Quality and Storage Stability of Yoghurt Produced from Pigeon Pea Milk Supplemented with *Propionibacterium freudenreichii*

BY

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PREFACE

The work described in this thesis was carried out at the Department of Biotechnology and Food Technology, Durban University of Technology, under the supervision of Dr Oluwatosin Ijabadeniyi and Prof Francis Shode.

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As the candidate's supervisors we agree to the submission of this thesis.

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DECLARATION

I, Amina Osizemeyele Yusuf, declare that:

1. The research reported in this thesis, except where otherwise stated, is my original research.

2. This thesis or any part of it has not been submitted for any degree or examination at any other university.

Signed: 

DEDICATION

This thesis is dedicated to my late mother

ALHAJA AJARA AHMED

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ABSTRACT

Pigeon pea (*Cajanus Cajan*) is consumed in many parts of Africa as a source of protein and carbohydrate. It is underutilised and mainly grown for subsistence. Researching on pigeon pea may enhance value addition and increase its utilization. In this study, a non-dairy probiotic yoghurt was prepared from pigeon pea milk. Yoghurt samples were prepared, using 100% pigeon pea milk, pigeon pea/soy milk in the ratio 50:50 and 100% soy bean milk. The yoghurts were inoculated with yoghurt starter cultures and divided into two equal parts. One part inoculated with *Propionibacterium freudenreichii* was referred to as probiotic yoghurt, while the other part served as the control. The nutritional, sensory and some functional properties of the yoghurt were determined. The microbiological quality of yoghurt samples stored at 4, 10 and 21°C, respectively, for 4 weeks, were monitored and analysed for *aerobic spores' formers*, *E. coli*, *total plate counts*, *mould* and *Propionibacterium freudenreichii* weekly. The protein contents of the yoghurt samples varied from 4.54-5.85% for 100% soymilk and 100% pigeon pea yoghurt respectively. The probiotic yoghurt showed slightly lower protein content than pigeon pea yoghurt alone. All the yoghurt samples had considerably high total solids (16.04-17.41%) and were fairly good sources of amino acids. Essential amino acids in the yoghurt samples were comparable to the FAO/WHO (2007) recommended amino acid requirement for adults. Anti-nutritional factors of yoghurt samples were significantly lower ($P \leq 0.05$) than their milk counterparts, which may be attributed to the fermentation process. Probiotic yoghurt samples showed higher firmness than non-probiotic samples. Total plate counts (log 7.01-7.46 CFU/ml) samples stored for 2 weeks at 4° C were similar. Predominant organisms were LAB and *Propionibacterium freudenreichii*. Storage temperature of yoghurt samples had an influence on the total plate count and LAB. Total plate count and LAB significant increased approximately by log 2 CFU/ml for the first two weeks of storage. However, moulds and *E. coli* were not detected in all samples. Beyond 2 weeks of storage, there was significant decline in total plate counts and LAB, while mould grew and increased. Aerobic spore formers and moulds were observed in the control

yoghurt. However, *E. coli* was not found in all yoghurt samples throughout storage period. The pH of the milk in which yoghurt mixtures were formulated, ranged from pH 7 to 6.8 for pigeon pea and soymilk declined significantly as a result of acidification. Decline in pH at 4, 10 and 21°C was significant ($p \leq 0.05$) with the rate higher at 21, 10 than 4° C. Decline in pH resulted in increased TTA values over storage temperatures and periods. Samples stored at 21°C and 10°C had significantly higher TTA values than samples stored at 4° C. The colour values evaluated were recorded as L*, b*, a* and ΔE* during 4 weeks storage at 4, 10 and 21° C. Significantly high values ($p \leq 0.05$) were recorded for L* yoghurt samples with soymilk. The colour scale defines positive (red) and negative (green) for a* and b* positive (yellow) and negative (blue). All a* values both positive and negative were less than 3. There was no negative value recorded for b*. Colour difference ΔE* values trends increased as storage time and temperature increased. There were significant ($p \leq 0.05$) differences between samples stored at same and different storage temperatures and periods. Water holding capacity was significantly different ($p \leq 0.05$) in all the yoghurt samples stored at 4, 10 and 21°C for 4 weeks. Formulation with 100% soymilk recorded higher values. Soy yoghurt and probiotic yoghurts (100 %) showed higher water holding capacity compared to pigeon pea yoghurt and pigeon pea/soymilk yoghurt. The addition *of Propionibacterium freudenreichii* did not significantly affect sensory properties of the yoghurts. Acceptable yoghurt was produced from pigeon pea with comparable quality to soy which serves as control. Proximate composition was comparable to previous reports. Microbial quality and profile of all the yoghurt samples were similar. The absence of pathogenic bacteria in all the yoghurt samples confirm their safety. Soy yoghurt was most acceptable amongst the yoghurt samples but all the samples had comparable ratings, and these ratings are within commercially acceptable range (4 to 9) for yoghurt. Storage at 4^{o}C should be the most acceptable, as storage at 21^{o}C encourage proliferation of contaminant

CHAPTER 1

INTRODUCTION

1.0 IMPORTANCE OF THE STUDY

The use of neglected and underutilized crops which are likely valuable as animal and human foods must be increased to maintain balance between population growth and agricultural productivity, particularly in the tropic and subtropical area of the world in order to ensure food security (FAOSTAT. 2010). Food security is defined as a measure of ensured access to essential nutrition (Barrett 2010). “Food security” refers to the availability of and the level of access that individual households, communities, regions and nations have to food. A household (or any of the other social grouping - referred to here) is considered “food security” (Pepple, 2010). It is key to highlight 2 common definitions of food security from the United Nation’s Food and Agricultural Organization (FAO) and The United States Department of Agriculture (USDA) respectively. Food security exists when all people, at all times have physical, social and economic access to sufficient, safe and nutritious food to meet their dietary needs and food preferences for an active and healthy life (FAO, 2011). Food security for a household means access by members at all times to enough food for an active, healthy life. Food security includes at a minimum (1) the readily availability of nutritionally adequate and safe foods and (2) an assured ability to acquire acceptable foods in a socially acceptable way (that is without resorting to emergency food supply), scavenging, stealing or other coping strategies (USDA, 2008). The United States Department of Agriculture (2010) also, defines food insecurity as "limited or uncertain availability of nutritionally adequate and safe foods or limited”. Food insecurity is a condition that occurs when there is a lack of access to safe and nutritious food; preventing people from living healthy and active lives worldwide. In accordance to FAO *et al*., (2010), a total of about 925 million people are chronically hungry due to extreme poverty, 2 billion people lack food security due to various degrees of poverty. Also, over 60% of the world undernourished people live in Sub-Saharan Africa and undernourishment prevalence rate in Sub-Saharan Africa is above 35% (FAO 2015). There is the need for increased awareness of the urgency for the production of more food in order to

improve food security (FAOSTAT. 2010). Hence, the improvement in production, processing, preservation as well as development of new crops and products through mechanization, prevention of food wastages as a result of poor handling and also by adopting new methods of processing are important (FAOSTAT. 2010). There is also a need to develop and encourage the use of neglected and underutilized crops such as pigeon pea for domestic needs and export (FAO *et al*., 2012).

Food insecurity remains a major challenge in Sub-Saharan Africa. One out of seven adults goes to bed hungry in Sub-Saharan Africa (Shisana, 2013). In South Africa, 1 out of 5 children do not eat breakfast before going to school (Shisana, 2013). Hunger and starvation have been known to contribute to malnutrition (Bain *et al.*, 2014). Plant protein sources have been traditionally considered as part of cheap meals throughout the world as they have a major role in the fight against malnutrition. It is therefore necessary that the low consumption level of plant protein sources in developing countries be increased (Kaheel *et al.,* 2002).

Protein-energy malnutrition is a wide spread problem all over the world. It has been reported to have both health and economic implications and also described to be the most common nutrient deficiency condition in developing countries (FAO/WHO 2001). Normally, foods of animal origin are relied upon to meet protein requirement (Yusuf *et al.,* 2006). The high cost and inadequate supply of animal protein however, have brought about more research efforts towards the study of food properties and the potential utilization of plant protein, especially the underutilized and neglected high protein legumes (Ayuk *et al.* 2014). The research carried out by *Kaheel et al. (2002),* reported that plant protein provides about 65% of the world protein for human. Cereals and legumes contribute about 45% to 50% of these plant proteins. Out of these, legumes have been reported as the foremost source of dietary protein ranging from 20 to 40g/100g dry matter (Kaheel *et al.,* 2002). Over the past few decades, the utilization of plant based protein foods has increased because of better knowledge of their nutritive value, functional and processing properties (Foster *et al.,* 1999).

Legumes are low-glycemic-index and low-energy-dense foods containing high proportions of dietary fiber, vegetable proteins, oligosaccharides and phenol (Tharanathan and Mahadevamna, 2003). Pigeon pea is an underutilized legumes

grown in South Africa and several region of Sub-Saharan Africa. Pigeon pea is one of the tropical legumes and it is highly drought resistant. Due to climate change, there is advocacy that drought resistant crops such as pigeon pea be cultivated in areas with dry land in order to improve their utilization (Grazzino Da-silver, 2012). Also, soybeans have been widely studied and used for a variety of dairy like products including yoghurt. Soybean has also been used more than other legumes. However, other underutilized legumes like pigeon pea should be developed into alternative superfoods. Lately, due to the awareness of nutritional benefits of plant based food by health conscious consumers, the demand for yoghurt from non-dairy based sources such as soy yoghurt has been on the rise. Apart from the health benefits, plant based food consumption can be promoted to increase emphasis on plant-based proteins in place of animal-based proteins and this may contribute to the reduction of environmental impact of the current agricultural practices (Carlsson-Kanyama *et al.,* 2009). When assessing the overall grams protein obtained per kg of greenhouse gases produced, cooked soybeans yield 12 times more protein per kg than beef, and 2 times that of chicken. It may therefore be economically and environmentally efficient to increase consumer demand for plant foods (Carlsson-Kanyama *et al.*, 2009).

Yoghurt is one of the oldest and most common fermented dairy products known and consumed all over the world (Saint-Eve *et al.,* 2008). As the rate of consumption continues to rise, producers are continually investigating value-added ingredients such as probiotics, prebiotics and others for health-conscious consumers (Allgeyer *et al.,* 2010). Yoghurts are classified according to their physical properties of gels which include set, stirred and drinking. Materials used for the preparation are full to low fat, additives such as sweeteners, fruits and flavouring. There are other variations of products, including frozen-concentrated, dried, low calories and pasteurised yoghurt (Tamime, 1981; Shah, 2007); and of recent probiotic yoghurt. This versatility coupled with their acceptance as healthy and nutritious foods has led to their popularity across all population sub groups all over the world (Rheometry, 2011).

Probiotic foods are foods that contain live microorganisms, which after digestion, actively enhance the health of consumers by improving the balance of microflora in the gut' (Fooks *et al.,* 1999).

Probiotic organisms are micro-organisms when consumed in adequate amounts; confer a health benefit on the consumer (FAO *et al*., 2006). To realize most of these health benefits, adequate amount of viable probiotic organisms must reach the intestines. Their viability in product has also been cited as another important prerequisite for achieving beneficial health effects (Lourens-Hattingh *et al.,* 2001b). In order to observe health effects, the consumption of a minimum level of live microorganism is required. This level, depending on strain used and the required health effect is usually between 10^8 to 10^{11}CFU/g (Vanderhoof *et al.,* 1998). If 100g of fermented dairy products is eaten on a daily basis, they should contain between 10^6 to 10^9CFU/g of the live bacteria at the time of consumption. Hence different forms of delivering matrix should be studied and optimized to ensure that probiotic are viable and delivered in adequate numbers before the end of product's shelf life (Kailasapathy *et al.,* 2001).

Recently, some probiotic yoghurts have received attention due to their numerous health benefits. These health benefits have been attributed to products containing probiotics organisms. Many researchers have some of these benefits well documented and established, while others have shown promising potentials in animal models (Donkor, 2007).

These benefits include among others, production of anti-microbial compounds, assimilation of cholesterol, alleviate lactose intolerance, modulate host immune system, inhibit *Helicobacter pylori*, prevent autoimmunity and exhibit anti-mutagenic properties gut infections (Parvez *et al.* 2006; Riedel *et al.* 2006; Paturi *et al.* 2007; Cousin *et al.* 2011; Moslemy *et al.* 2015). These beneficial effects have been linked to the production of propionic acid, folacin, bacteriocins (Kiatpapan *et al.,* 2001), (Kiatpapan and Murooka 2001; Cousin *et al.* 2012), vitamin B_2 and vitamin$B_{12,}$ (Skupin *et al.,* 2006) and the formation of exopolysaccharide (Mohammadi and Mortazavian 2011). The mechanism through which probiotic organism exerts a health benefit in vivo is not clearly understood (Donkor, 2007).

But it is known that some strains produce certain health promoting metabolites such as proteins and fatty acids which are helpful in the nutritional and physiological perspectives. However, it is noteworthy that ingestion of probiotics in fermented foods open up the possibility that the health promoting metabolites may also be produced in vivo (Tamime, 2008).

Some bacteria which have been used are mostly bifid bacteria and lactic acid bacteria, e.g. *Lactobacillus bulgaricus* is normally used in conventional yoghurt. However, studies have shown that many bacteria also display probiotic properties, some of which have a long history of use are the dairy *Propionibacteria* (Cousin *et al.,* 2011). These are food grade bacteria capable of utilizing carbon source to produced propionic acid as a main metabolic end product (Cousin *et al.,* 2011).

Lourens-Hattingh *et al.,* (2001b) stated that dairy and soy foods may serve as an ideal system for the delivery of probiotic bacteria to human gastro intestinal tract due to the provision of favourable environment that promotes growth and enhances the viability of the organisms. Foods that promote growth and enhance the probiotics viability are referred to as prebiotics. Prebiotics has been described as nondigestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of some bacteria in the colon such as *Bifidobacterium* (Ziemer *et al.,* 1998). The nutritional supplements combining probiotics and prebiotics in a form of synergism is however referred to as symbiotic (Ziemer *et al.,* 1998).

Pigeon pea yoghurt may serve as an ideal system for delivery of probiotic bacteria to human gastrointestinal tract (GIT). Since similar favourable environment that promotes growth and enhances the viability of these organisms also exist in pigeon pea milk as its composition is similar to that of soy milk (Table 3.3). Pigeon pea milk is an aqueous extract from dehulled pigeon pea seeds. On the other hand, pigeon pea milk yoghurt is a fermented pigeon pea milk with mixed starter cultures consisting of *Streptococcus thermophillus and Lactobacillus delbrueckii spp. bulgaricus*. The addition of probiotic organism with the yoghurt cultures as co-culture or supplement after or during fermentation makes it a probiotic yoghurt.

Pigeon pea milk based yoghurt may offer an alternative to consumers with dietary and health concerns. It has several nutritional advantages such as low level of

cholesterol and unsaturated fats as well as absence of lactose and allergies (Philips, 2014). In addition, pigeon pea contains raffinose, starchyose, pentanals, n-hexanal and oligosaccharides. These raffinose, starchyose, pentanals, n-hexanal, oligosaccharides are used during fermentation for bacteria growth and conversion into a range of products such as acetic acid, lactic acid, and different vitamins thus, removing the potential cause of flatulence and also reduce the levels of pentanals and n-hexanal which causes beany flavour (Desai *et al.,* 2002; Islam *et al,* 2011).

Propionibacterium freudenreichii displays a peculiar central metabolic pathway Wood Werkmann cycle results in production of propionates, acetate, succinate and carbon dioxide from any carbon source (Nik *et al.,* 2008). According to the studies carried out by Nagpal *et al.*,(2012), the addition of probiotic organism such as *Propionibacterium freudenreichii* in fermented products has the potential to improve the quality of the product and health status of the consumers (Nagpal *et al.,* 2012).
Pigeon pea is an under-researched and underutilized legume. It, however, contains high nutrients that could provide rich vegetable flour, nutritious milk, yoghurt and probiotics yoghurt. The milk can be potentially used as alternative source for yoghurt production. It may also be utilized as coffee creamer in chocolate-milk and other drinks. Although production of milk and yoghurt from other legumes have been reported in literatures. However there is no report on pigeon pea milk yoghurt, and pigeon pea milk yoghurt supplemented with *Propionibacterium freudenreichii*. This research therefore will contribute useful information on potential utilization of pigeon flour, and milk in food and related industries and could add value to this underutilized legume.

1.1 Aim

The aim of this work is to develop pigeon pea milk based yoghurt, using *Propionibacterium freudenreichii* as an adjunct culture, for health conscious consumers.

1.2 OBJECTIVES

The objectives of this study are:

To evaluate Pigeon pea, milk yield, suitability as a matrix for yoghurt, probiotic yoghurt processing, textural and nutritional profile of the yoghurt Samples

- To determine storage stability, pH and titrate-able acidity (TTA), colour, water holding capacity and syneresis of pigeon pea and soy milk yoghurt, and yoghurt supplemented with *Propionibacterium freudenreichii.*
- To determine the anti-nutritional factors of pigeon pea flour, pigeon pea and soy milk, pigeon pea and soy milk yoghurt, pigeon pea and soy milk yoghurt supplemented with *Propionibacterium freudenreichii.*
- To determine the consumer acceptability of pigeon pea and soy milk yoghurt and pigeon pea and soy milk yoghurt supplemented with *Propionibacterium freudenreichii.*

Chapter 2 of this thesis contains a thorough literature review of recent scientific knowledge on the proposed subject.

Chapter 3 contains milk yield, textural quality of yoghurt and nutritional quality of the flour, milk and yoghurt samples produced.

Chapter 4 reports on the microbiological quality and storage study, pH, TTA, colour, water holding capacity and syneresis of yoghurt with and without *Propionibacterium freudenreichii*.

Chapter 5 explores the influence of processing on the anti-nutritional components of the flour, milk and yoghurt produced.

Chapter 6 accesses the consumers' acceptability and principal component analysis carried out on yoghurt samples produced while chapter 7 focuses on general conclusion and recommendations.

CHAPTER 2

LITERATURE REVIEW

Legumes belong to the family *Leguminosae* in which the pods and seeds of the plant are eaten (Pszczola, 2009). They are among the most nourishing edible seeds eaten by man. Some 100 or more species of beans have been reported to be cultivated throughout the world (Pszczola, 2009). Legumes certainly have been described to have a "colourful" history, demonstrating a wide range of colours and colour combinations, different sizes, shapes, and flavours (Pszczola, 2009). The world production of legumes was 23.2 million metric tons in 2010, which represented an increase of almost 70% as compared to that in 1980. From 1980 to 2000, dry production was reported to have increased by 3.9 million metric tons. However, a considerably higher increase of 5.6 million metric tons was reported in only the ten year- period from 2000-2010 (FAOSTAT 2010).

Most regions of the world have experienced substantial increase in production and use of legumes during the past three decades. Africa, Asia and America had an increase of approximately 145, 36 and 49% respectively. However, a 36% decrease in legume production was reported in Europe (FAOSTAT. 2010).

Legumes occupy an important place in human nutrition, especially among low income group in the developing countries *(*Siddiq *et al*., 2010b). They are good sources of protein range (from 19.61 to 30.12%) dietary fibres, (4.42-5.0 %) carbohydrates (52.6364.20%), minerals (2.62-4.40%) and vitamins (Table 1). They are staple food and cheap source of protein in developing countries where Protein Energy Malnutrition (PEM) is prevalent (Siddiq *et al.,* 2010a). Legume crops are widely spread the world over, and they are reported to have demonstrated global adaptability, genotypic, phenotypic diversity and different means of preparation and dietary use (Uebersax *et al.,* 2012). Many factors have been reported to influence the use of legumes such as the type, cultivar selection, cropping environment, system and storage condition, handling infrastructure, processing and final product preparation (Uebersax *et al.,* 2012). Legumes are well known for their therapeutic properties. They contain large quantity of phenolic compounds and flavonoids (Yao *et al.,* 2004). These compounds have been linked to the therapeutic effect of legumes. For example, they have been reported to

have many beneficial physiological effects in control and preventing various metabolic diseases such as diabetes mellitus, coronary heart diseases and colon cancer (Tharanathan *et al,*. 2003).

Legumes are classified into oil seeds and pulses. The oil seeds are soya beans, peanuts, marama beans etc., while the pulses are Adzuki bean, Beach bean, Black bean, Black gram, Cow pea, Faba bean, Grass pea and Pigeon pea etc. (Hoover *et al.* 2010). Their different shapes, sizes, colours and colour combination are shown below Figure: 1) while their common and scientific names are presented (Table 2.2.). Pulses are dicotyledonous plant seeds which have 16,000 to 19,000 species in approximately 750 general (Ratnayake *et al.*, 2001). Chemical composition of some pulses, including Pigeon pea are presented (Table 2.1).

Table 2.1 Chemical composition of some pulses

Legume types	Species	Moisture	Protein	Fat	Ash	Fibre	Carbohydrate
Pigeon pea	Cajanus cajan[a]	4.91	20.50	3.80	4.20	5.00	64.20
Bambara	Vigna subterranean[b]	4.30	20.70	6.00	4.40	3.30	61.30
Cowpea	Vigna unguiculata[c]	11.55	19.61	1.40	3.20	2.92	63.24
Chick pea	Cicer arietiub[c]	8.01	22.83	5.43	3.04	3.50	57.19
Lentil	Lens culinaris[c]	9.14	31.12	0.81	2.62	3.68	52.63
Broad bean	vicia faba[c]	12.97	22.61	2.67	2.97	2.46	56.39
Kidney bean	Phaseolus vulgaris[c]	9.15	20.09	2.46	3.85	3.78	57.6

(Akande *et al*, 2010)[a] (Sexena *et al., 2008*)[b] (Anonymous, 2014)[c] (Qayyum *et al.,2012)*. Values are reported on % wet basis.

Table 2.2 Common and Scientific Name for Pulses

Common name	Scientific name
Azuki bean	Vigna ungu
Beach pea	Lathyrus maritimus
Black bean	Phaseolus vulgaris
Black gram	Phaseolus mung
Chick pea	Cicer arietinus
Cow pea	Vigna unguculata
Faba bean	Vicia faba
Grass pea	Lathyrus sativus
Horse gram	Dolichos biflorus
Jack bean	Phaseolus vulgaris
Kidney bean	Len culinaris
Lentil	Phaseolus lunatus
Lima bean	Phaseolus acontifolius
Moth bean	Phaseolus vulgaris
Navy bean	Phaseolus vulgaris
Northern bean	Phaseolus vulgaris
Pea(smooth)	Pisum sativum
Pea(wrinkled)	Pisum sativum
Pigeon pea	Cajanus cajan
Pinto bean	Phaseolus vulgaris
Velvet bean	Mucuna prurien
Yam bean	Pachyrhizus ahipa+

Source: Adapted from Hoover *et al.* (2010)

2.1 THE POTENTIALS OF LEGUMES

Legumes generally require dry cleaning and sorting, gentle handling to assure a minimum degree of mechanical damage, and soaking and blanching prior to filling and thermal processing (Siddiq *et al.*, 2012). The popularity of ease for the development of new legume products or legume formulations such as dehydration, extrusion, frozen and microwaveable food product, especially in developing countries should be encouraged.

The different grades of dry-legumes-based products used in industrialized regions, with wide market distribution channels, include package dry beans, canned beans (in brine or special sauce), pre-cooked legumes products which include the following pre-cooked, dehydrated, flakes powder, extruded and pasta types

products, specialized food ingredients, meal, flours, concentrate quick-cooking beans and frozen bean, are used in food formulations (Uebersax *et al.,* 2012). Such formulations include bread or cookies made from beans ready to use ingredients that include natural bean-powders and ground cooked beans (Uebersax *et al.,* 2012).

Legume powders can be added to foods such as baked foods snacks, soups, gravies, dips, cereals, and dry mixes without affecting taste or texture unless so desired (Pszczola 2009). Children, who do not like to eat vegetables can get up to full serving of vegetable in an unexpected ways, like in bread on their sandwich (Gregory, 2009). Legumes powders was reported to have been used to replace 7 to 30% of flour in bread, cakes, cookies, pizza crust, tortillas, dough and also in traditional uses' such as a refried bean dips, bean soup and puddings (Gregory, 009). Protein quality is generally not a nutritional problem for consumers who have mixed diets containing both vegetable and animal proteins. A diet high in legume can actually reduce the risk of developing a chronic disease (Krasaekoopt *et al.,* 2003). Chronic diseases are conditions that typically take many years (10-30 years) to develop and include certain types of cancer, diabetes mellitus, heart disease, and other diseases of the blood system (Siddiq *et al.,* 2012). The inclusion of dry legumes in daily diet has many beneficial effects in controlling and preventing various metabolic diseases such as diabetes mellitus, coronary heath disease and colon cancer (Flight *et al.,* 2006; Dilis *et al.,* 2010; Raju *et al,*. 2011). However, anti-nutritional factors are associated with legumes. On the other hand, they can be overcome with different strategies, including cereal-legume protein complementation (Hernandez *et al*., 1995). Digestibility and flatulence-producing components still present current restriction of their broad utilisation, especially when feeding legumes to children (Siddiq *et al.,* 2012). The use of appropriate preparation techniques such as soaking, cooking, dehulling, grinding and roasting whole beans, germination and fermentation however have been reported to improve digestibility and reduced flatus from legumes (Mubarak, 2005a). A number of traditional weaning food have been developed from legume and food mixtures have been prepared, using pre-heated legumes and cereals (Siddiq *et al.,* 2012). Prepared

drum- dried legume meal can provide both protein and energy to infants as well as offer preparation convenient for mothers (Uebersax *et al.,* 1991). (FAO 2015) has developed a detailed guide line for preparation and use of weaning food. Numerous United States public private sector groups (e.g. USAD, USAID, Gates foundation) have made significant impact on developing weaning mixes and guidelines (Siddiq *et al.,* 2012).

2.2 PIGEON PEA (*Cajanus Cajan*)

The origin of pigeon pea is uncertain. It is known to have been cultivated in Egypt before 2000 BC (Akande *et al.,* 2010). However, the crop was likely introduced into East Africa from India Immigrants in the 19th century (Odeny, 2007). The crop represents about 5% of the world legume production, with more than 70 % being produced in India (Sharma *et al.,* 2011). Substantial amount of pigeon pea is also produced in Africa (Odeny, 2007). Global annual production of pigeon pea was around 3.7 million metric tons in 2010 (FAOSTAT.2010). Pigeon pea is widely distributed all over Africa, including South Africa. Because of the subsistence nature of the crop, production figures are grossly underestimated (Odeny, 2007). They are grown in more than 25 tropical and subtropical countries, either as a sole crop or intermixed with cereals or with other legumes such as groundnut (*Arachis hypogaea*) (Odeny, 2007). Being a legume, it enriches the soil through symbiotic nitrogen fixation (Van der, 1995). They are drought resistant, and therefore can be grown in areas with less than 650mm annual rain fall (Fuller, 2006). Figure 2.1 below shows matured dried pigeon pea seeds.

Figure 2.1 Matured dried pigeon pea seeds two types of land races

Pigeon pea ranks 5th in importance among edible legumes of the world (Morton, 2011). Pigeon pea is considered an important legume grain for human nutrition in many of the protein-deficient tropical countries of the world as they serve as supplement to cereal based diet. The genotype, growth conditions, duration and condition of storage are some of the factors that determine a wide variability that exist in seed composition (Akande *et al.*, 2010b).

2.2.1 Nutritional value of Pigeon Pea

Pigeon pea seed have been reported to contain 85% cotyledon, 14% seed coat and less than 1% embryo (Saxena *et al.*, 2010). Carbohydrate and proteins are major constituents of the cotyledon, embryo, and the seed coat (Table 2.3). Quantitatively, the cotyledon (66.7%) and seed coat (58.7%) contain substantial amounts of carbohydrates**.** Protein (49.6%) constitutes a main portion of the embryo. Carbohydrates and fat are also present in significant quantities in embryo. About one third of seed coat is made up of fibres as shown in Table 2.3. The seed also contains amino acids, calcium, fibres and iron (Table 2.3).

The contents of methionine and cysteine, the sulphur-containing amino acids, range from 1-1.7% and they predominately reside in the cotyledon and embryo (Table 2.3). Calcium is predominantly in the seed coat and embryo. The protein and carbohydrate content of pigeon pea is comparable with other legumes. However, the fat and ash content are relatively low compared to those reported for cowpea (Table 2.3). Furthermore, the amino acid composition of pigeon pea has been reported to be compared with other legumes favourably. The lysine content is slightly higher than those reported for cowpea and soya bean (Table 2.5)

Table 2.3 Distribution of nutrients in mature pigeon pea seed

Constituent (%)	Whole seed	Cotyledons	Embryo	Seed coat
Carbohydrates	64.2	66.7	31.0	58.7
Protein	20.50	22.20	49.60	4.90
Fat	3.80	4.40	13.50	0.30
Fibre	5.00	0.40	1.40	31.90
Threonine	3.80	4.30	4.70	2.50
Ash	4.20	4.20	6.00	3.50
Lysine	6.80	7.10	7.00	3.90
Methionine	1.00	1.20	1.40	0.70
Cystine	1.20	1.30	1.70	-
Calcium	296	176	400	917
Iron	6.70	6.10	13.0	9.50
Thiamine	0.63	0.40	-	-
Riboflavin	0.16	0.25	-	-
Niacin	3.10	2.20	-	-

Source: Faris and Singh (1990)

Table 2.4: Comparative composition of Pigeon pea to other legumes

Legumes	Protein	CHO	Fat	Ash	Fibre
Bambara groundnut	32.40	51.79	7.36	5.78	2.68
Cow pea	24.13	56.60	4.37	4.73	0.97
Mung bean	26.37	59.80	1.10	4.30	4.30
Kidneybean	20.09	57.67	2.46	3.85	6.78
Soybean	35.90	26.80	24.90	6.60	5.00
Pigeon pea	22.40	64.20	3.80	4.20	5.00

[1]Values are reported in% dry basis [2]sources; Nwodo *et al*(2012); [b]Mazahib *et al*(2013); [c]Arawande *et al* (2010); [d]Amarteifio *et al*(1998); Qayyum *et al*(2012); Ologhobo *et al* (1984)

Table 2.5: Amino acid composition of some legumes protein

Legume type	Asp	Glu	Lys	His	Ile	Leu	Met	Val
Pigeon pea	11.5	9.23	7.79	3.66	3.47	6.78	1.19	5.85
Bambara	9.6	15.4	6.3	3.0	3.8	7.3	1.8	4.3
Soybean	11.4	16.9	6.1	2.5	4.6	7.7	1.2	4.6
Peanut	12.1	21.1	3.8	2.5	3.5	7.0	1.3	3.9
Cowpea	12.2	18.9	6.9	2.5	4.8	7.7	1.2	5.4
FAO/WHO			5.8	1.9	2.8	6.6	1.7	3.5

Amino acids are expressed in g/100, source: Kudre *et al* (2013); Ijarotimi *et al* (2009), Akande *et al* (2010). FAO/WHO: recommended pattern

2.2.2 Anti-nutritional factors of Pigeon Pea

As part of composition of legumes, pigeon pea contains some amount of antinutritional factors, such as raffinose, starchyose, verbascose, polyphenols, lectins and enzymes inhibitors (Saxena, Kumar and Sultana 2010). Singh (1988) studied a number of pigeon pea genotypes by quantifying the important anti-nutritional factors and toxic substances and found a large variation among genotypes for these traits. Amylase and trypsin inhibitors and polyphenols were said to be found in significant quantities. Flatulence causing sugars were reported to be found also in appreciable quantities. Unavailable carbohydrates which characteristically reduce the bioavailability of important nutrients have also been reported (Saxena *et al.,* 2010). The coat is rich in anti- nutritional factors and this is of great importance where whole seeds are eaten, especially in areas where de-hulling facilities are not on ground. In India where seed coats are removed, the large amounts of tannins present in the coloured pigeon pea, pose no problem in its consumption (Saxena *et al*., 2014).

White seeded cultivars which contain relatively less quantity of anti-nutritional factors compared with brown and light brown seeded pigeon pea are predominantly grown (Saxena *et al*., 2014). Williams *et al.* (1987) compared the anti-nutritional factors of the white, brown and light brown seeded and reported that quantity of polyphenols in the brown coloured line were more in white seeded. Enzymes

inhibition activity was also observed to be larger in the coloured pigeon pea compared to the white seeded (Williams *et al.,* 1987)**.**

Different modern processing methods and various traditional treatment such as soaking, cooking, dehulling, fermentation and germination have been employed to improve the nutritional quality of food legumes to various degrees (Trugo *et al.*, 2000; Mubarak 2005b). For example, cooking of pigeon pea have been reported to improve the bioavailability of nutrients and destroyed some anti-nutritional factors (Saxena *et al.,* 2002). Heat treatment also enhance starch digestibility. Lines of pigeon pea which takes a longer time to cook faces the danger of losing vital vitamins from the food. Cooking of pigeon pea after germination enhances the digestibility of starch and also lowers the levels of oligosaccharides (Reddy *et al.*, 1984). Fermentation of the seed has also been found to help in decrease of inhibitory activities of digestive enzymes (Singh *et al.,* 2014).

Soaking has been reported to reduce anti-nutritional factors, particularly oligosaccharides and raffinose family (Sharma *et al.,* 2011). Maximum loss of phytate occurs when pigeon pea is soaked in water. High content level of phytate is lost with the discarded soaking water. The amount of the loss increases with an increase in the time of soaking (Sharma *et al.,* 2011). The reduction in phytate contents of pigeon pea during soaking can be described to leaching out of the anti-nutrients into soaking media under the influence of the concentration gradient. Soaking pigeon pea in water for 24 hour at room temperature and at 55^{o}C decreased the phytate content by 50 % and about 90% respectively (Sharma *et al.,* 2011).

2.2.3 Medicinal Properties of Pigeon Pea

Pigeon pea has also been reported to have various medicinal properties due to the presence of polyphenols and flavonoids in different parts of the plant. In Africa, Asia and South America, different parts of the pigeon pea plants are used for management of various disorders such as joint pain, ulcer, diarrhoea, sores, cough, dysentery, hepatitis, measles (Grover *et al.*, 2002; Tarak *et al.,* 2011; Liang *et al.,* 2013). Other authors have reported that the plant can be used to treat diarrhoea, gonorrhoea, burns, eye infection, ear ache, sore throat, sore gums amongst others (Leevy *et al.*, 1976; Duke *et al.,* 1981; Van der Maesen, 2006). Extracts of pigeon

peas have been reported to display various pharmacological activities such as hypoglycaemic, hypolipidemic, hepatoprotective, nepehroprotective, activities (Risvik *et al.,* 1997) anticancer (Ashiddi *et al*., 2006, 2007, 2010; Luo *et al*., 2010), anti-plasmodia (Duker Eshun *et al.,* 2004), anti-sickle cell anaemia activity (Ekeke *et al.,* 1990), anti-osteoporotic activities (Zhang *et al*., 2007). Also, stilbenes extracts from pigeon pea have been said to have potential action in treatment of postmenopausal osteoporosis (Zhang *et al*., 2007).

2.2.4 Utilization of pigeon pea

The supplementation of cereals with protein rich legumes is one of the best keys to protein malnutrition in developing countries (Boye *et al.,* 2010). Pigeon pea protein may serve as diet for vegetarians and also in the development of imitation milk and their product. Production of dairy like product such as cheese analogue, milk chocolate, milk powder, buttermilk and yoghurt have been developed from peanut and cowpea (Isanga *et al.*, 2009; Aidoo *et al.*, 2010). To provide nutritious diet among vulnerable sections of the population in developing nations, pigeon pea flour has been studied to assess its high level of protein, iron and phosphorus content potentials (Harinder *et al.* 1999). Pigeon pea has been endorsed for making of porridge for school feeding programmes and other vulnerable sections of the population in developing nation (Harinder *et al.* 1999).

2.3 SOYBEANS (GLYCINE MAX)

The soybean originate in Southern China (Liang *et al.*, 2013). They belong to the family Leguminosae, subfamily papilonoidaa, and genus glycine. Cultivated in various geographical locations and under many different growing conditions responsible for their large genetic diversity.

2.3.1 Nutritional value of Soybean

Major component are protein, fat and carbohydrates. Protein makes up 40% dry weight of the soybean. Soy protein prior to processing is not highly bioavailable due to inhibition factors such as trypsin inhibitors, urease, and hemagglutinin (Singh *et al* 2008; Whent, 2013). Even traditional soy foods such as tofu or edamame are processed with heat before they are consumed. Soy protein contains all of the

essential amino acids for humans, which makes it unique among plant-based proteins (Singh *et al.*, 2014). Soybean contains an average of 15 to 20% oil by weight. The major fatty acids are palmitic, stearic, oleic, linoleic, and α-linolenic (Sugano, 2006). Soybeans also contain phenolic, and isoflavones, including tocopherols and carotenoids, which provide antioxidants as well as vitamins to the diet.

2.3.2 Medicinal value of Soybean

Soy foods have been known for their use in chronic disease prevention, especially against osteoporosis, coronary heart disease, and some types of cancer (Isanga *et al.*, 2008). Phenolic compounds are known to contribute to the antioxidant capacity of food. Diets high in phenolic are associated with reduced risk of heart disease and some types of cancer (Del Rio *et al.,* 2013). Although known as a health-enhancing food, soybeans and products produced from them can develop undesirable sensory properties which make them less acceptable to consumers (Chambers *et al.,* 1996). High content of polyunsaturated fats and oxidative enzymes inherent in soybeans may partially be responsible for the perceived poor flavour of soy products. Consumers usually consider flavour first when deciding to purchase a food. However, consumers may likely purchase a functional food if they know its health benefits (Guo *et al.,* 2015).

2.3.3 Utilization of Soybean

Soybeans and their products are mostly used as animal and human food products. Soybean oil, however, is extracted and used extensively in human foods. The U.S. Food and Drug Administration allowed a health claim for soy protein in 1999 (Huang *et al.,* 2014) and this helped to increase public awareness of soy as a health beneficial food. The use of soybean includes traditionally Asian foods (tofu, tempeh, natto, miso, and soymilk) (Golbitz & Jordan, 2006), as well as vegetarian foods and mock meats. The soy bean components such as oil, protein, lecithin, fibre, and sterols are used in processed foods to enhance both functionality and nutrition (Sugano, 2006).

2.4 LEGUME MILK

Vegetables do not secret milk like animals. However, there are products extracted from oilseeds, pulses and cereals that have the properties close to that of dairy milk. The most commonly used legume in milk production is soybean which has nutrient content close to dairy milk (Prado *et al.*, 2008). Extraction of milk from vegetable is an old technology that has been modified to produce dairy like milk and milk products. Some legumes that have been used are soybean, cowpea, wing beans, peanuts and melon seed, Chickpea, black graw, mung beans, coconut, lupine and sunflower (Quasem *et al.*, 2009; Rivera-Espinoza *et al.*, 2010). Some of these milk products have been used to produce different kinds of milk based like products such as coffee creamers and chocolate milk drink (Aidoo *et al.*, 2010).

Legume milk and their products have been found suitable for vegetarians, people who are lactose intolerant, those allergic to cow milk proteins, consumers on cholesterol free and dairy free diet (Granato *et al.*, 2010b). Legume milk is extracted, using similar methods. Akinyele and Akinlosotu (1991) explained the extraction of cowpea milk. The cowpea seeds were soaked overnight, dehulled and re-soaked again for 24hours. The re-soaked seeds were blended and milk obtained was heat treated and homogenised. The methods are only slightly modified, depending on the type of legume and the purpose of the milk. In production of milk from legumes the beans are heated to a temperature of about 98^{o} C for several minutes. The heating is done to deactivate trypsin inhibitors and Lipo-oxidase. This process enhances the removal of the beany taste linked with the legume milk (Brough *et al.*, 1993). Deactivation of trypsin inhibitors is essential because they have been reported to be the cause of soy milk indigestion in the stomach due to reduction of the enzyme trypsin activities in the stomach (Brough *et al.*, 1993; Quasem *et al.*, 2009; Murevanhema *et al.*, 2013). After grinding, the liquid is separated from solid and the resultant liquid is milk. The milk is then refrigerated (Aidoo *et al.*, 2010).

2.4.1 Adaptation of the legume milk for survival of probiotics

Vegetable milk being different from the dairy milk is treated in such a way to make it more favourable to yield a product similar to their dairy counterparts (Granato *et*

al., 2010a). The structural features of fruit and vegetables matrices need to alter in order for an ideal substrates for probiotic cultures to be realized. Such modifications include among others pH and fortification of culture media. Legume milk contains favourable nutrients, such as minerals, amino acids, vitamins and bioactive substance, while lacking the dairy allergy which will encourage consumption by certain segments of the population.

When culture stimulants are not added, the fermentation process is prolonged (Nsofor *et al.,* 1996) or starter concentration has to be increased (Tamime *et al.,* 1999). Ideal vegetable milk should be inoculated with organisms capable of fermenting galactose oligosaccharides such as raffinose and starchyose in soybeans (Nsofor *et al.,* 1996). Nsofor *et al.* (1996) investigated acid production rates in soymilk with 1% sucrose and cow milk, their results indicated a non-dependence of culture on milk constituents. Non-dairy stimulants, however, may be needed to achieve high activity at low starter concentration (1-3%) normally used for yoghurt production (Granato *et al.*, 2010b).

Yeast extract has been shown to prompt high activity in cheese starter culture, and may also stimulate vegetable milk starter culture (Uvere *et al.,* 1999). Several sub culturing stages in vegetable milk during its development may induce synthesis of enzyme that hydrolyses oligosaccharide which causes flatulence in humans as reported in soy milk (Quasem *et al.*, 2009). Repeated sub culturing may lead to great reduction of starchyose concentration in pigeon pea yoghurt, as reported for soy yoghurt (Nsofor *et al.,* 1996).

Propionic acid bacteria may play a crucial role in pigeon pea milk fermentation since they have been reported to reduce flatulence due to raffinose after consumption (Wu *et al.,* 2012). The type and quantity of carbohydrates available, and degree of hydrolysis of the milk proteins which in turn determines the availability of essential amino acids, plays a significant role during the fermentation process (Heller, 2001). Proteolytic and lipolytic properties of probiotics may be important for further degradation of proteins and lipids which affect the taste and flavour of the end product (Heller, 2001). Probiotics viability in the food matrix

depends on factors, such as pH, storage temperature, oxygen levels, and presence of competing microorganisms and inhibitors (Granato *et al.*, 2010a). It is important that the pigeon pea beverage formulation maintains the activity and viability of the probiotic for extended period of time.

2.5 MICROBIAL FOOD CULTURES

Microbial food cultures include bacterial food cultures, fungi and yeast. Bacterial food cultures is subdivided into "starter cultures" and "probiotics". "Starter cultures" are those traditionally used in the fermentation of food (Stevens *et al.*, 2009). Probiotics are live microorganisms which, when used in adequate amounts, confers health benefit on the host (FAO /WHO2006). The presence of living organisms in traditional fermented foods is well known and have been reported to be the subject of scientific research for over a century (Stevens *et al.*, 2009). The organisms in many cases are found to determine the quality of the fermented product, such as acidity, flavour, and texture, as well as the health benefits that is beyond simple nutrition (CFR, 2007). These characterizing organisms may be present as natural micro-flora of the food or as a result of the intentional addition of organisms as starter cultures in food fermentation process (Stevens *et al.*, 2009).

Lactic acid producing bacteria, as part of traditional fermented dairy food predate history (Sardine, 1979). The presence and action of bacteria have a basic effect on the level of acidity, moisture content, texture and shelf life of the fermented food. From the past studies, such bacteria naturally present in fermented food such as yoghurt can be said to be a natural part of the traditional food in the same way that acetic acid is natural in lemon juice. Neither the bacteria in the traditional fermented food nor the acetic acid in the lemon are reported to be ingredients of the food. They are, in all respects, part of the traditional food (FDA 2001).

In general, regulations state that a large number of bacteria, which provide a wide range of functions in fermented foods, are foods and thereby, not openly regulated (Wessels *et al.*, 2004). The use of these bacteria must align in most cases to general food safety standards such as the European General Food law (European Parliament and the council, 2002).

2.5.1 Probiotics

The term "probiotics" is from Greek, meaning for Life. It was first used to describe substance secreted by one microorganism which encourages the growth of another microorganism (Lilly *et al.,* 1965). Probiotics are live microorganisms which, when used in adequate amounts, offers health benefit on the host (FAO/WHO, 2006). Different species of genera *Lactobacillus* and *Bifidobacteria* are mainly used as probiotics. Other species that have also been reported used over the years include *Escherichia coli, Nissle, Saccharomyces boulardii*, *Streptococcus thermophillus*, *Enterococcus francium, Pedi coccus and Leuconostoc* (Boyle *et al.,* 2006; Shah 2007; Tharani, 2012). Apart from *Lactobacillus* and *Bifidobacterium* that are often used as probiotics, there are concerns regarding the safe use of these genera that has many pathogenic species such as Enterococcus amongst others (Senok *et al.,* 2005). Culturing bacteria used for yoghurt making such as *Streptococcus salivanus subsp. Thermophillus* and *Lactobacillus delbrukeii subsp. bulgaricus* which have been traditionally used in yoghurt production are not considered probiotics because they are not able to survive and grow in host intestinal tract and are regarded as bacteria cultures (Senok *et al.,* 2005; Tharani, 2012). *Lactobacillus, Streptococci* and *Bifidobacterium* which are part of intestinal micro flora that produce lactic acid as major metabolite, are currently used in probiotic preparations (Penner *et al.,* 2005). Lately some evidence has shown that dairy *Propionibacterium* strains possess potential probiotics effects and can therefore be used as probiotic organism. This organism has been reported to have various beneficial effects such as the production of propionic acid, folacin and bacteriocins (Kiatpapan *et al.,* 2001b), vitamin B2 and vitamin $B_{12,}$ (LeBlanc *et al.,* 2006) stimulation of *Bifidobacterium* growth and favourable effects on the lipid metabolism and immune system (Kiatpapan *et al.,* 2001a). The above characteristics have recommended their use as dietary supplement and reveal their favourable effects on improvement of the nutritional and therapeutic quality of the food, extension of shelf life of product, inhibition of growth of pathogenic and harmful micro flora (Al Zoreky *et al.,* 1993) and contribution to better organoleptic properties to the product (Mantere-Alhonen, 1995). Some food substances referred to as prebiotics may also be added to improve

on the nutritional, textural and therapeutic quality of yoghurt. These substances such as resistant starch are naturally present in legumes (Bernat *et al.,* 2014).

2.5.2 Prebiotics

In accordance to Ziemer *et al.* (1998) prebiotics are non-digestible food substance that beneficially affect the host by selectively stimulating the growth and/or activity of some bacteria in the colon such as *Bifidobacterium*. Their selectivity has been confirmed for *Bifidobacterium*, whose growth has been reported to be promoted by the uptake of substances such as fructo-oligosaccharrides, transgalactosylated oligosaccharides and some legume oligosaccharides such as in soybean (Schrezenmeir and Vrese, 2001). Beside their prebiotic properties, certain oligosaccharides have shown a number of functional effects on the gastrointestinal tract (GIT) physiology. These include, reduced-fat-and cholesterol absorption, modulation of microbial proliferation, which may subsequently reduce intestinal disturbances, cardiovascular disease and intestinal cancer (Ziemer *et al.,* 1998). According to some scholars, inulin and other fructo-oligosaccharides which are often used as prebiotics, resist digestion by gastric acid and pancreatic enzymes *in vivo* (Cummings *et al*., 2001). Inulin are hetrogenous blends of fructose polymer which are widely distributed in nature as plant storage carbohydrates (Niness, 1999). Their use as a functional food ingredient has been described to offer a unique combination of exciting nutritional properties and some important technological benefits (Roberfroid, 2004). Furthermore, addition of inulin to yoghurt plays an importance role in yoghurt as the prebiotic effect, which they exert on probiotics to enhance their action as probiotic cultures added to yoghurt (Niness, 1999). Inulin is a component of many regularly consumed vegetables, fruits and cereals, including leek, onion, garlic, wheat, chicory, artichoke, and banana.

Besides their role as development promoter, when allowed to ferment such as in Hi-maize, many species of these bacteria produce a range of potentially beneficial short-chain fatty acids (SCFA) such as acetate, propionate, and butyrate (Brown *et al*., 1998) SCFA that are normally produced in the colon, include butyrate which is involved in regulating intestinal cell functions and growth by suppressing tumour cells and decreasing the proliferation of colonic mucosal cells (Johnson and Gee,

1996). When probiotic and prebiotic are combined, for example, in yoghurt, it is known as symbiotic.

2.5.3 Symbiotics

Symbiotics refer to nutritional supplements combining probiotics and prebiotics in a form of synergism. When probiotics and prebiotics are rightly combined, they improve the survival and activity of the fermenting organism, for example fructans combined with a *Bifidobacterium* strain (Gibson and Roberfroid, 1995). The combination of prebiotic and probiotic result in synergistic effects, and in addition, the growth of inherent strains of beneficial bacteria in the colon is promoted. Symbiotics also act to improve the survival, implantation and growth of newly added probiotic organisms like when probiotic yoghurt is consumed. The symbiotic concept has been widely used by dairy drink and yoghurt manufacturers in many countries in Europe (Niness, 1999).

2.5.4 Classification of *Propionibacterium*.

The genus *Propionibacterium* has been reported to be divided into two groups based on their habitant of origin: Classical of dairy *Propionibacteria* and cutaneous *Propionibacteri*a (found in skin) (Downes *et al.*, 2009). Dairy 'dairy *Propionibacteria*' are made up of six species in the genus: *Propioni freudenreichii, Propionic acid propionici, Propioni jensenii, Propioni miroaerophilum*, *Propioni freudenreichii subsp freudenreichii,* and *Propioni thoeni.* They are also called classical species. They are mainly isolated from milk and their products and also display no pathogenic trait (Cousin *et al.,* 2011). They were found to have the ability to produce propionic acid during growth which led to the name *Propionibacterium* (Cousin *et al.,* 2011). They grow at a temperature range of 15 to 40^{o} C and pH of 5 to 8 with an optimal temperature growth at 30^{o} C at neutral pH. They have been shown to exhibit a peculiar carbon metabolic pathway - the Wood-werkmann cycle (Wood, 1981). *Propionibacterium* produces propionate, acetate, succinate and carbon dioxide from any carbon source and they have been used as dairy starter in cheese production apart from being used as vitamins producers and bio-preservative (Cousin *et al.*, 2011). Their safety is confirmed, by the wild spread consumption of Swiss-type cheese. Furthermore, they have received the GRAS (Generally

Recognised as Safe) status (MattilaSandholm *et al.,* 2002). This enables them to be used as probiotic foods, eye formation in cheese and bio preservation for fermented foods (Hatakka *et al.,* 2008). Also, they assist in flavour development and production of exo-polysaccharides (EPS) used for viscosifying, stabilizing, gelling and water binding agents amongst others (Gorret *et al.,* 2001).

Furthermore, the European food safety authority has granted "qualified presumption of safety" (QPS) status to species *P. freudenreichii* (Cousin *et al.,* 2011). Some of their features include, absence of antibiotic resistance, no virulence factors (Meile *et al.,* 2008), no cytotoxic effect in mouse coloncytes (Bandres *et al.,* 2009), and no indication of side effects from consumption of dairy *Propionibacteria* has been reported (*Cousin et al., 2011)*. They produce cheese - characterized by round "eyes" opening formation of eyes through production of carbon dioxide and synthesis of aroma compounds (Jijon *et al.*, 2004).

2.5.5 Habitat

Propionibacteria are present in raw milk as part of raw milk microbiota and used as an index for level of hygiene of the milk facilities. However, their presence in milk has decreased as a result of improvement in raw milk quality and the process of microbiological purification such as microfiltration and bactofugation (Moslemy *et al.,* 2015). They grow in milk because they use lactate as carbon substrate as well as lactose. Their growth in milk is not extensive due to their weak proteolytic activity (Moslemy *et al.,* 2015). They have been freeze dried and used as starter cultures for other types of cheese as well for flavour enhancer (Jijon *et al.,* 2004). In addition to the dairy technology use, their uses have increased tremendously, which include incorporation in commercial probiotics, freeze-dried culture as tablets or capsules that are intended for use to improve intestinal movement and comfort. These types of formulation have been reported to beneficially modulate gut microbiota and associated metabolic activities (Hatakka *et al.,* 2008). The dairy *Propionibacterium* is not human microbiota and as such, can be isolated from various habitat such as soil, grass fodder, rumen, raw milk and dairy products (Rinta-Koski *et al.,* 2001).

2.5.6 Peculiar Metabolism

They metabolize a variety of substrates like carbohydrates such as glucose, galactose, fructose, lactose, alcohol (glycerol, erythritol) and organic acids (lactate and pyruvate). They present a particular central carbon metabolic pathway, the propionic fermentation.

$$3\ \text{Lactates} \rightarrow 2\ \text{propionates} + 1\text{acetate} + 1\ Co_2 + H_2O$$

Propionic fermentation, in addition to glycolysis, pentose phosphate and EnterDoudoroff pathways relies on Wood-werkmann cycle (Cousin *et al.,* 2011). This reaction is catalysed by enzymes with specific cofactors, including vitamin B_{12} (cobalamin) and B_6 (folic acid) H (biotin) (Cousin *et al.,* 2011*).* Furthermore, *Propionibacterium* are not the only bacteria able to release short chain fatty acids (SCFAs) but the unique, Woodwerkmann cycle which results in production of short chain fatty acids from various compounds are regarded as beneficial. These compounds were thus described as nutraceuticals producers (Ruas-Madiedo *et al.,* 2002). Deficiency of the above vitamin causes anaemia and also have been reported to be involved in neurodegenerative disease. Furthermore, they have long been used in production of food grade B_{12}, with high yield and low cost and B_2 (riboflavin) vital for cellular process (Ruas-Madiedo *et al.,* 2002). Conversion of free linoleic acid into conjugated linoleic acid in vitro during fermentation and storage of milk is one of the features of *Propionibacterium freudenreichii* (Prandini *et al.,* 2007).

2.5.7 Modulation of Gut Microbiota

In vitro studies carried out by Collado *et al*. (2008) showed the antagonistic effects of *Propionibacterium freudenreichii* on pathogenic bacteria. This strain alone or in combination with other probiotic bacteria reduced adhesion of pathogens. This property may be due to its ability to aggregate with these pathogens (Collado *et al.,* 2008; Kajander *et al,* 2008). They have also been reported to inhibit adhesion of *Helicobacter pylori* to human epithelia cells and as well as prevent the leakages induced by *Helicobacter pylori* (Kajander *et al.,* 2008). The commonest feature- is its ability to enhance growth and thus the increase in number of intestinal bifid

bacteria. In vivo studies, consumption of yoghurt containing *B. bifid* and *Propionibacterium freudenreichii* have been reported to reduce coliforms while enhancing bifid bacteria population in rat faeces (Cousin *et al.,* 2011). In humans, several studies have shown that gut microbiota is modulated by dairy *Propionibacterium* (Cousin *et al.,* 2011). They have the ability to bind carcinogenic compounds such as aflatoxin B1 and delay, but not prevent, absorption in duodenal has been reported (Gratz *et al.,* 2003, 2005). Therefore, ingestion of dairy *Propionibacterium* may limit metabolisation of these compounds and so decrease cancer emergence risk. Independent studies have showed that consumption of *Propionibacterium freudenreichii* in various form, as whey cultures, or heat-inactivated or not (Nagahara *et al.,* 2001) or as freeze dried bacteria (Jan *et al.,* 2002) enhanced faecal bifid bacterial counts. In infants, eating yoghurt containing *Propionibacterium freudenreichii reduced* coliforms however bifidobacteria were enhanced (Sarkar. 2013).

2.5.8 Potentials use of *Propionibacteria*

Propionibacterium also have some advantages in relation to other probiotics used in various products, including dairy and non-dairy. Combined application of *Propionibacterium* and Bifid in milk and soymilk resulted in a good compatibility and reduced flatus after consumption (Wu *et al.,* 2012). *Propionibacterium* is capable of biosynthesis of low calories sugar.

Trehalose is reported to play a positive role in energy supply and also in stress protection mechanism (Moslemy *et al.,* 2015). Using trehalose producing strain could lead to improvement in viability and desired functionality against stress conditions. This may result in high number of this organism reaching the gastrointestinal system (Pophaly *et al.,* 2012). Conjugated linoleic acid (CLA) is also produced by *Propionibacterium freudenreichii*. *Propionibacterium freudenreichii* has been reported to covert free linoleic rumenic acid (Meile *et al.,* 2008) and have tremendous potential in CLA production other than various starter cultures, are health promoting fatty acid in human body (Jiang *et al.,* 1998). They also have anti fugal properties against pathogenic fungi (Dyrby *et al.,* 2007). It is

also known that whey fermented by *Propionibacterium freudenreichii* increased bifidobacteria population as a potent probiotic organism. The increased bifid population also resulted in alleviation of ulcerative colitis symptoms in animal and human model *(Pophaly et al., 2012)*. Furthermore, research has shown that this positive effects is related to DHNA (1,4-dihydroxy-2-naphthoic acid) production by *Propionibacterium* (Pophaly *et al.*, 2012).

2.6. Nutritional benefit of probiotic yoghurt

Live probiotics can provide nutritional benefits either during the preparation of fermented probiotic foods or in the digestive tract of the host. After fermentation, the texture and the flavour of the raw material can be significantly improved (Dherbecourt *et al.,* 2008; Meile *et al.,* 2008; Dherbecourt *et al.,* 2010). The adverse effects of some component in food can be reduced, such as food intolerance and allergies caused by some oligosaccharides and proteins (Viljanen *et al.,* 2005). Furthermore, the levels of amino acid and vitamins can be increased, leading to improved nutritive value of the food (Jiang *et al.,* 1998; Ruas-Madiedo *et al.,* 2002; Donkor, 2007). Sugars and other spoilage promoting components of food have also been reported to be removed which enhances the shelf life and ensure food safety (Al Zoreky *et al.,* 1993; Mantere-Alhonen 1995; Ekinci *et al.,* 2006; Ekinci *et al.,* 2008). Evidence has shown that bioavailability of calcium, zinc, iron, manganese, copper and phosphorous is increased in probiotic yoghurt compared to milk (Gilliland *et al.,* 1984).

There are many proof-supporting potential clinical usage of probiotics in the prevention and treatment of diseases of the gastrointestinal, respiratory and urogenital tracts (Gardiner *et al.,* 2002). Helping the body's ability to resist the invasion of pathogens and maintaining the host wellbeing (D'Aimmo, *et al.,* 2007), enhancement of immune system and reduction of lactose intolerance (Gilliland *et al.,* 1984), reduction in serum cholesterol levels and blood pressure (Rasic 2003), anti-carcinogenic activity (Rasic *et al.,* 1993; Collado *et al.,* 2008), improved utilisation of nutrient (Lourens-Hattingh *et al.,* 2001a) are other benefits of probiotics. Probiotics have also been widely used in therapeutic application such as

prevention of urogenital diseases (caused by *Candida vaginitis*), alleviation of constipation, protection against traveller's diarrhoea, prevention of fantile diarrhoea, reduction of antibody induced diarrhoea, control of influmtun bowel disease and irritable bowel syndromes (Gilliland *et al.,* 1984).

2.6.1 Viability of probiotic bacteria

In order to obtain the desired health effects, probiotic bacteria must be able to grow in food matrix and survive in sufficient numbers. It has been suggested that probiotic organisms should be present in food at a minimum concentration of 10^5-10^6 cfu/g (Dave *et al.,* 1997; Rybka *et al.,* 1997; Gomes *et al.,* 1999) or the daily intake should be about 10^8 cfu/g. Such high numbers have been recommended, but not compulsory, in order to compensate for possible losses in the numbers of the probiotic organisms during passage through the stomach and intestine.

2.6.2 Factors affecting viability of probiotic bacteria

Several factors, such as the strains selected, interactions between species present, acidity, pH and hydrogen peroxide due to bacterial metabolism, have been known to affect the viability of probiotic microorganisms during manufacture and storage of yoghurt (Lankaputhra *et al.,* 1996; Dave *et al.,* 1997). Other factors which include storage temperature, oxygen content, concentrations of acetic and lactic acids, nutrients limitations in milk to sustain growth, growth promoters and inhibitors, inoculation level, fermentation time and post-acidification have also been suggested to affect viability of probiotic organisms in yoghurt (Shah, 2000; McComas *et al.,* 2003; Tamime *et al.,* 2005; de Carvalho Lima *et al.,* 2009). The improvement of survival and viability may be achieved by selection of the right culture.

2.6.3 Tactics for improving viability of probiotic organisms

Growth of some probiotic bacteria has been reported to substantially improve in milk supplemented with dairy and non-dairy ingredients such as whey powder, whey protein concentrates; acid casein hydrolysate and whey protein hydrolysate (Dave *et al.,* 1997; McComas *et al.,* 2003). The addition of these supplements are

necessary because of poor proteolytic activity which limit probiotic bacteria growth in milk/soymilk (Dave *et al.,* 1997; Donkor 2007). The addition of the above supplement provided a readily available source of peptides and amino acids as growth factors vital for growth of probiotic bacteria, thereby shortening the incubation time considerably (Dave *et al.*, 1998; Donkor 2007; Ekinci *et al* 2008).

2.7 *Propionibacterium freudenreichii*

Like *Lactobacilli*, dairy *Propionibacterium* has been reported to have a long history of safe use as starter cultures in the food industry (Reinbold, 1985; Vorobjeva, 2005; Sarkar, 2013). The bacterium has also been found to confer health benefits to the host by improving its intestinal microbial balance when administered in adequate amounts (FAO/WHO, 2006). For it to provide beneficial health effect to the host, it must survive in the gastrointestinal tract in adequate number, tolerating acid, bile, gastrointestinal enzymes and be able to adhere and colonize in the intestinal epithelium cells (Huang and Adams 2004; Anastasiou *et al.* 2006). Thus the characteristics of probiotics such as gastrointestinal tolerance and adhesion to the epithelium are critical factors in maintaining probiotic efficacy as described by Burns *et al.*, (2011; Ranadheera *et al.* 2012 and Yonekura *et al.*, 2014).

Propionibacterium freudenreichii (P. freudenreichii) has been reported to develop stress adaptation mechanism which result in tolerance against severe injuries when an unfavourable environmental factor is sensed (Moslemy *et al.* 2015). As a result, it is able to cope with various technological stresses such as thermal treatment and acidification. Despite these stresses, it can survive and grow to a final population of about 10^9 CFU/g showing efficient adaptation mechanism (Leverrier *et al.,* 2003; Leverrier *et al.,* 2005). Regarding their potentials as probiotic organisms, *P. freudenreichii* also exhibits tolerance towards digestive stresses such as pH and bile exposure (Huang *et al.*, 2004; Suomalainen *et al.*, 2008). This may be attributed to a pre-treatment such as short exposure to a sub-lethal dose of stress (Anastasiou *et al.*, 2006). *P. freudenreichii* is also able to develop high tolerance toward digestive stress, including acidity and exposure to bile. Hence, it is able to survive at pH of 2

(Jan *et al.*, 2002) and also in the presence of bile salt concentration above those reported in the content of human colon (Leverrier *et al.*, 2003).

Yoghurt containing *Propionibacterium freudenreichii* and yoghurt starter cultures are known to have provided *Propionibacterium freudenreichii* with high tolerance toward acid challenge or bile salt challenge (Leverrier *et al.*, 2005). The consumption of the dairy vectors by human volunteers was reported to have resulted in improved survival and metabolic activity in the gut (Nagpal *et al.*, 2012). Therefore, *Propionibacterium freudenreichii* can conveniently be used as a probiotic organism in yoghurt preparations. Delivering of probiotics into host in suitable food matrix has been reported to be the most important means of maximizing probiotics efficacy (Ranadheera *et al.*, 2012).

2.8 YOGHURT

The South African regulations define yoghurt as the product obtained from pasteurized or reconstituted milk which has been inoculated with a yoghurt culture and which can ferment under controlled conditions (Murevanhema *et al.*, 2013)

Yoghurt is produced by blending fermented milk with various ingredients that provide flavour and colour. Yoghurt predates written history, but became popular within the last 30-40 years (Sardine, 1979). This is due to many factors, including the introduction of fruits and flavourings into yoghurt, the convenience of it as a readymade breakfast food, because of increase in demand of yoghurt market, as well as benefits of probiotics in human diet (Al Zoreky *et al.*, 1993).

2.8.1 Probiotic yoghurt

Recently, there has been increased interest in natural and biological products for health promotion and disease control, using microorganisms. This has led to investigation of novel fermentation processes that are more efficient for production of biological products such as propionic acid sources (Moslemy *et al.*, 2015). Recently, a combination of propionic, lactic and acetic acids has been recommended for food preservation. Their salts, such as calcium, sodium and potassium, including acids have been listed as preservatives that are Generally Recognized as Safe

(GRAS) (Leverrier *et al.,* 2003; Moslemy *et al.,* 2015). The use of probiotics have attracted the attention of food industries and consumers as a food supplement. Probiotics are live microorganisms which are added to food to enhance their characteristics apart from health benefits (Organization 2006; WHO 2006). Microbiologically, yoghurt is an end product of controlled fermentation of high solids whole milk with a symbiotic mixture *Streptococcus thermophillus* and *Lactobacillus delbrueckii* subp. *Bulgaricus* (Gasson *et al.,* 1994) and addition of probiotics makes it probiotic yoghurt (Saccaro *et al.,* 2009; Sadrzadeh-Yeganeh *et al.,* 2010). The two most commonly used probiotics in probiotic beverages include *Lactobacillus acidophilus* and *Bifidobacterium bifid.* However there are more than 400 strains of beneficial probiotics (Shimakawa *et al.,* 2003).

Recently, *Propionibacterium* have been reported to show potential probiotics effects. They are used in several fermented dairy products such as cheese where they derive their importance. They are also used on an industrial scale in acid and vitamin B_{12} production. In addition, they are able to produce bacteriocin, and are normally combined with lactic acid and /or Bifidobacterium in probiotic food production (Mantere-Alhonen 1995). Probiotics are becoming increasingly important due to their perceived health and nutritional benefits. Probiotics global market reached 19 billion dollar in 2013 alone and it is expected to increase (Moslemy *et al.,* 2015).

2.8.2 Quality of yoghurt

The chemical, microbiological and physical properties of yoghurt are evaluated in order to determine its quality. These properties are controlled under food legislation of each country. The protein content must be no less than 30 g/kg, while the pH of the product must be less than 4.50. In some European countries, the use of stabilizers is prohibited (De Vyust and Degeest, 1999). However, there are no legal requirements for the physical properties of the yoghurt products. It is important, that set yoghurt be firm, smooth in texture, free from lump or graininess and spoon able without any syneresis on the surface of the product (Tamime *et al.,* 2005). In addition, methods which include sensory and instrumental assessments have been used for assessing physical properties of yoghurts.

2.8.3 Physicochemical properties of yoghurt

During heat treatment at 95° C for 20 minute for yoghurt milk, protein denaturation was observed resulting in molecular rearrangement (Lucey, 2002). Furthermore, lactic acid bacteria have limited capacity to synthesize amino acids, using inorganic nitrogen, thus relied on performed amino acids. The enzymes inherent in Lactic acid bacteria, e.g. proteinase and peptidase hydrolyse milk proteins to free amino acids required for cell development. Casein is hydrolysed and hydrophobic peptides are increased during storage, suggesting increase in soluble nitrogen at the end of storage (Cruz *et al.,* 2007; Serra *et al.,* 2009a). On the other hand, the disaccharides are split by specific hydrolyses to monosaccharides such as lactose to galactose and glucose, sucrose to glucose and fructose. Glucose enters the major metabolic pathways (Ouwehand *et al.,* 1998; Salminen *et al.,* 1999; Donkor, 2007). In addition, sucrose may also contribute monosaccharides for exopolysaccharides formation in certain *P. freudenreichii* and in certain lactic acid bacteria (Salminen *et al.,* 1999).

2.8.4 Methods used in Yoghurt Processing

The four main steps in yoghurt processing are;

Milk preparation, Heat treatment, Inoculation and incubation and Treatment and handling after incubation (cooling and storage).

2.8.5 Milk preparation and heat treatment

Milk solids are reconstituted, with deionised water heated to 30-40°C prior to addition of milk, followed by heating the mix milk 50^{O}C with constant stirring for 30 min to dissolve solid particles. A similar procedure was also used for soy milk. Soy yoghurt manufacturing, heat treatment of soy milk was carried out at 95°C for 30 minutes (Ren *et al.* 2006). The level of heat treatment applied to legume is usually higher than used for pasteurization. Several benefits of using such a high level of heat treatment include (Ren *et al.* 2006):

- Killing of all pathogenic and most of spoilage bacteria,
- Inactivation of most enzymes which may cause undesirable effects to the final product,

- Removal of toxic compounds and a reduction in the oxidation-reduction potential of the medium suitable for the growth of starter cultures by removal oxygen.
- Improvement in acid production and in the quality of milk as a substrate conversion of calcium into a soluble form leading to a decrease in time for milk coagulation,
- Improvement in firmness and syneresis of yoghurt as a result of whey protein

Other changes which may also take in yoghurt production, include dissolution of colloidal calcium phosphate, hydrolysis of proteins to peptides and free amino acids, degradation of citrate to acetaldehyde and diacetyl (*Streptococcus thermophillus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*) and probiotic organisms (*Propionibacterium freudenreichii*) have been reported (Donkor 2007). These organisms are categorised as Gram-positive, non-spore-forming, non- motile, catalase-negative bacteria that grow under strictly anaerobic condition (Robinson *et al.* 2008). The basic process of yoghurt production and the changes of milk constituents are attributed to the fermentative action of the starter cultures, the secretion of nutritional and chemical substances by the microorganisms, as well as the presence of the microorganisms and microbial enzymes (Donkor 2007). The main biochemical reaction involved during the processing of yoghurt is the fermentation of lactose to mainly lactic acid by *Lactobacillus* (Hackett *et al.* 2013). Such fermentation is reported to result in the following changes: Acidification leads to a reduction in the pH of milk close to isoelectric point resulting in formation of smooth gel. The proteolytic activities of lactic acid bacteria (LAB) result in higher content of free amino acids in yoghurt. However, total amino acid content of yoghurt may not be substantially different from that of the milk in which it was made (Rasic *et al.* 1993).

2.8.6 Food storage stability and shelf life

A lot of changes take place in food during processing and storage. It is well known that conditions under which foods are processed and stored may adversely influence the quality attributes of foods (Man *et al.,* 1994). When foods are stored for a certain period, one or more quality attributes may reach undesirable state. At that instant, the food is considered unsuitable for consumption and it is said to have reached the end of its shelf life which is an important feature of all food (Man *et al.,* 1994). The Shelf life of food product may be defined as the time between the production and packaging of the product and the point at which it becomes unacceptable under definite environmental conditions (Ellis 1994). Storage and distribution are necessary links in the food chain quality and safety (Ellis 1994). There are concerns about the conditions and maximum duration of these links in the chain, although most food deteriorations take place gradually. A total quality method must embrace all aspects of food from its conception, through development and production to its consumption. For a manufactured food product, this will include (Ellis 1994):

- Product design (including hazard analysis and risk assessment to ensure safety.
- Specification and testing of ingredients and packaging material.
- Manufacturing process.
- Transport, storage and retail display.
- Storage at home and consumption.

Since the food must be safe and have an acceptable quality when consumed, the time for which this is maintained, shelf life is therefore an essential aspect of product design, the control of which is a requirement of Good Manufacturing Practice (GPM) (IFST, 1991) as well as a requirement of the international Standard for quality system, ISO 9001(ISO, 1987).

Shelf life determination for any new product often requires storage for significant period. This will include sampling from early development stages as well as initial production runs. Through the evaluation of stored samples, potential storage

problems can be identified and either eliminated or controlled before food goes into production (Man, Jones and Vasavada 1994). When in production, an ongoing quality assurance system is equally important. This involves assessment of freshly made products typically before the products will be released into distribution and sample stored up for shelf live determination (Man *et al.*, 1994). Furthermore, shelf life should also be evaluated, so that any change in the storage performance can be noted, and appropriate actions taken, if necessary.

2.8.7 Shelf life determination

Before shelf life is determined, it is essential to determine which factors limit the shelf life. Factor such as physical, chemical and biological changes which result in sensory changes in the food must be evaluated. If the limiting factors are not correctly identified, the subsequent studies will be imperfect.

a) Physical changes: are caused by mishandling of food during harvesting, processing and distribution leading to a reduction in shelf life of food. Bruising of fruits and vegetables during harvesting and post handling lead to the development of wilt. Crushing of dried snack foods during distribution seriously affect their quality. In case of frozen foods, fluctuating temperatures cause recrystallization of ice cream leading to undesirable sandy texture. Syneresis in yoghurt, water holding capacity and texture of yoghurt may be affected as a result of rearrangement of protein strands (Serra *et al.*, 2009b).

b) Chemical changes: During processing and storage of food, chemical changes occur that involve the internal food component and external environmental factors. These changes may result in food deterioration and reduce the shelf life. During yoghurt preparation, major constituents, including lactose and milk protein, soy proteins, raffinose, starchyose and other soy carbohydrates are utilized for the bacterial growth, which results in the conversion of fermentable materials into a range of products such as lactic acid, acetic acid, peptides, amino acids and different vitamins (Donkor 2007).

c) Microbial changes: are controlled using cold storage, heat treatment and addition of selected antimicrobial agents. However, environmental factors such as,

pH, temperature and nutrient still allow the growth of some spoilage and pathogenic organisms. Furthermore, many of these pathogens adapt and survive this condition (Leyer *et al.*, 1992.). Massa *et al.* (1997) studied the survival of *E. coli* in yoghurt during preparation and storage at 4°C. *E. coli* lost its viability slowly during refrigeration. Moreover, lactic starter cultures for yoghurt production, produce inhibitory compounds, and high numbers of lactic and probiotic bacteria compete with the pathogens for nutrients during processing and storage (Pitt *et al.*, 2000). Yet, spoilage and pathogenic microorganisms may be able to survive and proliferate in food from the time of production to the time of consumption.

2.8.8 Quality and safety of yoghurt during storage

Spoilage and pathogenic organisms may be able to survive processing, storage and environmental conditions in the absence of GMP (Good Manufacturing Practices) and GHP (Good Hygiene Practices) (IFST, 1991). The isolation of these bacteria such as *E. coli* from many acid foods has shown the potential of these pathogens to survive the harsh conditions such as low pH, low water activity and refrigeration storage. Yoghurt prepared in this manner may present risk of transmitting food infection (Guraya *et al.*, 1998). Growth and survival of microorganisms in fermented foods have been investigated by many workers over the years. According to Gulmez and Guven (2003), *E. coli* 0157:H7 survived 21 days during cold storage at 5°C. Moreover, pathogens and spoilage organisms can survive in yoghurt at various temperatures. However, their survival may depend on pH, lactic acid bacteria, probiotic organisms' metabolites and storage temperatures. The increase in level of culturing microorganisms and probiotics which result in rapid decline in pH, production of more organic acids and subsequent inhibition of pathogens and spoilage organisms may be responsible (Gulmez and Guven, 2003). The antagonistic activity of lactic acid bacteria and low pH, was much enhanced at higher temperatures. However, at higher temperatures yeast counts grew as high as 4 log cfu/ml. At these levels, yeast contributes to spoilage of yoghurts. At low temperatures the number of yeast remain low, increase in numbers as storage after prolonged storage not usually more than 2 log CFU/ml cause severe spoilage (Dublin-Green and Ibe, 2008). Yoghurt stored at ambient temperatures may be free

of pathogens but the quality may have deteriorated because of the growth of yeast and other spoilage agents.

2.9 Importance of Pigeon Pea Beverage to Food Security in Africa

Pigeon pea utilization in processing and value addition might encourage wider production. Also, preparation and fermentation of pigeon pea milk may increase the consumption of this viable crop and hence improve its commercial status. Furthermore, innovative value-added products such as the pigeon pea probiotic beverage have the potential to overcome Africa's great problems e.g. hunger, malnutrition, and gender inequality.

Pigeon pea is a low-cost crop, and grown by subsistence farmers for their annual income. A pigeon pea probiotic beverage would result in buoyant new market outlets for farmers and the boosting of income opportunities for rural areas. It is a crop that thrives very well in sites too hot and too dry where peanut, maize, or even sorghum will not grow well. It has also been described as a complete food owing to its exceptional nutritional quality (Akande *et al.* 2010). Compared with peanut, pigeon pea has less oil and a slightly less protein, but more carbohydrate, and anti-oxidative potential (Pale *et al.*, 1997). The overall combination with cereals nicely balances the food groups. Among legumes, pigeon pea has been suggested to have the best biological value (Sharma, Agarwal and Verma 2011) and also possesses healing properties thus regarded as food with nutraceutical properties. Pigeon pea could also be a veritable tool for attacking Africa's chronic malnutrition. Also, since it is mostly grown by women it offers a convenient way to economically empower women, thereby improving the lives of their families.

2.10 SENSORY EVALUATION

Sensory evaluation is defined as the science of judging and evaluating the quality of food by the use of human senses, for example, taste, smell, sight, touch and hearing (Eastman *et al.,* 2005). Sensory testing has developed into a precise, formal and structured methodology that is continually being updated and refined with existing techniques. The developed methods have been reported to serve economic interest and can establish the worth or acceptance of a commodity (Meilgaard *et al.,* 2006).

Before a product reaches the market, it must have been evaluated to determine its acceptability by the consumers. This is especially true in the food industry because of the many taste and social preferences (Meilgaard *et al.*, 2006). There are many questions that need to be answered before one is willing to invest hundreds of thousands of dollars producing, transporting, and marketing a new product. Some of these possible questions are:

(i) Will anyone like the product? If so, who?

(ii) Would anyone of those be willing to buy the product? If so, at what price?

(iii) How can the product be successfully marketed to those people? and

(iv) Will anyone prefer the product over another to capture some of the market share of that food category? If so, how much? (Bopp *et al.*, 1997).

Consumer testing by affective testing is a useful tool involved to try to answer questions about the success of a new product. Although there are many types of consumer tests, the affective test is the most common for basic consumer tasting of food (Donkor 2007). When Affective testing is done properly, the following are the results;

- Allow different actions to be tried to find the best accepted product.
- Break the masses of consumers down into smaller groups to allow an understanding of who will buy the product and how to market it to them.
- Assess the market share potential for the new product. In addition, other products can be improved upon by testing results.
- Data is -obtained by asking specific questions about a person’s age, sex, geographic location, nationality, religion, education and employment along with their preferences on the product being tested.
- It groups users based on these variables and learns the preferences of particular groups' eating habits (Bopp *et al.*, 1997).

Sensory tests have been used to select a product that optimizes value for money. It is also used as a practical tool in product development by helping in product matching, improvements, and grading (Donkor 2007). Research is another area where sensory evaluation is frequently used. Evaluation of a product may be needed to determine the effect an experiment has on its subject. Finally, quality control and marketing are others areas where sensory testing may be done (Stone *et al.,* 1991; Meilgaard *et al.,* 2006). Sensory evaluation is divided into two methods, subjective and objective testing. Subjective tests involve objective panellists, while objective testing makes use of laboratory instruments without using the senses. Both tests are essential in sensory evaluation and necessary in a variety of conditions (Meilgaard *et al.* 2006). The following are some sensory tests.

a) Hedonic scale

One such subjective test is the use of the hedonic scale method. This rating scale method measures the level of the liking of foods, or any other product where an affective tone is necessary. This test is based on people's ability to communicate their feelings of like or dislike. Hedonic testing is popular because it may be used with untrained people as well as with experienced panel members. A minimum amount of verbal ability is necessary for reliable results (O'Mahony 1986; Poste *et al.,* 1991).

In hedonic testing, samples are presented in succession and the subject is told to decide how much he likes or dislikes the product and to mark the scales accordingly. The nature of this test is simple compared to others. The hedonic scale is anchored verbally with nine different categories ranging from like extremely to dislike extremely. These phrases are placed on a line-graphic scale either horizontally or vertically (Lawless *et al.,* 2010). Many different forms of the scale have been reported to be successfully used. However, variations in the scale form is likely to cause marked changes in the distribution of responses and ultimately in statistical parameters such as means and variances (Guillou *et al.,* 1995). Characteristics of the subjects, the test situation, attitudes or expectations of the subjects can all have a

profound effect on results. A researcher needs to be cautious about making inferences on the bases of comparison of average ratings obtained in different experiment (Poste *et al.*, 1991; Stone *et al.*, 1991; Guillou *et al.*, 1995)

b) Facial scales

These scales were mainly intended for use with children and those with limited reading. They can be described as a series of line drawings of facial expressions ordered in a sequence from a smile to a frown, or they may depict a popular cartoon character (Carpenter, 2012). The facial expression may be accompanied by a descriptive phrase and may have five, seven or nine categories (Lawless *et al.*, 2010). For computational purposes, these facial expressions are converted to their numerical counterparts and treated statistically (Stone *et al.*, 1991).

2.10.1 Other sensory tests

There are a number of different sensory tests as indicated by the following series of definitions besides hedonic and face scales used in the sensory evaluation of a food product and they include;

a) **Difference tests:** In difference tests the panellists are merely asked if a difference exists between two or more samples (Lawless *et al.*, 2010).

b) **Preference tests:** Preference or acceptance tests determine representative population preferences and these tests inherently require many people on the panel (Lattey *et al.*, 2007).

c) **Triangle test:** In the triangle test, three coded samples are presented to the panellist. He/she is told that two samples are identical and he/she is asked to indicate the odd one (Bi, 2008).

d) **Duo-Trio test:** In the duo-trio test, three samples are presented to the taster. One is labelled "R"(reference) and the other two are coded. One coded sample is identical with "R" and the other is different. The panellist is asked to identify the odd sample (Donkor, 2007)

e) **Paired comparison test:** In the paired comparison test, a pair of coded samples that represent the standard or control and an experimental treatment are presented to the panellist, who is asked to indicate which sample has the greater or lesser degree of intensity of a specified characteristic, such as sweetness and hardness.

f) **Multiple comparison test:** In multiple comparison tests, a known reference or standard is labelled "R" and presented to the panellist with several coded samples. The panellist is asked to score the coded samples in comparison with the reference sample (Hochberg *et al.*, 2009).

Ranking: The panellist is asked to rank several coded samples according to the intensity of some particular characteristic (Meilgaard *et al.*, 2006).

Scoring: Coded samples are evaluated by the panellist who records his reactions on a descriptive graduated scale. These scores are given numerical values by the person who analyses the results (Kemp *et al.*, 2011).

Flavour-profile method: The flavour-profile method consists of a small laboratory panel of 6 or 8 people trained in the method measure of the flavour profile of food products. Descriptive words and numbers, with identifiable meaning to each panel member, are used to show the relative strength of each note on a suitable scale (Donkor, 2007).

Dilution tests: Dilution tests involve the determination of the identification threshold for the material under study. Determining the type of research that is being done, and the type of evaluation that is needed is crucial in obtaining accurate results from a sensory project (Poste *et al.*, 1991; Stone *et al.*, 1991).

2.11 Rheological and Textural Properties of Yoghurt

Rheology has been described as a science of deformation and flow of materials (Prentice 1992; Steffe 1996). Deformation is an important property of food materials since they have to deform before ingestion. Furthermore, several phenomena take place during eating, one of which is mouth feel. Mouth feel is defined as the sensory experience derived from the sensation in the mouth and tongue after ingestion of food (McKenna, 2003). Mouth feel is influenced by the textural and rheological characteristics of food (Guinard *et al.,* 1996).

2.11.1 Measurement of rheological and textural characteristics of yoghurt

Assessment of rheological and textural characteristics are carried out by various testing procedures and instruments. These procedures are classed into two categories which are;

a) Destructive or large deformation
b) Non-destructive or small deformation

The non-destructive deformation instruments are not used to assess mechanical characteristics of yoghurt such as yield stress since this provides little information on rheological properties of gel (Lucey, 2002).The Brookfield viscometer, texture analyser and physical rheometer are used for these types of test, which perform tests within the same machine. While destructive or large deformation occurs in practice during processing and eating of many food materials which have large extensional components, extensional flows are vital in mixing and milling/sheeting of pastry and dough (Dobraszczyk, 2003). Other types are converging and diverging flow, such as in extrusion and pumping, spreading of soft solids as in butter, cheese and pastes, and expansion of bubbles in foams as bread dough, cakes and heat extruded snacks (Dobraszczyk, 2003). Unfortunately, most tests in flow are carried out in shear under small deformations mainly because most conventional viscometers operate in shear and also because the equipment is readily available and the technique well established (Dobraszczyk, 2003). In the food industries, various rheological tests are

used extensively to assess the nature of the material, especially milk. Since it is converted to different milk based products with different physical properties

The studies of rheological properties in food industries are of great importance. This enables the understanding of rheological behaviour of food materials during the flowing stages involved in the preparation stage such as:

- Quality control of ingredients and finished products
- Design and evaluation of processing equipment unit operations and process parameters
- Time and temperatures selection/adjustment
- Characterization and development of yoghurt products for consumer's acceptability and structure and relationship structure and textural properties (Shaker *et al.*, 2000).

Flow behaviour of yoghurt: yoghurt is semi solid fermented milk gel product with a firm (set) and viscous (stirred) texture and has "viscoelastic" properties – (Lucey 2002). Materials that are referred to as viscoelastic are those that exhibit both the viscous property of a liquid and elastic property of a solid simultaneously under applied stress. They also behave as non-Newtonian fluid (Forterre *et al.*, 2008). Non Newtonian fluid is defined as a fluid exhibiting uniform flow, but where the relation between shear stress and rate of shear is not constant the viscosity is not constant (Steffe, 1996).

In this type of fluid, a minimum shear stress known as the "yield" must be exceeded before flow begins. This type of flow is common with food, such as tomatoes ketchup, mayonnaise and are known as plastic flow. Plastic refers to materials that exhibit yield stress and not synthetic plastics (Swaminathan *et al.*, 2006).

2.12: Conclusion/ knowledge gaps identified.

Pigeon pea is an under-researched and underutilized legume. Production of milk and yoghurt from other legumes have been reported in literatures. Pigeon pea milk yoghurt, and pigeon pea milk yoghurt supplemented with *Propionibacterium freudenreichii* however have not been reported.

CHAPTER 3
1ST RESEARCH CHAPTER
EVALUATION OF PIGEON PEA MILK YIELD, SUITABILITY AS A MATRIX FOR YOGHURT, PROBIOTIC YOGHURT PROCESSING AND NUTRITIONAL PROFILE OF THE YOGHURT SAMPLES

ABSTRACT

Pigeon pea (*Cajanus Cajan*) is consumed in many parts of Africa, including South Africa, as a dietary source of protein and carbohydrate. It is underutilised and mainly grown for subsistence. Research on pigeon pea may unlock the potential of the crop, enhance value addition and increase its utilization. In this study, non-dairy probiotic yoghurt was processed from pigeon pea milk. Yoghurt samples were prepared, using 100% pigeon pea milk, pigeon pea/soy milk in the ratio 50:50 and 100% soy bean milk. The yoghurt samples were inoculated with *Lactobacillus bulgaricus and Streptococcus thermophillus* and divided into two equal parts. One part inoculated with *Propionibacterium freudenreichii* was referred to as probiotic yoghurt, while the other part served as the control. The Milk percent yield, and proximate, amino acids, fatty acids composition and textural properties of the yoghurt were determined. The protein contents of the yoghurt samples varied from 4.54-5.85% for 100% soymilk and 100% pigeon pea yoghurt, respectively. Pigeon pea probiotic yoghurt showed slightly lower protein content than pigeon pea yoghurt alone. All the yoghurt samples maybe relatively high total solids (16.04-17.41%) and were fairly good sources of amino acids. The TS (16.4-18.7g), solid non-fat (16.0-17.4g) and moisture content (81.4-83.5g) of the yoghurt samples were not significantly different. The amino acids content of all the yoghurt samples were comparable to FAO/WHO (2007) recommended amino acid requirements. Probiotic yoghurt samples generally showed slightly lower amino acid profile compared to samples without *Propionibacterium freudenreichii*. All yoghurt samples showed increase in firmness with increase in storage period. *Propionibacterium freudenreichii* yoghurt samples showed significantly higher firmness (1.36-1.70 N) than yoghurt without *Propionibacterium freudenreichii* (0.1- 1.62). Pigeon pea milk yoghurt would provide not only beneficial health effects but also a new healthy, alternative snack.

3.1 INTRODUCTION

Yoghurt is a fermented food made from fresh, whole or skimmed milk which is carried out by the action of bacterial starter cultures (Falade *et al.,* 2014). It is traditionally consumed as a healthy food due to its nutritional properties (Shah 2007; Muniandy *et al.*, 2016). In accordance to Shah (2007), the health benefits derived from consuming yoghurt can be improved by incorporation of probiotic strains, e.g. lactic acid bacteria. The limitation of dairy products such as the presence of allergies, unfavourable cholesterol content and the demand for health-promoting non-dairy products have resulted in recent trend for non-dairy probiotics yoghurt (Bautista-Gallego *et al.,* 2013). Efforts have been made to produce yoghurt and similar

fermented products from vegetable sources such as soybean (Granato *et al.*, 2010a), corn milk (Yasni *et al.*, 2014) and Bambara groundnuts (Falade *et al.*, 2014). The beneficial role of yoghurt and yoghurt-like products produced from legume milk, may be further enhanced by supplementation with probiotics resulting in a product termed probiotic yoghurt (Donkor, 2007). These benefits include among others, immune system modulation (Mantere-Alhonen 1995), activation of immune competent cells (Perez-Chaia *et al* 1995), protective effects against the early stage of colon cancer (Burust Rowland 2000), suppressing unfavourable bacterial enzyme activities and reducing DNA damage (Ewaschuk *et al,* 2006), reduction in serum cholesterol levels (Somkuti and Johnson 1999), lactose intolerance, gut infections and inflammation diarrhoea (Gilliland *et al.,* 1984). Metabolites from yoghurt such as folic acid protects the body against some form of cancer (Hugenboltz *et al* 2002). Besides these, legume milk contains prebiotics such as oligosaccharides amongst others, which confer benefits such as increase in viscosity within food matrices encouraging probiotics survival during preparation and storage (Bernat *et al.*, 2014).

The nutritional quality of vegetable yoghurt may vary with milk source and other added nutrients. For instance, Sunny-Roberts *et al.* (2004), studied some quality parameters of fermented groundnut milk. The lysine content of fermented groundnut milk was found to increase by almost double compared with the unfermented type (Sunny-Roberts *et al.*, 2004). Improvement in the nutritional properties of vegetable yoghurts using probiotics such as *Propionibacterium* has been shown to have positive impacts on health status, function rate and comfort of the gut (Moslemy *et al.,* 2015). *Propionibacterium freudenreichii* is widely used as a starter cultures in food industries and is traditionally used during making of Swiss cheese (Hettinga *et al.,* 1972a; Vorobjeva *et al.,* 2008; Sarkar 2011). These organisms have a long history of safe use, they are generally recognized as safe (GRAS). Their safety is confirmed by the wild spread consumption of Swiss-type cheese. (Saarela *et al.,* 2000; Moslemy *et al.,* 2015). The benefits of *Propionibacterium* depend on their ability to produce propionic acid, bacteriocin, vitamin B12, growth stimulation of other bacteria (Cousin *et al.,* 2012) and the formation of exopolysaccharide (Mohammadi *et al.,* 2011).

Probiotic foods are known as "foods" that contain live microorganisms, which upon ingestion, actively enhance the health of consumers by improving the balance of microflora in the gut (FAO/WHO 2006). Although there are more research data on the use of *lactobacillus* and *Bifidobacterium* species as probiotics than *Propionibacteria*, the latter reported to have positive impacts on health status, function rate and comfort of the gut (Moslemy *et al.,* 2015). The application of probiotics is mostly limited to dairy products such as yoghurt, kefir and cultured drinks. Among them yoghurt is the most popular (Cruz *et al.,* 2010; Granato *et al.,* 2010a; Karimi *et al.,* 2011). As the rate of consumption is increased,

producers are stepping up research on value added ingredients such as probiotic, prebiotics and new matrices (Allgeyer *et al.,* 2010), such as pigeon pea milk. Legumes such as pigeon pea contain phytochemicals, dietary fibres and carbohydrates with low glycaemic index. However, their application has been limited by the presence of anti-nutritional factors. These compounds can however be eliminated by simple traditional and technological methods (Jiménez ‐Martínez *et al.,* 2003; Mubarak 2005b).

Pigeon pea is a legume grown in many parts of the world, including South Africa. Nutritionally, pigeon pea is rich in protein (20-32%) and carbohydrates (49-60%) (Saxena *et al.,* 2010) similar to those in cowpea (Oyeyinka *et al.,* 2013) and Bambara groundnut (Arise *et al.,* 2015; Oyeyinka *et al.,* 2015). The lysine content (7.79 g/100g) of pigeon pea is superior to that of soya (6.1 g/100 g) (Akande *et al.,* 2010a). However, pigeon pea remains an underutilized crop grown mainly for subsistence. The underutilization of many crops including pigeon pea has been attributed to lack of sufficient research to unlock their potential and value addition (Oyeyinka *et al.,* 2015). Focusing on neglected crops such as pigeon pea may facilitate their utilization beyond traditional use.

The objective of this work is therefore to evaluate the pigeon pea and soybean milk yield, suitability as matrix for yoghurt and probiotic yoghurt and nutritional profile of the yoghurt samples. In this study, probiotic yoghurt was formulated from pigeon pea and soybean milk (control) using yoghurt starter cultures and *Propionbacterium freudenreichii*.

3.2. MATERIALS AND METHODS.

3.2.1 Materials

The Pigeon pea grains were obtained from Agricultural Research Council of South Africa, while the Soya bean was obtained from Soya Bean Research Farm, Pine-town Kwazulu Natal, South Africa. Yoghurt cultures (*Lactobacillus delbrueckii* and *streptococcus thermophillus*) and *Propionibacterium freudenreichii* were purchased from Lake Foods South Africa.

3.2.2 Methods

3.2.3 Preparation of Pigeon pea flour

Pigeon pea seeds were screened to remove the bad ones and extraneous materials. Accurately, 250g of the screened Pigeon pea grains were soaked in 2.5 L of milli Q water with 0.5% $NaHCO_3$ overnight and were manually dehulled. The dehulled Pigeon pea seeds were thoroughly rinsed with milli Q water and further blanched in 0.5% NaHCo3 solution at

60^{o} C for 20 minutes. Thereafter, the seeds were rinsed in milli Q water, dried in a hot air oven at 30^{o}C, dry-milled and sieved to obtain the flour. The flour was stored in polythene bags and kept in a refrigerator for analysis.

3.2.4 Milk Preparation

Pigeon pea seeds were screened to remove the bad ones. 250 grams of Pigeon pea seeds were soaked 2.5 L of milli Q water with 0.5% $NaHCO_3$ for 24 hours, and manually dehulled. The dehulled Pigeon pea grains were thoroughly rinsed with milli Q water and further soaked in 0.5% $NaHCO_3$ solution at 60°C for 30 minutes, after which the grains were rinsed in milli Q water, milled into paste and mixed with milli Q water in ratio 1:1 (w-v) using a grinder (Waring laboratory model HGBTWG4 USA). The resulting slurry was centrifuged at 4000g using Heraeus Megafuge 40R (Centrifuge model: Thermofisher Scientific, England).

The milk obtained was heat treated for 20 minutes at 95^{o}C while stirring continuously with a glass rod to prevent burning. The recovered milk was homogenised, using Silverson laboratory homogeniser (P T 20, Fisher scientific water side, U.K.) at 60^{o}C and allowed to cool and then refrigerated.

Pigeon pea milk

3.2.2.1 Soymilk

Soy beans grain was screened to remove the bad ones and extraneous materials. Accurately

250grams of soybeans grains were soaked in 2.5 L of milli Q water, with 0.5% NaHco3 overnight and manually dehulled. The dehulled soy beans seeds were further soaked in milli

Q water with 0.5% $NaHCO_3$ solution at 100^oC for 20 minutes and rinsed with milli Q water, and then mixed in milli Q water in ratio 1:2 with soya beans (w/v) and thereafter transferred into a stainless steel blender (Waring laboratory model HGBTWG4 USA). The resulting paste was centrifuged at 4000 g using Heraeus Megafuge 40 R (Centrifuge model:

Thermofisher Scientific, England) and heat treated at 95^oC for 20 minutes. The milk obtained was homogenized with a laboratory Silverson homogeniser (P T 20, Fisher scientific water side, U.K), at 60^oC and allowed to cool and then refrigerated.

Soymilk

3.3 Experimental Design

This study examined the nutritional factors and amino acids profile of set yoghurt made with 16% (w/w) total solids. Yoghurts were fermented using starter cultures and *Propionibacterium freudenreichii* as a supplement. Six batches of set yoghurt were produced as shown in Table 3.1. All the batches were formulated thus 100% pigeon pea milk, 100% soymilk and 50/50% pigeon pea/soymilk for the starter culture and starter cultures/*Propionibacterium freudenreichii*.

3.3.1 Yoghurt preparation

Yoghurt was prepared following the methods of (Tamime and Robinson, 1999) with slight modifications in temperatures raised from 85 °C for 30 min to 95 °C for 20 min. Skimmed milk powder and sucrose were added to pigeon pea milk (approximately 12.5 grams/100 grams total solids) and soymilk (approximately 15 grams/100 grams total solids) to make a total solids of 16 grams/100 grams. Milk was heated to 65°C and homogenized at 25 MPa for 2 minutes using Silverson Homogenizer (PT 210, Fisher Scientific water side, UK). The temperature was further raised to 95°C for 20 minutes (Aminigo, Metzger and Lehtola 2009). The heat treated milks were thereafter cooled to 43°C in a water bath and inoculated with 2% starter cultures (*Lactobacillus bulgaricus and Streptococcus thermophillus*). The inoculated milk samples were divided into two equal portions, one portion was used as a control and the other inoculated with 1% probiotic (*Propionibacterium freudenreichii*) and were incubated at 43^{o}C for 5 hours until a pH of about pH 4.6 was reached. At the end of the incubation period, the yoghurts were cooled and refrigerated at 4^{o}C overnight.

Table 3.1: Yoghurt Formulations

Ingredient	PPY	SMY	PPY/SMY	PPYP	SMYP	SMY/PPYP
Pigeon pea milk	100%	100%	50%	100%	0%	50%
Soymilk	0%	0%	50%	0%	100%	50%
Skimmed milk	3.5g/100ml	1g/100ml	2.25g/100ml	3.5g/100ml	1g/100ml	2.25g/100ml
Sugar	5g/100ml	5g/100ml	5g/100ml	5g/100ml	5g/100ml	5g/100ml
Starter cultures	2%	2%	2%	2%	2%	2%
Probiotic	0%	0%	0%	1%	1%	1%

Where: **SM:** Soy milk, **PM:** Pigeon pea milk, **SM/PY**: 50% soymilk + 50% pigeon pea milk yoghurt, **SMYP**: Soymilk probiotic yoghurt, **SM/PYP**: 50% soymilk + 50% pigeon pea milk probiotic yoghurt, **PPYP**: Pigeon pea milk probiotic yoghurt, **SMY**: Soymilk yoghurt, **PPY**: Pigeon pea milk yoghurt.

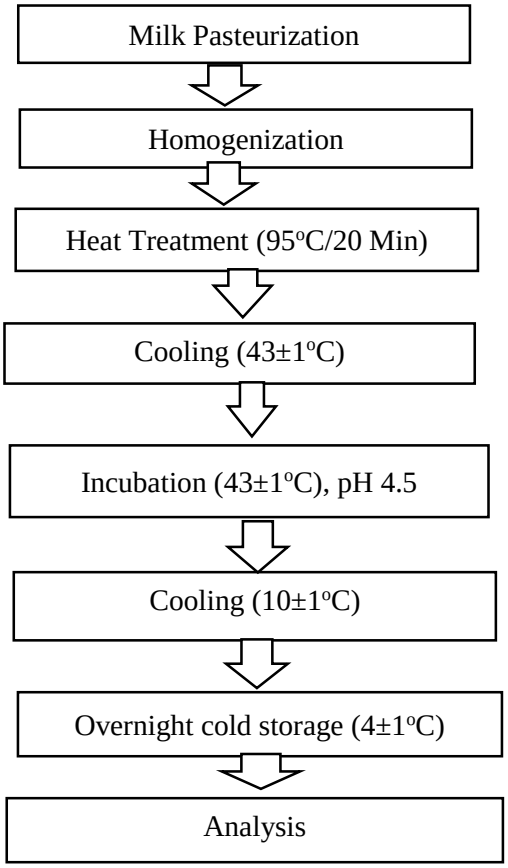

Figure 3.1 Flow Diagram for Yoghurt Processing

Figure 3.2: Yoghurt samples made from pigeon and soy milk

3.3.2 Proximate composition analysis.

Proximate composition of pigeon pea flour, pigeon pea milk, soy milk, pigeon pea and soy milk yoghurt and fermented pigeon pea and soy milk supplemented with *Propionbacterium freudenreichii* were determined.

3.3.3 Moisture content

The moisture content of the samples was measured according to the Association of Official Analytical Chemists International (AOAC) Official Method 934.01 (AOAC 2002), in which the samples of known weight were dried in a forced air oven set at 95ºC for 72 hours. The moisture content of the food products was determined by weight difference after freeze drying in a freeze drier (Mini Iyotrap LTE scientific Ltd United Kingdom).

3.3.4 Fat

The fat content of the samples was determined according to the Soxhlet procedure, using a Büchi 810 Soxhlet Fat Extractor (Büchi, Flawil, Switzerland) according to the AOAC Official Method 920.39C (AOAC, 2002). A 250 ml round bottom flask was weighed, cooled in a desiccator and the mass recorded. 3 grams of sample was weighed and the mass was

recorded. The sample was transferred to an extraction thimble, and the thimble was placed in an extraction chamber. 100 ml of petroleum ether was added to the flask and the flask was connected to the reflux chamber. The condenser was connected to the chamber and the tap was opened to allow for the water to steadily flow through the condenser. The heating mantle was turned on to a medium temperature setting. The fume cupboard extractor fan was turned on. The sample was extracted by refluxing the solvent for at least five minutes. The solvent was topped via the condenser chamber as soon as the solvent level dropped to below the reflux chamber level. The heating mantle was switched off just before all solvent evaporated from the round bottom flask. The round bottom flask was not allowed to run dry in order to prevent the fat extract from burning as this would affect the final extract mass. The flask containing the extract was cooled in a desiccator for 3 to 4 hours and the mass recorded as

$$\% \text{ fat} = \frac{\text{weight of residue (g)} * 100}{\text{weight of sample (g)}}$$

Where, weight of residue = original sample mass - mass of fat extract.

3.3.5 Ash

Ash was determined by combusting the samples in a furnace set at 550 ºC for four hours following the AOAC Official Method 923.03 (AOAC, 2005).

3.3.6 Protein

In clean dry digestion tubes, Three grams of samples were weighed. 4 grams of catalyst were added to the mixture and 25ml concentrated H_2SO_4. The digestion tubes were connected to NaOH trap for absorbing the noxious fumes. The vacuum was used to draw the fumes into the NaOH trap. With a cotton plug the unused opening was closed while heating commenced and maintained such that the sample boiled. The digestion was allowed to proceed for approximately 1-2 hours. Digestion was completed when the solution turns light clear green or looks greenish. The Buchi 321 distillation unit was switched on and allowed to preheat. Distillation vessel was inserted with digested sample. The holder was pressed downwards and released when tube was in place. The sample was diluted in water in approximate ratio of 1:3. The water switch was pressed until desired quantity was filled in. 250ml Erlenmeyer receiving flasks was prepared by adding 25ml 2% boric acid and six drops screened methyl red indicator). The Erlenmeyer receiving flask was placed under the long tubes. 32% sodium hydroxide solution was added to sample by pressing on NaOH switch. (Minimum volume of 32% sodium hydroxide required is 100ml until the solution turns dark brown in colour).

The distillation time was set to $2\frac{1}{3}$ -3 minutes and the distillation proceeded. The residue aspirations switch was set in ON position so that at the end of distillation, the distillation switch residues was aspirated off into the sink.

The distillate was titrated with standard 0.1N sulphuric acid. The end point was reached when the light blue solution should turn colourless to grey. The protein content was determined by using the equation:

$$\% \text{ Nitrogen} = \frac{\text{titration in ml} - \text{blank in ml} * 1.4 * 0.1}{\text{mass of sample}}$$

% Protein = Factor (6.2) x N

3.3.7 Total carbohydrate

Total carbohydrate was obtained by difference. The carbohydrate content (dry basis) was estimated according to the (AOAC, 1995) method as seen below: Carbohydrate % = (100 - moisture% + fat% + protein% + ash %).

3.3.8 Determination of milk yield

The percentage milk yield for pigeon pea and soybean milks were determined by taking the weight of slurry obtained after wet milling of dehulled pigeon pea and soy bean (Nwokolo, 1996). Milk yields were calculated from ratio of milk weight to the weight of slurry. The results were expressed as percent yield.

3.3.9 Amino acids

The amino acid profile of the samples was analysed by the Waters API Quattro Micro method which consists of a column C18, 1.7um, 2.1x 100mm and a binary solvent manager. Yoghurt samples (400 mg) were subjected to Waters AccQ Tag Ultra Derivatization kit; 10µl of the undiluted sample were added to the Waters AccQ Tag kit constituents and placed in a heating block at a temperature of 55^{O}C for 10 minutes. Injection volume was 1µl. 3.3.10

3.3.10 Texture analysis

The hardness of yoghurt was measured with a Trapeziumx EZ – SX (EZ Test) texture analyser (Shimadzu Scientific, China.) with an 11mm cylinder probe and N expressed in

newton. The probe (0.100N) was inserted into each yoghurt sample to a depth of 15 mm and speed 24mm/min. The instrument was fitted to a computer to measure the gel strength. The firmness was measured immediately after removal from refrigerated temperature at 4°C, Measurement was done at two point of each sample far away from each other and the means were calculated.

3.4 Results and Discussion

3.4.1 Yield of Milk from Pigeon pea and Soybean.

The Pigeon pea gave lower milk yield (48%) compared to soybean which gave a yield of about 60% (Figure 1). Pulses generally have been found to produce lower milk yield than oil seeds. For instance, Falade *et al.* (2014) reported higher milk yield (52.2%) for soybean compared to Bambara groundnut (47.0%). Similar trend of high milk yield from soybean compared to Bambara has also been observed by some authors (Beddows and Wong 1987; Brough, Azam-Ali and Taylor 1993). The higher milk yield from soybean suggests that milk extraction from soybean was more efficient compared to pigeon pea. Variation in milk yield among pulses may be attributed to source and cultivar differences.

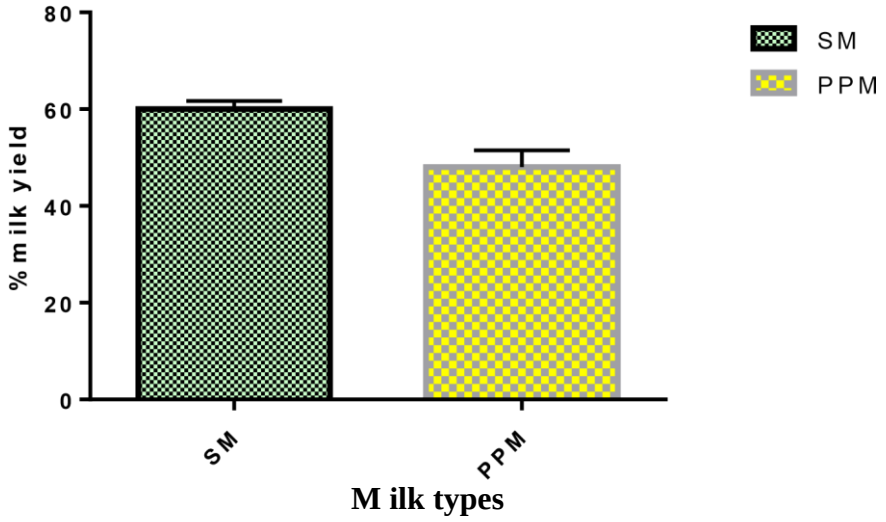

Figure 3.3 Milk Yield of Soybean (SM) and Pigeon pea (PPM).

3.4.2: Proximate composition of Pigeon pea flour

Table 3.2 presents the proximate composition of pigeon pea flour with the protein content value of 26% and crude fat 2%. This is expected as pigeon pea has been classified as protein rich food comparable to soy bean and groundnuts (Aremu *et al.,* 2006). Moreover, crude fat value was low as it is not an oil rich legume when compared to soybean (Salunkhe *et al.,* 1985; Aremu *et al.,* 2006). The ash, fibre and carbohydrate were 3.62, 8.75 and 50.19 respectively. These values were comparable to previously reported literature (Akande *et al.,* 2010a).

Table 3.2 Proximate composition of Pigeon pea flour

Sample	Moisture	Crude protein	Crude fat	Crude fibre	Ash	Carbohydrate
Flour	8.74	25.66	2.32	8.75	3.62	50.91

3.4.3 Proximate composition of milk and yoghurt supplemented with and without *Propionibacterium freudenreichii*

The protein contents (9.49-12.30 %) of milk samples were generally higher than their yoghurt counterparts (4.54-5.85 %) (Table 1). Pigeon pea milk showed significantly lower protein (9.49 %) content than soybean milk (12.30 %). Similarly, the fat content of soybean milk was higher (approx. 4 times) than that of pigeon pea milk. Soybean is an oil seed and is known to be richer in protein than most pulses.

The protein (approx. 9%) and fat (approx. 2%) contents of soymilk were higher compared to that of pigeon pea milk which was approximately 7 and 0.6% respectively (Table 3.3). However, pigeon pea milk showed higher (7 times) carbohydrate content than soymilk. The low fat content in pigeon pea milk is expected since it is not an oil seed. Total solids (TS) content of soymilk (15%) and pigeon pea (13%) milk are within the recommended range of 9g/100g in low fat yoghurt to as high as 30g/100g in all other types of yoghurt (Tamime *et al.,* 1999). According to Tamime *et al.* (1999), good yoghurts are made from milks with TS content ranging between 15 and 16 g/100g. Therefore, prior to making yoghurts, the TS of the milks were increased to 16% by adding skimmed powdered milk and sucrose, and hence water/bean ratio 1:1 and 1:2 for pigeon pea and soybean. Soybean is an oil seed and is known to be richer in protein than most pulses.

Table 3.3 Proximate composition of milk, yoghurt and yoghurt supplemented with *Propionibacterium freudenreichii*

Samples	Total solid	Solid-non fat	Moisture	Crude fat	Crude protein	Ash	CHO
SM	15.016^{a}	13.03±.97^{a}	84.9±16^{a}	2.00±.04^{a}	12.3±.50^{a}	0.65±.00^{bc}	0.52±.00^{a}
PM	12.54±.25^{b}	12.20±.57	87.46±25^{a}	0.56±.00^{a}	9.49±.63^{a,b}	0.58±.08^{b}	3.70±1.70^{b}
SMY	18.29±.00^{c}	16.13±.23^{b}	81.71±.00^{a}	1.99±.24^{b}	4.54±.05^{ab}	1.03±00^{c}	10.74±.19^{c}
PPY	16.43±.95^{d}	16.26±.1.07^{b}	83.46±.06^{a}	0.03±.01^{b,c}	5.85±.07^{ab}	0.83±.06^{c}	9.57±1.05^{cd}
SM/PY	18.43±.37^{d}	17.41±.66^{b}	81.83±.09^{a}	0.76±.67^{cd}	5.00±.00^{ab}	0.72±.01^{c}	11.70±.69^{cd}
SMYP	18.44±.69^{d}	16.04±.84^{b}	81.57±.09^{a}	2.40±.16de	5.77±.45^{bc}	0.49±.00^{c}	10.10±.71^{cd}
PPYP	18.17±00^{d}	16.92±.18^{b}	81.83±.00^{c}	1.25±.18de	5.02±.04^{c}	0.61±.00^{b}	11.26±.18^{cd}
SM/PYP	18.71±.08^{d}	17.14±.15^{b}	81.36±01^{d}	1.58±.06^{e}	5.02±.02^{d}	0.62±.01^{b}	11.44±.02^{e}

Where: **SM:** Soy milk, **PM:** Pigeon pea milk, **SM/PY**: 50% soymilk + 50% pigeon pea milk yoghurt, **SMYP**: Soymilk probiotic yoghurt, **SM/PYP**: 50% soymilk + 50% pigeon pea milk probiotic yoghurt, **PPYP**: Pigeon pea milk probiotic yoghurt, **SMY**: Soymilk yoghurt, **PPY**: Pigeon pea milk yoghurt.

In general, the protein contents of the formulated yoghurts were substantially lower compared to their milk counterparts (Table 3.3). LAB (Lactic Acid Bacteria) have limited ability to use inorganic nitrogen and therefore depend on amino acids present in the growth medium as nitrogen sources. Proteolytic system in dairy LAB is of scientific importance due to its activities in milk fermentation (Donkor 2007). The synthesis of proteolytic enzymes by starter culture is a vital requirement for rapid acid production in milk (Donkor 2007). These microorganisms have a number of proteinase and peptidase which hydrolyse soy and pigeon

milk protein to essential amino acids required for growth, free amino acids for processes such as protein synthesis, generation of metabolic energy and recycling of reduced co-factor (Donkor 2007). However, soy and pigeon pea yoghurt supplemented with *Propionibacterium freudenreichii* alone showed similar protein contents (approx. 6%) and were slightly higher than those without *Propionibacterium freudenreichii.*

Proteolysis during yoghurt fermentation may result in two effects in the product: increase in the digestibility that is diet criterion and decrease in firmness or viscosity That is technology criterion (Sarkar *et al.,* 2010). The protein content of the yoghurt samples which ranged between 4.54 and 5.85 is similar to values previously reported (Diarra *et al.,* 2005; Ranadheera *et al.,* 2012). Unlike the protein content which seems to decrease after fermentation, the carbohydrate content of the yoghurt significantly increased. The addition of skimmed milk and sucrose may have contributed to the higher carbohydrate of the yoghurt samples. Furthermore, the digestion of oligosaccharide, unavailable carbohydrates and production of exopolysaccharides and ability of dairy propionibacteria to biosynthesise trehalose a low calorie sugar may also have contributed to the higher content of carbohydrates in the yoghurt samples supplemented with *Propionibacterium freudenreichii* compared to non-supplemented (Pophaly *et al.,* 2012). Besides, addition of 1g/100ml of lactose is found to significantly improve the growth of bacteria in soymilk (Messina *et al.*, 1997). Furthermore, addition of sweetener is one of the techniques to overcome the problem of objectionable beany in the product and toning down acidic taste (Sarkar, 2011). This has also been reported to indirectly affect physical properties of yoghurt such as viscosity and consistency of the finished product (Tamime *et al.,* 1999). Supplementation with 2 % glucose influenced the production of lactic acid (Favaro Trindade *et al*., 2001).The TS (16.418.7g), solid non-fat (16.0-17.4g) and moisture content (81.4-83.5g) of the yoghurts samples were not significantly different.

The level of solid-non-fat (SNF) 12.50 in Pigeon pea milk and soymilk were also not significantly different (Table 3.3). This may be attributed to protein and fat composition of the milk. However, percentage of solids-non-fat is regulated by legal standard of the country in question. The minimum requirement is that solids- non- fats should be in the range of 8.2 to 8.6g/100g. This is done to protect the consumers.

Therefore, solids- non-fat should be roughly comparable to the levels found in liquid milk (Tamime *et al.,* 1999). Nevertheless, the levels of solids-non-fat in Pigeon pea milk and soymilk were above the recommended range of 8.2 to 8.6 for yoghurt preparation. The fat content of yoghurt with starter cultures (*Streptococcus thermophillus* and *Lactobacillus delbrueckii subsp bulgaricus*) were significantly $P \leq 0.05$) lower to those containing *Propionibacterium freudenreichii* and that of the milk in which they were prepared. Although, low fat content of pigeon pea yoghurt was expected since it is not an oil rich legume. Similar results were observed in fermented ground nut milk (Sunny-Roberts *et al.,* 2004). However, decrease in fat may be due to their utilization by yoghurt starter culture (*Streptococcus thermophillus* and *Lactobacillus delbrueckii subsp bulgaricus)* to yield energy. Homogenization is also implicated in the low fat content of some of the yoghurt samples. Extent of lipolysis in milk that was homogenized is more than that which was not homogenized. This is largely due to large measure destruction of protective layer of the fat globules membrane (Tamime *et al.,* 2005). However, homogenization improves stability of

plant milk by disrupting aggregation and lipid droplets and thus decreases particle size

distribution (Nik *et al.,* 2008). Nevertheless, reduction in fat content may also contribute to the keeping quality of yoghurt, as chances of rancidity would be greatly reduced (Sunny-Robert *et al*., 2004). Furthermore, the higher fat content of probiotic yoghurt may also be linked to the ability *of Propionibacterium freudenreichii* to produce conjugated linoleic acid (CLA) which are health promoting fatty acids in the human body (Bensmira *et al.,* 2012).

Furthermore, the ash contents were generally low (0.5-1%). The higher levels of ash of the yoghurt samples than the skimmed milk in which they were prepared may be due to the addition of milk and sucrose prior to fermentation (2%).

3.7.4 Amino acid profile of flour and milk

Table 3.4 depicts the amino acids profile of pigeon pea flour and milk. Amino acids profile of the milk was significantly $p \leq 0.05$ higher than that of the flour and comparable to FAO standard 2007. The significant differences in amino acids profile the milk and flour suggest processing such as extraction and centrifugation to obtain the milk.

Lysine and glutamic acids were most abundant in milk and aspartic and glutamic acids in flour. Similarly, Akande *et al*. (2010a) reported higher lysine content in roasted pigeon pea flour compared to raw pigeon pea flour (Akande *et al.*, 2010a). Generally, most amino acids were heat stable and concentration increased with heat processing (Akande *et al.*, 2010a). Glutamic acid was found to have the highest value followed by aspartic acid.

Pigeon pea flour and milk were found deficient in sulphur containing amino acid. The relatively low concentration of sulphur containing amino acids has been reported in literature (Apata *et al.,* 1994; Aremu *et al.,* 2006). However, the high protein genotypes which have been developed with protein content as high as 32% (Reddy *et al.,* 1979; Saxena *et al.,* 2002; Saxena *et al.,* 2010) and contain about 25% higher sulphur containing amino acids (Pophaly *et al.,* 2012). Amino acids profile of pigeon pea are comparable with other plant protein sources such as soybean, (Akande *et al*., 2010) Bambara ground nuts, (Kudre *et al*., 2013) amongst others.

Table 3.4 Amino acid profile of pigeon pea flour and milk

Samples (g/100g)	Flour	Milk	FAO/WHO STD(2005)
Lysine	1.537	4.089	4.2
Histidine	0.790	2.237	
Arginine	1.242	3.647	2.0
Aspartic acid	2.218	5.702	-
Threonine	0.834	2.231	2.6
Serine	1.11	2.959	-
Glutamic acid	4.815	12.299	-
Glycine	0.816	2.231	-
Alanine	0.958	2.616	-
Cystine	0.025	0.138	-
Valine	1.000	2.698	4.2
Methionine	0.197	0.573	2.2
Isoleucine	0.807	2.247	4.2
Tyrosine	0.610	1.819	1.4
Phenylalanine	2.326	5.690	2.8
Proline	1.070	2.817	-
Leucine	1.658	4.570	4.2

Amino acid composition of pigeon pea milk and flour compared with FAO (2005).

3.4.5 Amino acids of yoghurt samples

Glutamic and aspartic acid, which may include glutamine and asparagine respectively, were the major amino acids in the yoghurt samples (Table 3.5). These amino acids were also the most abundant in milk samples (Table 3.4). Probiotic yoghurt samples generally showed slightly lower amino acid profile compared to samples without *Propionibacterium freudenreichii*. During fermentation process such as in yoghurt production, the amino acid and peptides are used by LAB (Lactic acid bacteria) for cell growth and survival, protein synthesis, generation of metabolic energy and recycling of cofactors (Nielsen *et al.,* 2001). Furthermore, Donkor (2007), reported that LAB (Lactic acid bacteria) depend on free amino acids for metabolic activities during fermentation. Thus, the slightly lower amino acid content of the probiotic yoghurt could be linked with the activities of the LAB (Lactic acid bacteria). The *Propionibacterium freudenreichii* may have utilised the amino acids for growth since they must be alive to exert their probiotic function. This may account for the reduction in the protein content of probiotic pigeon pea yoghurt (Table 3.3). The lysine, isoleucine, leucine, methionine, phenylalanine, valine, threonine and histidine contents of the yoghurt samples were comparable to the FAO/WHO (2005) recommended amino acid requirement for adults (Table 3.5). Essential amino acids are those essential for the synthesis of body proteins that can only be obtained from the diet (Isanga *et al.,* 2009).

Table 3.4: Amino acids composition of yoghurt sample

Samples(g/100g)	PPY	SM/PYP	SMY	PPY	SM/PY	SMYP	FAO/WHO
Histidine	1.10± 0.01^{b}	.95±0.01^{a}	1.03±0.09^{a,b}	1.12±0.06^{b}	.97±0.02^{a}	1.02±0.05^{a,b}	1.6
Serine	1.70±0.01^{a}	1.66±0.03^{a}	2.00±0.03^{b}	1.70±0.01^{a}	1.70±0.00^{b}	2.00±0.02$_{b}$	
Arginine	1.73±0.01^{a}	2.06±0.00^{b}	3.00±0.14^{c}	1.74±0.01^{a}	2.11±0.05^{b}	2.80±0.0^{c}	
Glycine	1.0±0.01^{a}	1.20±0.01^{b}	1.60±0.11^{d}	1.03±0.04^{a,b}	1.18±0.0^{c}	1.60±0.0^{d}	
Aspartic acid	2.65±0.01^{a}	3.00±0.05^{a}	3.70±.07^{a}	2.47±0.39^{a}	3.08±0.01^{a}	3.07±0.42^{a}	
Glutamic acid	6.02±0.00^{b}	5.84±0.03^{a}	6.33±0.01^{d}	6.17±0.01^{d}	5.88±0.02^{c}	6.09±0.12^{c}	
Threonine	1.23±0.00^{a}	1.30±0.04^{a}	1.47±0.03^{b}	1.26±0.03^{a}	1.30±0.03^{a}	1.45±0.12^{b}	0.9
Alanine	1.20±0.09^{a}	1.30±0.01^{a}	1.35±0.11^{a}	1.20±0.02^{a}	1.27±0.03^{a}	1.32±0.01^{b}	
Proline	2.00±0.03^{b}	1.80±0.01^{b}	1.95±0.00^{c}	2.00±0.01^{c}	1.80±0.00^{c}	1.94±0.07^{c}	
Lysine	2.01±0.03^{b}	1.91±0.00^{a}	2.09±0.00^{c}	2.04±0.00^{b,c}	2.00±.02^{bc}	2.00±.06^{c}	1.6
Tyrosine	1.03±0.03^{a}	1.10±0.00^{ab}	1.57±.01^{b}	1.43±,00^{b}	1.06±04^{b}	1.35±01^{c}	
Valine	1.43±0.03^{b}	1.39±0.00^{a,b}	1.57±0.02^{c}	1.35±0.00^{b}	1.35±0.0^{a}	1.56±0.02$_{b}$	1.3
Isoleucine	1.24±0.03^{a,b}	1.30±01^{ab}	1.60±01^{bc}	1.24±.01^{c}	1.30±01^{d}	1.54±01^{e}	1.3
Leucine	2.53±06^{a}	2.50±01^{a}	3.00±0.03^{b}	2.55±0.45^{a}	2.50±0.05^{b}	2.75±0.02^{b}	1.9
Phenylalanine	2.75±0.01^{b,c}	2.20±0.01^{a}	2.06±0.20^{a,b,c}	2.84±0.16^{c}	2.22±0.04^{a,b,,c}	2.75±0.02^{a,b}	1.9

Where: **SM/PY**: 50% soymilk + 50% pigeon pea milk yoghurt, **SMYP**: Soymilk probiotic yoghurt, **SM/PYP**: 50% soymilk + 50% pigeon pea milk probiotic yoghurt, **PPYP**: Pigeon pea milk probiotic yoghurt, **SMY**: Soymilk yoghurt, **PPY**: Pigeon pea milk yoghurt. FAO/WHO Standard (2007).

Table 3.5 Fatty Acid composition of pigeon pea flour and milk

Fatty acid (%)	Flour	Milk
Tetradecanoic acid, methyl ester	00.00	00.01
Rentodecenoic, methyl ester	00.02	00.03
Cylopropaneoctanoic acid, methyl ester	28.00	31.10
ISTD (17)	10.30	06.30
9, 12 Octadecadeinioic acid(2,2) methyl ester	40.70	40.40
Tras-9-octadecanoic acid methyl ester	11.80	11.00
Eicosanoic acid, methyl ester	00.60	00.00
Heneicosanoic, acid methyl ester	00.00	00.00
Docosanoic acid, methyl ester	00.70	01.00
Tricosanoic acid,metyl ester	00.00	00.20
Oleic acid, methyl ester	02.70	02.00
Octadecadienoic acid, methyl ester	04.60	06.00
9, 11-octadecadienoic acid, methyl ester	00.00	00.02
Hexadecanoic acid, methyl ester	28.80	31.10

3.4.6: Textural (firmness (N) characteristics of yoghurt samples stored at 4°C for 4 weeks

The texture of the formulated yoghurt samples stored at 4°C for 4 weeks were assessed using a texture analyser. All yoghurt samples showed increased firmness with increase in storage period (Table 3.7). Yoghurt with *Propionibacterium freudenreichii* texture were significantly ($P \leq 0.05$) different from those without *Propionibacterium freudenreichii*. The increased firmness during storage may explain the increase in WHC during storage (Figure 3.7). Domagała (2009) studied the textural properties of yoghurt prepared from goat, cow and sheep milk. The yoghurt samples similarly showed increase in firmness during a 14 day storage period (Domagała 2009). Furthermore, probiotic yoghurt samples showed significantly higher firmness (1.36-1.70 N) than yoghurt without *Propionibacterium freudenreichii* (0.15-1.62 N). The higher firmness of probiotic samples may be explained by the production of extracellular slime, an exopolysaccharide which contributes to increased viscosity of liquid cultures (Skogen. et all 1973Skogen, Reinbold and Vedamuthu 1974; Ekinci and Barefoot 2006; Ekinci and Gurel 2008). Ekinci and Gurel (2008), reported firmness value in the range of 0.19 to 0.31 N for probiotic yoghurt prepared from cow milk.

These values were much lower than values obtained in this study. Differences in total solids content of yoghurt and the degree of protein-protein interactions may explain the variation among reported data (Tamime and Robinson 1999; Ekinci and Gurel 2008; Domagała 2009). Furthermore, the variation in the composition of fats and other components in the respective milk may have contributed to the differences in the firmness of the yoghurt samples. This may be due to soy protein gels higher capacity to entrap water within its three dimensional network (Kpokodo, 2014).

Table 3.6 Textural characteristic of yoghurt samples stored at 4°C

Week of storage	SMYP	SMY	SM/PY	SM/PYP	PPYP	PPY
1	1.63±0.01^{e}	1.46±0.02^{d}	0.48±0.01^{c}	1.37±0.01^{c}	1.36±0.01^{c}	0.48±0.01a
2	1.66±0.01 ^{e}	1.47±0.02^{d}	0.51±0.02^{b}	1.39±0.01^{c}	1.38±0.01^{c}	0.15±02^{a}
3	1.70±0.01^{a}	1.51±0.20^{a}	0.530±0.02^{a}	1.42±0.01^{c}	1.420±.01^{a}	0.18±0.01^{a}
4	1.72±0.01^{e}	1.62±0.01^{c}	0.580±0.01^{b}	1.46±0.02^{c}	1.47±0.02^{c}	0.29±0.06^{a}

Where: **SM/PY**: 50 % soymilk + 50 % pigeon pea milk yoghurt, **SMYP**: Soymilk probiotic yoghurt, **SM/PYP**: 50 % soymilk + 50 % pigeon pea milk probiotic yoghurt, **PPYP**: Pigeon pea milk probiotic yoghurt, **SMY**: Soymilk yoghurt, **PPY**: Pigeon pea milk yoghurt.

3.8 Conclusion and Recommendation

The study revealed that plain and probiotic yoghurt with comparable nutritional and textural qualities to soy yoghurt can be produced from pigeon pea milk. However, since the yoghurts were produced without additives such as colours, stabilizer, preservatives the qualities of the yoghurt could be improved upon by utilising the above additives.

CHAPTER 4

2ND RESEARCH CHAPTER

STORAGE STABILITY OF PIGEON PEA AND SOYMILK YOGHURT, PIGEON PEA AND SOY MILK YOGHURT SUPPLEMENTED WITH *PROPIONIBACTERIUM FREUDENREICHII.*

Abstract

Yoghurt is a nutritious food product containing carbohydrates, fats, minerals, vitamins and most of the essential amino acids. However, it is also a suitable environment for the growth of microorganisms, including spoilage and harmful types. The presence of selected microorganisms, including *E. coli,* was therefore determined in yoghurt samples during storage at different temperature conditions. Yoghurt was prepared from 100% pigeon pea milk, 100% soymilk and 50/50% pigeon pea/soymilk. The milks were inoculated with yoghurt cultures (*Streptococcus thermophillus and Lactobacillus* d*elbrukeii*) and divided into two equal parts, one served as control and the other part inoculated with *Propionibacterium freudenreichii*. Three yoghurt samples were produced. The microbiological quality of yoghurt samples stored at 4°, 10° and 21°C respectively for 4 weeks was monitored by taking aliquot weekly from each yoghurt sample and analysed for aerobic spore formers, *E. coli,* total plate counts, mould and *Propionibacterium freudenreichii* using standard plating technique: Total plate counts (log 7.01- 7.46 cfu/ml) of samples stored for two weeks at 4°C were similar. Predominant organisms were LAB (Lactic Acid Bacteria), with increase in storage temperature from 4°C through 10°C to 21°C, total plate and LAB significantly increased approximately by log 2 CFU/ml for the first two weeks. Moulds however were not detected in all samples during the first two weeks. Beyond two weeks of storage, there was a significant decline in total plate counts and LAB, while moulds increased. Aerobic spore formers and moulds were observed in control. *E. coli,* however, was not found in all yoghurt samples throughout storage period. The pH values of the milk in which yoghurt mixtures were formulated were pH 7 and pH 6.8 for pigeon pea and soymilk, respectively, declining significantly as a result of

acidification. Differences in decrease in pH at 4°, 10° and 21° C were significant ($p \leq 0.05$), the rate was higher at 21°C, 10°C than 4°C. Decreasing pH resulted in increased TTA values over storage temperatures and periods. The TTA values which range from 0.95-1.35, 0.95-1.34 and 0.95- 1.75 for samples stored at 4° C, 10° C and 21°C. Samples stored at 21and 10 had significant higher TTA values than samples stored at 4°C. The colour values evaluated were recorded as Hunter L*, b*, a* and colour difference (ΔE*) during 4 weeks storage at 4°C, 10°C and 21°C. Significantly, high values ($p \leq 0.05$) recorded for L*in the yoghurt samples with soymilk. The colour scale defines positive and negative for a* and b*. All a* values both positive and negative were less than 3. There were no negative value (blueness) recorded for b*. Colour difference ΔE* values trends increased as storage time and temperature increased. There were significant ($p \leq 0.05$) differences between samples stored at the same and different storage temperatures and periods. Water holding capacity was significantly different ($p \leq 0.05$) in all the yoghurt samples stored at 4°C, 10°C and 21°C for 4 weeks. Formulation with 100% soymilk recorded higher values. Yoghurt with acceptable microbial safety and functional properties were produced from pigeon pea and soybean milk.

4 .1 Introduction

In recent years legume milk has been successfully converted into low-cost edible products such as yoghurt and yoghurt like product (Aidoo *et al.,* 2010). Yoghurt is a fermented product obtained from lactic acid fermentation of milk by *Lactobacillus delbrueckii subsp bulgaricus and Streptococcus thermophillus* with *Propionibacterium freudenreichii* as a supplement or co-culture (Ekinci *et al.,* 2008).

Yoghurt is usually made with heat-treated milk of standardised composition after inoculation and incubated at temperatures of 45^{o}C (Rasic and Kurman, 1978). Apart from the traditional yoghurt cultures, probiotics are sometimes used with starter cultures to produce probiotic yoghurt. Yoghurt is a nutritious food product with most of the essential amino acids, carbohydrates, fats minerals and vitamins (Rasic and kurman, 1978). The presence of these nutrients in yoghurt and probiotics yoghurt promote the growth of pathogens. The nutrients in yoghurt are essential for microbial growth with glucose serving as carbon and energy source (Jimenez-Matinez *et al.,* 2003). Although yoghurt cultures are known to provide defence against food-borne pathogens, studies have shown that pathogens are able to survive during fermentation and storage of yoghurt. The survival of food borne pathogens in yoghurt and yoghurt like products have been attributed to their ability to adapt to the low pH (Tamime and Robison, 1985). Cormac (1996) reported the enhanced survival of acid-adapted pathogenic strains in foods containing lactic acid such as yoghurt (Gahan *et al.,* 1996).

Recently, there has been increased concern regarding the presence of pathogens in the dairy products in the industries. The common sources of these contamination are workers and equipment (Simonne *et al.,* 2010). Yoghurt is one of the products considered safe because the milk is heat treated and it's acidity is supposed to be an effective barrier for survival of many food borne pathogens. Many fermented products, such as yoghurt and yoghurt-like, are meant to be consumed with no additional heat treatments. As such, contaminants may persist through manufacturing, distribution, storage and eventually reach consumers unabated at consumption (De Vries, 1996). Typically, pathogenic microbes and the toxins they produce are the major food safety hazards associated with dairy products (Bachrouri *et al*., 2006).

Over the years, microorganisms such as *Staphylococcus aureus, Salmonella spp., Escherichia coli, Shigella spp., Listeria Monocytogens and Yersinia enterocolitica* have been reported as most common foodborne Pathogens that are present in many foods and also survive in milk and milk products (Gulmez and Guven 2003; Collado *et al*., 2008).

The spoilage of yoghurt by yeast has appeared as an important problem (McKay, 1992). The occurrence of yeast as contaminant in yoghurt is encouraged by the high acidity, sugar content and low storage temperature (Dublin-Green and Ibe, 2008). In fermented milk products such as yoghurt and yoghurt supplemented with *Propionibacterium freudenreichii* micro flora is kept alive until sale to the customers and may not contain any pathogenic microorganism (IDF, 1988). Lactic acid bacteria and *Propionibacteria freudenreichii* exert strong antagonistic activity
against many spoilage and pathogenic microorganisms. This is achieved by the production of various metabolites such as organic acids, diacetyl, hydrogen peroxide and bacteriocin during fermentations (Nasib *et al.*, 2006).

Apart from the production of inhibitory compounds, high numbers of lactic acid bacteria and *Propionibacteria freudenreichii* (10^6 cfu ml^{-1}), also compete with the pathogens for nutrient during fermentation process (Pitt *et al*., 2000). The combined influence of large numbers of competing lactic acid bacteria and low pH have been reported to produce an unfavourable environment for many pathogens and food spoilage organisms to thrive (Pitt *et al.*, 2000). Adhesion of pathogenic bacteria to mucosal surface is considered to be the first step of intestinal infections (Tuomola *et al*., 1999). Probiotic bacteria with beneficial health effects have also been found to adhere to the intestinal mucosal, and have been reported to inhibit mucosal adherence and invasion by pathogens (Tuomola *et al*., 1999). Each antimicrobial compound produced during fermentation provides an additional obstacle for pathogens and spoilage bacteria to overcome before they can survive and or multiply in the food from time of production to the time of consumption.

The use of lactic starter cultures and probiotics for the production of yoghurt and yogurt like products has been reported to provide a measurable defence against pathogens and spoilages microorganisms. However, as indicated earlier, many of these spoilage microorganisms tolerate the acidic conditions created by these starter cultures and probiotics thus thrive in these products (Devereux *et al.*, 2003).

Yoghurt is stored at 4^{o} C after it has been produced. Many researchers have observed that some foodborne pathogens are able to survive in the yoghurt stored at this low temperature (Bachrouri et al., 2006). In Africa, many of her communities lack refrigeration facilities and thus store yoghurt at ambient temperatures (21-37^{o} C). Furthermore, most people do not know the correct storage temperature for yoghurt. In everyday life, due to the following reasons, yoghurt is stored incorrectly (Ashenafi, 2002). After purchase, yoghurt is sometimes left in the car for up to six hours. Furthermore, electricity interruption for hours is also an issue. During this period, the temperature of the yoghurt increases providing opportunity for microorganisms to grow. Diverse storage temperatures and times have been implicated to be responsible for changes in quality characteristics of yoghurt (Obi *et al*., 2010; Osman and Razig 2010). Improper storage of yoghurt can lead to spoilage within a short period of time (Mataragas *et al.*, 2011). The storage conditions under which yoghurts are stored are important.

Further, viability of probiotic microorganisms during storage and health benefits are of prime importance, although there are reports where non-viable cells have shown health benefits (Salminen *et al.*, 1999; Ouwehand & Salminen 1998). This research aimed at studying storability, the changes in pH and titrate able acidity, microbial quality, colour, water holding capacity and syneresis of yoghurt and probiotic yoghurt (fermented soy and pigeon pea milk, and fermented soy and pigeon pea milk supplemented with *Propionibacterium freudenreichii*) during 28 days storage period.

4.2 Materials and methods

4.2.1 Materials

Pigeon pea seeds were obtained from Agricultural Research council South Africa. Soya bean from soya bean research farm Pinetown. Yoghurt cultures were purchased from Lake Foods South Africa.

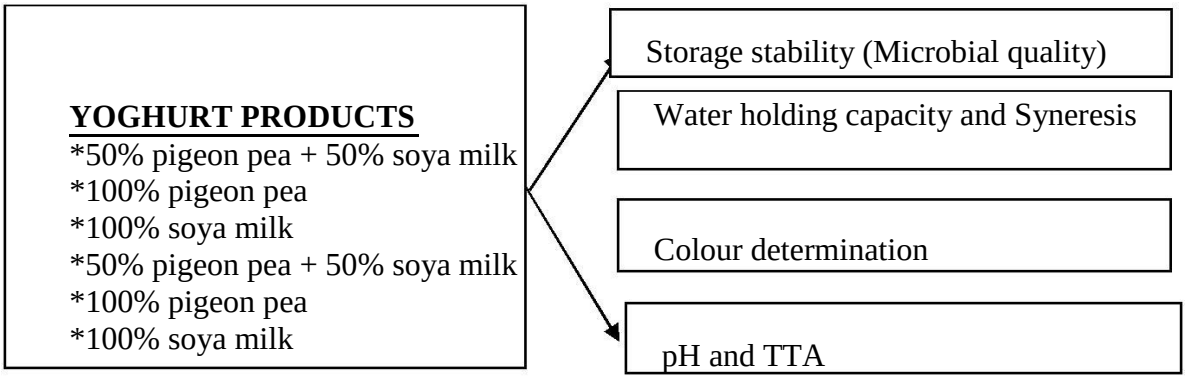

Figure 4.1 Experimental design

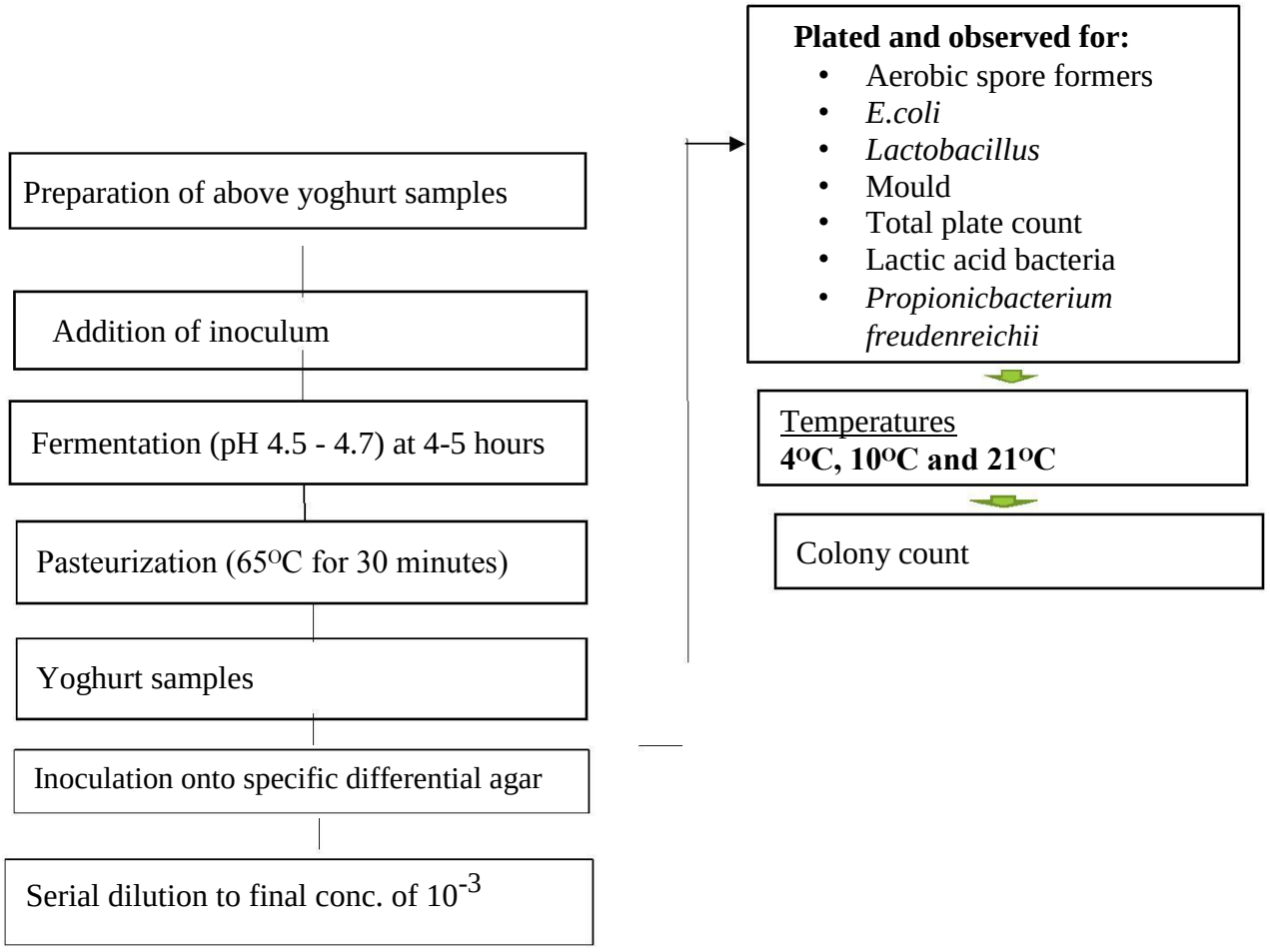

Figure 4.2: flow chart showing process for microbial quality

4.2.2 Milk Preparation

Pigeon pea seeds were screened to remove the bad ones. 250grams of Pigeon pea seeds were soaked 2.5 litres of milli Q water with 0.5% $NaHCO_3$ for 24 hours, and manually dehulled. The dehulled Pigeon pea seeds were thoroughly rinsed with milli Q water and further soaked in 0.5% NaHCo3 solution at 60°C for 30 minutes, after which the seeds were rinsed in milli Q water, milled into paste and mixed with milli Q water in ratio 1:1 (pigeon pea in g/water in ml) using a grinder (Waring laboratory model HGBTWG4 USA). The resulting slurry was centrifuged at 4000g using Heraeus Megafuge 40R (Centrifuge model: Thermofisher Scientific, England).

The milk obtained was heat treated for 20 minutes at 95^{o}C while stirring continuously with a glass rod to prevent burning. The recovered milk was homogenised, using Silverson homogeniser laboratory mixer emulsifier machine (P T 20, Fisher scientific water side, U.K.) at 60^{o}C and allowed to cool and refrigerated.

4.2.3 Soymilk

Soy beans seed were screened to remove the bad ones and extraneous materials. 250 grams of soybeans seeds were soaked in 2.5litres of milli Q water, with 0.5% NaHco3 overnight and manually dehulled. The dehulled soy beans seeds were further soaked in milli Q water with 0.5% $NaHCO_3$ solution at 100^{o} C for 20 minutes and rinsed with milli Q water, and then mixed in milli Q water in ratio 1:2 with soya beans (soya beans in g/water in ml) and thereafter transferred into a stainless steel blender (Waring laboratory model HGBTWG4 USA). The resulting paste was centrifuged at 4000g using Heraeus Megafuge 40 R (Centrifuge model: Thermofisher Scientific, England) and heat treated at 95^{o} C for 20 minutes. The milk obtained was homogenized using Silverson homogeniser laboratory mixer emulsifier machine (P T 20, Fisher scientific water side, U.K), at 60^{o} C and allowed to cool and refrigerated.

4.2.4 Yoghurt preparation

Pigeon pea milk (approximately 12.5 grams/100 grams total solids) was added 3.5grams/100grams skimmed milk powder. This was followed by stirring in powdered milk in pigeon pea milk and raising the temperature 43^{o} C for 30 minutes and 3.5grams/100 ml sucrose was added as sweetener. The milk was then heated to 65° C and homogenized at 25 MPa (Silverson Homogenizer model 18319).

The temperature was further raised to 90° C for 20minutes. The heat treated milk was cooled to 43°C in a water bath then inoculated with 2% starter cultures (*Lactose bacillus bulgaricus and Streptococcus thermophillus*). The inoculated milk was divided into two equal portions, one portion was used as control and the other was inoculated with 1% probiotic cultures (*Propionibacterium fruendenchii*) and were incubated at 43°C for 4 hours for portion with probiotic and 5 hours for starter cultures until a pH of about pH 4.6 was reached. At the end of the incubation period, the yoghurts were cooled and refrigerated at 4°C overnight.

4.2.5 Microbial Quality

The microbiological quality of six yoghurt samples produced were determined. The samples were stored at different temperatures: 4°C, 10^{O}C and 25°C. The microbiological quality of the samples were monitored by taking aliquots, weekly, from each sample and analysed for aerobic spore formers, *E. coli*, total coliform counts and moulds, using the standard plating technique. A 1 ml aliquot of each yoghurt sample was serially diluted and inoculated onto specific differential and selective media, to analyse for specific microbial types.

4.2.5.1 Determination of aerobic spore-formers

Tryptone Soy Agar was prepared, sterilised and kept in a water bath at 50°C until use. Serial dilutions were pour plated. A set of plates were incubated aerobically at 37°C for 48 hours. Three replicates of each sample were analysed. This was placed in water bath at 75°C, and held at that temperature for 20 minutes. 1 ml of serial dilutions were plated in duplicates. And inverted set plates were incubated aerobically at 35°C for 48 hours. Three replicates of each sample were analysed.

4.2.5.2 Determination of *E. coli*

A 1ml aliquot of yoghurt was inoculated into 10 ml double strength of Lauryl Sulfate Tryptose (LST) broth and incubated for 24hours. After 24hours, a 1ml aliquot was transferred into 10 ml of Brilliant Green Lactose (BGLB) broth and incubated at 35°C for 24hours. After 24hours, 1ml aliquot was transferred into *Escherichia Coli* (EC) broth tubes in a water bath at 45°C. This was transferred into Eosin Methylene Blue (EMB) agar, which was incubated in a water bath at 45°C. After 24hours, EMB plate was incubated at 35°C for 24hours and observed for typical non-mucoid, nucleated, dark centred colonies with or without a metallic sheen, which are indicative of *E. coli* (Feng *et al*., 2002). Three replicates of each sample were analysed.

4.2.5.3 Determination of total plate count.

Using plate count agar, yoghurt samples were serially diluted and inoculated into petri dishes. The plates were incubated for 24 to 48hours. Colonies were then counted and

expressed as log cfu/ml (Chako, 2010). Three replicates of each sample were analysed.

4.2.5.4 Determination of moulds

Determination of the presence of moulds was done according to the method of Beuchat (1992) and modified. Potato dextrose agar (PDA) was prepared and sterilised and kept in a water bath at 50°C until use. Exactly 25mL of PDA was poured into petri dishes and allowed to set. Yoghurt samples were serially diluted and inoculated onto petri dishes and swirled. The petri dishes were incubated at room temperature for 48-72 hours. Three replicates of each sample were analysed.

4.2.5.5 Determination of lactic acid bacteria

DeMann Rogosa-shape (MRS) was prepared and cooled to 45°C. The agar was poured into a Petri dish, where appropriate diluted yoghurt with peptone water was transferred into the molten MRS agar. The mixture was evenly mixed by gentle tilting and swirling and allowed to solidify. Then after, the plates were inverted, and incubated at 37° C for 48 hours. Three replicate of each sample were analysed.

4.2.5.6 Determination of *Propionibacterium freudenreichii*

Sodium lactate agar was prepared and cooled to 45°C and was poured into petri dish and allowed to set. Appropriately diluted yoghurt with peptone water were cultivated on sodium lactate agar incubated anaerobically at 32°C for 96 hours as reported in Ekinci and Barefoot, 2006). Three replicate of each sample were analysed.

4.2.6 pH and titratable acidity

The pH and titratable acidity (TTA) of the yoghurt samples were monitored at a week interval. Using pH Meter (3510). TTA was measured by the samples against 0.1 NaOH and expressed as percentage lactic acid. Phenolphthalein was used as an indicator (Amerine *et al.,* 1967).

4.2.7 Water holding capacity

The water holding (WHC) of yoghurt was carried out by the method reported (Harte, luedecke, Swanson, and Bar-bosa-canovas, 2003) with slight modifications. Accurately 100grams of yoghurt was centrifuged for 15minutes at 8000xg at a temperature 4°C. Using Eppendorf centrifuge 5810R. Water holding capacity was calculated as:

$$WHC\ (\%) = (1- {}^{w1}/_{w^2}) \times 100/1.$$

Where W1 is the weight of whey after centrifugation

W2 is the weight of yoghurt.

4.2.8 Susceptibility to syneresis

Susceptibility to syneresis was measured by placing 100ml of yoghurt on a funnel lined with filter paper. After the drainage, the volume of the whey collected in a beaker was measured and used as an index of syneresis. The following formula was used to calculate susceptibility to syneresis (STS).

$$STS\ (\%) = ({}^{v^1}/_{v^{2)}}) \times 100/1$$

Where V1 = volume of whey collect after drainage,

V2 = volume of yoghurt samples

4.2.9 Colour determination

The colour of the yoghurt sample (50 mL) yoghurt samples was measured using a Hunterlab Colorflex Spectrophotometer after overnight refrigeration, and 1,2,3,and 4 weeks, at different temperatures of 4, 10 and 21^0C respectively over a 28 day storage period. The spectrophotometer was standardised using the black and white tiles. Results were reported as L*, a*, b* and ΔE, values. The yoghurt samples were placed in glass samples holder and reflectance measured for L*a*b* and ΔE, L* is lightness with 100 as maximum indicating a perfect reflecting diffuser and the minimum is zero, which is black. The a* and b* axes have no specific numerical limits, negative a* is green, positive is red. Negative b* is blue, positive b* is yellow (Aidoo *et al*.,

2010) and Δ which is differences in colour. A Lab scan EZ spectrophotometer (Hunter Association Laboratory Inc. Reston, V.A).

The equation for calculated Δ is as follows:

$$\Delta = \sqrt{(\Delta *) 2 + (\Delta * 2) + (\Delta *) 2}$$

Where Δ ∗, Δ∗, Δ ∗, are the difference of the L*, a* and b* between storage samples and day 1 samples.

4.3 RESULTS AND DISCUSSION

4.3.1 Microbial quality

The microbiological quality of the yoghurt samples stored at different temperatures: 4°C, 10°C and 21°C for 4 weeks were investigated. Samples were monitored by taking aliquots, weekly, from each sample and analysed. Colonies from incubated plates were counted using colony counter (Colonu DOC.IT imaging station, made in USA) and expressed as CFU/mL (Table 4.1- 4.4). The predominant organisms in all the fermented yoghurt were Lactic acid bacteria (LAB) and followed by *Propionibacterium freudenreichii.* This may be attributed to the fact that the starter cultures were used under proper condition of fermentation for yoghurt production. With increase in storage temperature from 4 through 10 to 21°C, the total plate, LAB and *Propionibacterium freudenreichii* counts increased by approximately, log 2 CFU /mL. Moulds, *E. coli* and aerobic spore formers were not detected in the samples at week 1 and 2. Findings from this study is similar to previous reports where higher plate and LAB and *Propionibacterium freudenreichii* counts were observed for soy yoghurt stored at higher temperature of 37°C compared to those stored at refrigerated conditions (Mahala and Mohammed 2010; Hackett *et al.* 2013; Falade *et al.* 2014) Chako *et al.,* 2010). According to Sengupta *et al.* (2013), LAB grow well at temperatures between 20° and 40° C with an optimum temperature of 32° C. Also the optimal growth at higher temperatures may have accounted for the increase in the LAB count for the yoghurt samples in this study. However, yoghurt samples stored at 21° C showed signs of spoilage instead of semi- liquid state it became liquid. Viljoen *et al.* (2003) observed maximum counts of 6.95 log units in

fruit yoghurt at temperatures of 15 to 25° C. With the exception of moulds growth which was detected in the non-probiotic yoghurts (SM/PY, SMY and PPY) stored at 4° C after 3 weeks, the total plate, LAB, *Propionibacterium freudenreichii*, *E. coli* and aerobic spore former counts remained unchanged. Mould, total plate and Lab counts increased with increase in temperature. However, beyond the 2nd week of storage, there was a decline in total plate and LAB and *Propionibacterium freudenreichii* counts, while the mould count increased. Many other authors similarly reported decline in LAB counts of yoghurts samples after storage for few weeks (Aminigo *et al.,* 2009; Laye *et al*., 1993). Furthermore, aerobic spore formers and moulds were observed in all yoghurt samples except the probiotics. These organisms increased throughout the storage period of 4 weeks. The growth of moulds may have resulted from increase in acidity during fermentation process which may have provided favourable conditions for mould growth (Sengupta *et al.*, 2013). Their presence including yeast in yoghurt has been associated with contamination (Dublin-Green and Ibe, 2008). Furthermore, yeast as contaminant of yoghurt has been attributed to their ability to ferment added sweeteners such as sucrose and lactose (Suriyarachchi and Feet, 1981).

Expectedly, coliforms including *E. coli* were not found in all the yoghurt samples throughout the storage period. This indicates that the yoghurts were prepared under hygienic conditions. Starter cultures and probiotics produce organic acids such as lactic, acetic, propionic acid and other secondary metabolite such as bacteriocin that act against the growth of spoilage and pathogenic bacteria during fermentation and storage (Moslemy *et al*., 2015). These acids have been implicated in preventing the growth of aerobic spore formers and mould in yoghurt samples. The absence of moulds and aerobic spore formers in the probiotic yoghurts may thus be linked with these acids as previously reported (Moslemy *et al.*, 2015).

However, *Propionibacteria* has been reported to possess antibacterial and antiviral properties. This is an important aspect in the production of bacteriocin which inhibit bacteria activities (Mantere-Alhonen, 1995). Furthermore, production of bacteriocin-like compounds known as "Microgard'™ has been associated to *Propionibacterium*

Prevented the growth of mould and aerobic spore formers. Jenseniin G is one of the bacteriocins that is produced from dairy *propionibacteria* that is used to control acid - formation and post acidification during refrigerated storage of yoghurt from shelf to table (Weinbrenner *et al*., 1997). Moreover, pasteurization which destroy enough microorganisms and extend shelf life (Rustom *et al*., 1995), thus milk was also heat treated to 95°C for 20 minutes which is higher than 80°C for 20 minutes previously reported to eliminate undesirable microorganisms and makes legume milk suitable for fermentation (Aminigo *et al.*, 2009). *Bacillus* spores population could have been reduced by tyndallisation with CO2 injection which involves heating to 80°C to activate spore germination followed by heating to 95°C (Kim *et al*., 2012).

Table 4.1 Storage stability for yoghurt samples

Week 1	4°C (log/CFUx10^6)		
Product	Total plate count	LAB	P. freudenreichii
SMYP	8.34±.01^c	8.78±.01^c	8.65±01^d
S/PYP	8.88±.00^e	8.78±.01^c	8.62±.01^c
PPYP	8.73±.02^c	8.58±01^a	8.58±.01^b
SMY	8.77±.01^d	8.76±.01^b	ND
SM/PY	8.86±01^e	8.73±.01^b	ND
PPY	8.59±.01^b	8.57±.01^a	ND

Week Product	Total plate count	LAB	P. *freudenreichii*
10°C			
SMYP	8.34±.01^c	8.78±.01^c	8.65±.01^d
S/PYP	8.88±.00^e	8.78±.01^c	8.78±.01^c
PPYP	8.73±.02^c	8.57±.01^a	8.58±.01^d
SMY	8.77±.01^d	8.76±.01^b	ND
SM/PY	8.86±.01^e	8.73±.01^b	ND
PPY	8.57±.01^b	8.57±.01^a	ND

Week 1 Products	21° C Total plate count	(log/CFU× 10^6) LAB	*P. freudenreichii*
SMYP	8.78±.01[a]	8.86±.01[d]	8.84±.02[d]
S/PYP	8.88±.01[b]	8.58±.01[a]	8.52±.01[b]
PPYP	8.85±01[b]	8.78±.01[c]	8.66±.01[c]
SMY	8.75±.01[a]	8.67±.02[b]	ND
SM/PY	8.88±.01[b]	8.78±.01[c]	ND
PPY	8.76±.01[a]	8.78±.02[c]	ND

SM/PY: 50 % soymilk + 50 % pigeon pea milk yoghurt, **SMYP**: Soymilk probiotic yoghurt, **SM/PYP**: 50 % soymilk + 50 % pigeon pea milk probiotic yoghurt, **PPYP**: Pigeon pea milk probiotic Yoghurt, **SMY**: Soymilk yoghurt, **PPY**: Pigeon pea milk yoghurt, **Mould, aerobic spore former and *E. coli* were not detected**

Table 4.2 Storage stability for yoghurt samples

Week 2	4 ° C	(log/CFU×10^6)	
Product	Total Plate Count	Lactic acid bacteria	*P. freudenreichii*
SMYP	7.36±.01^{d}	7.35±.01^{b}	7.66±.01^{bc}
S/PYP	7.46±.01^{e}	7.45±.02^{c}	7.68±.01^{c}
PPYP	7.11±.01^{b}	7.34±.01^{a}	7.62±.02^{b}
SMY	7.01±.01^{a}	7.32±.01^{a}	ND
SM/PY	7.38±.01^{d}	7.53±.03^{b}	ND
PPY	7.22±.01^{c}	7.38±.02^{b}	ND

Week 2	10 ° C	(log/CFU×10^6)	
Product	Total Plate Count	Lactic acid bacteria	*P. freudenreichii*
SMYP	8.86±.01^{b}	8.78±.01^{bc}	8.70±.01^{b}
S/PYP	8.76±.01^{a}	8.72±.02^{b}	8.74±.02^{c}
PPYP	8.96±.01^{c}	8.80±.02^{d}	8.76±.01^{e}
SMY	8.86±.01^{b}	8.84±.01^{e}	ND
SM/PY	8.87±.01^{c}	8.76±.01^{bc}	ND
PPY	8.84±.01^{b}	8.63±.01^{a}	ND

Week 2	21° C	(log/CFU×10^6)	
Product	Total Plate Count	Lactic acid bacteria	*P. freudenreichii*
SMYP	8.86±.01^{b}	8.78±.01^{bc}	8.70±.01^{b}
S/PYP	8.76±.01^{a}	8.72±.02^{b}	8.74±.02^{c}
PPYP	8.96±02^{c}	8.80±.01^{d}	8.76±.01^{e}
SMY	8.86±.01^{b}	8.84±.01^{e}	ND
SM/PY	8.87±.01^{c}	8.76±.01^{bc}	ND
PPY	8.84±.01^{b}	8.63±.01^{a}	ND

Table 4.3 Storage Stability of Yoghurt Samples

Week 3 Product	4° C Mould	Log/CFU×10^6 Total plate count	Lactic acid bacteria	P. freudenreichii	Aerobic spore former
SMYP	ND	6.82±.01^b	6.35±.02^a	6.28±.01^b	ND
S/PYP	ND	6.85±.07^b	6.97±.02^c	6.75±.01^c	ND
PPYP	ND	6.85±.02^b	6.89±.04^c	6.77±.01^d	ND
SMY	1.14±.01^c	6.56±.01^b	6.82±.02^b	ND	.079±.01^c
SM/PY	1.20±.02^b	6.82±.02^b	6.93±.02^c	ND	0.84±.01^c
PPY	1.06±.01^b	6.82±.02^b	6.78±.01^c	ND	0.84±.01^c

Week 3 Product	10°C Mould	Log/CFU×10^6 Total plate count	Lactic acid bacteria	P. freudenreichii	Aerobic spore former
SMYP	ND	6.32±.01^a	6.91±.01^a	6.58±.01^a	ND
S/PYP	ND	6.86±.01^c	6.84±.02^c	6.74±.01^b	ND
PPYP	ND	6.87±.03^d	6.08±.01^a	6.75±.01^b	ND
SMY	1.36±.01^b	6.80±.01b	6.92±.01^d	ND	0.94±.01^b
SM/PY	1.32±.01^c	6.85±.01^c	6.91±.01^a	ND	1.0±.01^d
PPY	1.29±.01^a	6.92±.01^e	6.87±.01^c	ND	1.20±.01^c

Week 3 Product	21° C Mould	Log/CFU×10^6 Total plate count	Lactic acid bacteria	P. freudenreichii	Aerobic spore former
SMYP	ND	6.34±.02^a	6.78±.01^a	6.50±.01^b	ND
S/PYP	ND	6.86±.01^a	6.84±.01^b	6.75±.01^c	ND
PPYP	ND	6.86±.01^a	6.82±.01^a	6.74±.01^b	ND
SMY	1.38±.01^d	6.90±.01^b	6.86±.01^b	ND	0.95±.02^b
SM/PY	1.36±01^b	6.85±.01^a	6.86±.91^b	ND	1.2±01^c
PPY	1.32±.01^b	6.90±.01^b	6.86±.01^b	ND	0.91±.02

SM/PY: 50% soymilk + 50% pigeon pea milk yoghurt, SMYP: Soymilk probiotic yoghurt, SM/PYP: 50% soymilk + 50% pigeon pea milk probiotic yoghurt, PPYP: Pigeon pea milk probiotic yoghurt, SMY: Soymilk yoghurt, PPY: Pigeon pea milk yoghurt, E. coli was not detected. ND: Not detected.

Table 4.4 Storage Stability of Yoghurt Samples

Week 4 Product	4° C Mould	(Log/CFU×10^6) Total plate count	Lactic acid bacteria	P. freudenreichii	Aerobic spore former
SMYP	ND	6.04±.04^a	6.38±.01^a	6.20±-01^b	ND
S/PYP	ND	6.80±.01^c	6.96±.01^d	6.64±.02^d	ND
PPYP	ND	6.00±.01^c	6.82±.01^b	6.53±.02^c	ND
SMY	1.65±.02^c	6.52±.04^b	6.82±.01^b	ND	1.20±.01^b
SM/PY	1,59±.01^b	6.82±.01^c	6.91±.01^c	ND	1.21±.01^b
PPY	1.62±.01^b	6.79±.01	6.84±.01^b	ND	1.53±.01^c

Week 4 Product	10° C Mould	(Log/CFU×10^6) Total plate count	Lactic acid bacteria	P. freudenreichii	Aerobic spore former
SMYP	ND	6.32±.01^a	6.79±.01	6.43±.33^d	ND
S/PYP	ND	6.83±.01^{ab}	6.79±.01	6.80±.13^c	ND
PPYP	ND	6.78±.02^b	6.05±.01	6.03±04^b	ND
SMY	1.67±.02^c	6.81±.01^{ab}	6.91±.02	ND	1.52±.01^c
SM/PY	1.65±.02^c	6.80±.01^{ab}	6.91±.01	ND	1.25±.01^b
PPY	1.67±.01^c	7.89±.01	6.84±.01	ND	1.53±.01^C

Week 4 Product	21° C Mould	Log/CFU×10^6) Totalplate count	Lactic acid bacteria	P. freudenreichii	Aerobic spore former
SMYP	ND	6.34±.02^a	6.73±.01^a	6.61±.01^b	ND
S/PYP	ND	6.86±.01^d	6.74±0.1^a	6.63±.02^b	ND
PPYP	ND	6.84±.01^d	6.82±.01^b	6.70±.01^c	ND
SMY	1.72±.02^b	6.71±.01^b	6.82±.01^b	ND	1.89±.01^c
SM/PY	1.86±.01^c	6.84±.01^d	6.81±.01^b	ND	1.79±.01^b
PPY	1.74±.01^b	6.75±.02^c	6.87±.01^c	ND	1.88±.01^c

SM/PY: 50% soymilk + 50% pigeon pea milk yoghurt, SMYP: Soymilk probiotic yoghurt, yoghurt, SMY: Soymilk yoghurt, PPY: Pigeon pea milk yoghurt, E. coli was not detected. ND: Not detected. SM/PYP: 50% soymilk + 50% pigeon pea milk probiotic yoghurt, PYP: Pigeon pea milk probiotic

4.3.2 Effects of storage time on pH and titratable acidity

The pH of yoghurt samples stored at different temperatures of 4°, 10° and 21° C for a 4 week period were analysed. Generally, the pH of the yoghurt samples were expectedly lower compared to their milks from which they were prepared (Fig 4.2 a, b and c). The pH of the milk was pH 7 and pH 6.8 for soy and pigeon pea milk respectively. Soymilk pH was higher than previously reported (Park *et al.,* 2005). However, there were significant differences ($P \leq 0.05$) in pH of all the yoghurt samples at termination of fermentation.

This may be attributed to acidification caused by the presence of organic acids such as lactic, acetic and propionic acids which were produced during fermentation (Moslemy, 2015). However, there was minimal variation in the pH of the yoghurt samples as storage period and temperature increased. During yoghurt preparation, the pH of the milk declined from 7 and 6.8 to 4.4 and 4.88 with starter culture and probiotic bacteria during incubation, resulting in formation of smooth gel near isoelectric point (Ekinci *et al*., 2008; Moslemy *et al.,* 2015). The decline in pH can be an effective way to inhibit spoilage and pathogenic microorganisms (Lind 2010). *Propionibacteria* are resistant to low pH. Furthermore, they have adaptation to milk and other food substrates (Mortazavian *et al*., 2012). The pH units for the samples at 0 week or termination pH were: SM/PY, SMYP, SM/PYP, PPYP, SMY, and PPY 4.48, 4.56, 4.83, 4.50, 4.88 and 4.44 respectively while 4.04, 4.35, 4.35, 3.55, .4.14.and 4.45 were pH units recorded at the end of storage period for the 4th week. These indicate that there was a decline in pH for all the yoghurt samples produced, as the storage temperature and storage time increased. The pH of the yoghurt samples increased after overnight refrigeration at 4°C and then declined slightly during cold storage, an effect commonly known as "post acidification" (Ng *et al.*, 2011). However, during refrigeration storage at 4° C there was a slight decline in pH and it remained almost unchanged. During the first two weeks of cold storage at 4° C the pH remained unchanged in the control soy yoghurt. On the other hand, the change in the probiotics soy yoghurt occurred in the first week of storage. This stability in pH units was as a result of lower activities of starter culture and probiotics during refrigeration storage at 4° C. Stijepec *et al.* (2013) reported stable

pH for probiotic soy yoghurt during storage of up to 20th day at 4 °C. Furthermore, Murevahame (2012) observed a fairly stable pH for fermented Bambara probiotic yoghurt stored at 5°C. However, storage at 10° and 21°C resulted in significant decline in pH units over the storage period. Furthermore, the rate of decline in pH units was higher than samples stored at 4°C. This indicates the higher activities of yoghurt cultures and probiotics cultures at storage temperatures of 10° and 21° C. Falade *et al*. (2014), reported a similar result in which the pH of yoghurt stored at 27°C was significantly lower than those stored at 7° C. Nevertheless, by the end of storage periods the pH of all the samples were significantly lower than recorded at termination of fermentation of control yoghurts and probiotics respectively. It is interesting to note that end pH had no effect on the extent of pH decrease during storage at the vary temperatures at which the samples were stored, but on the temperatures, days and the metabolic activities of organisms used as cultures and probiotics.

In this study, all yoghurt samples declined in pH units over storage periods indicating the development of acidity. However, the pH recorded did not fall below levels which is generally considered detrimental to the survival of probiotics organism (Dave and Shaah, 1997) for samples stored at 4 and 10 °C. Furthermore, sensitivity of probiotics to low pH are species and strain specific. According to Moslemy *et al.* (2015), fermented milk process is efficient in inducing acid tolerance response in *Propionicbacteria*. Hence, a multi-tolerance response in *Propionibacterium freudenreichii* can be induced leading to efficient protection toward bile salt and parallel to heat stress. At the end of storage, the decline in pH, range which are suitable for restricting pathogenic and spoilage organism were similar and were no different between probiotics and control samples during 28 days storage at different temperatures (Bilgidi *et al*., 2006). The pH of yoghurt samples, SM/PY, SMYP, SM/PYP, PPYP, SMY, and PYP stored at 4° C were within 4.55-4.2. These values are within the recommended pH units of yoghurt (Tamime and Robison, 1999).

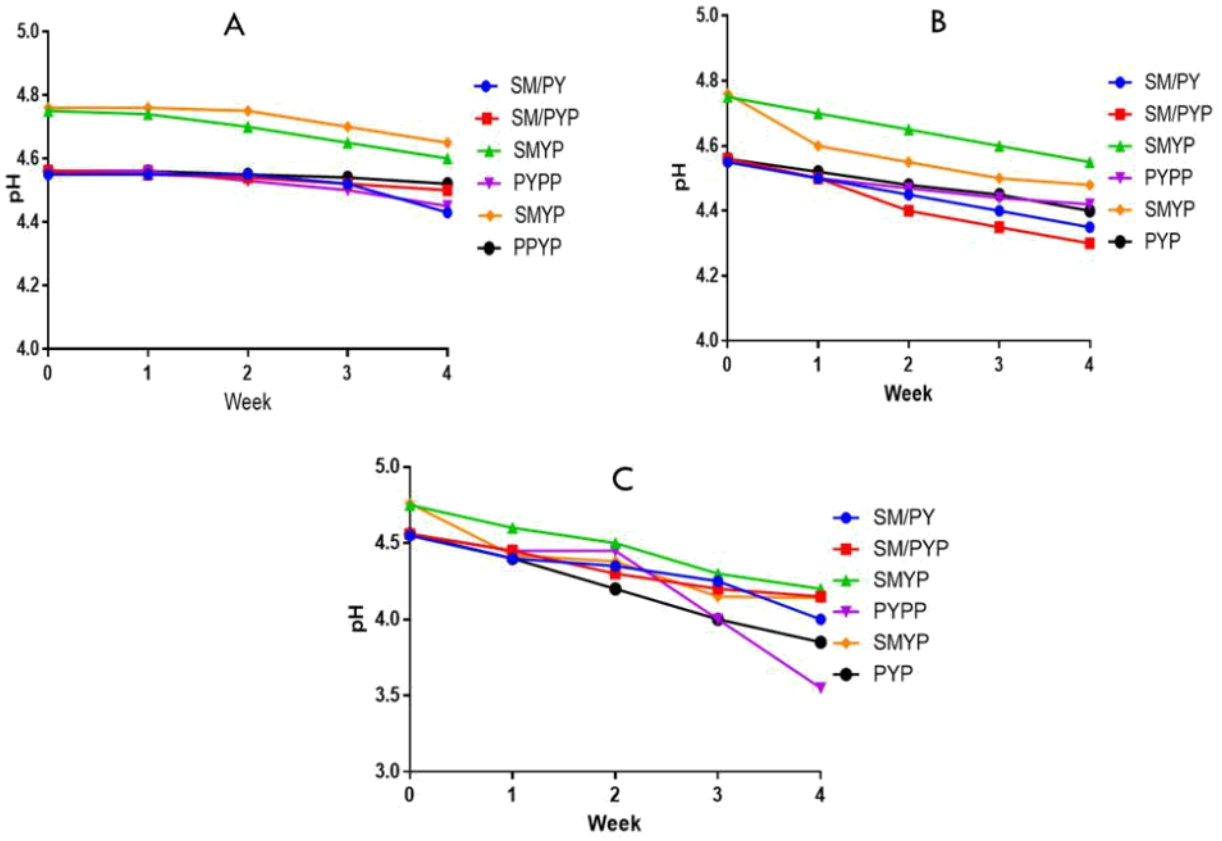

Figure 4.3 a, b and c: Yoghurt samples stored at 4, 10 and 12°C

SM/PY: 50% soymilk + 50% pigeon pea milk yoghurt, **SMYP**: Soymilk probiotic yoghurt, **SM/PYP**: 50% soymilk + 50% pigeon pea milk probiotic yoghurt, **PPYP**: Pigeon pea milk probiotic yoghurt, **SMY**: Soymilk yoghurt, **PPY**: Pigeon pea milk yoghurt.

4.3.3 Titratable acidity

Expectedly, decline in pH of all the samples resulted in simultaneous increased titratable acidity (TTA) values over storage period and storage temperature. As shown in Fig 4.3 a, b and c the mode of metabolic activity in the form of TTA actually depends on storage temperatures and substrate over a period of storage (Dave and Sha, 2002). TTA of yoghurt sample stored at 4° C increased for SM/PY, SM/PYP, SMY, PYPP SMYP and PPYP. The TTA of yoghurt samples stored at 10 and 21^{0}C significantly ($p \leq 0.05$) increased during storage (Fig: 4.3 a, b and c). The TTA of probiotic and non-probiotic stored at 21° C were significantly higher than yoghurt stored at 4° C. Osundahunsi *et al.* (2007), reported increase in acidity of

plain soy yoghurt stored at 6° C for 8 days. Their values were similar to values obtained in our studies. Furthermore, Stijepić *et al.* (2013) observed stable TTA values for plain soy yoghurt stored at 4° C for 20 days. Bambara groundnut milk beverage stored at 5°C, was found to gradually increase in TTA. However, samples stored at 15 and 25°C recorded a faster increase in TTA (Murevanhema *et al.,* 2013). The decline in pH and simultaneous increase in TTA in yoghurt samples during storage periods could be due to the starter cultures activities such as post acidification resulting from lactic acid (Osundahunsi *et al.,* 2007; Murevanhema *et al.,* 2013) acetic and propionic acid production (Moslemy *et al.,* 2015). Low organic acid production in soy and pigeon pea milk was observed as shown in fig (4.3 a, b and c). This offers a better environment for cell growth during fermentation and storage compared to milk. Findings in our study were similar to those reported by Liu Yao *et al.* (2004).

The absence of moulds and aerobic spore formers in the probiotic yoghurts may thus be linked with these acids as previously reported (Moslemy *et al.,* 2015). In our current study, the activity during cold storage at 4° C in probiotics and non-probiotics yoghurt were very low when compared to the reports of (Dave *et al.,* 1997). However, this may be as a result of reduced activities of culture and probiotics at that storage temperature, and also limited hydrolysable proteins in the form of milk proteins. Furthermore, our study indicates lower amount of organic acids production in soy and pigeon pea milk even though the culture grew well. The TTA of yoghurt with *Propionibacteria freudenreichii* ranged from 0.95 to 1.35, 0.95 to 1.34 and 0.95.to 1.75 at 4°, 10° and 21°C respectively. However, samples stored at 10° and 21°C recorded higher increase in acidity.

Furthermore, Murevanhema (2012) reported a gradual increase in titratable acidity of Bambara ground nut milk beverages stored at 5° C in contrast, a faster increase was recorded for samples stored at 15° and 25° C respectively. As the storage week and temperatures increased there was increased acidity as the storage period increased. The increase in TTA resulted in drop of pH. Similarly, Bensmira and Jiang (2012); and Mataragara *et al*. (2011) observed such trends. Probiotic and non-probiotics yoghurt had a similar metabolic trend but there were little or no increase

in their levels of acidity. The rate of metabolism reduced after the respective peak growth periods possibly due to diminishing nutrient supply in medium. A similar trend of cell population decrease was noticed during storage at 4°C for 28 days in (Table 4.1-4.4). Acidification which is as a result of growth rate and activities of yoghurt starter cultures and probiotics resulted in decline in the pH (Zare *et al.*, 2012). Liu (1997) reported that LAB grew well in soy milk but produced less organic acids. Low organic acid production in soy and pigeon pea milk as shown in Fig. 4.3 a, b and c offer a better environment for cell growth during fermentation and storage compared to milk. This finding is similar to that reported by Liu (1997). However, in our study during incubation of soy and pigeon pea milk with yoghurt cultures and probiotics organisms, there was a decline in pH units and increased TTA values as shown in figure (Fig. 4.3 a, b and c) thus making pigeon pea a potential medium for bacterial growth. Besides, addition of 1g/100ml of lactose was found to significantly improve the growth of bacteria in soymilk (Liu 1997).

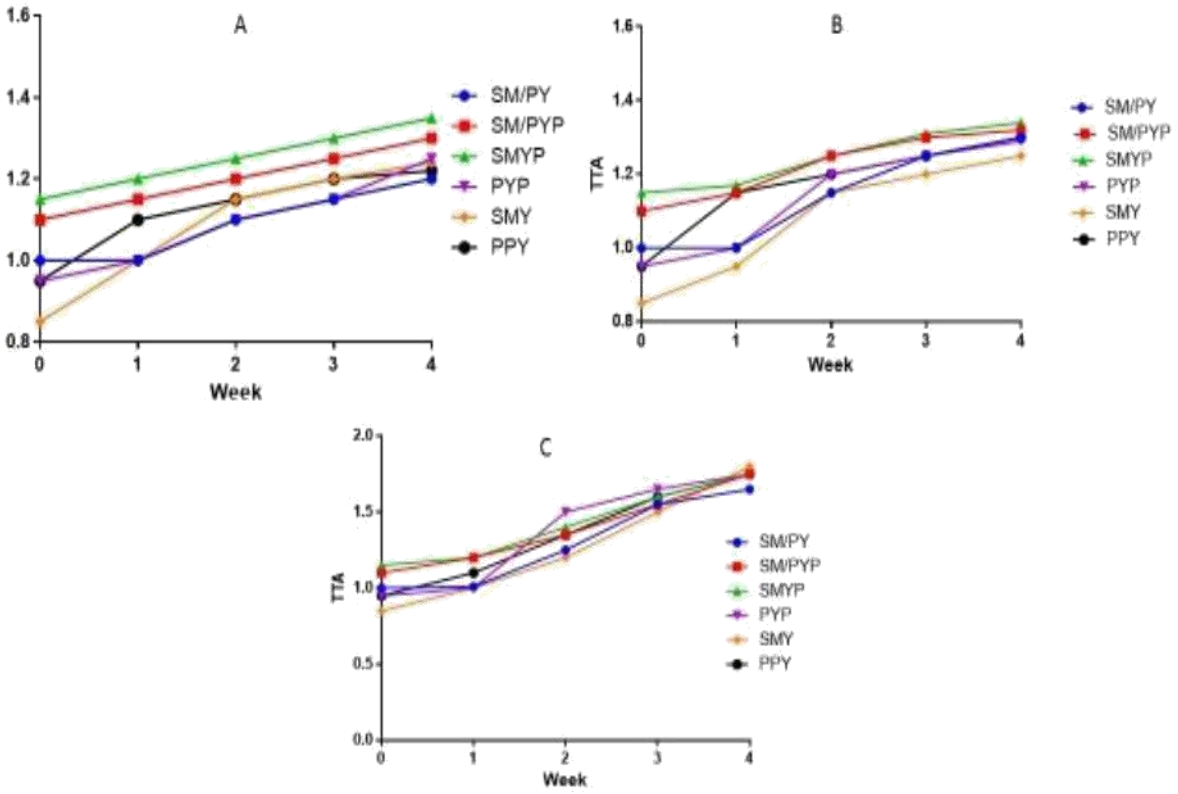

Figure 4.4 A, B and C TTA values of yoghurt samples stored at 4, 10 and 21°C

SM/PY: 50% soymilk + 50% pigeon pea milk yoghurt, **SMYP**: Soymilk probiotic yoghurt, **SM/PYP**: 50% soymilk + 50% pigeon pea milk probiotic yoghurt, **PPYP**: Pigeon pea milk probiotic yoghurt, **SMY**: Soymilk yoghurt, **PPY**: Pigeon pea milk yoghurt.

4.4: Effect of storage temperature and time on the colour of yoghurt samples

4.4.1: L* value of yoghurt samples

The L* (lightness) values of yoghurt samples ranged from 77.64 to 88.64, 78.00 to 88.32 and 76.42 to 86.12 respectively at 4, 10, and 21^{o}C storage temperature for 4 weeks. The slight difference recorded for L* may be due to various milk's colours 80 and 70.52 for soy milk and pigeon pea milk respectively, from which the yoghurt samples were prepared.

The fermentation process increased L* (lightness) when compared to L* values for soy and pigeon pea milk and compared to colours of yoghurts stored at different temperatures. Significant differences ($P \leq 0.05$) observed in all 6 samples of yoghurt ($P \leq 0.05$) might be due to the effect of yoghurt cultures and probiotics

used. The heat treatment at 95^{o}C for 20 minutes for milk before fermentation has been reported to enhance colour stability and is suitable for fermentation. L* values remained unchanged. However, significant differences was observed as storage period was prolonged as shown in figure 4.5 a, band c. Colour is one of the most important visual attributes in food. Changes in physiochemical and microbiological characteristics of yoghurt affect shelf life and cause colour deterioration (Coggins *et al.,* 2010)

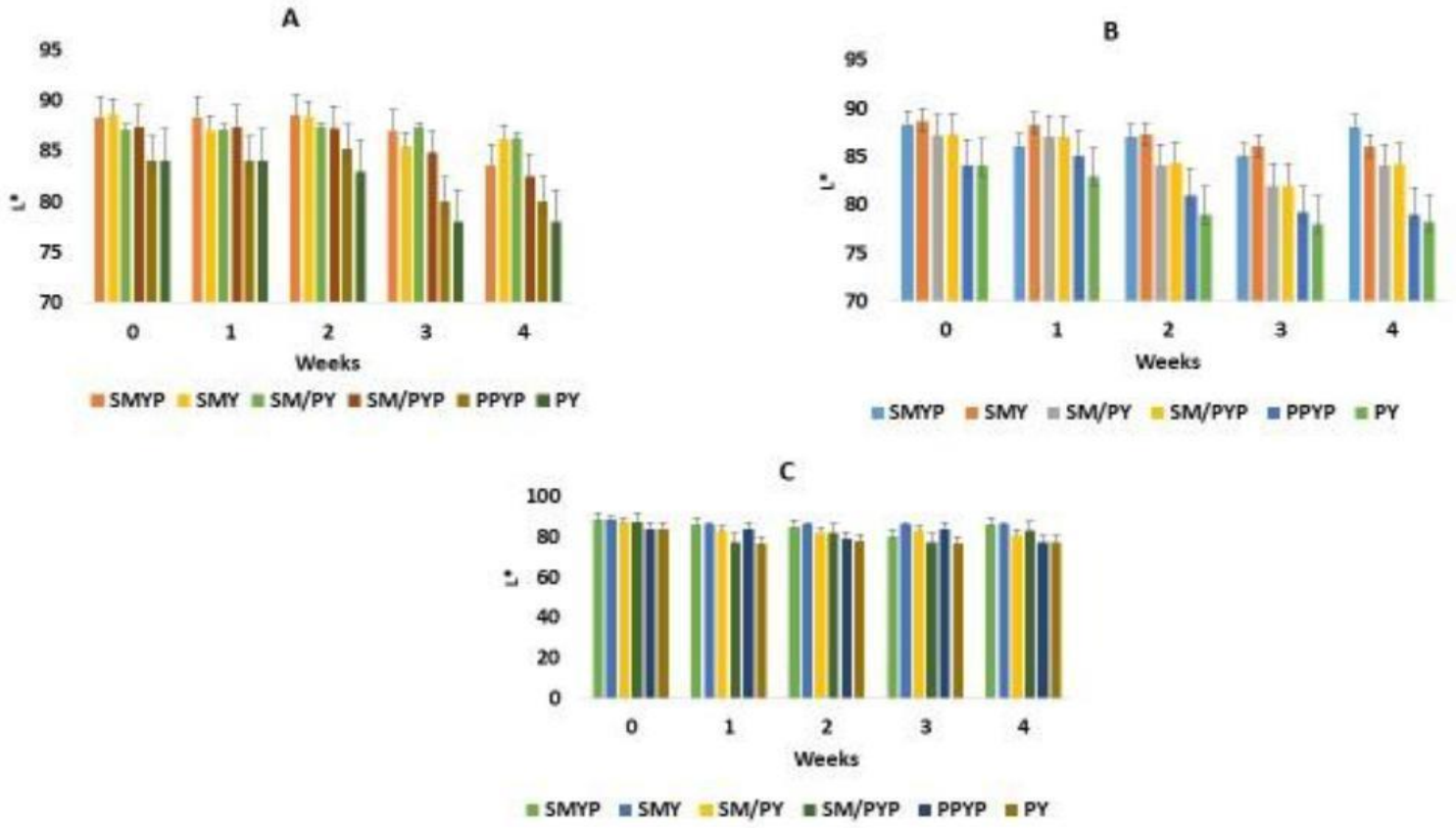

Figure 4.5 A, B and C: L* values of yoghurt samples stored at 4, 10 and 21°C
SM/PY: 50% soymilk + 50% pigeon pea milk yoghurt, **SMYP**: Soymilk probiotic Yoghurt, **SM/PYP**: 50% soymilk + 50% pigeon pea milk probiotic yoghurt, **PPYP**: Pigeon pea milk probiotic yoghurt, **SMY**: Soymilk yoghurt, **PPY**: Pigeon pea milk yoghurt.

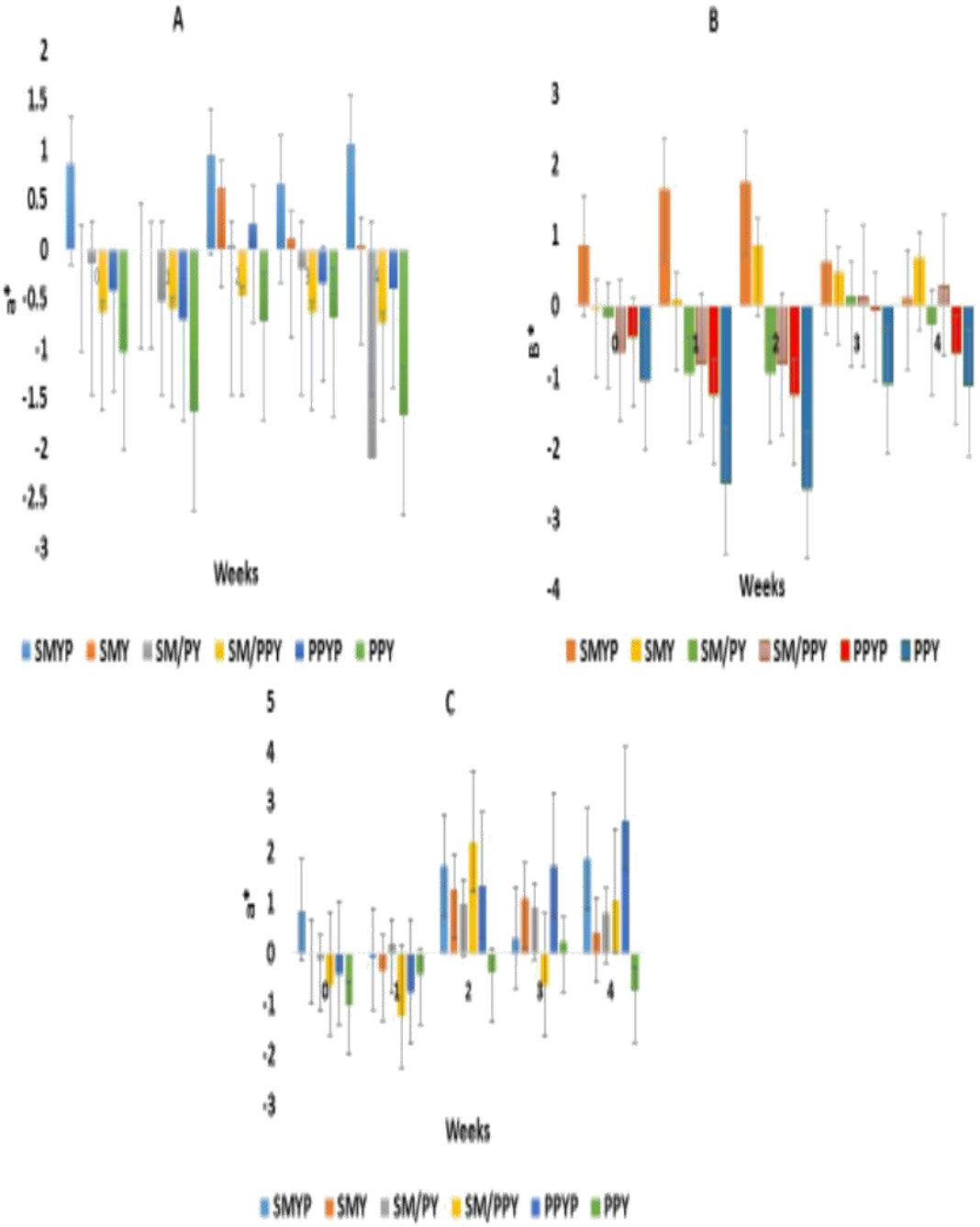

Figure 4.6 A, B and C: a* values of yoghurt samples stored at 4°C, 10°C and 21°C
SM/PY: 50% soymilk + 50% pigeon pea milk yoghurt, **SMYP**: Soymilk probiotic yoghurt, **SM/PYP**: 50 % soymilk + 50% pigeon pea milk probiotic yoghurt, **PPYP**: Pigeon pea milk probiotic yoghurt, **SMY**: Soymilk yoghurt, **PPY**: Pigeon pea milk yoghurt.

4.4:3 b* values of yoghurt samples

b* values of yoghurt samples stored at temperature of 4°, 10° and 21°C for 4 weeks respectively were evaluated for their colour values: b* (Figure 4.7 a, b and c). Similarly, negative values b* defines blueness while positive values b* defines yellowness. In our studies negative values were not recorded for b*. The positive values indicate the yellowness of the samples which ranged from 14.67 to 25.96, 14.92 to 24.96 and 16.01 to 25.62 for samples stored at 4°, 10° and 21°C. This is expected because of differences that existed between the milk colours soy and pigeon pea. Significant differences ($P \leq 0.05$) in samples stored at the same temperatures and at different temperatures were expected as the yoghurt samples were made of different milk formulations.

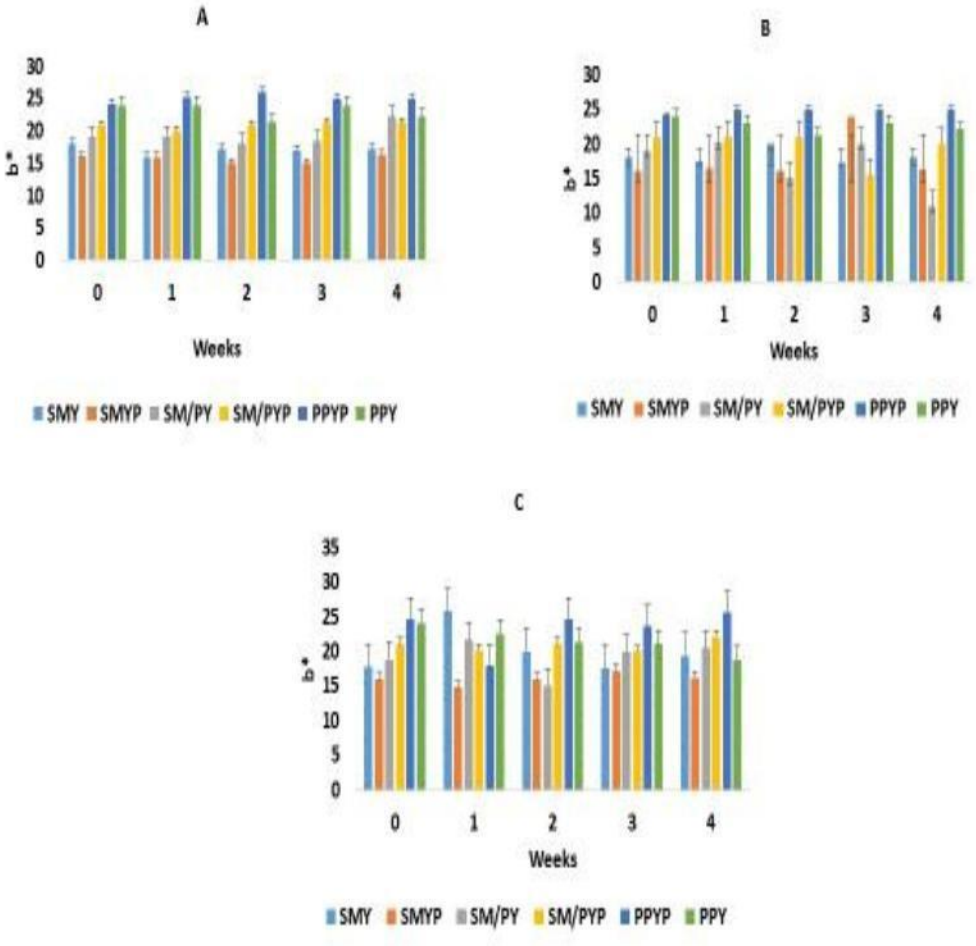

Figure 4.7 A, B and C: b* values of yoghurt samples stored at 4, 10 and 21°C

SM/PY: 50% soymilk + 50% pigeon pea milk yoghurt, **SMYP**: Soymilk Probiotic yoghurt, **SM/PYP**: 50% soymilk + 50% pigeon pea milk probiotic Yoghurt, **PPYP**: Pigeon pea milk probiotic yoghurt, **SMY**: Soymilk yoghurt, **PPY**: Pigeon pea milk yoghurt

4.4:4 Colour difference ΔE ∗values

The L*, a* and b* were converted to total colour differences Δ ∗ value using the formular in (4.2.9.) The Δ ∗values (table 4.5A, B and C) had a trend to increase as storage time and temperature increased. However, considering the L* values remained unchanged during the 4 week storage at 4°, 10° and 21°C respectively. The slight increase in Δ * may be attributed to an increase in a* and b* values due whey released over storage time as shown in (tables 4.5, 4.6, and 4.7)

Table 4.1 Difference in colour ΔE of yoghurt sample stored at 4°C

Samples	Week 0	Week 1	Week 2	Week 3	Week 4
SMYP	16.26±.01^{b}	15.30±.01^{b}	14.63±.01^{a}	16.44±.01^{a}	17.40±04^{c}
SMY	14.60±.02^{a}	13.31±.01^{a}	15.75±.01^{b}	16.50±.01^{b}	13.40±01^{b}
SM/PY	17.70±.01^{c}	17.30±.00^{c}	20.50±.01^{c}	19.30±.01^{c}	12.40±01^{a}
SM/PYP	20.18±.01^{d}	20.03±.o1^{d}	21.02±.01^{d}	21.00±.00^{d}	18.50±.01^{d}
PPYP	24.30±.00^{e}	24.93±.01^{e}	26.40±.00^{e}	27.00±.01^{e}	22.02±.01^{f}
PPY	23.80±.00^{f}	23.40±.01^{f}	25.72±.01^{f}	26.00±00^{f}	18.72±.01^{e}

Table 4.1 Difference in colour E of yoghurt sample stored at 10° C

Samples	Week 0	Week 1	Week 2	Week 3	Week 4
SMYP	16.26±.01^{a}	13.51±.00^{b}	16.00±.01a	16.00±.01^{a}	16.40±.02^{a}
SMY	14.60±.02^{b}	13.51±.02^{a}	15.05±.01a	16.00±.01^{a}	17.00±.02^{b}
SM/PY	17.70±.01^{c}	17.00±.00^{c}	20.00±.01c	20.00±.14^{c}	20.40±.01^{d}
SM/PYP	20.18±.00^{d}	20.06±.01^{d}	23.00±.01d	20.00±01^{b}	20.00±.01^{c}
PPYP	24.30±.00^{e}	25.00±.01^{f}	24.00±.01f	23.27±.04^{d}	25.00±.01^{f}
PPY	23.80±.00^{f}	22.40±00^{e}	27.00±.01^{e}	27.20±.01^{e}	25.00±.01^{e}

Table 4.3 Difference in colour∆E of yoghurt sample stored at 21°C

Samples	Week 0	Week 1	Week 2	Week 3	Week 4
SMYP	16.26±.01^{b}	25.00±00^{c}	25.50±.01^{c}	27.00±01^{f}	20.30±04^{b}
SMY	14.60±.02^{a}	27.00±.01^{d}	27.40±01^{d}	15.00±00^{a}	16.00±.01^{a}
SM/PY	17.70±.01^{c}	28.00±.03^{d}	33.00±01^{f}	22.20±00^{c}	21.00±01^{c}
SM/PYP	20.18±.00^{d}	21.00±.00^{d}	21.50±01^{b}	24.43±01^{d}	23.00±.02^{d}
PPYP	24.30±.00^{e}	21.00±01^{e}	30.00±.01^{e}	18.46±.03^{b}	24.00±.00^{f}
PPY	23.80±.00^{f}	18.00±.01^{a}	18.20±01^{a}	26.50±01^{e}	23.50±00^{e}

Where: **SM/PY**: 50% soymilk + 50% pigeon pea milk yoghurt, **SMYP**: Soymilk Probiotic yoghurt, **SM/PYP**: 50% soymilk + 50% pigeon pea milk probiotic , **PPYP**: Pigeon pea milk probiotic yoghurt, **SMY**: Soymilk Yoghurt, **PPY**: Pigeon pea milk yoghurt.

4.4.5 Water holding capacity

Water holding capacity (WHC) and Susceptibility to syneresis (STS) of yoghurt and probiotics yoghurt were studied over 28 days of yoghurt samples stored at 4°, 10° and 21°C respectively (Fig 4.7).

All yoghurt samples showed an increase in the ability to entrap water with an increase in storage period. This suggests a strong protein network within the yoghurt samples. Probiotic yoghurt prepared from 100% soymilk displayed a higher tendency to hold water, while 100% pigeon pea yoghurt showed the lowest ability. Aguirre-Mandujano *et al.* (2009) reported that variations in the protein matrix of different yoghurt mixtures may lead to differences in their water holding capacity. Further, formulations containing high protein contents have been associated with the formation of stable protein gels (Kpodo *et al.* 2014). These authors hypothesised that strong protein-protein interactions have the tendency to produce gel structure with higher WHC (Kpodo *et al.* 2014). In this study, the higher WHC of 100% soybean probiotic yoghurt could be attributed stronger protein-protein interactions than in other samples. Similar reports have been documented by early studies (Kpodo *et al.* 2014). Soy protein gels have been found to have better capability to entrap water within its three dimensional network (Kovalenko and Briggs 2002).

The WHC of a protein gel is an important parameter in yoghurt manufacturing, since it is related to syneresis. According to literature, syneresis is due to breakdown of many protein strands and structural rearrangement which result in expulsion of whey (Serra *et al.* 2009).

Thermal treatment given to the milk improve suspension (Diarra *et al*., 2005) while homogenization improves the stability of plant milk substitutes by disrupting aggregation and lipid droplets resulting in decrease of particles size distribution (*Nik et al*., 2008). Both homogenization and thermal treatment have been reported to alter the molecular properties such as structural rearrangement which result in whey expulsion (Kivela *et al*., 2011, 2010). When the protein network of yoghurt system exhibits low WHC, STS occurs and this is undesirable. Variation in temperatures as indicated (Table 6-8 correct this) with different protein matrix of yoghurt mixtures resulted in differences in WHC and STS values recorded during storage at 4°, 10° and 21°C. The WHC values recorded at 4 through 10 to 21°C were significantly different ($P \leq 0.05$). As indicated in (Table 6-8 correct this\), it thus appears as if the storage temperature and time had significant effect on WHC, including the matrix of the various yoghurt mixtures. Furthermore, syneresis produced by funnel drainage does not represent the usual breakage of yoghurt matrix but reflects the ability of the entire gel structures to retain water.

Syneresis are closely related to whey released due to network rearrangement triggered by changes in pH as storage time and temperature increased. Boeneke and Aryana (2008) observed significant ($P \leq 0.05$) differences in syneresis over 5 weeks of storage period in lemon yogurts fortified with folic acid. Similarly, Aportela *et al*. (2005) found a significant influence of storage time on the syneresis of piña colada yogurt systems fortified with fiber and calcium. The physical attributes of yogurts, including the lack of visual whey separation, are vital aspects of the quality and overall sensory consumer acceptance (Lee and Lucey, 2010).

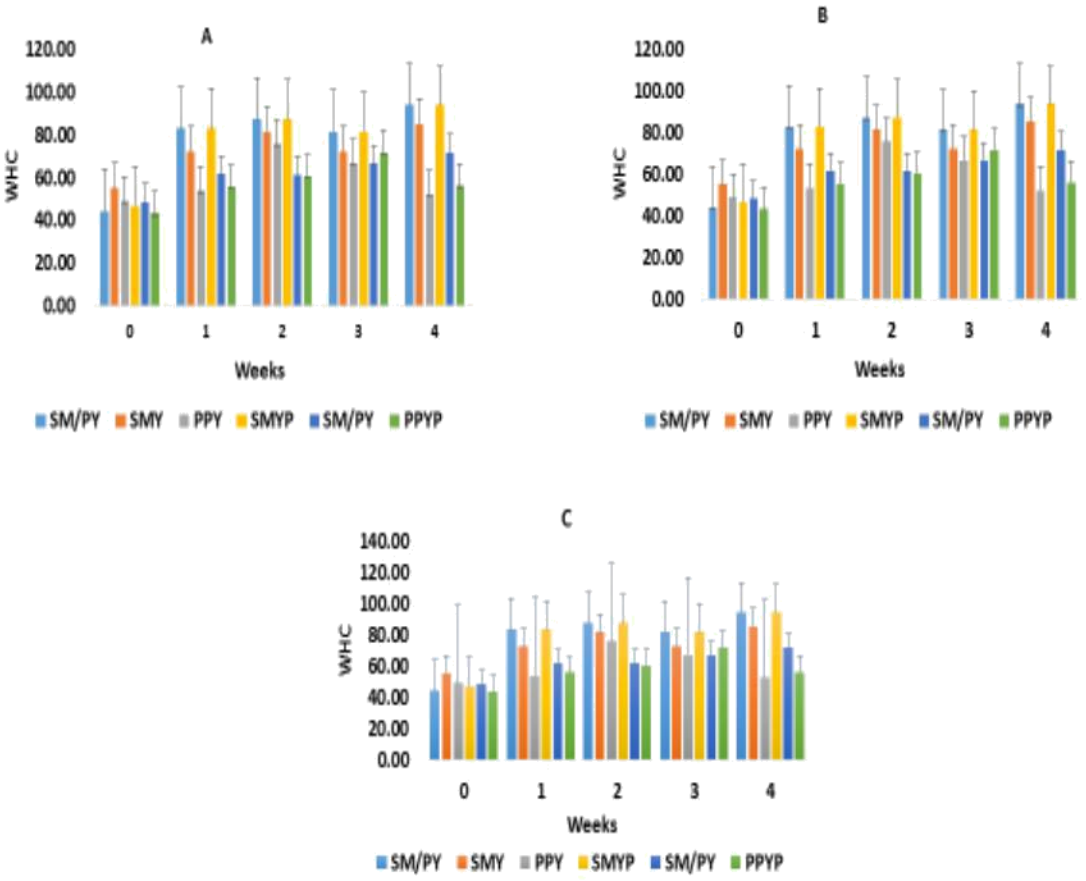

Figure 4.8 A, B and C: Water holding capacity (WHC) of yoghurt samples

SM/PY: 50% soymilk + 50% pigeon pea milk yoghurt, **SMYP**: Soymilk probiotic yoghurt, **SM/PYP**: 50% soymilk + 50% pigeon pea milk probiotic yoghurt, **PPYP**: Pigeon pea milk probiotic yoghurt, **SMY**: Soymilk yoghurt, **PPY**: Pigeon pea milk yoghurt.

4.5 Conclusion

Improved microbial quality and functional properties can be realised using pigeon pea milk as matrices and *Propionibacterium freudenreichii* as supplement the in making of set-type of non-dairy yoghurt production without affecting microbial and functional qualities. During the first two weeks LAB and *Propionibacterium freudenreichii* increased by approximately log 2 CFU/ml and *E. coli* was not detected in all samples throughout storage period. Low organic acid production in soy and pigeon pea milk as shown offers a better environment for cell growth during fermentation and storage compared to milk. The pH of yoghurt samples, stored at 4°C were within 4.55-4.2. These values are within the recommended pH units of

yoghurt. The colour differences that exist between the yoghurt samples resulted as milk colours from soy and pigeon pea were different. Storage at 4°C is the most acceptable, as storage at 21°C encourage proliferation of contaminants. The absence of pathogenic bacteria in all the yoghurt samples confirm their safety. Therefore, owing to the positive result in both microbial and functional qualities, the obtained product might be considered a new functional food with potential health benefits suitable for vegetarians, lactose-intolerant and people allergic to milk proteins.

CHAPTER 5

3RD RESEARCH CHAPTER
EFFECTS OF PROCESSING ON SOME ANTI- NUTRITIONAL FACTORS IN PIGEON PEA FLOUR, MILK AND YOGHURT.

Abstract

Raw plant matter is part of the diet of most human populations. However, the majority of these plants are processed by cooking, blanching, fermentation, and sprouting, prior to consumption. These treatments have been suggested to increase the range of plant foods for humans such as legume seed, tubers and leafs which otherwise would be too toxic or indigestible. In this study, pigeon pea was processed into flour, milk and yoghurt, while soybean was processed into milk and yoghurt. The anti-nutritional factors (phytate and trypsin) content were investigated. The phytate content range between 0.2-0.5 mg/g for phytate while trypsin 0.2- 3 TIU/mg. There was significant differences in all the samples investigated. Probiotic yoghurt was significantly ($P \leq 0.05$) lower in anti-nutritional factors investigated than non-probiotics yoghurt. While milk was significantly ($P \leq 0.05$) lower in anti-nutrients than flour. Yoghurt with acceptable anti-nutritional factors was produced.

5.1 Introduction

The nutritive value of legumes have been suggested to impact positively on human physiology (Tharanathan and Mahadevamna, 2003). A larger portion of the world population depends on legumes for dietary protein, particularly in countries where animal proteins are scarce and expensive (Tharanathan and Mahadevamna, 2003). Legumes are an important source of proteins, energy, dietary fibre, minerals and vitamins required by humans. Lately, research suggests frequent consumption of legumes. More than 4 times in a week may beneficially reduce the risk of cardiovascular disease, such as cancer, diabetes and osteoporosis amongst others (Flight and Clifton 2006; Compos-Vega *et al.*, 2009). However, legumes contain a lot of bioactive substances such as enzyme inhibitors, lectins, phatate, oligosaccharide and phenolic compounds. These compounds play a metabolic role in persons who frequently consume them. The role of these substances in

metabolism may be regarded as positive, negative or both (Champs, 2002). Some of these bioactive substances are referred to as anti-nutritional factors due to their effects on diet quality (Compos-Vega *et al.,* 2009). Anti- nutritional factors such as phytic acid which exhibits antioxidant properties and protects Deoxyribonucleic acid (DNA) damage have been suggested to have complementary and overlapping mechanisms of action (Compos-Vega *et al.,* 2009. Legumes contain several of these heat stable and heat labile anti-nutritional factors that interfere with digestion, causing gastrointestinal distress and flatulence (Rockland and Nishi 1979). In many cases, the contents of inhibitory factors are not considered to be absolute and may vary, depending on the variety and/or cultivar, climatic condition, location, irrigation conditions, types of soil and period of year during which they are grown (Miller and Woodrow; Singh and Sedeh 1979). Anti-nutritional factors are substances that adversely affect the overall nutritional value of all foods consumed by interfering with body processes such as body's metabolic rate and leading to toxicity (Sandberg, 2002).

These chemical compounds found in plants are defence mechanism used by plants to inhibit the action of digestive enzymes of attacking insects (Lee and Leegood, 1999). They have also been linked to unavailability of nutrients, indigestibility and binding of divalent elements when food is consumed by humans (Sandberg, 2002).

(Sunny-Roberts, Otunola and Iwakun 2004) reported greater reduction in trypsin inhibitor after fermentation of the milk. Legumes such as pigeon pea contain phytochemicals, dietary fibres and carbohydrates with low glycaemic index and anti-nutritional factors. However, their application has been limited by the presence of anti-nutritional factors. These compounds can however be eliminated by simple traditional and technological methods such as soaking, dehulling, heat treatment and fermentation amongst others (Jiménez-Martínez, Hernández-Sánchez and Dávila- Ortiz 2003; Mubarak 2005).

In addition, the activities of the fermenting organism is also known to contribute to the reduction of anti-nutritional factors in foods. The effects of dietary properties of phytic acid is known posse antioxidant properties and protective effects (Philips, 2003;

Boye *et al.*, 2009). Several authors have reported that the dietary protective effect of phytic acid includes the inhibition of the iron induced radical generation and lipid per-oxidation (Grag, Shamsuddin and Ullah, 2005).

Anti-nutritional factors such as trypsin inhibitors have been implicated in the formation of insoluble complexes with proteins, amino acids divalent and trivalent mental (Mune *et al.* 2011). These complexes result in impaired digestion and affects bioavailability of nutrients in the body. The reduction in these anti-nutritional factors may be associated with several factors such as soaking and blanching pre-processing steps employed during milk preparation. Adequate heat treatment has also been found to inactivate trypsin inhibitors in Bambara groundnut (Mune *et al.* 2011).

This study investigates the influence of processing/fermentation on the anti-nutritional content of pigeon pea flour, and pigeon pea and soybean milk and yoghurts.

5.2 Materials and Methods.

The Pigeon pea seeds used were obtained from Agricultural Research council South Africa, while the Soy originated bean from soya bean research farm, Pinetown, South Africa. Yoghurt cultures and probiotics were purchased from Lakes foods South Africa.

5.2.1 Methods

5.2.2 Preparation of Pigeon pea flour

Pigeon pea seeds were screened to remove the bad ones and extraneous materials. 250g of the screened Pigeon pea seeds were soaked in 2.5 litres of milli Q water with 0.5% $NaHCO_3$ overnight and were manually dehulled. The dehulled Pigeon pea seeds were thoroughly rinsed with milli Q water and further blanched in 0.5% NaHCo3 solution at 60°C for 20 min. Thereafter, the seeds were rinsed in milli Q water, dried in a hot air oven at 30^{O}C, dry-milled and sieved to obtain the flour. The flour was stored in polythene bags and kept in a refrigerator for analysis.

5.2.3 Milk Preparation

Pigeon pea seeds were screened to remove the bad ones. Pigeon pea seeds (250g) were soaked in 2.5 litres of milli Q water with 0.5% for 24 hours, and manually dehulled. The dehulled Pigeon pea seeds were thoroughly rinsed with milli Q water

and further soaked in 0.5% $NaHCO_3$ solution at 60°C for 30 min., after which the seeds were rinsed in milli Q water, milled into paste and mixed with milli Q water in ratio 1:1 (pigeon pea in g/water in ml) using a grinder (Waring laboratory model HGBTWG4 USA). The resulting slurry was centrifuged at 4000 × g using Heraeus Megafuge 40R (Thermofisher scientific, England). The milk obtained was heat treated for 20 minutes at 95 °C, while stirring continuously with a glass rod to prevent burning. The recovered milk was homogenised using Silverson homogeniser laboratory mixer emulsifier machine (PT. 210, Fisher scientific water side, U. K.) at 60°C and allowed to cool and refrigerated.

5.2.4 Soymilk

Soy beans seeds were screened to remove the defectives ones and extraneous material. Soybeans (25 g) seeds were soaked in 2.5L of milli Q water, with 0.5% NaHCO3 overnight and manually dehulled. The dehulled soy beans seeds were further soaked in milli Q water with 0.5% NaHCO3 solution at 100°C for 20 min and rinsed thoroughly with milli Q water, and mixed milli Q water in ratio 1:2 (soya beans in g/water in ml) and transferred into a stainless steel blender (Warring laboratory model HGBTWG4 USA). The resulting paste was centrifuged at 4000×g, using Heraeus Megafuge 40R (Thermofisher scientific, England) the resulting milk cooked at 95°C for 20 min. The milk obtained was homogenised and pasteurised and refrigerated.

5.2.5 Preparation of Yoghurts

Pigeon pea milk (approximately 12.5g/100g total solids) was added 3.5g/100 g together with skimmed milk powder. This was followed by stirring in powdered milk in pigeon pea milk and raising the temperature 43°C for 30 min thereafter 3g/100 ml sucrose was added as sweetener. The milk was then heat treated to 65°C and homogenized at 25 MPa using Silverson Homogenizer model (P T. 210, Fisher Scientific water side, U.K.). The temperature was further raised to 90°C for 20 min. The heat treated milk was cooled to 43°C in a water bath, then inoculated with 2% starter cultures (*Lactose bacillus bulgaricus and Streptococcus. thermophillus*). The inoculated milk was divided into two equal portions, one portion was used as control and the other was further supplemented with 1% probiotic culture (*Propionibacterium fruendenchii*) and samples were incubated at 43°C for 5 h until a pH of about 4.6 was reached.

At the end of the incubation period, the yoghurts were cooled and refrigerated at 4˚C overnight. Six types of set yoghurt were prepared from pigeon pea and soymilk. Three with fermented with yoghurt cultures and the other three with yoghurt cultures and *Propionibacterium fruendenchii.*

5.2.6 Determination of phytate

The phytic acid content in the yoghurt was determined using the spectrophotometric method described by Omotoso (2006) and Wheeler and Ferrel (1971) with slight modifications. Phytic acid reacts with a coloured complex such as Fe (III)-sulphosalicylate to form a colourless Fe (III)-phytate complex. This method thus measures the Fe (II) content which correlates to the phosphorus content (4:6) and the phosphorus content correlates to the phytic acid content (1:1).

A standard curve was prepared using Fe (II) in the range 0.025-2 mg/ml Accurately 5g of fridge dried 6 different yoghurt samples were added to 50 ml of 3% trichloroacetic acid (TAA). The samples were then placed in a shaking incubator for 30 min at constant speed (156 rpm). The suspensions were then centrifuged at 10 000 rpm for 15 min and the supernatants (2.5 mL each) were transferred to 15mL centrifuge tubes. Two millilitres of FeCl3 solution (2 mg/mL) was added to each sample. All six tubes containing the samples were heated for 45 min in a water bath (90-100ºC). The solutions were then centrifuged (10 000 rpm for 15 min) and the supernatants poured off.

The pellets were then washed twice by adding 10-15 ml 3% TCA solution, heating for 5-10 min and centrifuging (10 000 rpm for 15 min). The resulting pellets were washed once with distilled water and re suspended in 1 mL distilled water and 1.5 ml of 1.5 N NaOH solution and stirred. The volume was made up to 15 mL with distilled water and then heated in boiling water for 30 min and centrifuged (10 000 rpm for 15 min). The solution was filtered while hot with Whatman No. 2 filter paper. The residues were washed with 30-40 ml of hot distilled water and the filtrate discarded. The precipitates left in the paper were dissolved in 20 mL 3.2 N solution of HNO3 and transferred to a 50 mL volumetric flask. The samples were cooled at room temperature

and made up with distilled water. A 2.5 ml of each sample was transferred to a volumetric flask and diluted to 35 ml with distilled water. Thereafter, 10 ml of 1.5 M potassium thiocyanate (KSCN) solution was added and the solution made up to 50ml with distilled water. The absorbance of Fe (II) in the samples was read within 1 min at 480 nm using a spectrophotometer (UltraspecII-LKB, Biochrom, England).

5.2.7 Determination of trypsin inhibitors

Trypsin activity of yoghurt samples were first determined using the reaction: tyrosine produces trypsin in the presence of a casein substrate, as outlined by Jayaraman (1981).

Trypsin inhibition in the yoghurt was indicated by trypsin activity falling below the tyrosine standard activity. The control 0.9 mL of the casein bovine substrate and 0.1 ml of 0.25% trypsin were added to a test tube, mixed well and incubated for 30 min at room temperature. Then 1.5 mL of 10% TCA was added to stop the reaction. After 15 min, the test tube was put into boiling water for 10 min and filtered using Whatman no. 1 filter paper. The filtrate (0.5 mL) was added to the test tube together with 1 mL of distilled water 0. 5 mL of 0.5 N NaOH and 1.5mL of diluted Folin Ciocalteau (FC) reagent. Five grams of each yoghurt samples was added to 200 ml of distilled water and allowed to shake for 24 h at 37°C. After 24 h, the yoghurt samples were filtered with Whatman no.1 filter paper. Each of the yoghurt samples (0.1 ml) was added to 0.9 mL of the substrate and 0.1 mL of 0.25% trypsin. Tyrosine (Sigma, USA) was used as a standard to measure trypsin activity. A blank was prepared by adding 1.5 mL of water to 5 mL of 0.5 N NaOH together with 1.5 mL of Folin Ciocalteau (FC) reagent. Absorbance (Abs) was read at 650 nm.

The activity of trypsin was calculated as follows:

$$\text{Activity of trypsin} = \frac{\text{Abs of test} - \text{Abs of (+ve) control}}{\text{Abs of the standard} - \text{Abs of the blank}} \times \text{dilution factor}$$

5.3 Results and Discussion

5.3.1 Phytate

Phytate and trypsin content of yoghurt samples analysed ranged from 0.17 – 0.49 TIU and 0.35- 1.85mg/g and they were significantly lower ($p \leq 0.05$) in probiotic yoghurt compared to non- probiotic and their milk counterpart (Figure 5.1)

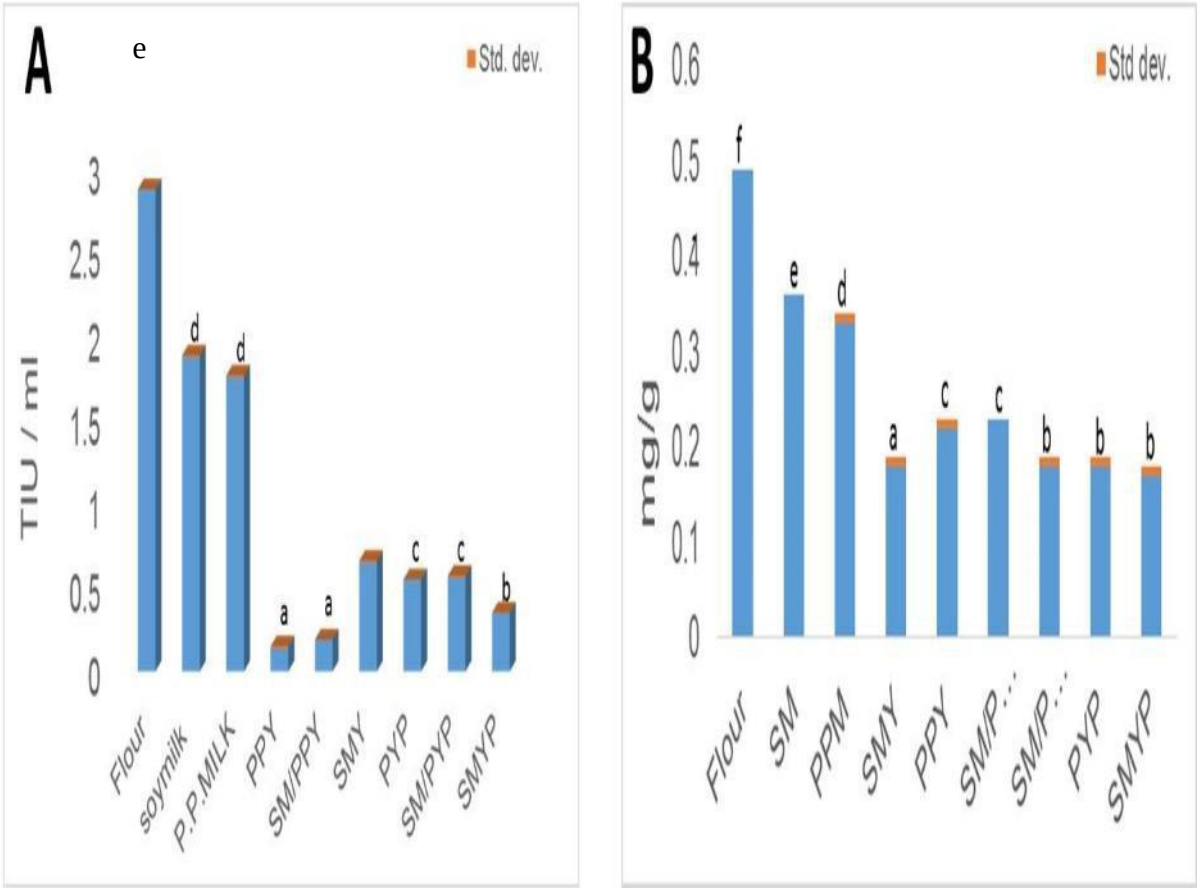

Figure5.1: A and B: Trypsin and Phytate

Where: **SM/PY**: 50% soymilk + 50% pigeon pea milk yoghurt, **SMYP**: Soymilk probiotic yoghurt, **SM/PYP**: 50% soymilk + 50% pigeon pea milk probiotic yoghurt, **PPYP**: Pigeon pea milk probiotic Yoghurt, **SMY**: Soymilk yoghurt, **PPY**: Pigeon pea milk yoghurt.

Phytate content of flour, milk and yoghurt samples ranged between 0.2 -0.05mg in yoghurt, milk and flour. Phytate content in yoghurt samples were significantly ($P \leq 0.05$) lower than the flour and also from milk which yoghurt was prepared. Anti-nutritional factors such as phytate have been implicated in the chelating of divalent mineral such as calcium, iron and magnesium (Mune *et al.* 2011). This results in reduced bioavailability of these nutrients in the body. The reduction in these anti-nutrients may be associated with several factors such as soaking and blanching pre-processing steps employed during milk preparation. According to Sharma, Agarwal and Verma (2011) soaking of pigeon pea in water for 24 hrs at room temperature and at 55°C decreased phytate content by 50% and 90% respectively. Flour and milk were found to contain the highest concentration of phytic acid. However concentration in milk was significantly higher than t yoghurt' in which it was prepared. The low concentration of phytic acid in milk in this study was as a result of different types of pre-treatment steps such as soaking in $NaHCO_3$ solution, dehulling , soaking $NaHCO_3$, heat treatment and fermentation in which legume milk were subjected to (Mubarak 2005). Of note, were the low concentrations of phytic acid recorded for yoghurt samples. However, yoghurt samples with *Propionibacterium freudenreichii* were significantly ($P \leq 0.05$) different from yoghurt samples without *Propionibacterium freudenreichii.* This suggests metabolic activities of *Propionibacterium freudenreichii* which further reduced the level of anti-nutritional factors in the matrix.

5.3.2 Trypsin inhibitions

Trypsin inhibitions range from 0.2 to 3 (trypsin inhibition units) TIU/mg for yoghurt, milk and flour samples, There were significant ($P \leq 0.05$) differences in (trypsin inhibition units) TIU/mg of the flour, milk and yoghurt samples. Trypsin inhibitor contents of yoghurt samples were generally lower than the milk from which they were prepared (Figure 5.1A). In addition, the activities of the fermenting organism are also known to contribute to the reduction of anti-nutritional factors in foods. For instance, the trypsin inhibition contents in unfermented pea nut milk reduced from 4.13 to 3.03 TIU/mg protein after fermentation (Sunny-Roberts, Otunola and Iwakun 2004).

However, as earlier observed, the effect of treatments before and during fermentation is most likely resulted in reduction and non-activities of these enzymes associated with activity of the inhibitors (Singh *et al.,* 2014). Trypsin inhibitors, according to Venter and Van Eyssen (2001) are compounds that interfere with protein digestion, they cause pancreatic enlargement and enhance chemically induced pancreatic tumours. However, the trypsin inhibitors are heat-labile in nature and this suggests that they can be inactivated by cooking (Prathibha *et al*., 1995). It has also been reported that in well cooked foods, the trypsin inhibitors may not interfere with digestion (Adane *et al.* 2013).

Conclusion

The elimination of the anti-nutritional factors in pigeon pea and soy bean, is possible by preliminary steps such as soaking, blanching, dehulling and by manipulation of starter culture and probiotic combination, and also, are some of the measures to be taken in order to overcome the problem of objectionable beany flavour in the final product.

CHAPTER 6

4TH RESEARCH CHAPTER
CONSUMER ACCEPTABILITY OF PROBIOTIC YOGHURT PRODUCED FROM PIGEON PEA MILK SUPPLEMENTED WITH *PROPIONIBACTERIUM FREUDENREICHII*

Abstract

Consumer demand for healthy non-dairy products due to their awareness of nutritional benefits and potential functionalities in fermented legume milk has been on the increase. The development of new matrices and conducting consumer acceptability test are necessary to determine whether consumers will like and consume the product. In this study, sensory acceptability of yoghurt samples supplemented with *Propionibacterium fruendenchii* and without *Propionibacterium fruendenchii* were investigated. Sensory evaluation was performed using 50 of untrained panellists using a 9 point hedonic scale. Aroma, taste and consistency were not significantly different. Pigeon pea yoghurt showed slightly higher rating for colour compared to other yoghurt samples. Overall acceptability was significantly higher in soy milk yoghurt compared to all other samples. Principal component analysis indicates the variation of the sensory characteristics of the six yoghurt samples assessed. PC1 accounted for 57% of the total variation and differentiated yoghurt samples based on their formulation. PC2 accounted for approximately 26% of the total variation and differentiated yoghurt sample with soymilk based. Principal component analysis indicated variation in the different sensory characteristics of yoghurt samples. Overall, our study suggests that pigeon pea milk's sensory properties can be improved and *Propionibacterium freudenreichii* and it can be used in preparation of non-dairy yoghurt.

6.1 Introduction

Regular yoghurt is generally popular among consumers because the characteristic beany taste associated with legume milk is unacceptable (Ranadheera, 2012). This however may be reduced substantially during processing of legume based yoghurt samples. Several authors have reported that consumers usually consider flavour first when deciding to purchase food but may likely purchase a functional food if they

know the health benefits (Wansink, Ittersum and Painter 2004). Currently, consumers are becoming more health conscious and interested in looking for foods with functional properties besides their nutritive values (Hekmat and Reid 2006). Probiotics dairy products are well-thought-out to pose functional properties, as they are added to the traditional yoghurt cultures to offer therapeutic benefits (Hekmat and Reid 2006).

Furthermore, fermentation using bacterial starter cultures other than lactic acid bacterial may influence the characteristics, including sensory properties, of the product (Ekinci and Gurel 2008). Thus probiotic bacteria are used in combination with lactic acid cultures to produce probiotic food products (Moslemy *et al.,* 2015). Combined use of these bacteria in soy milk has been reported to have resulted in a good compatibility and reduced flatus due to anti-nutritional factors digestion after consumption (Wu *et al.,* 2012). The final product that is the yoghurt would have modified nutritionally by reducing anti-nutritional factors amongst others, preserve and improve flavour, texture and enhancement of health beneficial properties. Abundant health benefits of probiotic organisms have been reported and they include, reduction of blood- cholesterol, improvement of immunity, alleviation of symptoms of lactose intolerance, treatment of diarrhoea, anti-carcinogenic and antihypertensive properties and biotransformation of isoflavones phytoestrogen to improve hormonal balance in postmenopausal women (Fuller, 1992; Nagata *et al*., 1998; Shah, 2000a; Lourens-Hattingh and Viljoen, 2001; Setchell *et al*., 2003).

Fermented milk products are normally considered as probiotic source (Leverrier *et al.* 2003). Probiotics foods are defined as "food" that contain live microorganisms, which upon digestion, actively enhance the health of consumers by improving the balance of micro flora in the gut such as yoghurt (FAO/WHO, Organization 2006). Yoghurt is a fermented food made from fresh milk whole or skimmed milk which is carried out by the action of yoghurt starter cultures (Falade *et al.* 2014). Attempts have been made to produce this type of product from substrates such as soymilk (Granato *et al.* 2010) corn milk, and Bambara milk (Falade *et al.* 2014). Addition of probiotics makes probiotic yoghurt. The application of probiotics is mostly limited to dairy products such as yoghurt, kefir and little attention has been given to

utilization of other milk sources such as pigeon pea milk and their use for dairy-like products such as yoghurt. The sensory quality of vegetable yoghurt may vary with milk source and other added nutrients. Falade *et al.* (2014) reported similar consistency values for Bambara and soybean yoghurt. However, Bambara yoghurt showed superior aroma, colour, taste and overall acceptability than that of soybean (Falade *et al.* 2014). The use of pigeon pea milk in production of fermented milk and fermented milk supplemented with *Propionibacterium fruendenchii* may serve to increase utilization of this neglected legume.

Human gastrointestinal tract harbours 500 different species of bacteria which play beneficial roles. As a person ages, the number decreases and potentially pathogenic organisms increase. Therefore, consumption of probiotic yoghurt may serve to adjust this and replenish the organisms lost through defecation (Hekmat and Reid, 2006). Despite all the potential health benefits, there has been little work done to determine the sensory characteristics and acceptability of yoghurt fermented with these bacterial probiotics such as *Propionibacterium freudenreichii.*

The objective of this study is to assess and compare the sensory properties of yoghurt samples supplemented with *Propionibacterium freudenreichii and yoghurt without Propionibacterium freudenreichii.*

6.2 Materials and Methods

Pigeon pea seeds were obtained from Agricultural Research council South Africa, Soya bean from soya bean research Farm Pinetown. Yoghurt cultures and probiotics were purchased from Lakes foods, South Africa.

Figure: 6.1 Raw Dehulled pigeon pea

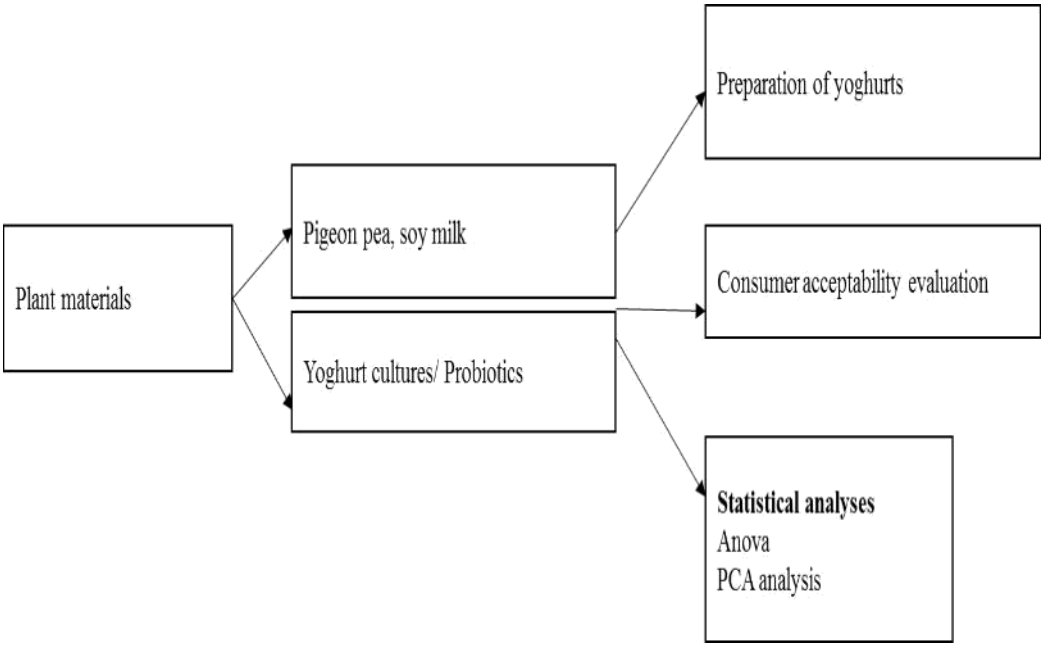

Figure: 6.2 Experimental design

6.2.1 Milk Preparation

Pigeon pea seeds were screened to remove the bad ones. Pigeon pea seeds (250 g) were soaked 2.5 l of milli Q water with 0.5% $NaHCo_3$ overnight, and manually dehulled. The dehulled Pigeon pea seeds were thoroughly rinsed with milli Q water and further soaked in 0.5% $NaHCo_3$ for three hours, after which the seeds were rinsed in milli Q water, milled into paste and mixed with milli Q water in ratio 1:1 (pigeon pea in g/water in ml) using a grinder (Warring laboratory model HGBTWG4. The resulting slurry was the milk obtained and cooked for 20 minutes at 95°C, while stirring continuously with a glass rod to prevent burning. The recovered milk was homogenised in a homogeniser laboratory mixer (P.T, 210, Fisher scientific water side, U.K.) at $60^{o}C$ and allowed to cool and refrigerated.

6.2.2 Soymilk

Soy bean seeds were screened to remove the defective and extraneous material. 25 g of seeds were soaked in 2.5 l of milli Q water, with 0.5% NaHco3 overnight and manually dehulled. The dehulled soy beans seeds were further soaked in milli Q water with 0.5% NaHCo3 solution at $100^{o}C$ for 20 min and rinsed thoroughly with milli Q water, and mixed milli Q water in ratio 1:2 (soya beans in g/water in ml) and transferred into a stainless steel blender (name of the manufacturer, USA). The

resulting paste was centrifuged at 4000×g and resulting milk cooked at 95ºC for 20 min. The milk obtained was homogenised and pasteurised and refrigerated at 4°C temperature.

6.2.3 Analysis of milk

6.2.3.1 Determination of total solids

10 g of soy and pigeon pea milk was weighed into pre-weighed crucible and placed in hot air oven at 105ºC for 48 hours. Dried residue was weighed after cooling in a desiccator to calculate total solids based on this moisture content was determined.

6.2.3.2 Preparation of Yoghurts.

Pigeon pea milk (approximately 12.5g/100g total solids) was added 3.5g/100g skimmed milk powder. This was followed by stirring in powdered milk in pigeon pea milk and raising the temperature 43°C for 30 minutes and 3g/100 mL sucrose was added as sweetener. The milk was then heat treated to 65°C and homogenized at 25 MPa Silverson Homogenizer (P T. 210, fisher scientific water side, U.K.). The temperature was further raised to 90°C for 20min. The heat treated milk was cooled to 43°C in a water bath then inoculated with 2% starter cultures (*Lactose bacillus bulgaricus and Streptococcus. thermophillus*). The inoculated milk was divided into two equal portions, one portion was used as control and the other inoculated with 1% probiotic cultures (*Propionibacterium fruendenchii*) and were incubated at 43°C for 5 h for until a pH of about 4.6 was reached. At the end of the incubation period, the yoghurts were cooled and refrigerated at 4°C overnight.

Six types of set yoghurt were prepared from pigeon pea and soymilk. Three fermented with yoghurt cultures and the other three with yoghurt cultures and *Propionibacterium fruendenchii* and subjected to sensory evaluation.

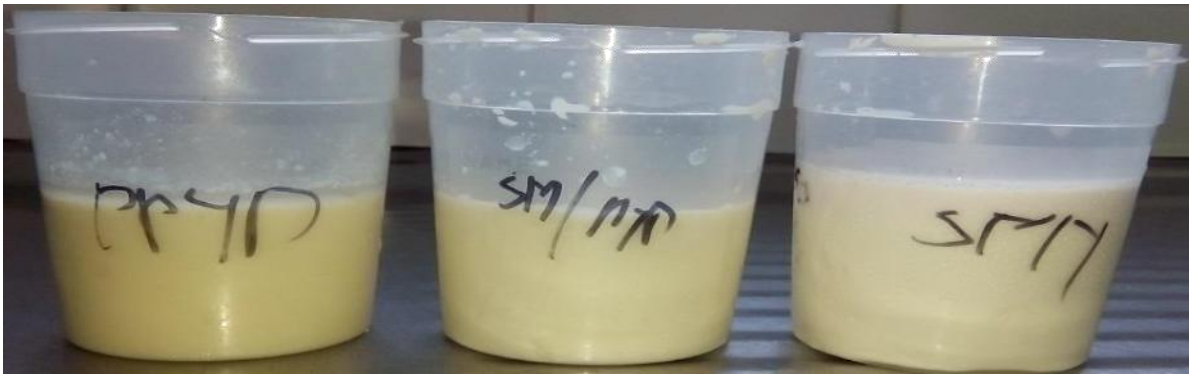

Figure 6. 3: Pigeon /Soymilk Yoghurt

6.3 Ethical consideration

The ethical approval to carry out this study was obtained from Durban University of Technology Durban and Ethics Committee. The panellist members signed the consent forms to indicate their consent to participate in the study and to confirm that they understood the purpose of study. The participation of the panellist in the study was voluntary and they were free to withdraw from the study at any stage. Personal information in the consent forms were made confidential.

6. 4 Sensory evaluation

Sensory evaluation was carried out on the freshly prepared yoghurt samples and yoghurt samples supplemented with *Propionibacterium fruendenchii*. The sensory attributes of yoghurt samples were evaluated using 9-point hedonic scale where 1 represents dislike extremely and 9 to like extremely.

Fifty (50) regular consumers of yoghurt were recruited in Durban University of Technology. The consumer panel members were made of 29 females and 21 males with no allergies to soy. The six samples of yoghurts and yoghurts supplemented with *Propionibacterium fruendenchii* were presented to the panellist in booths (Figure 6.4a, b and c). The panellists were seated apart to prevent them influencing one another. The samples were randomly labelled with three-digit codes obtained from a Table of Random Numbers. The yoghurt and yoghurt supplemented *with Propionibacterium fruendenchii* samples were served to the panellist for evaluation using the protocol of Anyango *et al.* (2011). The yoghurt and yoghurt supplemented with *Propionibacterium fruendenchii* samples were served to each of the panellist

in a randomized order, which was determined from a Table of Random Permutation of Nine.

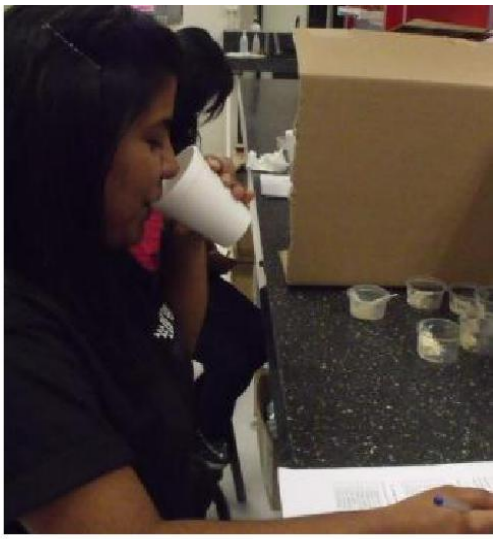

Figure 6.0 a, b, and c: Panellists during sensory evaluation session

6.5 Results and Discussion

The addition of probiotic to yoghurt samples seems not to influence aroma, taste and consistency since there was no significant difference in these parameters ($P < 0.05$). (Table 6.1). However, pigeon pea yoghurt (PPY) showed slightly higher rating (approx. 7.3) for colour compared to other yoghurt samples. Previous findings similarly did not observe much variation in taste of milk soured with *Propionibacteria freudenreichii spp* compared to the milk without the *Propionibacteria freudenreichii* (Mantere-Alhonen 1995). The soured milk was reported to have good sensory property, flavour and consistency. Generally, the taste of yoghurt has been attributed to proteolytic changes which produce aroma precursor and compounds (Moslemy *et al.* 2015)Small peptides may also interfere with Ca and Mg ions to produce a broth like aroma which also contributes to yoghurt flavour (Moslemy *et al.* 2015). Furthermore, induced diacetyl and acetone production have also been reported to play a role in yoghurt flavour (Sakar and Misra, 2006). Also during fermentation tartness and green flavour of yoghurt produced have been attributed to *L. bulgaricus* (Oliverial, 2000). These are perceived both through aroma and taste, non-volatile acids such as acetic, propionic and volatile as butyric and thermal degradation compounds contribute also to taste and aroma. However, taste acceptability of yoghurt samples were very similar regardless of milk type used (Table 6.1). Also, the taste acceptability of yoghurt samples was not significantly influenced by cultures and *Propionibacterium freudenreichii* used for fermentation since no significant differences.

Furthermore, aroma was also not affected by the use of types of organism used for fermentation. However, according to Awobusayo *et al*. (2015) the use of starter culture improves aroma of fermented products due to the release of aromatic compounds. During spontaneous fermentation, flavour and aroma compounds are also released by starter culture (Awobusuyi *et al.*, 2015). *Propionibacterium freudenreichii* has been deliberately developed to produce better aroma profile and flavour substances and such the beany taste was not perceived (Moslemy *et al.* 2015). Addition of sweetener altered taste and viscosity which was perceived as the mouth feel. Mouth feel which is also the physical and chemical interaction with mouth were not significantly different (Table 6.1).

The gelatinisation of starch aided the perceived mouth feel as expected due to added sucrose and thermal treatment. Fermentation improves textural characteristics.

Table 6.1 Sensory acceptability of yoghurt samples

Sample	Colour	Aroma	Taste	Mouth Feel	Overall acceptability
PPYP	$6.88\pm1.8^{a,b}$	5.98 ± 2.3^{a}	6.04 ± 2.0^{a}	6.22 ± 2.0^{a}	$6.08 \pm 2.0^{a,b}$
SMY	$6.82\pm1.8^{a,b}$	6.29 ± 2.0^{a}	6.12 ± 2.4^{a}	6.67 ± 2.0^{a}	6.80 ± 2.1^{b}
SMYP	7.02 ± 1.9^{b}	6.35 ± 1.9^{a}	6.20 ± 2.0^{a}	6.82 ± 1.5^{a}	$6.55 \pm 1.8^{a,b}$
SM/PY/P	$6.42\pm1.8^{a,b}$	6.06 ± 2.1^{a}	5.94 ± 2.0^{a}	6.42 ± 2.2^{a}	5.80 ± 2.4^{a}
PPY	7.27 ± 1.4^{b}	6.35 ± 1.6^{a}	5.76 ± 1.9^{a}	6.88 ± 1.3^{a}	$6.49 \pm 1.7^{a,b}$
SM/PY	6.10 ± 2.7^{a}	6.08 ± 2.4^{a}	6.08 ± 2.3^{a}	6.60 ± 1.8^{a}	$6.54 \pm 2.3^{a,b}$

MEANS ± SD (n=50). LSD ($p<0.05$ Means values are followed by different superscript letters not significantly different. Where: **SM/PY**: 50% soymilk + 50% pigeon pea milk yoghurt, **SMYP**: Soymilk probiotic yoghurt, **SM/PYP**: 50% soymilk + 50% pigeon pea milk probiotic yoghurt, **PPYP**: Pigeon pea milk probiotic yoghurt, **SMY**: Soymilk yoghurt, **PPY**: Pigeon pea milk yoghurt.

The colour acceptability of yoghurt samples with starter culture and those with *Propionibacterium freudenreichii* were not significantly different from their milk counterparts (Table 6.1). As previously reported in chapter 4, colour of milks in which the yoghurts were prepared were significantly different. The significant differences ($P \leq 0.05$) in colour (Table 6.1) of the yoghurt samples were due to formulation of different yoghurt mixtures. The lighter colour of yoghurt samples compared to their corresponding milk previously reported may be linked to fermentation which resulted in lowered of pH which in turn increased the lightness (L* value) of the yoghurts (Nnan, 1997).

Although, soy milk yoghurt was the most acceptable among the yoghurt samples, with an overall acceptability rating of 6.8, all the samples had comparable ratings to that of soy yoghurt, and these ratings were within commercially acceptable range (4 to 9) for yoghurt by the Karl Ruther nine points scheme (Tamime *et al.* 2005).

6.6: Principal component analysis

PCA indicates the variation in the sensory characteristics of the six yoghurt samples. The first two principal components (PC1 and PC2) accounted for 83% of the total variation in the sensory attributes data (Fig 6.1: A and B). PC1 accounted for 57% of the total variation and differentiated yoghurt samples based on their formulation. PC2 accounted for approximately 26% of the total variation and differentiated

yoghurt sample with soymilk based. The taste attribute was clearly separated from the other attributes such as colour, mouth feel, texture and overall acceptability of yoghurt samples (Fig 6.1a and b). Therefore, taste appears to be the main sensory attribute influencing the overall acceptability of the yoghurt samples. Although, as shown in (Table 6.1) there was no statistical difference in means of descriptive sensory data in consumers' liking for taste of yoghurt samples with *Propionibacterium freudenreichii* and without *Propionibacterium freudenreichii*.

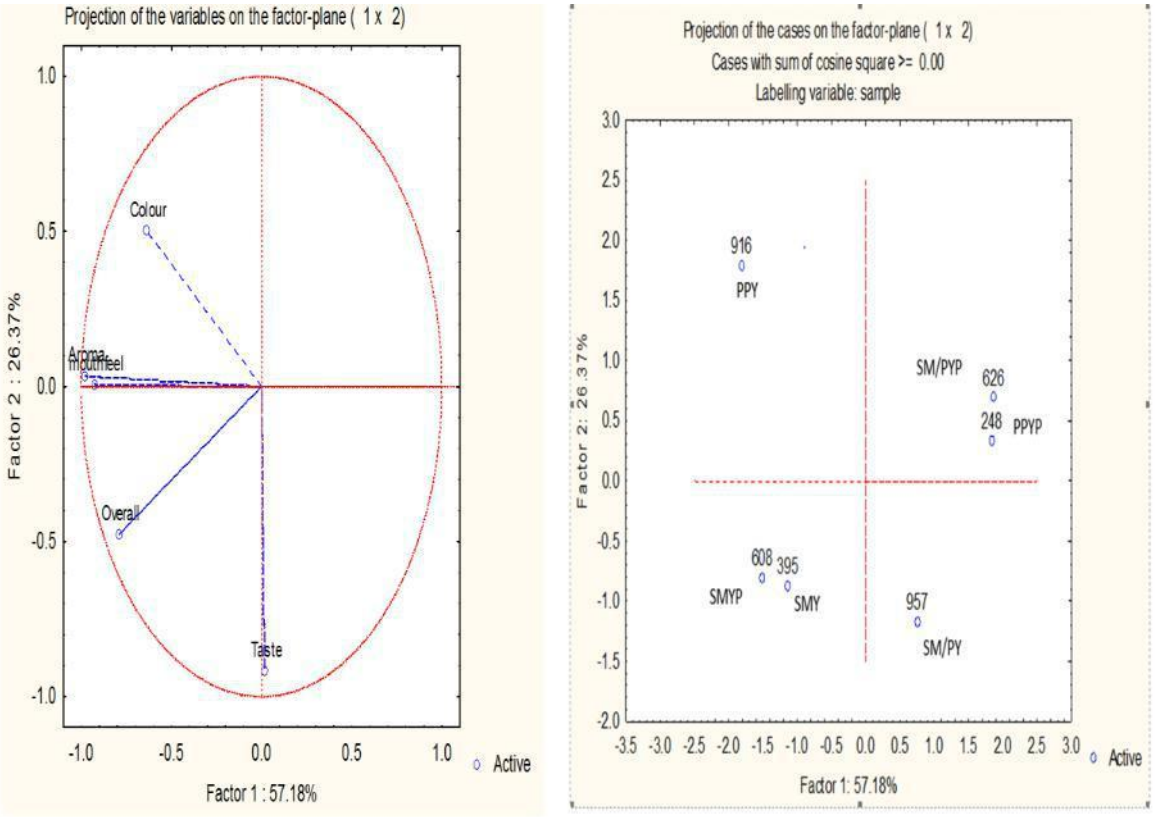

Figure: 6 A and B Principal Components analysis (PCA) for consumer acceptability of yoghurt. A indicating vectors loadings for sensory attributes and B components scores for yoghurt samples

6.6 **Conclusion**

In conclusion, probiotic yogurt containing *Propionibacterium freudenreichii* was successfully prepared and found by sensory evaluation to be comparable in appearance, flavour, texture, and overall acceptability to the standard yogurt. All the samples had comparable ratings to that of soy yoghurt, and these ratings are within commercially acceptable range (4 to 9) for yoghurt by the Karl Ruther nine points scheme suitable for human consumption.

CHAPTER 7

7.1 GENERAL DISCUSSION

Legumes are low-glycemic-index, low-energy-dense foods containing high proportions of dietary fibres, vegetable proteins, oligosaccharides, and phenolics (Tharanathan and Mahadevamna, 2003). Pigeon pea is one of the underutilised legumes in South Africa. Lately, due to the awareness of nutritional benefits of plant based food by health conscious consumers', demand for yoghurt from non-dairy based sources has been on the increase. In this study, probiotic yoghurt was produced from pigeon pea milk using *Propionibacterium freudenreichii.* The use of soy milk in yoghurt production has been well documented. Therefore, the study design combined different blends of pigeon pea milk and soy milk. The milk was fermented with bacterial cultures (*Lactobacillus delbrueckii Streptococcus thermophillus,* and *Propionibacterium freudenreichii).* Proximate composition, anti–nutritional factors, and amino acid profile of the flour, milk, fermented milk and fermented milk supplemented with *Propionibacterium freudenreichii*, were determined.

The use of pigeon pea in the production of probiotic yoghurt may serve to increase the utilization of this neglected legume. Thus this research has the potential to reduce food insecurity through increased utilization of pigeon pea in South Africa and Africa at large.

Yoghurt is normally made from cow's milk by *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus salirivarius* subsp. *thermophilus* (Lourens-Hattingh *et al.,* 2001). Attempts have been made to produce this type of product from different food sources such as soya milk (Granato *et al*., 1996) and corn milk (Supavititpatana *et al*., 2008). Incorporation of probiotic organisms such as *Lactobacillus acidophilus, Bifidobacterium* sp. and *Propionibacterium jensenii702* in fermented products could improve the quality of the product and may be beneficial to the health status of consumers. Pigeon pea milk based yoghurt may provide additional nutritional benefits that are beyond simple nutrition for consumer due to their hypolipidemic, hypoglycaemic, anti-cholestrolemic and anti-atherogenic properties and reduced food allergenicity as reported by (Habib *et al*., 2012; Mesial *et al.*, 1994; Lopez-Lazaro, *et al*., 2002), in soy milk, unfortunately soy bean has been listed as one of the foods which have allergies (Philips, 2014).

This study is innovative since the use of pigeon pea milk for yoghurt and probiotic yoghurt has not been reported. In addition, the effects of probiotics in pigeon pea milk is not known. Therefore, this study explores the use of pigeon pea in probiotic yoghurt production.

7.2 Findings and conclusions

Acceptable yoghurt was produced from pigeon pea with comparable quality to soy which serve as control. The higher milk yield from soybean suggests that milk extraction from soybean was more efficient compared to pigeon pea. Variation in milk yield among pulses may be attributed to source and cultivar differences.

Proximate composition of pigeon pea flour with the protein content value of 26% and crude fat 2%. This is expected as pigeon pea has been classified as protein rich food comparable to soy bean and groundnuts (Aremu *et al.,* 2006). Moreover, crude fat value was low as it is not an oil rich legume when compared to soybean (Salunkhe *et al.,* 1985; Aremu *et al.,* 2006). The ash, fibre and carbohydrate were 3.62, 8.75 and 50.19 respectively. These values were comparable to those reported in the literature (Akande *et al.,* 2010).

Proximate composition of yoghurt samples was comparable to previous reports and amino acids composition of both milk and yoghurt were within the recommended requirement for adults (FAO/WHO, 2007).

Microbial quality and profile of all the yoghurt samples were similar. The absence of pathogenic bacteria in all the yoghurt samples indicates their safety. Storage at 4^{o}C is the most acceptable, as storage at 21^{o}C promoted proliferation of contaminants. Differences in decline in pH at 4°C, 10°C and 21°C were significant ($p \leq 0.05$), the rate was higher at 21°, 10° than 4°C. Decrease in pH resulted in increased TTA values over storage temperatures and periods. The colour values evaluated were recorded as L*, b*, a* and ΔE* during 4 weeks storage at 4, 10 and 21°C. Significantly, high values ($p \leq 0.05$) recorded for L*(lightness) yoghurt samples with soymilk. The metabolic activities of starter and probiotics were maintained throughout 28 days storage period such that probiotic survival were within the recommended level to exert the potential health benefits. Production of acid is important in yoghurt preparation. Starter cultures used played the important role of acid and flavour enhancer to the products. The probiotic bacteria on the other hand were seemingly responsible for metabolising acrylic aldehydes that are responsible for beany flavour and produced a low concentration of acetic acids which provide a vinegary flavour.

Soy yoghurt was most acceptable among the yoghurt samples but all the samples had comparable ratings, and these ratings are within commercially acceptable range (4 to 9) for yoghurt. Pigeon pea yoghurt compared to that of soy yoghurt suggesting that it can be used to deliver probiotics to consumers. The positive results of nutritional, anti-nutritional factors, microbiological analyses as well as consumer's acceptability test showed that the obtained yoghurt samples might be considered a new functional food with possible health benefits suitable for different groups such as vegetarian and people allergic to animal proteins.

7.3 Recommendations

Since consumer acceptability test was carried out on adults, it is recommended that it should also be evaluated on children of school going age and weaning mothers who can also benefit from pigeon pea milk during breastfeeding. For the pigeon pea yoghurt, the use of flavours and sweeteners can be encouraged for further appeal to consumers. Determination of bioactive compounds in both milk and yoghurts should also be done. Determination of proteolytic activity, determination of angiotensin-converting enzyme inhibitory activity in probiotic yoghurt should also be carried out. Also, for further study the use of metabolomics to study microbial communities and metabolities such as fatty acids, etc. should be tried.

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