encoded MiSeq-based high throughput amplicon sequencing (HTS) of their V3 regions and analysis using QIIME. There were significant changes ($P \leq 0.05$) in LAB and yeast counts as fermentation time increased. From rep-PCR, identified LAB species include *Weissela cibaria, Lactococcus lactis, Leuconostoc lactis, Leuconostoc messenteroides,* and *Lecleria adecarboxylata* while yeast species are *Saccharomyces cerevisiae, Pichia fermentans* and *Torulospora delbrueckii*. In addition to the genera revealed in rep-PCR, HTS showed the presence of *Bacillaceae* and other bacteria involved in spontaneous and kefir fermentation of soymilk. Alpha diversity and weighted unifrac PCoA biplots also showed the diversity and interrelationship of microrganisms as fermentation progressed. The microbial communities of spontaneous and kefir fermentation of sprouted and unsprouted soymilk samples were adequately compared through these approaches and HTS revealed more detailed information. LAB and yeast isolated could find further applications as starter cultures.

Keywords: Soymilk, Fermentation, rep-PCR, High throughput sequencing, Lactic acid bacteria, Yeast.

7.1. Introduction

Fermentation, as a processing operation in the production of soy-based foods, has been traditionally employed as a means of reducing or removing the limitations of soybeans consumption, including beany flavour, allergenic potentials and indigestible polysaccharides such as raffinose and stachyose. (Wang *et al.*, 2006, Jooyandeh, 2011). Fermented soy-based foods are therefore gaining wide interests due to their beneficial functional and health related properties (Yang *et al.*, 2011, Ng'ong'ola-Manani *et al.*, 2015). Soymilk, which is water extract of soybeans (Murugkar, 2014), is a soy-based food with the capacity to adequately serve as an alternative to dairy milk, due to its protein quality, functional characteristics and absence of lactose and cholesterol (Zhao and Shah, 2014b, Battistini *et al.*, 2017). However, soymilk, like every other soy-based food, has some inherent limitations, including antinutritional factors and beany flavour.

Fermentation could bring about the modification and improvement of physical and chemical properties of soymilk (Akabanda *et al.*, 2010). Fermentation of soymilk has been extensively achieved through the use of single or mixed microbial cultures of lactic acid bacteria, bifidobacteria and other microbial strains. Studies on lactic acid bacteria (Rekha and Vijayalakshmi, 2010b, Marazza *et al.*, 2012, Xiao *et al.*, 2015), bifidobacteria (Hou *et al.*, 2000, Shimakawa *et al.*, 2003, Zhao and Shah, 2014b), mixed culture (Wang *et al.*, 2006, Battistini *et al.*, 2017) fermentation of soymilk, showed improvements in their nutritional properties, antioxidant potentials and functional health benefits. In most of these works, cultures were deliberately added to sterile soymilk and fermentation was conducted under controlled conditions. Similar to that of dairy milk, spontaneous fermentation of soymilk, which involves the activities of naturally occurring fermenting microorganisms in an uncontrolled microbial condition, could be achieved (Obadina *et al.*, 2013). However, spontaneous fermentation of soymilk has been sparsely documented and properties and characteristics of the microbial community in naturally fermented soymilk has been scarcely studied. Apart from single and mixed culture fermentation, soymilk has also been fermented with kefir grains (Guzel-Seydim *et al.*, 2011). Kefir grains are regarded as a discrete matrix of mixed group of microorganisms, including LAB and yeast, which are embedded in a semi-hard, slimy polysaccharide matrix (Liu *et al.*, 2006, Guzel-Seydim *et al.*, 2011) and could be recovered after fermentation.

Therefore, kefir fermentation does not result from the metabolic activity of a single species of microorganisms. Kefir is believed to be a functional food because of its health benefits, coupled with its nutritive value (Farnworth and Mainville, 2003). Soymilk fermented with kefir (soymilk-kefir) has been suggested to have antioxidant and anti-mutagenic effects (Liu *et al.*, 2005). The microbial community of kefir grains had been previously determined (Dobson *et al.*, 2011, Kesmen and Kacmaz, 2011, Nalbantoglu *et al.*, 2014), although, little is known about the changes in microflora of soymilk-kefir as a result of fermentation.

Sprouting of soybeans before processing has been explored to improve the nutritional and functional properties of resulting soy-based foods (Jiang *et al.*, 2013, Murugkar, 2014). Also, optimization of sprouting conditions had also been employed to further enhance the improvement of these beneficial properties of soy-based foods from sprouted soybeans (Guo *et al.*, 2011, Huang *et al.*, 2014). We have optimized the sprouting conditions of soybeans (Chapter 3) using response surface methodology and produced soymilk at these optimized conditions of sprouting. From these findings, optimal improvement of selected nutritional and functional properties of soymilk at 12 h soaking and 52 h germination, compared to soymilk produced with unsprouted beans, was achieved.

Thus far, a culture-dependent approach has been the only method used in the determination of microbial diversity of naturally fermented soymilk (Obadina *et al.*, 2013), while the microbial characteristics of naturally fermented soymilk and soymilk-kefir produced with sprouted soybeans is yet to be known. In this study, we attempted to determine the microbial community of naturally fermented soymilk and soymilk-kefir produced from soybeans, sprouted at the optimized conditions previously suggested, using culture-independent repetitive extragenic palindromic polymerase chain reaction (rep-PCR) and metagenomic approaches. The diversity of microorganisms in sprouted fermented soymilk products and their respective controls produced from unsprouted beans, was determined using these molecular and next generation sequencing approaches to understand their microbial communities and to determine the accuracy and applicability of each of these methods.

7.2. Materials and methods

7.2.1. Naturally fermented soymilk (*soy nono*)

Sprouted soymilk was produced with sprouted soybeans at optimized sprouting conditions of 12 h soaking and 52 h germination according to the method described in Chapter 3. Control samples (unsprouted fermented soymilk) were also produced from unsprouted soybeans. Thereafter, the modified method of Obadina *et al*. (2013) was used to produce naturally fermented soymilk. Sucrose (5%) was added to 100 ml soymilk samples and the mixture was loosely covered with aluminum foil covered and incubated at 25±2°C to ferment in conical flasks. Samples were drawn at 6 h intervals from 0-48 h and stored at 4°C, -20°C and -80°C for further analyses.

7.2.2. Soymilk-kefir

Water-kefir grains (5 g) were added to 100 ml of soymilk samples (sprouted and unsprouted) and the mixtures were incubated at 25±2°C (Liu *et al*., 2005). Samples were also drawn at 6 h interval and stored as appropriate for microbial analyses. Kefir grains were recovered from fermented soymilk-kefir and stored for later use.

7.2.3. Enumeration and isolation of pure of lactic acid bacteria (LAB) and yeast cultures

The method of Akabanda *et al*. (2013) was modified for LAB and yeast isolation. Serial dilutions (up to 10^{-6}) were made for each sample of naturally fermented soymilk and soymilk-kefir, and 0.1 ml of 10^{-3} to 10^{-6} dilutions were inoculated into MRS (Merck, USA) agar plates (LAB) and MYGP media, containing malt extract, yeast extract and gelatin peptone for yeast [with added 2ml stock solution of chloramphenicol (100 mg/l MYGP) and 10 ml of chlortetracycline (50 mg/l MYGP)] already prepared, using spread plate techniques. MRS plates were anaerobically incubated using anaerocult (Merck, USA) for 48-72 h at 30°C while MYGP plates were aerobically incubated at 25°C. Colonies were counted and recorded in each case. To purify colonies, plates were divided into 4 sectors and at least, 8 colonies with different appearances were re-streaked continuously on MRS and MYGP agar plates until pure cultures were obtained.

7.2.4. DNA extraction and identification of LAB and yeast isolates

Isolated pure cultures were collected from agar plates into Eppendorf tubes. There were 103 LAB and 17 yeast pure isolates in all. Extraction of DNA from pure isolates was performed using InstaGene DNA extraction kit following the manufacturer's instruction and were stored at -20°C. Rep-PCR fingerprinting was performed by mixing 3 μl of DNA from pure cultures with 22 μl of PCR mixture, containing PCR mastermix (Thermo Fisher Scientific, USA), GTG_5 primer and sterile MilliQ water and were run in SureCycler PCR system (Agilent, USA) using the thermocycling conditions of 95°C for 7 min, 30 cycles of 95°C for 1 min, 45°C for 1 min and 65°C for 8 min, followed by 65°C for 16 min and 4°C overnight. Thereafter, PCR products were visualized in 1.5% agarose gel after 120V and 2.5 h electrophoresis run (Nielsen *et al*., 2007). Clustering of rep-PCR profiles on the gel was done using Bionumerics 7.0 (Applied Math, Belgium) and construction of dendrograms were based on Dice coefficient of similarity and UPGMA (unweighted pair group method with arithmetic mean). Representative isolates were selected through the groupings from images of cluster analysis of rep-PCR products, for sequencing of their 16S rRNA (LAB) and D1/D2 region of 26S rRNA (yeast) genes (Jespersen *et al*., 2005, Akabanda *et al*., 2013). Before submission for sequencing at the MacroGen (Netherlands), amplification of the 16S genes was done using 27f (5'-AGA GTT TGATYM TGG CTC AG-3') and 1540R (5'-TACGGYTACCTTACGACT-3') primers and for the D1/D2 region of 26S genes, NL1 and NL4 primers were used, according to the method already described by Nielsen et al (2007). Sequences obtained were filtered and trimmed for alignment using the CLC Genomics Workbench version 11 (QIAGEN Bioinformatics) and then compared with the GenBank database using BLAST (NCBI, United States) to obtain the identities of LAB and yeast species.

7.2.5. Metagenomic DNA extraction from fermented soy-based foods

Aliquots of 1ml was taken from each sample and centrifuged at 300 rpm for 15 min in Eppendorf tubes. Supernatants were transferred into new tubes and centrifuged at 5000 rpm for 15 mins to obtain pellets from samples. Pellets were then washed with 1ml sterile water and centrifuged at 12000 rpm for 3 mins and supernatants discarded.

From the washed pellets obtained, whole genome DNA was extracted using the Bead-Beat Micro AX Gravity kit (A&A Biotechnology, Gdynia Poland), containing bead beat tubes, lysis buffer, wash buffers, equilibrating solutions, neutralizing solutions and Proteinase K, according to the manufacturer's instructions. Bead beating was performed at maximum speed using FastPrep-24™ 5G bead beater (MP Biomedicals, CA, USA). Samples were stored at -20°C for metagenomic DNA sequencing.

7.2.6. Illumina high throughput sequencing (HTS) of whole metagenomic DNA.

The methods described by Krych *et al*. (2018) with slight modifications were used. The bacterial community of the whole genome DNA from fermented soy-based food samples was determined using tag-encoded MiSeq-based high throughput sequencing (Illumina, CA, USA). Primers NXt_388_F (5'- TCGTCGGCAG CGTCAGATGT GTATAAGAGA CAGACWCCTA CGGGWGGCAG CAG-3′) and NXt_518_R (5'-GTCTCGTGGGC TCGGAGATGTG TATAAGAGAC AGATTACCGC GGCTGCTGG-3′) from Integrated DNA Technologies (Leuven, Belgium) was used to amplify the V3 region of the 16S rRNA gene. PCR reactions were carried out in SureCycler 8800 using cycling conditions of 95°C for 2 min; 33 cycles of 95°C for 15 s, 55°C for 15 s and 68°C for 30 s; followed by final step at 68°C for 5 min. Primers with adapters and indexes were then incorporated into PCR products by mixing 2 µl PCR product, 12 µl Phusion High-Fidelity Mastermix (Thermo Fisher, MA, USA), 2 µl of corresponding P5 and P7 primer (Nextera Index Kit) and nuclease free water and then cycling at 98°C for 1 min; 12 cycles of 98°C for 10 s, 55°C for 20 s and 72°C for 20 s; and 72°C for 5 min. Amplified fragments were purified with AMP Pure XP beads (Beckman Coulter Genomics USA). Pure products were quantified with Qubit Fluorometer (Invitrogen, Carlsbad, USA) followed by library pooling. Illumina MiSeq sequencing was then performed according to the manufacturer's instruction. Merging and trimming of raw sequence data was performed using fastq_mergerpairs and fastq_filters scripts in UPARSE pipeline, which was also used to purse dataset of chimeric reads and construct Operational Taxonomic Units (OTUs) (Edgar, 2013). The minimum length of trimmed reads, as well as merged reads was 150 bp.

Quantitative Insight into Microbial Ecology (QIIME) software (versions 1.7.0 and 1.8.0) was used to conduct subsequent analysis (Caporaso *et al*., 2010), Jackknifed beta diversity workflow was used to conduct the principal coordinate analysis (PCoA) and green genes (13.8) 16S rRNA gene collection was used as the reference database (McDonald *et al*., 2012).

7.3. Results and discussion

7.3.1. LAB and yeast count of naturally fermented soymilk and soymilk-kefir.

The plate counts (log cfu/ml) of lactic acid bacteria and yeast grown on appropriate cultures are as shown in Table 1. There was significant decrease ($p \leq 0.05$) in LAB count as fermentation time increased in unsprouted fermented soymilk. There was a general decrease in LAB count as fermentation time increased from 6 to 12 h for all samples, followed by an increase in LAB as fermentation time increased from 12 to 48 h, except for unsprouted fermented soymilk, where consistent decrease was observed with increase in fermentation time. Changes in LAB count (increase or decrease) were generally significant in all samples within the 6 h fermentation interval studied. It was also observed that the LAB counts for sprouted products (naturally fermented soymilk and soymilk-kefir) were higher for all sampling intervals, compared to the unsprouted ones. LAB dominated as the fermenting microorganisms in all naturally fermented soymilk and soymilk-kefir samples, with the LAB counts significantly higher than yeast counts in soymilk-kefir samples and the absence of yeast growth for naturally fermented soymilk samples. The highest LAB count was obtained in sprouted soymilk-kefir sampled at 6 h (9.91 log cfu/ml). Yeast count increased progressively ($p \leq 0.05$) in unsprouted soymilk-kefir. It was observed that there were no yeast counts in sprouted soymilk-kefir beyond the 6th hour fermentation, where yeast count was 7.3 log cfu/ml. The inhibitory effects on yeast growth noticed in naturally fermented soymilk samples and in sprouted soymilk-kefir samples after the 6th hour fermentation could be due to the production of lactic acid, acetic acid in high amounts, coupled with propionic and butyric acids produced in trace quantities during LAB fermentation (Schnürer and Magnusson, 2005, Alvarez-Martin *et al.*, 2008).

The accumulation of these organic acids in fermenting food systems bring about the decrease in pH, leading to the availability of undissociated acids for the neutralization of electrochemical proton gradients of cell membranes of yeasts, especially at pH below 4.5, causing the death of susceptible yeast cells (Schnürer and Magnusson, 2005).

Table 1: Microbial counts of LAB and yeast from natural and kefir fermented soymilk samples

	LAB (log cfu/ml)			
	6 h	**12 h**	**24 h**	**48 h**
Unsprouted soymilk	7.30^{a}	6.56^{b}	6.74^{b}	6.00^{c}
Sprouted soymilk	7.88^{b}	5.20^{c}	7.74^{b}	8.39^{a}
Unsprouted soymilk-kefir	9.64^{a}	7.58^{b}	7.69^{b}	NIL
Sprouted soymilk kefir	9.91^{a}	6.95^{c}	8.68^{b}	NIL
	Yeast (log cfu/ml)			
Unsprouted soymilk	6.42^{b}	6.56^{b}	8.09^{a}	NIL
Sprouted soymilk	7.30^{a}	0	0	NIL

Different superscripts represent significant difference $p \leq 0.05$ within sample ranges across different fermentation times

7.3.2. Identification of LAB and yeast isolates using rep-PCR

Following the growth of LAB and yeast on appropriate media, distinct pure cultures of these isolates were obtained by re-streaking on new media several times. One hundred and three (103) pure isolates were obtained for lactic acid bacteria from all samples while 17 yeast isolates were obtained only in soymilk-kefir samples, since there was no yeast growth in naturally fermented soymilk samples (Table 1). Rep-PCR amplification of DNA extracted from each isolate was used to differentiate these isolates using their fingerprints obtained after agarose gel electrophoresis. Kesmen and Kacmaz (2011) considered this approach as a cost effective and reliable method of classification of microbial isolates from LAB. A dendrogram was constructed after cluster analysis of gel images of DNA fingerprints of isolates using UPGMA (unweighted pair-group method with arithmetic averages) based on Dice's coefficient of similarity (Fig 1 and 2). Representative fingerprints selected from distinct clusters of similar fingerprints, potentially representing microorganisms with similar traits, sequenced after amplification of their 16S rRNA and D1/D2 regions of their 26S rRNA and BLASTed using the NCBI database revealed the identities of LAB and yeast with 99-100% identity match.

Lactic acid bacteria obtained are *Lactococcus lactis* (23 isolates), *Leuconostoc lactis* (27 isolates), *Leuconostoc messenteroides* (25 isolates), *Weissella cibaria* (19 isolates) and *Lecleria adecarboxylata* (9 isolates) for all samples while the yeast strains identified include *Pichia fermentans*, *Saccharomyces cerevisiae* and *Torulospura delbruekii*.

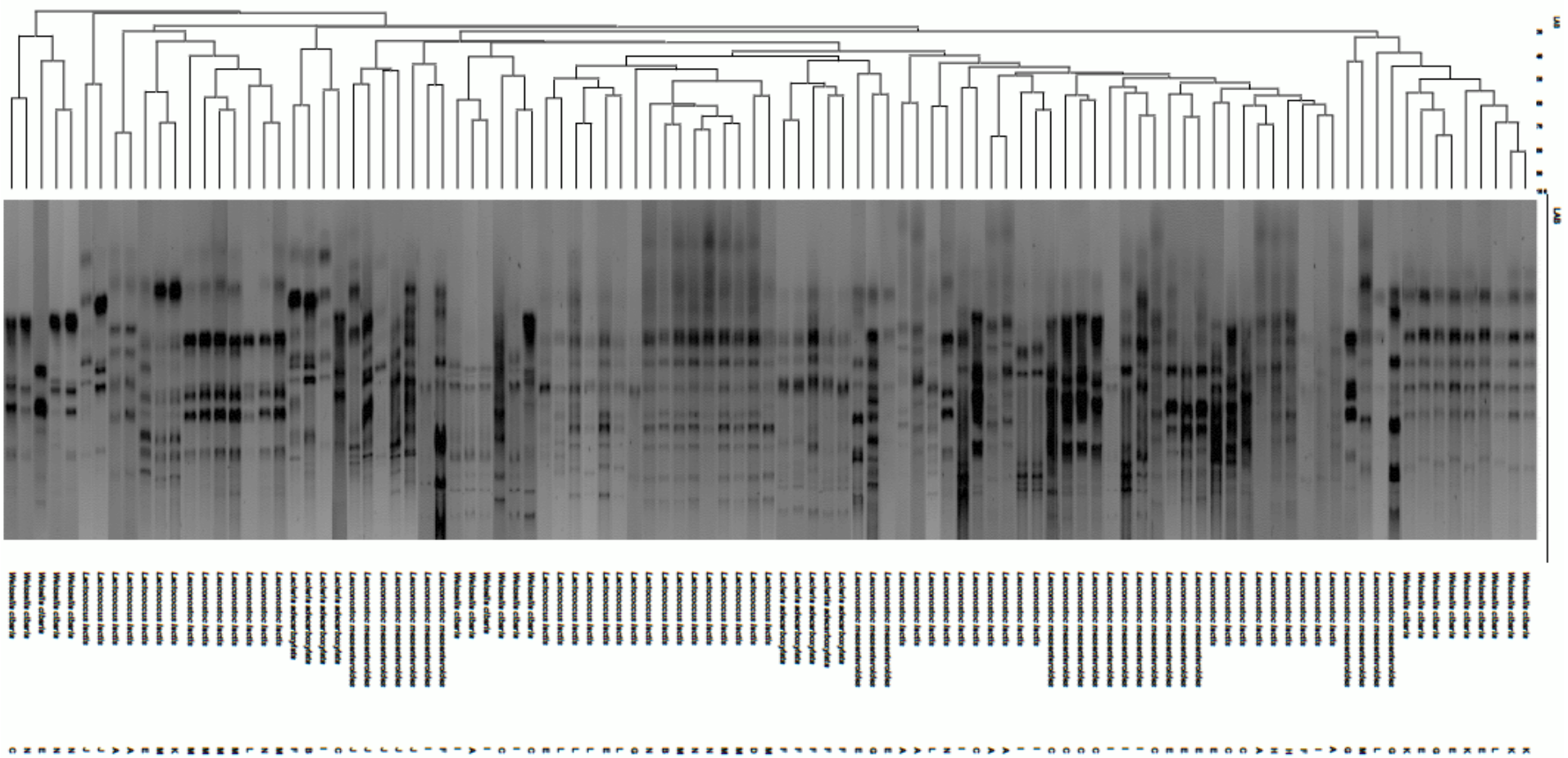

Figure 1: Dendrogram for cluster analysis of rep-PCR (GTG_5) fingerprints of pure LAB isolates. Dendrogram construction is based on Dice's coefficient of similarity using UPGMA (unweighted pair group method with arithmetic averages [A: sprouted fermented soymilk 6 h B: unsprouted fermented soymilk 6 h C: sprouted fermented soymilk 12 h D: unsprouted fermented soymilk 12 h E: sprouted fermented soymilk 24 h F: unsprouted fermented soymilk 24 h G: sprouted fermented soymilk 48 h H: unsprouted fermented soymilk 48 h I: sprouted fermented soymilk-kefir 6 h J: unsprouted fermented soymilk-kefir 6 h K: sprouted fermented soymilk-kefir 12 h L: unsprouted fermented soymilk-kefir: 12 h M: sprouted fermented soymilk-kefir 24 h N: unsprouted fermented soymilk kefir 24 h].

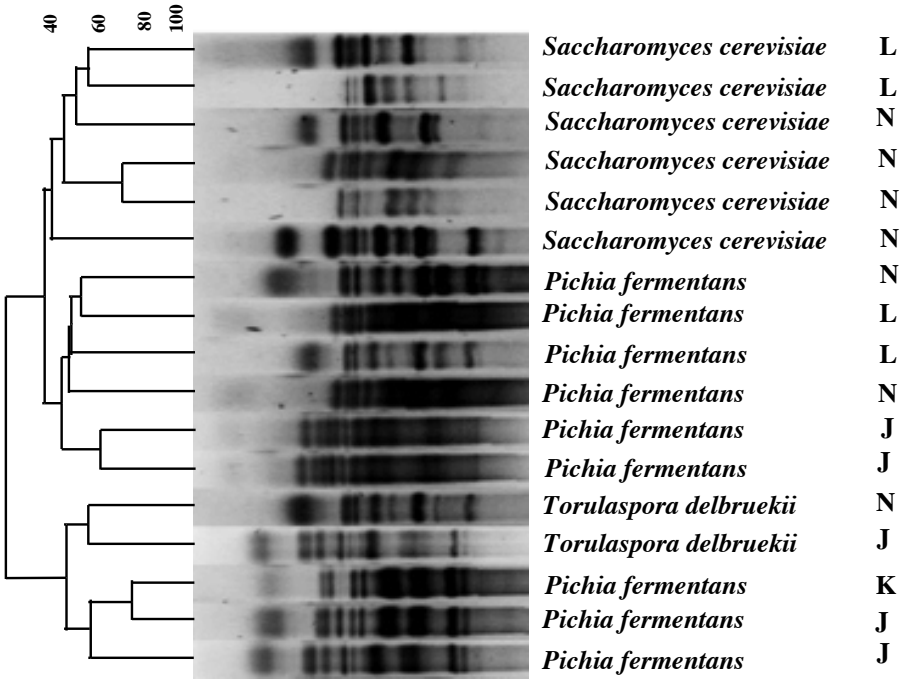

Figure 2: Dendrogram for cluster analysis of rep-PCR (GTG_5) fingerprints of pure yeast isolates. Dendrogram construction is based on Dice's coefficient of similarity using UPGMA (unweighted pair group method with arithmetic averages [J: unsprouted fermented soymilk-kefir 6 h K: sprouted fermented soymilk-kefir 12 h L: unsprouted fermented soymilk-kefir: 12 h N: unsprouted fermented soymilk kefir 24 h].

All isolated LAB strains were present in sprouted and unsprouted products, however, fingerprint clusters revealed higher population of lactic acid bacteria in all sprouted fermented soy-based foods for each sampling period, compared to their respective controls (Fig. 1). This is in line with the higher LAB count obtained for sprouted products (Table 1). Weiss *et al*. (2007) reported progressive increase in LAB count in unfermented sprouts of sesame from 0 h (2 log cfu/g) to 72 h (6.20 log cfu/g) germination. Booysen *et al*. (2002) attributed the increase in lactic acid bacteria count in sprouted wheat to the continual hydration of seeds during germination, enhancement of microbial growth in the seed bed due to favourable temperature and availability of fermentable sugars for optimal activity of enzymes and microorganisms. Soybean sprouting involves the breakdown of seed constituents into smaller, fermentable polysaccharides and sugars, thereby increasing the availability of fermentable substrates and hence, the proliferation of LAB. Lower LAB counts in fermented soymilk-kefir (unsprouted) (Table 1), compared with the fermented soymilk-kefir (sprouted), could explain the competitive survival of yeast cells in the unsprouted fermented products.

For yeast fermentation in soymilk-kefir products, *Pichia fermentans* and *Saccharomyces cerevisiae* were dominant, with 6 and 9 isolates, respectively (Fig. 2). It follows from the results obtained from microbial count that yeast cultures were externally introduced into the fermenting soymilk-kefir products from water kefir samples. Together with lactic acid bacteria, yeasts were also prominent part of the microbial community of water kefir. Gulitz *et al*. (2011) isolated *Saccharomyces cerevisiae, Zygotorulaspora florentia, Lanchacea fermentati* and *Hanseniaspora valbyensis* and Marsh *et al*. (2013) isolated *Dekkera bruxellensis* from water kefir samples. In this study, *Saccharomyces cerevisiae, Pichia fermentans* and *Torulaspora delbruekii* were isolated from soymilk-kefir samples.

7.3.2. Microbial diversity as revealed by high throughput sequencing

Natural and kefir fermentation of foods are expected to involve complex microorganisms, many of which may not be culturable (Nalbantoglu *et al*., 2014). High throughput sequencing was used in this study to holistically determine all the bacteria community of fermenting soymilk and soymilk kefir, to confirm if there would be changes in the fermenting bacteria community of sprouted products relative to the unsprouted and to understand the changes that these organisms may undergo as fermentation time increased. Sequencing of the V3 region of whole genome DNA extracted from all fermented soymilk samples was performed and reads were analyzed using QIIME. Alpha rarefaction plots of observed species and phylogenetic diversity (PD) (Fig. 3) were constructed to confirm the adequacy of sequence coverage used in reliably describing the diversity of operational taxonomic units (OTUs) present in the samples and to measure the complete phylogenetic distance within the bacterial community of each sample (Bokulich *et al*., 2012). Generally, five bacterial phyla namely *Actinobacteria, Bacteroidetes, Cyanobacteria, Firmicutes* and *Proteobacteria* and a group of unclassified bacteria were obtained from all samples, with *Cyanobacteria* having the highest count. Jung *et al*. (2014) found these phyla in the exterior part *doenjang-meju*, a Chinese fermented soybean. *Cyanobacteria*, whose class is *Chloroplast* and order is *Streptophyta* was predominant in samples taken at 6-12 h fermentation and their quantity gradually disappeared as the fermentation time increased (Fig 4.).

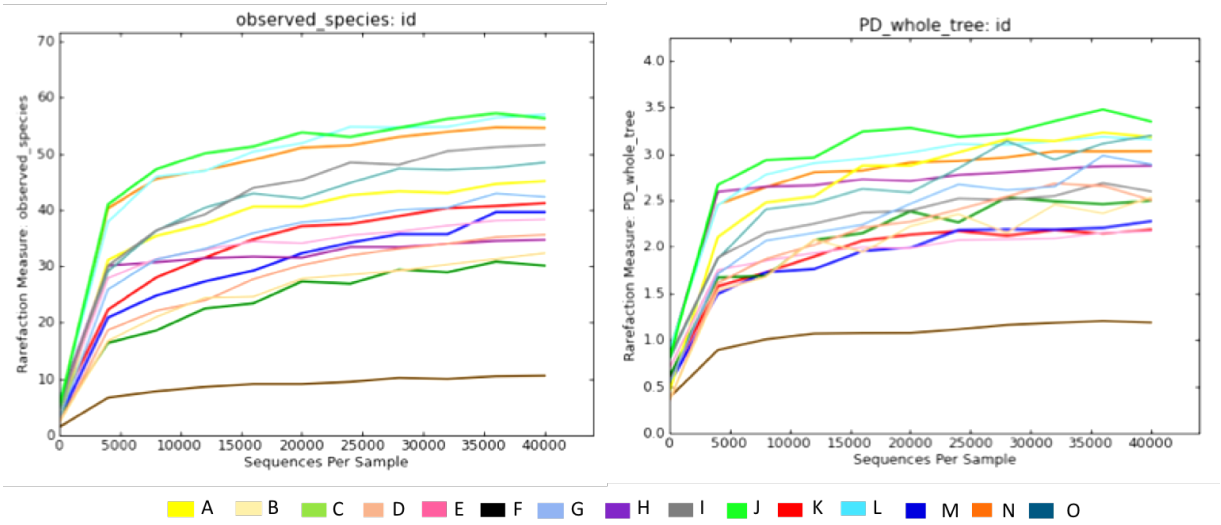

Figure 3. Observed species and phylogenetic diversity whole tree alpha rarefaction of barcoded amplicon sequences of samples [A: sprouted fermented soymilk 6 h B: unsprouted fermented soymilk 6 h C: sprouted fermented soymilk 12 h D: unsprouted fermented soymilk 12 h E: sprouted fermented soymilk 24 h F: unsprouted fermented soymilk 24 h G: sprouted fermented soymilk 48 h H: unsprouted fermented soymilk 48 h I: sprouted fermented soymilk-kefir 6 h J: unsprouted fermented soymilk-kefir 6 h K: sprouted fermented soymilk-kefir 12 h L: unsprouted fermented soymilk-kefir: 12 h M: sprouted fermented soymilk-kefir 24 h N: unsprouted fermented soymilk kefir 24 h].

Cyanobacteria are phototrophic prokaryotes and they contribute to the photosynthetic activity of plants through oxygen production (Hamilton *et al*., 2016). Therefore, they constitute the natural microflora of plants and disappear as fermenting microorganisms colonize foods. Jung *et al*. (2014) found *Cyanobacteria* in *doenjang-meju* in trace quantities despite 42 days fermentation, and Nakatsu *et al*. (2014) reported their presence in fecal samples of postmenopausal women fed with soy bar. The microbial diversity as measured by alpha rarefaction plots of observed species and phylogenetic diversity (Fig. 3) revealed unsprouted soymilk fermented spontaneously for 24 h (Sample F) to have the least diversity of microorganism while sprouted soymilk-kefir fermented for 12 h (Sample J) has the richest and most diverse community. As fermentation progressed, other bacteria phyla, especially *Firmicutes* became significantly expressed leading to the gradual reduction of *Cyanobacteria* (Fig. 4), with *Leuconostoc, Weissella* and *Lactococcus* genera exhibiting enhanced expression depending on the type of fermentation (natural of kefir), type of pretreatment used (sprouted or unsprouted) and the fermentation time (Table 2).

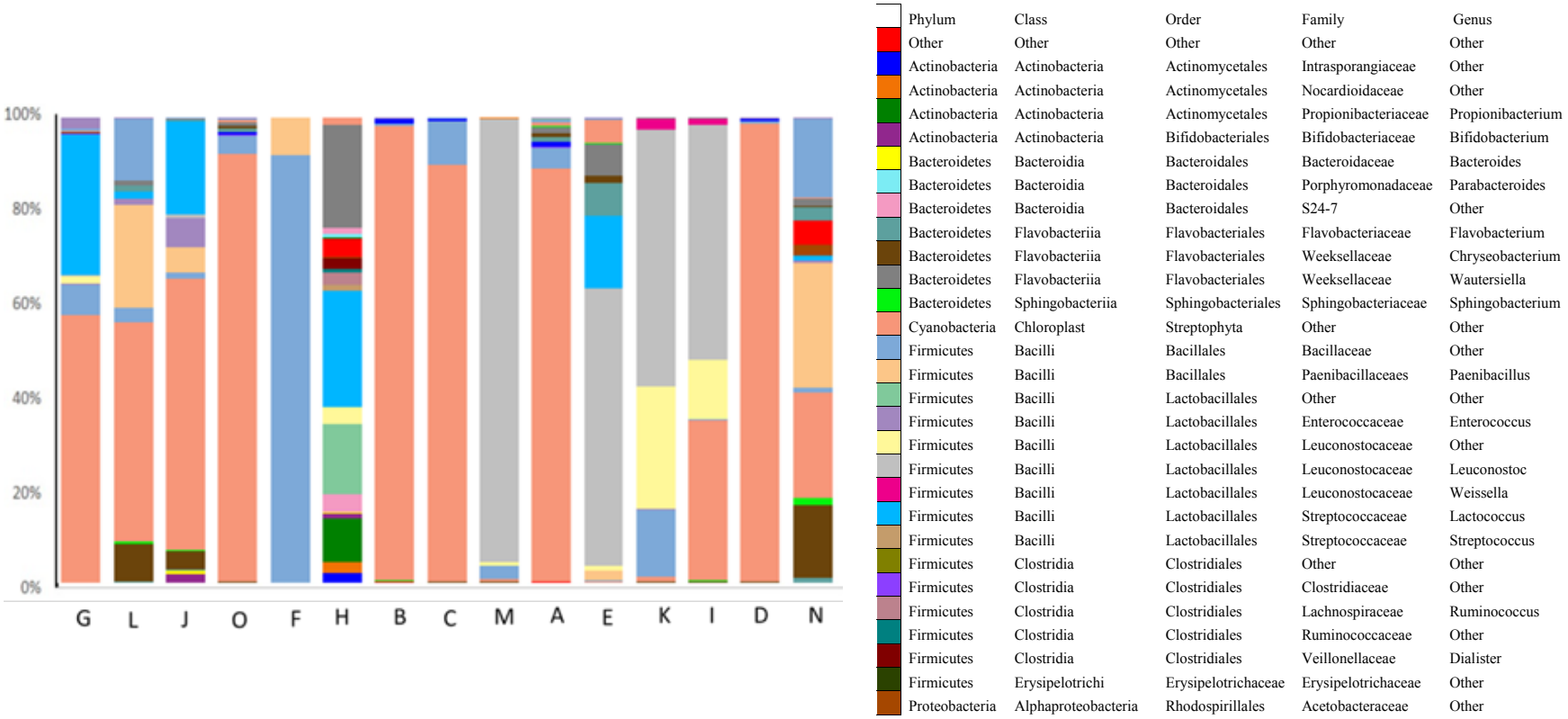

Figure 4: Bacterial diversity structure of fermented soymilk and soymilk-kefir products determined by high through-put sequencing of the V3 regions 16S rRNA [A: sprouted fermented soymilk 6 h B: unsprouted fermented soymilk 6 h C: sprouted fermented soymilk 12 h D: unsprouted fermented soymilk 12 h E: sprouted fermented soymilk 24 h F: unsprouted fermented soymilk 24 h G: sprouted fermented soymilk 48 h H: unsprouted fermented soymilk 48 h I: sprouted fermented soymilk-kefir 6 h J: unsprouted fermented soymilk-kefir 6 h K: sprouted fermented soymilk-kefir 12 h L: unsprouted fermented soymilk-kefir: 12 h M: sprouted fermented soymilk-kefir 24 h N: unsprouted fermented soymilk kefir 24 h].

Also present in the community of fermenting organisms is *Bacillales* family of phylum *Firmicutes* and this suggests that alkaline fermentation process may be occurring simultaneously in natural and kefir fermented soy-based foods in this study. Parkouda *et al*. (2009) reported the presence of *Bacillales*, especially *Bacillus subtilis* in the alkaline fermentation of many spontaneously fermented soy-based food of African origin and Sarkar *et al*. (2002) isolated *Paenibacillus alvei* as one of the major alkaline fermenting organisms in *soumbala*, an African locust beans indigenous to Burkina Faso. Minor to trace populations of bacterial genera including *Propionibacterium, Bifidobacterium* (belonging to *Actinobacteria*), *Bacteroides, Parabacteroides, Flavobacterium Chryseobacterium, Sphingobacterium* (belonging to *Bacteroidetes*), *Citrobacter, Acinetobacter* and *Pseudomonas* (belonging to *Proteobacteria*) were also observed across samples. Relationships among samples, as regards phylogenetic diversity, was calculated from weighted unifrac distances between samples and principal coordinate analysis (PCoA) plot (Fig. 5) was constructed to depict this relationship. This approach was also used by Bokulich *et al*. (2012). The PCoA plot also shows the shift in dominant microorganism with changes in fermentation time and conditions.

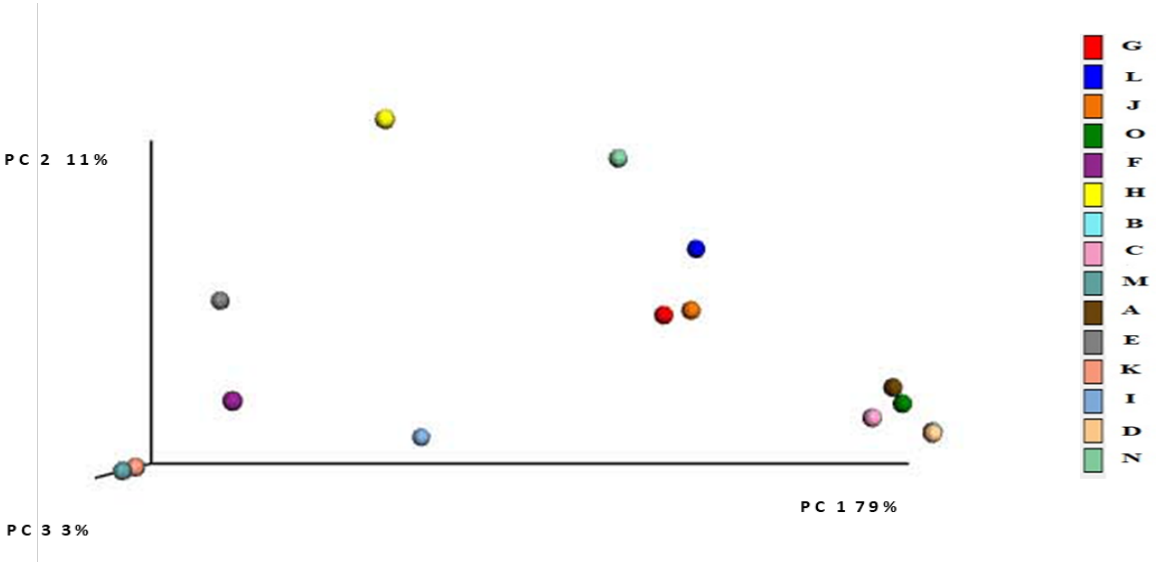

Figure 5: Jackknifed Weighted UniFrac PCoA biplot displaying the relationship among samples with respect to microbial diversity [A: sprouted fermented soymilk 6 h B: unsprouted fermented soymilk 6 h C: sprouted fermented soymilk 12 h D: unsprouted fermented soymilk 12 h E: sprouted fermented soymilk 24 h F: unsprouted fermented soymilk 24 h G: sprouted fermented soymilk 48 h H: unsprouted fermented soymilk 48 h I: sprouted fermented soymilk-kefir 6 h J: unsprouted fermented soymilk-kefir 6 h K: sprouted fermented soymilk-kefir 12 h L: unsprouted fermented soymilk-kefir: 12 h M: sprouted fermented soymilk-kefir 24 h N: unsprouted fermented soymilk kefir 24 h.]

Chapter 3 of this thesis was designed to optimize the sprouting parameters (soaking and germination) of soybeans to produce soy-based foods (fermented and unfermented) with improved quality attributes, as compared to the unsprouted ones. This present study evaluated the microbial community of fermented soy-based foods produced at the optimized conditions obtained from our previous study, using rep PCR and high throughput sequencing. It was observed from Table 2 that the percentage count of OTUs of genera of fermenting bacteria, including *Paenibacillus* and other unidentified Bacillus genera, *Leuconostoc, Weissella* and *Lactococcus* in sprouted fermented soy-based foods (soymilk and soymilk-kefir) was higher than those of unsprouted ones. It was also observed that Bacillaceae was more expressed in the spontaneous fermentation of soymilk as compared to soymilk kefir, suggesting the occurrence of alkaline fermentation, together with other fermentation processes. *Leuconostoc* was predominant in soymilk-kefir, with trace amounts of *Bacillaceae*. In spontaneously fermented soymilk, the highest expression of *Bacillaceae* was obtained in control fermented soymilk 24 h (91.9%) while *Leuconostocaceae* (*Leuconostoc*) was more predominant for sprouted fermented soymilk 24 h and sprouted fermented soymilk kefir 24 h. *Streptococaceae* (*Lactococcus*) was also well expressed in the spontaneous fermentation of soymilk, especially at 24 and 48 h. Sprouting of seeds before fermentation favoured the proliferation of lactic acid bacteria and this is also supported by the LAB counts (Table 1). The increase in LAB and shift in dominant fermenting microorganisms in soy-based foods could be attributed to the conditions of germination, such as temperature and high humidity, as earlier explained (Booysen *et al*., 2002) and supported by Justé *et al*. (2014) who found only 18% similarity in the microbial communities of germinated and ungerminated barley.

Table 2: Genus level taxonomy for bar-coded amplicon sequencing of V3 region of DNA of fermented soy-based foods

								Sample (%)													
	Phylum	Class	Order	Family	Genus	count	Total	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
	Other	Other	Other	Other	Other	0	0.1	0.2	0.2	0.1	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1
	Actinobacteria	Actinobacteria	Actinomycetales	Intrasporangiaceae	Other	0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	Actinobacteria	Actinobacteria	Actinomycetales	Nocardioidaceae	Other	0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	Actinobacteria	Actinobacteria	Actinomycetales	Propionibacteriaceae	Propionibacterium	0	0.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	9.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	Actinobacteria	Actinobacteria	Bifidobacteriales	Bifidobacteriaceae	Bifidobacterium	0	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.0	0.0	1.9	0.0	0.0	0.0	0.0	0.0
	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.3	0.0	0.6	0.0	0.0	0.0	0.0	0.0
	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Parabacteroides	0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0
	Bacteroidetes	Bacteroidia	Bacteroidales	S24-7	Other	0	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	3.8	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	0	0.1	0.1	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.3	0.0	0.3	0.0	1.0	0.0
	Bacteroidetes	Flavobacteriia	Flavobacteriales	Weeksellaceae	Chryseobacterium	0	1.9	0.1	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.2	3.9	0.1	7.9	0.2	15.5	0.0
	Bacteroidetes	Flavobacteriia	Flavobacteriales	Weeksellaceae	Wautersiella	0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.1	0.0
	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	Sphingobacterium	0	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.3	0.2	0.1	0.5	0.1	1.7	0.0
	Cyanobacteria	Chloroplast	Streptophyta	Other	Other	7	45.9	88.7	97.9	89.6	98.5	0.2	0.1	57.5	0.0	34.4	58.5	1.0	47.2	0.4	22.6	92.1
	Firmicutes	Bacilli	Bacillales	Bacillaceae	Other	1	9.4	4.4	0.5	9.4	0.6	0.4	91.9	6.5	0.0	0.3	1.2	14.5	3.1	2.9	1.0	3.8
	Firmicutes	Bacilli	Bacillales	Paenibacillaceaes	Paenibacillus	1	4.3	0.0	0.0	0.0	0.0	2.0	8.1	0.0	0.0	0.0	5.5	0.0	22.2	0.0	26.8	0.0
	Firmicutes	Bacilli	Lactobacillales	Other	Other	0	1.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	15.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	Firmicutes	Bacilli	Lactobacillales	Enterococcaceae	Enterococcus	0	0.6	0.0	0.0	0.0	0.0	0.1	0.0	0.3	0.0	0.0	6.3	0.1	1.2	0.0	0.6	0.0
	Firmicutes	Bacilli	Lactobacillales	Leuconostocaceae	Other	0	3.1	0.0	0.0	0.0	0.0	1.1	0.0	1.6	3.8	12.7	0.0	26.5	0.0	0.7	0.0	0.0
	Firmicutes	Bacilli	Lactobacillales	Leuconostocaceae	Leuconostoc	3	17.4	0.0	0.0	0.0	0.0	59.5	0.0	0.1	0.0	50.6	0.7	55.1	0.1	95.3	0.0	0.0
	Firmicutes	Bacilli	Lactobacillales	Leuconostocaceae	Weissella	0	0.2	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0	1.1	0.0	2.3	0.0	0.1	0.0	0.0
	Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	Lactococcus	1	6.2	0.0	0.0	0.0	0.0	15.6	0.0	30.4	24.8	0.0	20.0	0.0	1.6	0.0	0.9	0.0
	Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	Streptococcus	0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	Firmicutes	Clostridia	Clostridiales	Other	Other	0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	Other	0	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.2
	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Ruminococcus	0	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Other	0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	Firmicutes	Clostridia	Clostridiales	Veillonellaceae	Dialister	0	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	Firmicutes	Erysipelotrichi	Erysipelotrichaceae	Erysipelotrichaceae	Other	0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0
	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Acetobacteraceae	Other	0	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.4	0.0	0.0	0.0	0.0	0.0	2.3	0.0
	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Acetobacteraceae	Other	0	0.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	3.9	0.0	0.0	0.0	0.0	0.0	5.3	0.0
	Proteobacteria	Alphaproteobacteria	Rickettsiales	mitochondria	Other	0	0.3	0.9	1.0	0.7	0.4	0.0	0.0	0.1	0.0	0.1	0.1	0.0	0.1	0.0	0.0	0.7
	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Other	0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0
	Proteobacteria	Betaproteobacteria	Burkholderiales	Other	Other	0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Other	0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Delftia	0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	Ralstonia	0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.9	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	Proteobacteria	Deltaproteobacteria	Myxococcales	Other	Other	0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	Proteobacteria	Gammaproteobacteria	Other	Other	Other	0	0.8	1.0	0.0	0.0	0.0	6.9	0.0	0.0	0.0	0.1	0.1	0.0	1.2	0.0	2.9	0.6
	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Other	0	0.2	0.8	0.0	0.0	0.0	1.6	0.0	0.1	0.1	0.0	0.0	0.0	0.0	0.0	0.1	0.5
	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Other	0	2.3	1.4	0.0	0.0	0.0	7.0	0.0	0.1	22.1	0.1	0.4	0.1	0.9	0.0	1.7	0.9
	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Citrobacter	0	0.0	0.4	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.2
	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Erwinia	0	0.5	0.7	0.0	0.0	0.0	5.0	0.0	0.1	1.6	0.0	0.0	0.0	0.0	0.0	0.0	0.4
	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Acinetobacter	0	2.2	0.4	0.1	0.1	0.1	0.3	0.0	0.7	0.0	0.0	0.1	0.1	13.2	0.0	17.0	0.1
	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Other	0	0.0	0.1	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.1
	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Other	0	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1
	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	0	0.3	0.3	0.1	0.1	0.1	0.1	0.0	2.2	0.0	0.0	0.1	0.0	0.3	0.1	0.2	0.2
	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Other	0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

7.3.3. Comparison between rep PCR and HTS approaches

High throughput amplicon sequencing (HTS) of metagenomic DNA of fermented soy-based foods was more effective in revealing the community of bacteria involved in their fermentation, compared to rep-PCR. Detailed information about the entire bacterial community of spontaneous and kefir fermentations, including the possibility of the progress of alkaline fermentation (through the presence of *Bacillaceae* and *Paenibacillus*), the presence of *Cyanobacteria* phylum and its reduction as fermentation time increased, as well as the presence of unculturable and unassigned bacteria, were obtained through the amplification of V3 region and high throughput sequencing of metagenomic DNA of samples. Whereas, only lactic acid bacteria including *Leuconostoc, Lactococcus* and *Weissella* genera could be identified through rep PCR, more detailed information of the presence of other fermenting microorganisms were revealed through HTS. Culture-independent rep-PCR or PCR-DGGE approaches have been widely used to characterize the microbial diversity of fermented foods, including those involving kefir fermentation (Nielsen *et al.*, 2007, Kesmen and Kacmaz, 2011, Adimpong *et al.*, 2012, Akabanda *et al.*, 2013). However, such analyses may not provide complete information of the microbial community, due to the drawbacks of conventional methods, including trial of several growth media for pure culture isolation, thereby leading to errors and under-assessment of the entirety of microorganisms (Nalbantoglu *et al.*, 2014). These methods have also been found to erroneously determine species and strains, missing over a half of the microbial diversity (Hong *et al.*, 2009). The use of HTS helped to extensively reveal the community and diversity of fermenting and non-fermenting bacteria present in naturally fermented soymilk and soymilk kefir (sprouted and unsprouted), to compare their abundance and also to clearly monitor changes in microbial diversity and dominance with fermentation time.

7.3.4. Conclusion

Through the use of rep-PCR and high throughput amplicon sequencing, the microbial community and diversity of spontaneously fermented soymilk and soymilk-kefir produced from sprouted soybeans (at optimized sprouting conditions) and unsprouted soybeans was adequately determined.

The major genera of lactic acid bacteria identified included *Leuconostoc, Weissella* and *Lactococcus* (as revealed by rep-PCR and HTS) and yeast included *Saccharomyces, Pichia* and *Torulaspora*. The presence of *Bacillaceae* (as revealed only in HTS) also suggests the progress of alkaline fermentation. Other bacteria not involved with fermentation was adequately captured in HTS. The major fermenting LAB and yeast were detected using rep-PCR and cluster diagrams showed their relationships. However, there were limitations of culture separation of unculturable microorganisms present in the fermentation, which were resolved in HTS. Generally, there were higher quantities of fermenting organisms, especially LAB, present in sprouted fermented soymilk and soymilk-kefir for every fermentation time tested, as revealed by the preliminary microbial counts and HTS results. Fermenting microorganisms obtained from this study, especially LAB, have not been widely employed in controlled fermentation of soymilk compared to *Lactobacillus acidophilus, Lactobacillus plantarum, Streptococcus thermophilus* and *Bifidobacterium*. Hence, they can be made into starter cultures for controlled fermentation of soymilk. High throughput sequencing of the community of yeasts was not captured in this work and could be a subject for further studies.

Chapter 8: General Discussion

This study was conducted to expand the versatility and functionality of soybeans for the production of 'superfoods' through sprouting. The processes are non-heat food processing operations which harness the usefulness of enzymatic activities and microbial metabolism in achieving desired characteristics in foods. Soybean is an important plant source of many nutrients and phytonutrients, and consumption of soy-based foods has been linked to many health-related benefits (Jooyandeh, 2011, Kim *et al.*, 2016). However, there are concerns such as beany flavour, anti-nutritional contents, allergenic reactions and digestibility with the consumption of soy foods. Sprouting is one of the methods that has been used to reduce these adverse properties of soybeans (Jiang *et al.*, 2013, Guo *et al.*, 2011). Metabolic activities occurring during sprouting are often triggered by stress conditions on the sprouting seeds and are associated with enzymatic breakdown of seed reserves, as well as synthesis of other components (Zhang *et al.*, 2015). This leads to the improvement of nutritional quality, digestibility and overall functionality of sprouted beans, and their products (Fernandez-Orozco *et al.*, 2008). Sprouting of soybeans, like many other seeds, depends on a number of factors including germination time, germination temperature, soaking conditions, relative humidity and seed variety (Paucar-Menacho *et al.*, 2010a, Huang *et al.*, 2014). A number of attempts have also been made to optimize these sprouting conditions, using both traditional methods and response surface methodology (RSM), towards the improvement in the nutritional, phytochemical and functional attributes (Guo *et al.*, 2011, Jiang *et al.*, 2013, Murugkar, 2014, Huang *et al.*, 2014). This section discusses the importance of optimization of pre-process sprouting of soybeans (Chapters 3), nutritional advantages (Chapter 4) and functional characteristics (Chapters 5 and 6) of soy-based foods produced with optimized sprouted soybeans and the diversity of microorganisms in fermented soy-based foods (Chapter 7).

8.1 Importance of optimization of pre-process sprouting of soybeans (Chapter 3).

Optimization of process parameters using response surface methodology helps to understand the interrelationship between at least two factors responsible for the changes in attributes (responses), as against traditional methods of optimization, where the effect of one factor can be reported at a time (Baş and Boyacı, 2007, Bezerra *et al.*, 2008).

In this study, soaking and germination times, as factors of soybean sprouting, were optimized using RSM central composite rotation design. The effect of soaking, as a factor of sprouting has been sparsely studied, either singly, or in combination with other factors. Soybeans were subjected to soaking (12-24 h) and germination times (48-96 h) and used to produce soymilk in 17 experimental runs. Quality attributes (responses) obtained from experimental runs were fitted into second order polynomial regression model. Optimum conditions of soaking (12h) and germination (52h) were obtained. Soymilk produced at these optimized conditions of soybean sprouting had improved nutritional characteristics, reduced antinutritional properties and no significant difference in colour and rheological attributes when compared with its control sample (soymilk from unsprouted beans). The enhancement in quality attributes is of importance in sprouted soymilk as a functional food (superfood) on its own and as the intermediate raw material in the production of other soy-based foods studied (tofu, naturally fermented soymilk and soymilk-kefir). Many attempts at the optimization of soybean sprouting parameters did not consider soaking conditions and also produced varying results for the factors considered (usually germination temperature and time) (Paucar-Menacho *et al.*, 2010a, Paucar-Menacho *et al.*, 2010b, Jiang *et al.*, 2013, Guo *et al.*, 2011, Huang *et al.*, 2014). Also, these attempts suggested higher germination times (Jiang *et al.*, 2013, Murugkar, 2014) or varying germination times for optimal production of different attributes (Paucar-Menacho *et al.*, 2010a, Paucar-Menacho *et al.*, 2010b). Soymilk produced at the suggested optimized conditions of sprouting could be a plant-based source nutritious milk, and a desirable intermediate raw material in the production of other soy-based foods.

8.2 Nutritional advantages of soy-based foods produced with optimized sprouted soybeans (Chapter 4)

The effectiveness of optimization of sprouting conditions of soybeans in improving the nutritional and functional attributes of soy foods was investigated by analyzing the nutritional properties (including proximate composition, *in vitro* protein digestibility, amino acid contents, total flavonoids and total condensed tannins) of resulting soy-based foods. Sprouted soymilk served as the intermediate raw material in the production of tofu, naturally fermented soymilk and soymilk-kefir samples, while their control samples were also produced using similar processes.

Comparing sprouted soy-based foods with those from unsprouted soybeans (as controls) has been explored as means of clearly showing the effects of pre-process sprouting (Jiang *et al.*, 2013, Murugkar, 2014). Changes in the nutritional characteristics of foods produced with sprouted seeds have been generally attributed to various biochemical reactions occurring during sprouting. Nutritional changes, including increase in protein contents (Murugkar, 2014), improved protein digestibility (Dikshit and Ghadle, 2003, Shimelis and Rakshit, 2007), improved flavonoid contents (Zhu *et al.*, 2005) and reduced tannin contents (Sangronis and Machado, 2007), have been reported. Also, antinutrients such as phytic acid and tannins have been reported to leach into soak water, thereby reducing their composition in sprouted soybeans (Rakić *et al.*, 2007, Liang *et al.*, 2009). These attributes are further enhanced in fermented sprouted soy-based foods (Khattab and Arntfield, 2009). For this study, soy-based foods including tofu, naturally fermented soymilk and soymilk-kefir were obtained from soymilk produced from optimized sprouted soybeans using the modified methods of Rekha and Vijayalakshmi (2010b), Obadina *et al.* (2013) and Liu *et al.* (2005) respectively and then, changes in their nutritional attributes were investigated. Protein contents, *in vitro* digestibility and total flavonoid contents were significantly higher, especially in fermented sprouted products, as compared to their respective controls. Increased total flavonoid contents were also observed in sprouted soy-based foods, suggesting the enhancement of their antioxidant potentials, since isoflavones, which are potent antioxidants, are the major flavonoids present in soybeans (Lee *et al.*, 2011). There was significant reduction in condensed tannin contents for all sprouted soy-based food samples majorly due to soaking and enzymatic activities of sprouting, while the reduction in total solid contents could be attributed to the loss of nutrient reserves during sprouting (Rakić *et al.*, 2007, Sangronis and Machado, 2007). Although, there was no significant difference in the amino acid profile for all soy-based foods, contents were slightly higher in unsprouted soy-based foods (controls). Germination times beyond the suggested periods may lead to greater loss of amino acids, especially for sprouted fermented soy-based foods (Mbithi-Mwikya *et al.*, 2000, Yang *et al.*, 2012). Overall, products obtained from sprouted soybeans at the suggested optimized sprouting conditions had improved nutritional qualities.

8.3 Functional characteristics of soy-based foods produced with optimized sprouted soybeans (Chapters 5 and 6)

Consumption of antioxidant-rich foods such as legumes, pulses and peas could boost the inherent defense systems of the body against adverse physiological effects of free radical production (Pająk *et al.*, 2014). Soybean is one of the most commonly consumed legume seeds worldwide (Sęczyk *et al.*, 2017). Consumption of soy-based foods is gaining rapid popularity, especially in countries outside Asia. Soybeans contain high amounts of bioactive compounds such as polyphenols and flavonoids and consumption of these compounds could help prevent the occurrence of chronic diseases (Rodríguez-Roque *et al.*, 2013a, Morales-de La Peña *et al.*, 2010, Sęczyk *et al.*, 2017). Sprouted seeds are rich sources of phenolics and flavonoids, with high antioxidant activities (Pająk *et al.*, 2014). This study investigated the improvement in the antioxidant activities of sprouted soy-based foods tested with different antioxidant assays, including 2,2-diphenyl-1-picrylhydrazyl (DPPH), ferric reducing antioxidant activity (FRAP), superoxide scavenging, hydroxyl radical scavenging and metal ion chelating assays. Different concentrations of sample extracts from 0.5-5 mg/ml were used to test their reactivities against antioxidant assays. Samples were extracted with appropriate buffers for each assay, therefore, their antioxidant activities could be the combined effects of phenolic contents, isoflavones or bioactive peptides. Significant increase ($p \leq 0.05$) in radical scavenging activities was observed in all sprouted products relative to their controls. These antioxidant potentials were further enhanced by fermentation, as naturally fermented soymilk and soymilk-kefir had the highest anti-radical potentials on all assays tested. Soybeans naturally contain superoxide dismutase and isoflavones, which are potent superoxide scavengers (Marazza *et al.*, 2012, Chien *et al.*, 2006) and higher superoxide scavenging activity in sprouted foods could be attributed to the improvement of the activities of superoxide dismutase due to stress-induced conditions of sprouting (Fernandez-Orozco *et al.*, 2008). High hydroxyl radical scavenging activity has also been linked to reduced duration of germination, such as was used in this study (Vale *et al.*, 2014). Metal ion chelating ability of sprouted soy-based foods suggests that their consumption could provide a preventive measure against the production of toxic free radicals in the body. Ferric reducing antioxidant power (FRAP) of sprouted foods were not significantly different ($p \leq 0.05$) from those produced with unsprouted ones, except for sprouted soymilk-kefir. There was no DPPH radical scavenging activity detected for naturally fermented soymilk.

This could be due to the inability of their antioxidant moieties, including phenol, to access the DPPH radical active site, thereby impeding the hydrogen atom transfer reaction (Schaich *et al.*, 2015). The ability of processed food products to retain their bioactivity in formulated foods is an important step towards the development of novel functional foods. Therefore, sprouted soy-based products have potential to be used as functional food ingredients for preventing diseases or ameliorating disease symptoms.

The major threat to soybean consumption has been the presence of antinutritional factors and allergic reactions by consumers and these can be adequately removed or sufficiently minimized by proper processing (Cervantes-Pahm and Stein, 2010). The major proteins of soy products are glycinin and β-conglycinin, with molecular weights of approximately 360 kDa and 220 kDa respectively (Wilson *et al.*, 2005). They have been regarded as potent allergens (Krishnan *et al.*, 2009) and they account for over 50-90% of soybean storage proteins, depending on the concentration of proteins after processing, from soy flours (50%) to soy protein isolates (90%) (Plumb *et al.*, 1994, Lallès *et al.*, 1999). In this study, potentials for improved soy storage protein breakdown and allergen reduction in soy-based foods produced with soybeans sprouted at optimized conditions was investigated using 2-dimensional gel electrophoresis (2-DE) for separation of soy soluble proteins according to their molecular weights and isoelectric points, and identification of this protein using matrix assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry. Sprouting of soybean seeds at optimized conditions of soaking and germination suggested, has the capacity for reduction of the large molecular weight of its storage proteins into their subunits, through the activities of enzymes inherent in seeds and activated during germination. This is demonstrated by the significantly higher soluble protein concentration ($p \leq 0.05$) of sprouted soy-based foods per unit volume of proteins. Vernaza *et al.* (2012) found increase in soluble protein concentration in sprouted Brazilian soybeans as a result of breakdown of higher molecular weight storage proteins. The reduction in soluble protein content of sprouted fermented soymilk (48 h) could result from the increased rate of metabolism of reduced proteins during extended fermentation (Seo and Cho, 2016).

The enhanced reduction of soy storage proteins to their lower molecular weight subunits, facilitated by sprouting, suggests increased chances of peptide bioavailability and possible increase in the quantity of bioactive peptides that would be available after *in-vivo* pepsin and pancreatic digestion (Lallès *et al.*, 1999). Drastic reduction in quantity of soybean storage protein subunits by fermentation implies further breakdown of storage protein subunits, with possible implications of improved bioavailability of lower molecular weight peptides (below 11 kDa). Sprouting of soybean seeds at the suggested optimized conditions, as well as natural and kefir fermentation of soy-based foods produced with these sprouted seeds, have potential for increased bioactive peptides availability and reduced allergenic effects in soy-based foods.

8.4 Diversity of microorganisms in fermented soy-based foods

Fermentation brings about the modification and improvement of physical and chemical properties of soymilk (Akabanda *et al.*, 2010). Fermentation of soymilk has been extensively achieved through the use of single or mixed microbial cultures of lactic acid bacteria, bifidobacteria and other microbial strains. Although, soymilk fermentation has been achieved through the use of starter cultures (Marazza *et al.*, 2012, Zhao and Shah, 2014a) spontaneous fermentation of soymilk, which involves the activities of naturally occurring fermenting microorganisms in an uncontrolled microbial condition, could also be achieved (Obadina *et al.*, 2013). Apart from single and mixed culture fermentation, soymilk has also been fermented with kefir grains (Guzel-Seydim *et al.*, 2011, Liu *et al.*, 2005). The microbial community of spontaneously fermented soymilk and soymilk-kefir was investigated using two distinct approaches, namely; repetitive extragenic palindromic polymerase chain reaction (rep-PCR) and high throughput amplicon sequencing (HTS). Soymilk produced with soybean seeds sprouted at optimized conditions of soaking (12 h) and germination (52 h) were subjected to natural fermentation (for 48 h) and kefir fermentation (for 24 h), with samples drawn at 6 h interval while fermented soymilk from unsprouted beans served as the control sample in each case. LAB dominated as the fermenting microorganisms in all naturally fermented soymilk and soymilk-kefir samples, with the LAB counts significantly higher than yeast counts in soymilk-kefir samples and the absence of yeast growth for naturally fermented soymilk samples.

The inhibitory effects on yeast growth noticed in naturally fermented sprouted fermented soymilk and soymilk-kefir samples, could be due to the production of lactic acid, acetic acid in high amounts, coupled with propionic and butyric acids produced in trace quantities during LAB fermentation (Schnürer and Magnusson, 2005, Alvarez-Martin *et al.*, 2008). Rep-PCR of DNA extracted from pure cultures of LAB and yeast isolates -were carried out to differentiate these isolates, using their fingerprints obtained after agarose gel electrophoresis. Identities of lactic acid bacteria obtained are *Lactococcus lactis* (23 isolates), *Leuconostoc lactis* (27 isolates), *Leuconostoc messenteroides* (25 isolates), *Weissella cibaria* (19 isolates) and *Lecleria adecarboxylata* (9 isolates) and they were present in all samples. However, higher population of lactic acid bacteria was obtained in all sprouted fermented soy-based foods for each sampling period. This trend was also supported by the plate counts of LAB and yeast. Booysen *et al.* (2002) attributed the increase in lactic acid bacteria count in sprouted wheat to the continual hydration of seeds during germination, enhancement of microbial growth in the seed bed due to favourable temperature and availability of fermentable sugars for optimal activity of enzymes and microorganisms. *Pichia fermentans*, *Saccharomyces cerevisiae* and *Torulaspora delbruekii* were identified in fermented soymilk-kefir samples and their presence may be due to kefir fermentation, since no yeast growth was recorded during the spontaneous fermentation of soymilk.

High throughput sequencing revealed the presence of five bacterial operational taxonomic units (OTUs) with phyla namely *Actinobacteria, Bacteroidetes, Cyanobactera, Firmicutes* and *Proteobacteria* with *Cyanobacteria* having the highest count. *Cyanobacteria* are phototrophic prokaryotes and they contribute to the photosynthetic activity of plants through oxygen production (Hamilton *et al.*, 2016), hence, they constitute the natural microflora of plants and disappears as fermenting microorganisms colonize foods. In addition to the identification of *Leuconostoc, Weissela* and *Lactococcus* genera, more detailed information about the entire bacterial community of spontaneous and kefir fermentations, including the possibility of the progress of alkaline fermentation (through the presence of *Bacillaceae* and *Paenibacillus*), were obtained mainly because HTS involved the amplification of V3 region of metagenomic DNA.

The presence of bacterial genera including *Propionibacterium, Bifidobacterium* (belonging to *Actinobacteria*), *Bacteroides, Parabacteroides, Flavobacterium Chryseobacterium, Sphingobacterium* (belonging to *Bacteroidetes*), *Citrobacter, Acinetobacter* and *Pseudomonas* (belonging to *Proteobacteria*) in minor or trace populations were also observed across samples. The advantages of metagenomic approaches over molecular methods had earlier been explained by (Bokulich *et al.*, 2012, Nalbantoglu *et al.*, 2014). The microbial community of naturally fermented soymilk and soymilk-kefir, especially for LAB and yeast, were adequately characterized using Rep-PCR and HTS approaches, however, HTS was more effective than rep-PCR in revealing the community and diversity of fermenting and non-fermenting bacteria present in naturally fermented soymilk and soymilk kefir (sprouted and unsprouted) and also helped in monitoring the changes in microbial diversity and dominant microorganisms during fermentation time period.

8.5 General conclusions and recommendations

Through process optimization of soybeans sprouting using response surface methodology, the optimum soaking and germination times obtained were 12 and 52 h respectively. Soy-based foods produced from sprouted soybeans at these optimized conditions had improved nutritional attributes. Sprouting of soybeans brought about improvement in the antioxidant activities of soy-based foods as confirmed through different concentrations of samples and different antioxidant assays. Further enhancement of antioxidant activity could be achieved through fermentation. Sprouting at optimized conditions enhanced the reduction of allergenic soy storage proteins to their lower molecular weight subunits, suggesting increased chances of peptide bioavailability. Also, reduction in quantity of protein subunits by fermentation implies further breakdown of storage protein subunits, with possible implications of improved bioavailability of lower molecular weight bioactive peptides (below 11 kDa). The use of rep-PCR in characterizing the microbial community of spontaneously sprouted and un-sprouted fermented soymilk and soymilk-kefir revealed the presence of lactic acid bacteria (LAB) including *Leuconostoc messenteroides*, *Leuconostoc lactis*, *Lactococcus lactis* and *Weissella cibaria* and yeast including *Pichia fermentans*, *Saccharomyces cerevisiae* and *Torulaspora delbruekii*.

Further investigation of bacterial community using high throughput sequencing revealed a more detailed characteristic of spontaneous and kefir fermentation of sprouted and unsprouted soymilk, including the presence of *Bacillaceae* (suggesting the progress of alkaline fermentation) and *Bifidobacterium*. Overall, this study demonstrated the potentials for extended use of soybeans, through the application of sprouting as a cheap pre-processing operation, in the production of highly nutritional and functional soy-based 'superfoods'.

Further studies may be required in understanding the effects of the interrelationship between other sprouting parameters on the quality attributes of soy-based foods. Also, research gap exists in the study of antioxidant and allergenic properties of bioactive components of optimized sprouted soybeans, such as isoflavones and peptides. The potentials of lactic acid bacteria isolated in this study in the production of fermented soymilk products such as yoghurt may also be critically explored, with the possibility of developing them into starter cultures. Also, *in vitro* simulated digestion and animal studies may be possible areas of research in evaluating the potentials of optimized sprouted soy-based 'superfoods' in improving nutrient digestibility, imparting functional characteristics and enhancing overall system performance after consumption.

Chapter 9: References

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