APPLICATION OF SELECTED PROBIOTIC BACTERIA IN THE FERMENTATION OF COWPEA (*Vigna unguiculata* L. Walp) MILK

ADUOL KEVIN OMONDI ONYANGO

MASTER OF SCIENCE

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Application of Selected Probiotic Bacteria in the Fermentation of Cowpea (*Vigna unguiculata* L. Walp) Milk

Aduol Kevin Omondi Onyango

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DECLARATION

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

Signature: ……...……..... Date: ……...……

Kevin Omondi Aduol Onyango

This thesis has been for examination with our approval as the university supervisors.

Signature: ……...……..... Date: ……...……

Prof. Arnold Onyango, PhD

JKUAT, Kenya

Signature: ……...……..... Date: ……...……

Dr. Samuel Imathiu, PhD

JKUAT, Kenya

DEDICATION

I dedicate this work to my late parents, Mr. Lucas Newton Onyango and Mama Mellenia Atieno Aduol, for their dedication and support in my early years of education.

To my wife Winnie Atieno and sons Leon Omondi and Ivan Omondi for their patience, support and enduring my absence during my studies.

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May the Almighty God bless you all abundantly.

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LIST OF ABBREVIATIONS

ANOVA	Analysis of Variance
CFU	Colony Forming Units
CIAT	Centro International Agricultural Tropicale
CRD	Complete randomized design
FAO	Food and Agriculture Organization
GC	Gas Chromatography
HCN	Hydrocyanic acid
HPLC	High Performance Liquid Chromatography
IITA	International Institute of Tropical Agriculture
KALRO	Kenya Agricultural Livestock Research Organization
KEBS	Kenya Bureau of Standards
LAB	Lactic acid bacteria
LDL	Low Density Lipoproteins
NICHE	Netherlands Initiative for Capacity Building in Higher Education
PDA	Potato Dextrose Agar
PEM	Protein Energy Malnutrition
TPC	Total Plate Count
WHO	World Health Organization

ABSTRACT

There is mounting evidence that probiotic microorganisms improve human health in diverse ways, including suppressing the growth of pathogenic bacteria in the gastro-intestinal tract, and producing beneficial metabolites such as short chain fatty acids that reduce the risk for various non-communicable diseases. Fermented foods are a good vehicle for probiotics and their metabolites. Legumes, such as cowpeas, which are less expensive, are alternative sources of proteins especially for the economically vulnerable. Besides their traditional way of consumption as cooked whole seeds, they are also processed into legume 'milks' as a substitute for animal milks. The current study investigated the suitability of probiotic bacteria in the fermentation of cowpea milk, in terms of the extent of formation of beneficial short chain fatty acids in the fermented milks; the reduction of oligosaccharides, which contribute to the problem of flatulence upon consumption of legumes; the sensory acceptability of the fermented products; and changes in the physicochemical and microbial characteristics of the products during storage. Fermentation of cowpea milk was carried out using three mixed starter cultures containing (i) *Lactobacillus acidophilus, Bifidobacterium spp,* and *Streptococcus thermophilus* (ABT-5) (ii) *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* (YF-L 903) or (iii) *Lactobacillus rhamnosus* GR-1 and *Streptococcus thermophilus* (Yoba fiti). After fermentation at 45˚C for 14 hours, the population of the bacteria in cowpea milk attained more than 10^6 colony forming units (CFUs), which is the minimum accepted for a food product to be considered probiotic. There was a slight increase in microbial population during the first two weeks of storage (refrigeration at 4˚C) followed by a decline. However, the population did not drop below 10^6 CFUs by the 28^{th} day of storage.The oligosaccharides and short chain fatty acids were determined by high performance liquid chromatography and gas chromatography, respectively. Cowpea milk fermentation caused 67-100% reduction in raffinose and 20-70% reduction in stachyose in a culture-dependent manner. The stachyose content of raw cowpea (1.39±0.23g/100g) was higher than raffinose (0.22±0.06 g/100g), while verbascose was not detected. All the cultures produced propionic acid, butyric acid and valeric acid in differing concentrations but only Yoba fiti and ABT-5 produced isovaleric acid. The product fermented with YF-L 903 attained 2430 ppm of propionic acid, which was four times and ten times higher than the concentrations produced by the ABT-5 and Yoba fiti cultures respectively. Proximate analyses of samples: ash, moisture, crude fiber and protein content were performed according to Association of Official Analytical Chemist (AOAC) official methods 923.03, 925.09, 978.10 and 979.09. Total lipids were determined by modified Bligh and Dyer method. The carbohydrate concentration was determined by difference between 100 and total sum of the percentage of ash, moisture, fiber, fat and protein. All the analyses were performed in triplicates. Crude fat decreased significantly ($P<0.01$) after fermentation except for Yoba fiti culture which led to 33.2% increase. Crude fiber was not detected in all the samples. Fermentation with Yoba fiti also led to increase in protein content, although this was not significant. A decrease was observed for carbohydrate content, after fermentation, with YF-L 903 culture leading to the highest decrease of 7.1%. Evaluation of the acceptability of the fermented products in terms of taste, texture, aroma and appearance was done on a nine-point hedonic scale where 1 = like extremely, 5 = neither like nor dislike and 9 = dislike extremely. The study

demonstrates suitability of probiotic bacteria in the fermentation of cowpea milk. However, further work should be done to improve the sensory characteristics of these products.

CHAPTER ONE

INTRODUCTION

1.1 Background of the study

In developing countries, chronic protein deficiency is a major problem and increasing cases of malnutrition and many infant deaths have been attributed to it (Briend *et al.,* 2015). Non-communicable diseases such as diabetes and cardiovascular diseases are also increasing in prevalence throughout the world, especially in low income countries (Kwak *et al.,* 2014; Gowshall and Taylor-robinson, 2018). One of the recommended strategies for reversing this trend is adoption of health-promoting diets particularly plant-based foods (Tuso *et al.,* 2013; Somasundaram and Kalupahana, 2016). After considering the importance of legumes in the world's diet, the United Nations declared 2016 as the international year of leguminous (grain legumes) as "nutritive seeds for a sustainable future" (Ferreira *et al.,* 2019).

Cowpea is one of the most popular legumes consumed in Kenya, both as a vegetable and as a grain but most consumers have reported occurrence of intestinal gas or flatulence after legume consumption (Winham and Hutchins, 2011), which often hinders utilization. Cowpea seed is a good source of protein and also contains bioactive compounds such as flavonols and hydroxybenzoic acids, that can reduce the risk for physiological disorders such as obesity, dyslipidemia and cardiovascular complications (Sreerama *et al.,* 2012).

Cowpea milk, while not as well balanced in amino acids as cow's milk, can offer a low-cost alternative to those who are less fortunate, especially in areas where milk supply is scarce (Sethi *et al.,* 2016). Probiotic foods are a form of nutraceutical that contains a large number of health-promoting microorganisms (Nagpal *et al.,* 2012). Probiotics were originally described as microorganisms that, when consumed, could withstand the harsh conditions of the stomach and exert beneficial effects on the digestive organs. 10^6 settlement framing units in the small intestine and 10^8 in the colon are required for the centralization of probiotics required for clinical effects (Kwak *et al.,* 2014). In either case, the beneficial impact of microorganisms in the oral

cavity has recently been highlighted (Bermudez-Brito *et al.,* 2012). Probiotics have a number of medicinal advantages, including reducing the generation of inflammatory factors by pathogens and inhibiting the growth of pathogenic microorganisms (Bermudez-Brito *et al.,* 2012). Probiotics deliver metabolites that help kill bacteria, such as short chain unsaturated fats. Propionic and butyric acids have been shown to promote colon cancer cell apoptosis and to lower the risk of obesity, insulin resistance, diabetes, hypercholesterolemia, and cardiovascular dysfunction (Sivaprakasam *et al.,* 2016; Koh *et al.,* 2016; Canfora *et al.,* 2015; Thapa and Tamang, 2015). Such useful metabolites are called postbiotics (Klemashevich *et al.,* 2014; Cicero *et al.,* 2011). Legumes including cowpeas have a high potential as nutraceuticals (Trinidad *et al.,* 2010).

This study was therefore intended to determine the suitability of cowpea milk for the growth of different probiotic bacteria, associated chemical changes induced by such fermentation of cowpea milk, and the sensory acceptability of the fermented cowpea milk. Cowpea milk was fermented with three blended lactic acid bacteria starter cultures. All the cultures contained *Streptococcus thermophilus*, and, furthermore, one of the cultures contained *Lactobacillus delbrueckii* subs *bulgaricus*, the second contained *Lactobacillus rhamnosus* GR-1 strain, and the third contained *Lactobacillus acidophilus* and *Bifidobacterium* sp. All these microorganisms have past cases of probiotic impacts (Bermudez-Brito *et al.,* 2012; Reid, 2017).

1.2 Statement of the problem

Hunger and malnutrition remain a serious problem in third world countries like Kenya. Non-communicable diseases (NCDs), which were formerly regarded to be diseases of affluence, have also recently emerged as a major health problem to even resource-disadvantaged members of the society, especially in developing countries. Nutraceuticals, defined as foods or food constituents with health benefits beyond the traditional nutrients, are increasingly sought (Schmitt and Ferro, 2013; Kapravelou *et al.,* 2014) to improve the treatment of the NCDs. Probiotic foods, containing probiotic bacteria, constitute an important class of nutraceutical products. Currently, fermented dairy milk products containing probiotic bacteria are the major dietary sources of

probiotics. However, dairy milk is not affordable to economically disadvantaged people in the developing world, who mainly consume plant-based diets. Hence, this category of people, who may easily miss out on the benefits of the probiotics, even though they are vulnerable and need the probiotic-associated health benefits. There is also a high prevalence of protein deficiency among these people, and substituting milk with cereal-based porridge as a carrier of the probiotics will help to perpetuate protein deficiency.

1.3 Objectives

1.3.1 Main objective

To determine the suitability of cowpea milk as a food carrier of probiotic microorganisms in three mixed starter cultures containing *Streptococcus thermophilus* and *Lactobacillus* subs *bulgaricus*; *Lactobacillus acidophilus* La-5, *Bifidobacterium animalis* Bp-12 and *Streptococcus thermophilus;* and *Lactobacillus rhamnosus* GR-1 and *Streptococcus thermophilus*; and to determine how fermentation by these microorganisms affects the chemical and sensory characteristics of the cowpea milk

1.3.2 Specific objectives

1. To determine the growth of the different probiotic microorganisms in the cowpea milk and changes in their populations during refrigerated storage for 28 days.
2. To determine the formation of the short chain fatty acids such as propionic acid, butyric acid, valeric acid and isovaleric acid during fermentation of cowpea milk by the different starter cultures.
3. To determine the effect of fermentation with the different starter cultures on the oligosaccharide contents of cowpea milk.
4. To determine the sensory acceptability of cowpea milk fermented by different probiotic starter cultures.

1.4 Research hypotheses

1. H_0: Cowpea milk does not support the growth of the selected probiotic bacteria to final populations of $\geq 10^6$ colony forming units and there is no change in their populations in fermented cowpea milk during refrigerated storage for 28 days.
2. H_0: The starter cultures do not produce short chain fatty acids during cowpea milk fermentation
3. H_0: The starter cultures do not reduce cowpea milk oligosaccharides during fermentation.
4. H_1: The fermented cowpea milk products are of high sensory acceptability

1.5 Justification of the study

There is a need to develop food products that address both nutrition and health. Cowpea is an alternative source of cheap protein, and thus fermentation of its milk with probiotics can contribute to alleviation of protein energy malnutrition, while at the same time affording the health benefits associated with probiotics. Cowpea milk has additionally been reported to contain bioactive compounds such as polyphenols, flavonols and hydroxybenzoic acids that reduce the risk of non-communicable diseases, for instance, by moderating postprandial glucose by inhibition of starch digestion enzymes α-amylases and glucosidases, in management of diabetes. Cowpea milk fermentation with probiotic microorganisms may enhance its health benefits not only because of the live microorganisms but also because of beneficial metabolites produced by these microorganisms in the milk. Moreover, fermentation has been reported to reduce the content of oligosaccharides which contribute to flatulence in some individuals when they consume cowpeas.

CHAPTER TWO

LITERATURE REVIEW

2.1 Cowpea origin and description

There are about 7,000 plant species used worldwide as food providing about 95% of global plant derived energy and protein intake (Hamid *et al.,* 2016). Legumes are plants that belong to the family *Leguminosae*, which includes all types of beans, peas, peanuts, among others and serve as food for a large number of people (Stijepić *et al.,* 2013). Cowpea (*Vigna unguiculata*) is one of several species of the widely cultivated genus *Vigna* (Okereke and Hans-anukam, 2015). Four subspecies are recognised, of which three are mostly cultivated: *V. textilis*, *V. pubescens*, and *V. sinensis* (Ibrahim *et al.,* 2014).

Nigeria is believed to be the place of early domestication of cowpea (Oyarekua, 2011). While it may be uncertain when cultivation began, remains of charred cowpeas from rock shelters in Central Ghana have been dated to the second millennium (Fabbri and Crosby, 2016). In 2300 BC the cowpea is believed to have made its way into South East Asia where secondary domestication events may have occurred (Akibode and Maredia, 2011).

The seed pods of cowpeas contain 8 - 18 seeds per pod and are cylindrical and curved or straight. The seed coat varies in texture (e.g., smooth, rough, or wrinkled), colour (e.g., white, cream, green, buff, red, brown, black), and uniformity (e.g., solid, speckled, or patterned). Seeds of the most well-known cowpea types, such as 'black-eyed' and 'pink-eyed,' are white with a round irregularly shaped black or red pigmented area encircling the hilum that gives the seed the appearance of an eye (Gaafar *et al.,* 2016). The open display of flowers above foliage and the presence of floral nectaries contribute to the attraction of insects that aid in pollination.

Figure 2.1: Graphic design of a cowpea plant (Source: Okereke and Hans-anukam, 2015)

2.2 Global cowpea production

Cowpea is cultivated on about 14.5 million acres worldwide annually yielding about 6.5 million metric tons (Okereke and Hans-anukam, 2015). Globally, Africa produces 83% of total cowpeas, with 80% coming from West Africa. Nigeria is the leading producer at 45% followed by Niger (15%), Brazil (12%) and Burkina Faso (5%) (Boukar *et al.,* 2015).

2.3 Cowpea production in Kenya

In Kenya, cowpea production is characterized by low yields with a range of 102-239 kg/ha compared to potential yield of 1200-1800 kg/ha as reported by Mhango *et al.,* (2016). It is widely produced for its grain in Eastern Kenya, Coast and Nyanza regions and for its leaves in Western Kenya (Rusike *et al.,* 2013). In 2012, Eastern Kenya (Kitui, Makueni and Machakos counties) produced 90% of total national production. Nationally, it is grown on 214,492ha with 187,910ha in Eastern province (MoALF, 2015).

There was an increase in area under production and total output of cowpeas in Kenya by 18% and 13% in 2011, respectively compared to 2010 (MoALF, 2015) (Table 2.1). This was attributed to the increase in area under production and the fact that the Ministry of Agriculture availed seeds on time (MoALF, 2015).

Increase in production from 133,756 tons to 138,673 tons was observed in 2014 as compared to 2013, with the area under cowpea increasing from 250,798ha in 2013 to 281,877ha in 2014 (Table 2.1). This was attributed to the campaign by the Ministry of Agriculture and other partners on the adoption of drought tolerant crops (MoALF, 2015).

Table 2.1: Annual cowpea production in Kenya between years 2010 to 2014

Year	2010	2011	2012	2013	2014
Area(ha)	168,273	197,980	215,269	250,798	281,877
Production 90 kg bags	803,046	905,938	1,266,238	1,486,180	1,540,813
Tons	72,274	81,534	113,961	133,756	138,673
Yield (kg/ha)	405	414	531	531	495
Consumption	..	650,000	1,066,667	…	…
Price/90 kg bag (Ksh)	…	3,934	6,220	…	…

Source: MoALF (2015) ... = data not available … = data not available yet

2.4 Nutrient composition of cowpea grain and effects of processing on the nutrient content

Cowpea grains are excellent source of carbohydrates (50-60%) and an important source of protein (18-35%) (Ojokoh *et al.,* 2013). Cowpea grains have been shown to contain about 50% starch and 0.5-2.2% oil. Dried seeds contain 22% protein and 61% carbohydrates (Tuso *et al.,* 2013). Sucrose concentration of cowpea grains contributes to its taste (Tchiagam *et al.,* 2011). Abate *et al.,* (2012) reported that stachyose (3.43%), sucrose (2.97%) and raffinose (1.24%) are the predominant sugars in non-fermented cowpea flour. However, verbascose was not detectable in their research study. Ejtahed *et al.,* (2011) observed a decrease in proximate and mineral characteristics of cowpea milk compared to that of cowpea flour. He attributed the decrease to various factors such as soaking, natural fermentation during soaking, heat treatment, filtration and blending. Niyibituronsa *et al.,* (2019) reported that lactic acid bacteria fermentation of cowpea milk led to 79.7%, 5.9% and 12.8% decrease in verbascose, stachyose and raffinose, respectively. The author also observed an

increase in sucrose and fructose by 41.9% and 43%, respectively. The researcher also noted an increase in thiamin, niacin, phosphorous, energy and protein.

2.5 Factors influencing cowpea quality

2.5.1 Pre-harvest factors

Environmental conditions under which the grain grows affects its germination rate and general performance in terms of yields (Abate *et al.,* 2012). Good grain health can be achieved through proper grain production practices, such as control of pests and diseases during grain production as well as elimination of diseased crops during growth (Ntombela, 2012).

2.5.2 Post-harvest factors

High physical quality grains can be achieved through cleaning and sorting as well as proper storage to avoid damage by storage insects (Abate *et al.,* 2012). Shaban, (2013) explained that high grain moisture content leads to loss of viability thereby affecting the longevity of the grain. Cowpea grain health is determined by presence or absence of disease causing microorganisms such as fungi, bacteria, virus, nematodes and insects (Oyarekua, 2011) and fumigation during storage (Ntombela, 2012). Pathogen infected grains are small in size, rotten, shrunken and discolored (AATF, 2012).

2.6 Different ways of cowpea utilization

Cowpea's high protein content, adaptability to different types of soils and intercropping systems, resistance to drought and ability to improve soil fertility makes it economically important in many developing countries (AATF, 2012). It is a multipurpose crop used for food, fodder and a source of income (Mathu *et al.*, 2013). Cowpea haulms are dried on the rooftops and used as fodder during dry season and when the fodder is sold it provides household income for improved living standards (Drăghici *et al.,* 2016).

The crop is important in farming systems due to its ability to fix atmospheric nitrogen through symbiotic relationship with *Bradyrhizobia* species of bacteria capable of

nitrogen fixing at an average rate of 240 KgN/ha per annum (Drăghici *et al.,* 2016; Ntombela, 2012) which allows it to grow and improve poor soils.

Cowpeas helps in reducing food insecurity when used as a major vegetable and the grains sold in markets for household income (Abukutsa-Onyango, 2014). Its leaves are used as vegetable and are more popular than the grains in some areas since the leaves mature faster than the grains (Mamiro *et al.,* 2011).

Globally, Indians use cowpea grains to make *kozhukattai* (steamed sweet dumplings) prepared with cooked and mashed cowpeas mixed with jiggery and *ghee*. In Korea cowpeas is used in making rice-cakes (Jung *et al.,* 2014). In Kenya, cowpea grains are used as protein source in *githeri* (a cooked mixture of maize and cowpea grain) and leaves used as vegetable (Awika *et al.,* 2011). In Zimbabwe, *Nyemba* (Cowpea milk) is consumed after soaking the cowpea grains overnight and blending to form a smooth paste followed by filtration through a cheesecloth (ASHC and N2Africa, 2014). Cowpea milk is believed to originate from Nigeria and it's mostly consumed in West African countries as a beverage after its fermentation (Ibrahim *et al.,* 2014; Madodé *et al.,* 2013). In Malawi, cowpea flour together with maize flour are used to prepare a complementary porridge (Ngoma *et al.,* 2018).

2.7 Consumer concerns about cowpeas

Cowpeas are nutritionally beneficial to humans and animals and have been referred to as the poor man's source of protein in many rural and urban homes (Agbogidi, 2010). However, their wider acceptability and utilization is still limited due to presence of oligosaccharides such as raffinose and stachyose, considered to cause flatulence (Zartl *et al.,* 2018).

In a study carried out by Sreerama *et al.,* (2012) on 448 families, 40% reported abdominal discomfort in terms of ingestion, vomiting, diarrhea, increased belching, bad breath, flatulence, constipation and sleepiness, after consuming cowpeas cooked at atmospheric pressure. In the same study, 72.5% of respondents complained of abdominal discomfort after consuming undehulled cowpeas as opposed to 42.5% with dehulled cowpeas.

In another study to determine the effects of cowpea oligosaccharides on gas production in adult rats, it was found that 50% of total gas was produced after 7 hours when whole cowpeas was ingested by the rats (Zartl *et al.*, 2018). Madodé *et al.*, (2013) found that adult human beings produced high hydrogen concentration after consuming a cowpea porridge breakfast.

2.8 Application of lactic acid fermentation in food production

Lactic acid bacteria (LAB) are majorly rod-shaped Lactobacilli or the lactic cocci of genus Streptococcus. Some of the microorganisms involved in fermentation are *Lactobacillus thermophilus, L. lactis, L. bulgaricus, L. acidophilus, L. diacetilactis, L. plantarum, L. cremoris, L. rhamnosus, Streptococcus thermophilus* among others (Starzyńska–Janiszewska, 2011). Slashinski *et al.*, (2012) explained that lactic acid bacteria (LAB) from genera *Lactobacillus*, *Streptococcus* and *Leuconostoc* are the predominantly used probiotics in the food fermentation. Pranoto *et al.*, (2013) also reported that the most commonly used probiotics belong to the heterogeneous group of LAB (*Lactobacillus*, *Enterococcus*) and to the genus *Bifidobacterium.* However, yeast and other microbes have also been recently suggested as potential probiotics (Petruláková and Valík, 2015). However, a review by Rezac *et al.*, (2018) on use of probiotics in the fermentation of commonly consumed fermented foods reports that lactic acid bacteria is the most used probiotic.

During fermentation, LAB converts some of the available carbohydrates in foods to organic acids and lowers the pH of the food. This helps in food preservation as the low pH creates unfavourable growth conditions for pathogenic bacteria (Oyarekua, 2011). The acids as well as other flavour compounds which include diacetyl, acetaldehyde and acetoin contribute to the desired taste and flavour of the food (Pandya, 2016). Thus, fermentation helps improve taste of food, digestibility and ensures a proper balance of bacteria in the intestines (Madode *et al.*, 2013). Generally, fermentation improves food safety by inhibiting growth of pathogenic bacteria due to antimicrobial properties of lactic acid (Oyarekua, 2011) and by detoxifying microbial toxins such as aflatoxin (Chaves-Lopez *et al.*, 2014). Lactic acid fermentation is

carried out by lactic acid bacteria (LAB) producing lactic acid as the major byproduct (Zartl *et al.,* 2018;Turgut and Cakmakci, 2018).

Yoghurt and other cultured dairy products are generally good dietary sources of probiotics according to Panahi *et al.*, (2016) although other foods such as vegetables, meat, cereal products and legume products can also undergo fermentation by probiotics (Marco *et al.,* 2017). In a survey of 335 adults, yoghurt was found to be the main food associated with probiotic bacteria (Stanczak and Heuberger, 2009). However commercial *Lactobacilli* have also been added to meat products, snacks, and fruit juices (Heller, 2018; Ranadheera *et al.,* 2010). When selecting a probiotic microorganism to be used in fermenting a given food, its viability through food processing, packaging and storage conditions must be considered before selecting it for fermentation (Kubo *et al.,* 2011). For example, a food product's pH affects probiotic's survival and growth (Kechagia *et al.,* 2013).

2.9 Fermentation of cowpea milk

Growing awareness of nutritional and health benefits of plant-based foods by consumers has led to production of legume milk such as cowpea milk (Rezac *et al.,* 2018). Production of fermented cowpea milk has been exploited by a number of researchers (Sethi *et al.,* 2016; Afoakwa *et al.,* 2010) and proved to be a prospective alternative to animal milk because of its health claims (Mamiro *et al.,* 2011). The possibility of producing fermented cowpea milk for use was studied by Oyarekua, (2011) and the result showed that the milk was accepted by consumers with preference given to banana and strawberry flavours after the fermented milk was flavoured for taste. The milk can be made from soaked cowpea grains that are then dehulled, blended and strained using a cheese cloth (Murevanhema and Jideani, 2015). The strained milk is pasteurized, culture added and incubated to obtain a fermented cowpea milk product (Oyarekua, 2011).

2.10 Effects of lactic acid fermentation on cowpea milk

Cowpeas are important sources of macronutrients, micronutrients and antioxidant compounds with a great potential for human and animal nutrition (Ojinnaka *et al.,*

2013). However, they contain several anti-nutrients such as phytates, which limit their consumption and affect the digestibility and bioavailability of nutrients (Awika *et al.,* 2011).

Fermentation is seen as one of the oldest and most economical methods of food production and preservation (Indarti *et al.,* 2011). Several experiments have demonstrated that fermentation of legumes enhances their nutritive value and antioxidant properties; reduces some anti-nutritional endogenous compounds such as phytic acid, and exerts beneficial effects on protein digestibility and biological value of legumes (Nkhata *et al.,* 2018).

Lactic acid bacteria fermentation and controlled microbial fermentation of cowpea milk leads to a decrease in the final pH and an increase in the titratable acidity (Ferreira *et al.,* 2019; Kapravelou *et al.,* 2014; Zartl *et al.,* 2018). It leads to increase in crude protein, total ash, calcium, potassium and phosphorus in the fermented cowpea milk (Oyarekua, 2011). The increase in protein is associated with the increase in the biomass brought about by the fermenting microorganisms (Kapravelou *et al.,* 2020; Afoakwa *et al.,* 2010). However, fermentation leads to decrease in lipids, crude fibre and moisture (Kasangi *et al.,* 2010). Ojokoh *et al.,* (2013) argued that reduction in lipids after fermentation could be attributed to fermenting microorganisms utilizing them to yield energy. Reduction in crude fibre and moisture could be attributed to loss of dry matter during fermentation (Ojokoh *et al.,* 2013). Fermentation of cowpea milk leads to increase in vitamin levels, especially, riboflavin, niacin and thiamine (Anino *et al.,* 2019). It also leads to increase in amino acids in the fermented product (Alain *et al.,* 2013). Fermentation causes reduction in levels of anti-nutritional factors such as tannins, saponins, lectins and trypsin inhibitors (Starzyńska–Janiszewska and Stodolak, 2011). The levels of raffinose and stachyose, which are the major flatulence factors in cowpea have also been reported to reduce in fermented cowpea milk (Oyarekua, 2011).

2.11 Health benefits of probiotics in fermented foods

2.11.1 Antimicrobial properties

Species of LAB isolated from fermented milk products produce bacteriocin and nisin that have antimicrobial properties against pathogenic bacteria (Khan *et al.,* 2010; Gaggia *et al.,* 2011; Jiang *et al.,* 2012; Grosu-tudor and Zamfir, 2013). Strains of LAB such as *L. lactis* BH5 and *L. citreum* GJ7 isolated from *kimchi* (Korean salted and fermented vegetable) produce bacteriocins (Jung *et al.,* 2014). Bacteriocins show a strong antimicrobial activity against a wide range of pathogenic microorganisms such as *Listeria monocytogenes*, *Staphylococcus aureus*, *E. coli* and *Salmonella typhimurium* (Abubakr *et al.,* 2012). Mitra *et al.,* (2010) reported that *Lactococcus lactis* isolated from *dahi*, Indian curd, produces nisin Z that inhibits *L. monocytogenes* and *S. aureus.* Lesan *et al.*, (2017) reported that *Lactobacillus acidophilus* and *Bifidobacterium* spp. have antifungal properties that enable them fight harmful microbes and maintain healthy gums and teeth.

2.11.2 Antioxidant activity

Ping et al., (2012) reported that many Asian fermented soybean foods have antioxidant properties, e.g. *natto*, a *Bacillus*-fermented soybean food of Japan and *douchi*, a fermented soybean food of China (Wang *et al.,* 2007). Antioxidant activity of LAB has also been observed in *kimchi* (Park *et al.,* 2011) and yoghurt (Farvin *et al.,* 2010). The antioxidant activities in the fermented foods include 1,1-diphenyl-2-picryl hydrazyl (DPPH) radical scavenging activity and 3-ethylbenzo-thiazoline-6-sulfonic acid (ABTS) radical scavenging activity (Abubakr *et al.,* 2012; Liu and Pan, 2010).

2.11.3 Peptide and enzyme production

Bioactive peptides have immunomodulatory functions and are formed during food fermentation by proteolytic microorganisms (Qian *et al.,* 2011). They also have antithrombic activities (Anand *et al.,* 2014) and antihypertensive properties (Phelan and Kerins, 2011). Qian *et al.,* (2011) reported that species of *Bacillus* are involved in enzymatic hydrolysis of proteins producing peptides and amino acids.

During food fermentation microorganisms produce enzymes such as amylase, cellulase and catalase to break down complex compounds to simple bio-molecules (Chettri and Tamang, 2014). Several bacteria isolated from fermented foods such as *Bacillus subtilis, B. amyloliquefaciens* (Chang *et al.,* 2012; Zeng *et al.,* 2013) , *Vagococcus carniphilus, V. lutrae, Entrococcus faecalis, E. faecium* (Anand *et al.,* 2014) and *Virgibacillus halodenitrificans* SKI-1-3-7 isolated from fish sauce fermentation (Montriwong *et al.,* 2012), produce fibrinolytic enzymes that directly degrades fibrinogen or fibrin which act as thrombin inhibitor.

2.11.4 Degradation of anti-nutritive compounds

Some microorganisms present in fermented foods degrade anti-nutritive substances such as phytates (Tamang, 2015). Cyanogenic glycoside linamarin and lotaustralin in cassava tubers are detoxified by species of *Leuconostoc, Lactobacillus* and *Streptococcus* during traditional fermentation of *fufu* and *gari* in West Africa (Lambri *et al.,* 2013; Bamidele *et al.,* 2015; Omolara, 2014). Niyibituronsa *et al.,* (2019) reported that fermentation of cowpea milk led to decrease in flatulence-causing indigestible oligosaccharides, verbascose by 79.7%, stachyose by 5.9% and raffinose by 12.8%.

2.11.5 Synthesis of nutrients

Food fermentation leads to increase in vitamins, essential amino acids and other bioactive compounds (Thapa and Tamang, 2015). Anino *et al.,* (2019) reported that vitamin B_{12} is synthesized by non-pathogenic strains of *Klebsiella pneumonie* and *Citrobacter freundii*. Riboflavin and niacin contents are increased in many *Bacillus*-fermented Asian foods such as *tempe, kimchi* and *idli* (Choi *et al.,* 2007). Riboflavin and folic acid were found to be synthesized in *kimchi* by *L. mesenteroides* and *L. sakei* (Young *et al.,* 2013). Free amino acids are found to increase in fermented soybean foods (Dajanta *et al.,* 2011).

2.11.6 Prevention from cancer and gastrointestinal disorders

Some LAB-fermented foods have antimutagenic and anticarcinogenic activities as reported by Lee, (2004). Sauerkraut, fermented German vegetable, contains *s*-methylmethionine which reduces risk of tumor growth in the human stomach (Sivamaruthi *et al.,* 2020). Consumption of fermented milk products containing live cells of *L. acidophilus* decreases levels of enzymes that catalyze conversion of procarcinogens to carcinogens, such as β-glucuronidase, azoreductase and nitroreductase (Macouzet *et al.,* 2009). Cancer preventive potential of *L. plantarum* has been studied by Kwak *et al.,* (2014) in *kimchi* and *dahi.* Kilara and Chandan, (2013) reported that consumption of yoghurt can reduce chances of bladder, colon and cervical cancers considerably.

Some strains of Lactobacillus have been shown to improve the inflammatory bowel disease and ulcerative colitis (Orel and Trop, 2014). *L. rhamnosus* GG is effective in the treatment of acute diarrhea (Thapa and Tamang, 2015) and has immunomodulatory capacity (Granier *et al.,* 2013).

2.11.7Alleviation of lactose malabsorption

Lactose malabsorption is a condition in which lactose, the major carbohydrate in milk, is not completely digested into glucose and galactose due to lack of β-D-galactosidase (Nkhata *et al.,* 2018). Figueroa-gonzález *et al.,* (2011) reported that *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* used in production of yoghurt contain substantial quantities of β-D-galactosidase which helps in improving lactose malabsorption in lactose intolerant people. Tuso *et al.,* (2013) demonstrated that consumption of fresh yoghurt, with live microorganisms, leads to better lactose digestion and absorption than consumption of pasteurized milk products. *Kefir* was found to minimize symptoms of lactose intolerance by providing extra source of β-galactosidase in a study done by (Usui *et al.,* 2018).

2.11.8 Anti-allergic reactions

Lactobacillus species isolated from *kimchi* modulate Th1/Th2 balance by producing a large amount of IL-12 and IFN-γ alleviating atopic dermatitis and food allergy (Won *et al.,* 2011). Fermented fish oil, which is rich in omega 3 polyunsaturated fatty acids can reduce sensitization of allergy (Han *et al.,* 2012). *Lactobacillus kefiranofaciens* M1 isolated from *kefir* grains has been demonstrated to have anti-allergic effect (Hong *et al.,* 2010). Probiotics modify structures of antigens, reducing their immunogenicity, generation of pro-inflammatory cytokines and intestinal permeability (Figueroa-gonzález *et al.,* 2011). *Lactobacillus rhamnosus* has been shown to alleviate food allergy symptoms and reduce risk of development of allergic diseases (Kechagia *et al.,* 2013; Goyal *et al.,* 2012; Mamedov *et al.,* 2013).

2.11.9 Reduction of blood pressure

Probiotics improve total cholesterol and low-density lipoprotein cholesterol levels (Kechagia *et al.,* 2013; Patel *et al.,* 2010; Guo *et al.,* 2011). Studies have shown that *Lactobacillus rhamnosus, L. acidophilus, Bifidobacterium* spp., *S. thermophilus, L. delbruicki* ssp. *bulgaricus* and *L. kefiri,* have blood pressure reducing effect (Kechagia *et al.,* 2013; Rerksuppaphol and Rerksuppaphol, 2015; Ekhlasi *et al.,* 2017). They cut down cholesterol levels by inhibiting devono synthesis and reducing intestinal absorption of dietary cholesterol by binding, assimilation or degradation (Kechagia *et al.,* 2013). *Bifidobacterium animalis* has been shown to deconjugate cholesterol to the bile acids therefore reducing the aggregate body pool (Bordoni *et al.,* 2013; Lee *et al.,* 2017; Margolles and Sanchez, 2012).

2.11.10 Prevention of urogenital infections (Bacterial vaginitis)

Probiotic capsules of *L. rhamnosus, L. crispatus, L. gasseri, L. vaginalis, L. acidophilus, L. reuteri* and *S. thermophilus* have shown to be effective for preventing recurrent bacterial vaginosis (Shamshu *et al.,* 2017; Morelli, 2014; Siroli *et al.,* 2017). It is reported that *Lactobacilli* produce biofilms that cover the urogenital cells and often causes protection against uropathogens (Abatenh *et al.,* 2018; Kechagia *et al.,* 2013; Uriot *et al.,* 2017).

2.11.11 Other benefits of probiotics

Lactobacillus rhamnosus GG has shown to prevent antibiotic- or healthcare-associated diarrhea (AAD) and improve the symptoms of acute gastroenteritis (AGE) among children in Europe (Szajewska and Hojsak, 2020; Sharif *et al.,* 2017). *Bifidobacterium animalis* BB-12 help in obesity management by slowing down the conversion of excess glucose to glycogen and improves epithelial integrity by rebalancing dysbiotic state induced by an obesogenic diet (Uusitupa *et al.,* 2020; Barengolts *et al.,* 2019). However, there was minimum evidence that it can treat respiratory infections (Szajewska and Hojsak, 2020). Morelli, (2014) reported that risk of developing common cold after treatment with *Lactobacillus bulgaricus* was 2.6 times reduced in the treated group than in the placebo group. He also observed a significant increase in natural killer cell activity in the treated group. Merenstein *et al.,* (2015) reported that *Bifidobacterium* ssp. helps in prevention of inflammatory bowel disease. Mirghafourvand *et al.,* (2016) reported that consumption of yoghurt containing *Bifidobacterium* spp. and *Lactobacillus* spp. helped in alleviating constipation symptoms such as straining, anorectal obstruction, consistency and color of stool.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Materials

Five kilograms of M66 variety cowpeas were purchased from Muthurwa market in Nairobi, Kenya. The cowpeas were packaged in an eco-friendly recyclable paper bag and transported to the laboratory, where they were kept in a lockable cabinet at room temperature (20-25˚C) awaiting experiment.

Three probiotic cultures namely ABT-5 (Lactobacillus acidophilus La-5 + Bifidobacterium animalis Bp-12 + Streptococcus thermophilus), YF-L 903 (Streptococcus thermophilus + Lactobacillus bulgaricus subs. debulgaricus) and Yoba Fiti (Lactobacillus rhamnosus GR-1 + Streptococcus thermophilus), were obtained from ProLab Limited, Nairobi, Kenya and used for the fermentation of cowpea milk. The starter cultures were purchased in frozen form in sachets and stored according to manufacturer's instructions. They were stored at -4˚C in a freezer prior to use except Yoba fiti that was kept at room temperature (20 -25˚C).

The analytical and chromatographic grade chemicals and solvents used in this analysis were all used. Standards for oligosaccharides (stachyose, raffinose, and verbascose) were purchased from Sigma-Aldrich (United Kingdom). Sigma-Aldrich (Germany) provided short chain fatty acid (butyric, valeric, and isovaleric) standards, while Fluka Analytical provided acetic acid standards (Germany).

3.2 Experimental design

The study adopted the experimental study design in order to achieve the objectives. The study involved four (4) samples, three treated with different starter cultures and one without to act as the control. The proximate experiments were performed in triplicate and the average values used for the purpose of the study data. The proximate experiment had 6 factors: moisture content, crude protein, crude fat, crude fiber, ash and carbohydrate. Mineral analysis was done in triplicate for zinc, calcium, iron and magnesium, and average values used as data.

Lactic acid bacteria growth and survival was determined during storage period of 28 days at 4˚C to determine suitability of the cultures in fermentation of cowpea milk. Oligosaccharide and short fatty acid content were determined by high performance liquid chromatography (HPLC) and gas chromatography (GC). Oligosaccharide analysis had three factors: stachyose, raffinose and verbascose. Short chain fatty acid determination had four factors: propionic acid, butyric acid, valeric acid and isovaleric acid and. The experiments were done in triplicate and average values used for study data. The unfermented cowpea milk (with no culture added) was the control in all the experiments.

The consumer acceptability was done by use of a complete randomized design (CRD) approach. Fermented cowpea milk and the control samples were flavoured using strawberry and vanilla flavours. Randomized number codes were assigned to the fermented flavoured cowpea milk samples for blinding purposes. Sample arrangement on trays was randomized for each panelist. The evaluators came to the evaluation room at random to evaluate the samples for acceptability purposes ensuring the evaluation process was randomized.

3.3 Methods

3.3.1 Preparation of cowpea milk

Cowpeas (400g) were soaked for 12 hours at room temperature in 4 liters of distilled water (20°C-25°C). The steeping water was decanted, and the cowpeas were washed in tap water before being fully dehulled by hand. Water was used to blend (Von Hotpoint Blender, HB241CW) for 3 minutes at high speed in a 1:10 (w/v) ratio, followed by filtration through a cheese cloth (Figure 3.1). This blending ratio resulted into 2 liters of cowpea milk after filtration. The resulting filtrate was pasteurized at 70˚ C for 20 minutes (Anino *et al.,* 2019). Heating was done while stirring frequently to prevent the content from sticking on the equipment. The milk was allowed to cool to an incubation temperature of 45˚C and then divided into four portions.

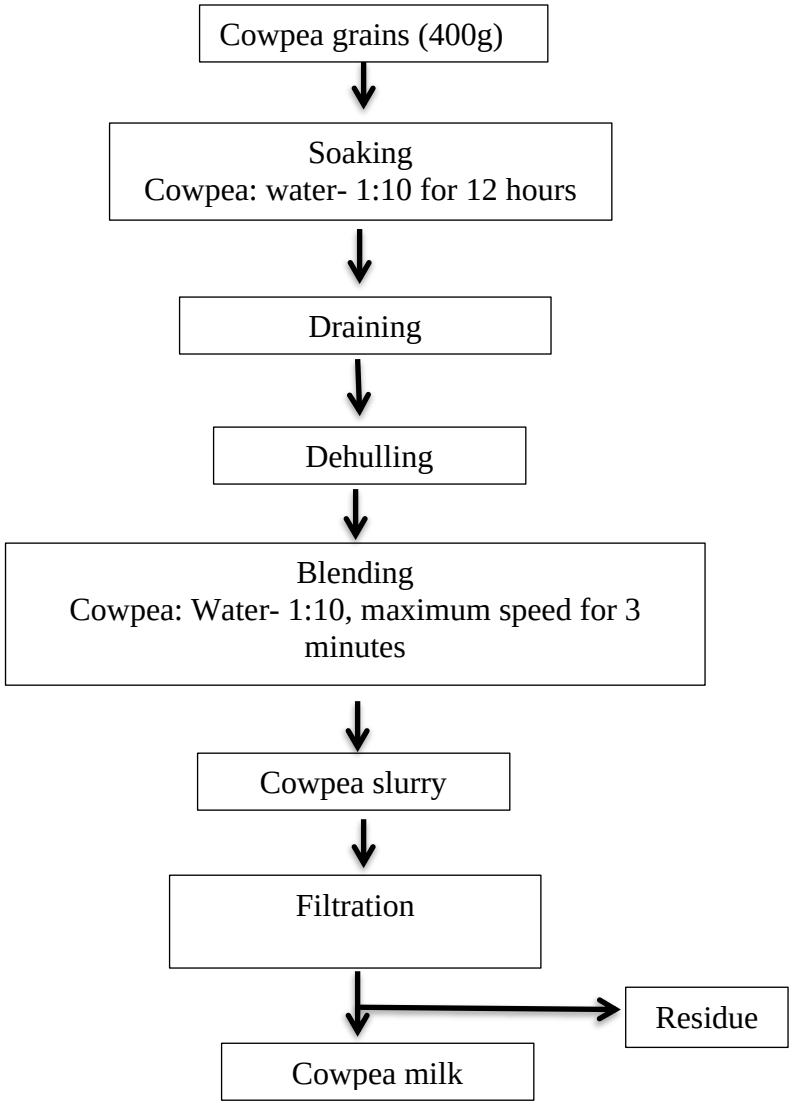

Figure 3.1: Cowpea milk preparation flow diagram. Source: modified from (Anino *et al.*, 2019)

3.3.2 Preparation of fermented cowpea milk

Having divided the cowpea milk into four portions of 0.5 liter each, every culture was added to a portion, leaving one portion without a culture. This was the control during fermentation. A 1.0g of each culture was poured onto a teaspoon and added directly to the cowpea milk, in 2-liter capacity stainless steel containers according to the manufacturer's instructions, while stirring according to the manufacturer's instructions. The portions of the cowpea milk were then incubated in an electric incubator; model IB-09 (Mitamura Riken Kogyo MRK Inc. Japan) at 45°C

anaerobically for 14 hours. Three independent fermentations were done to ensure replications.

3.3.3 pH measurement of fermented cowpea milk

The pH of the incubated milk was monitored during fermentation after every 2 hours in order to study the pH changes and to determine the optimum fermentation time for the cultures under investigation. The pH was determined at room temperature (20˚C-25℃) using a digital pH meter (HANNA, H18519N). The pH meter was calibrated with buffer standards of pH 4.0 and pH 7.0 before use. 50 ml of fermented cowpea milk for each sample was placed in a beaker and pH value recorded and sample discarded. The desired final pH was 4.5. The probe was rinsed thoroughly with distilled water before use on another sample (Niyibituronsa *et al.,* 2019). Independent measurements were taken in triplicate and average values calculated.

3.3.4 Lactic Acid Bacteria survival determination during storage of fermented cowpea milk

Changes in the bacteria counts during storage were determined by modified AOAC (2000) method 996.02. Lactobacillus MRS Agar and M17 Agar Base were used for Yoba fiti and ABT-5, respectively. YF-L 903 needed modification of the MRS Agar with 0.05% L. cysteine. For probiotic counts, 0.3 ml of fusidic acid was added into 500 ml MRS agar and mixed thoroughly before pouring into plates. The plates were then incubated at 30˚C in an incubator for 24 hours. Survival of probiotic microorganisms in the fermented cowpea milk was monitored for a period of 28 days and plating was done weekly.

Plates with ≤ 300 colonies were counted and the number of viable cell concentrations expressed in colony forming units (CFU)/ml of the fermented milk using the formula from International Dairy Federation method (IDF, 2018) as follows: Log C = $\sum x/n_1+(0.1n_2)$ x d

Where C = colony forming units (CFU/ml)

x = number of colonies in plates

n = reciprocal of dilution factor

d = dilution factor used

3.3.5 Proximate analyses of fermented cowpea milk

3.3.5.1 Moisture content determination in fermented cowpea milk

Moisture content was determined using oven drying procedure (AOAC, 2000), Method 925.09. A 10 ml of the sample was weighed into a moisture dish and transferred to a hot-air-oven previously heated to 105˚C for 3 hours. The final weight of the sample was taken after the drying period and cooling done in a desiccator. The moisture content of the sample was expressed as a percentage of the initial weight of the sample using the following formula:

$$\%\text{moisture content} = \frac{\text{weight of wet sample} - \text{weight of dry sample}}{\text{weight of wet sample}} \times 100$$

3.3.5.2 Crude protein determination in fermented cowpea milk

Crude protein was determined by the microKjeldahl method (AOAC, 2000) method 979.09. A 10 ml of sample was weighed into a digestion flask together with a catalyst composed of 5 g of K_2SO_4 and 0.5 g $CuSO_4$ and 15 ml of concentrated H_2SO_4. The mixture was digested in a heating block at 400 ˚C for 90 minutes until the contents became blue. This signified the end of the digestion process. The digest was cooled, transferred to a 100ml volumetric flask and topped up to the mark with distilled water. A blank digestion with the catalyst and acid was also done. A 10 ml of diluted digest was transferred into the distilling flask and washed with about 2 ml distilled water, 15 ml of 40% NaOH was added and this was also washed with about 2 ml distilled water. Distillation was done to a volume of about 60 ml distillate. The distillate was titrated using 0.02N HCl to an orange colour which signified the end point (Inobeme *et al.*, 2014).

The % nitrogen was calculated using the formula:

$$\% \text{ Nitrogen} = (V1 - V2) \times N \times f \times 0.014 \times 100/V \times 100/S$$

Where: V1 = titer for sample (ml); V2 = titer for blank (ml)

N = Normality of standard HCl solution (0.02)

f = factor of standard HCl solution

V = volume of diluted digest taken for distillation (10 ml)

S = weight of sample taken (g)

The crude protein was attained by multiplying the % nitrogen by a factor (6.25).

3.3.5.3 Crude fat determination in fermented cowpea milk

Total lipids were determined by modified Bligh and Dyer method (Difo *et al.*, 2014). A 10 ml of sample was weighed into 100 ml glass-stoppered centrifuge tube and denatured over boiling water (100^{o}C) for 3 minutes. A 4ml of water and 15 ml of 2:1(v/v) methanol-chloroform mixture were added. The mixture was shaken thoroughly and left at room temperature (20-25°C) for 2 hours on a shaker. All the contents were transferred into a 100 ml centrifuge tubes and centrifuged (Centrifuge model H-2000C Shimadzu Corp., Kyoto, Japan) at 3000 rpm for 10 minutes and the supernatant decanted into 50 ml centrifuge tube. Residue was re-suspended in 9.5ml of Methanol-chloroform-water (2:1:0.08 v/v) with continuous shaking to homogenize and then centrifuged. The supernatant was combined with the first extract and 7.5 ml each of chloroform and water added, shaken and centrifuged. The chloroform phase was withdrawn using a pipette and put into pre-weighed pear-shaped 50ml flask. Evaporation to dryness was done using a vacuum rotary evaporator (model RE 100, Bibby Co. Ltd) at low temperature.

The lipid content was calculated using the following formula:

$$\% \text{ lipids} = \frac{\text{weight of lipid}}{\text{weight of sample}} \times 100$$

3.5.4 Crude ash content determination in fermented cowpea milk

Ash content was determined using AOAC (2000) method 923.03. 10 g of samples were measured into crucibles and incinerated at 500-550˚C for 6 hours in an electric muffle furnace (model KL-420, Advantec) to constant weight. The samples were then cooled in a desiccator and weighed. Crude ash content was determined using the following formula:

$$\% \text{ ash} = \frac{\text{weight of ash}}{\text{weight of sample}} \times 100$$

3.3.5.5 Total carbohydrate content determination in fermented cowpea milk

Carbohydrate content was determined by subtracting the sum of weights of protein, lipid, ash and moisture from the total wet matter basis (Inobeme, *et al*., 2014).

% Carbohydrate = 100 – {Protein (%) + Lipid (%) + Ash (%) + Moisture (%)}

3.3.5.6 Crude fiber determination in fermented cowpea milk

Crude fibre was determined by the AOAC, (2000) method 978.10. 10 g of sample was transferred into a 750 ml Erlenmeyer flask and 0.5 g of asbestos added. 200 ml of boiling 1.25% H_2SO_4 was also added and the flask immediately set on a hot plate. The mixture was boiled for 1 hour thereafter filtered with Whatman filter paper No. 1 in a funnel and subsequently washed with boiling water.

The sample was washed back into the flask with boiling 1.25% NaOH solution. It was then filtered and thoroughly washed with boiling water. The residue was transferred into a clean crucible with a spatula and the remaining particles washed off with 15 ml ethanol into the crucible. The crucible with its content was taken into a furnace at 600 ˚C for 3 hours, cooled and reweighed. The loss in weight gave the crude fibre content and was expressed as a percentage of the initial weight of the sample.

$$\% \text{ DF} = \frac{\{(\text{wt of cc + sample beore ignition}) - (wt\ of\ cc{+}ash)\}}{\text{weight of fresh sample}} \times 100$$

Where, cc = crucible

3.3.5.7 Determination of minerals in fermented cowpea milk

Atomic absorption spectroscopy (AAS AA-7000, Shimadzu Cop. Japan) was used to determine the minerals; iron, zinc, calcium and magnesium. The four minerals were chosen for analysis because of their public health importance as minerals with higher prevalences of deficiency. Mineral analysis was done from the ash obtained in proximate analysis.

5 ml aliquot of sample in a 100 ml volumetric flask and 50 ml of 24% (w/v) tricarboxylic acid (TCA) was mixed. The samples were shaken for 30 minutes at 5 minutes intervals and filtered using Whatman filter paper No. 1. To a 5 ml aliquot of the filtrate transferred to a volumetric flask, 1 ml of 5% (w/v) lanthanum solution were added and made to volume with distilled water. A mixed standard containing 5.0 mg/l Fe, 5.0 mg/l Ca, 0.6 mg/l Mg, 1.6 mg/l Zn, 500 mg/l La and 1.2% (w/v) TCA were prepared. All determinations were made versus a reagent blank containing 500 mg/l La and 1.2% TCA (Anino *et al.*, 2019). The analyses were performed in triplicates.

3.3.6 Preparation of standard solutions for determination of short chain fatty acids and oligosaccharides in fermented cowpea milk

3.3.6.1 Preparation of oligosaccharide standards

For raffinose and stachyose standards, 10mg/ml stock solutions were prepared. 0.1g of each standard was measured into 10 ml volumetric flask and volume made up to the mark using 50% acetonitrile solution. Other concentrations of 8, 6, 4 and 2 mg/ml were prepared using the formulae $C_1V_1=C_2V_2$ (Niyibituronsa *et al.*, 2019).

Where C_1 = concentration of sample

C_2 = concentration of blank

V_1 = volume of titer for sample

V_2 = volume of titer for blank

For verbascose standard, 2mg/ml stock solution was prepared. 5mg of the standard was measured into 2.5ml flask and volume made up to the mark with 50% acetonitrile solution.

3.3.6.2 Preparation of short chain fatty acids standards

A 1% stock solution was prepared for butyric acid, isovaleric acid, valeric acid and propionic acid standards. 1ml of each standard was measured into 100 ml volumetric flask and distilled water added to the mark. For the mixed stock solution, 1ml of each standard was measured into one 100ml volumetric flask and distilled water added to the mark.

3.3.7 Determination of oligosaccharides in fermented cowpea milk

Flatulence causing oligosaccharides (stachyose, raffinose and verbascose) were extracted by the method of Borejszo and Khan, (1992) with few modifications. 5 g of sample was measured and 20 ml of ethanol added. It was then heated in a reflux apparatus (model SF-6, Sanshin Industrial Co. Ltd) for 1 hour and then allowed to cool. The samples were then concentrated using Rotary Vacuum Evaporator (model RE 100, Bibby Co. Ltd) by evaporating off the volatile solvents and then filtered through 0.45µm pore-size membrane syringe filter. A 2µl of each sample and standards were injected into high performance liquid chromatography (HPLC) machine model LC-20 AD/T (Shimadzu Corporation, Kyoto Japan), separately.

Analysis of oligosaccharides was done through High Performance Liquid Chromatograph unit model LC-20 AD/T (Shimadzu Corporation, Kyoto Japan), coupled with refractive index detector model RID-10A, degassing unit DGU-$20A_{5R,}$ column oven CTO-$10AS_{VP}$, communication bus module CBM-20A and auto sampler SIL-20A HT. Column used: Hypersil GOLD Amino column (4.6 ×160 mm). The mobile phase consisted of 65% acetonitrile and 35% distilled water and was maintained at a flow rate of 1 ml/min isocratically; injection volume: 20µl. The peak

areas of standard solutions were identified to determine the oligosaccharide content in the fermented cowpea milk samples.

3.3.8 Determination of short chain fatty acids in fermented cowpea milk

Short chain fatty acid content of cowpea milk fermented with the three starter cultures was determined and the cowpea milk with no culture added served as the control. Extraction of organic acids from cowpea milk samples was carried out according to the procedure by Shukla *et al.* (2010) with few modifications. 10g of each sample was centrifuged (Centrifuge model H-2000C Shimadzu Corp., Kyoto, Japan) at 10,000 rpm for 30 minutes and then filtered with Whatman filter paper No. 1. Subsequently, 1.2 µl of 2% H_2SO_4 was added to 2.1 ml of the filtrate and filtered through 0.45µm pore-size membrane syringe filter. Finally, 2µl of each sample and standards were injected into Gas Chromatography (GC) machine (Model GC-14B, Shimadzu Corporation, Japan) separately.

The Gas Chromatography analysis conditions were as follows: flame ionization detector (FID) and Shimadzu C-R6A Chromatopac; column: 10% SUPELCOWAX capillary column (30m × 0.53mm × 0.5µm film thickness); Initial temperature: 150°C, final temperature: 230°C; injection temperature: 220°C, detection temperature: 240°C and carrier gas: Nitrogen (100cm^3); Programme rate: 5°C/min; Range: 10^2; injection volume: 2µl; split ration 10 (0.5 µl); Column initial temperature: 1 minute; Column final temperature: 2 minutes; run time: 19 minutes. SUPELCOWAX capillary column was used because of its high efficiency, polar sample separation compatibility and their ability to maintain low pressure inside them. Run time was set at 19 minutes because of the efficiency of the column used in the analysis.

3.3.9 Sensory evaluation of fermented cowpea milk

The consumer acceptability was done by use of a complete randomized design (CRD) approach. Fifty-four (54) untrained panelists were involved in sensory evaluation after signing consent forms informing them about the samples and to ascertain their personal commitment in participating in the sensory evaluation. Each panelist was provided with samples of fermented cowpea milk of strawberry and vanilla flavours, a carrot

and a glass of water to cleanse their palates before and in between the tasting. Carrot was used because of its balanced taste of sweet, fruity and bitter flavours making it suitable for cleansing palates in between tasting. They rated their degree of liking for appearance, aroma, taste and texture on a nine-point hedonic scale where 1 = like extremely, 5 = neither like nor dislike and 9 = dislike extremely (Fidelis *et al.,* 2014) Cowpea milk fermented with each of the cultures (ABT-5, YF-L 903 and Yoba fiti) were flavoured with strawberry and vanilla flavours.

3.3.10 Data management and analysis

Data was analyzed using Analysis of Variance (ANOVA) with Stata version 12. Mean comparisons for treatments were made using Bonferroni tests. Significance level was set at $P \leq 0.05$.

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 Growth of probiotic bacteria in starter cultures

Fermentation of cowpea milk with probiotic bacteria may produce benefits related to live bacteria in the body and/or beneficial metabolites produced by the bacteria in the food. For a product to be considered probiotic based on live microorganisms, the product should have a high population of the microorganisms, not less than $\log_{10}$ 6.0 cfu/ml to ensure survival of a good number as they travel through the harsh environment in the stomach (Faria *et al.*, 2011). Thus, the populations of bacteria that were attained by cowpea milk fermentation were determined, as well as the changes in populations during storage. Initial inocula ranged from $\log_{10}$ 3.1±0.3 cfu/ml to $\log_{10}$ 4.7±0.5 cfu/ml (Figure 4.1). No culture was added to the control samples.

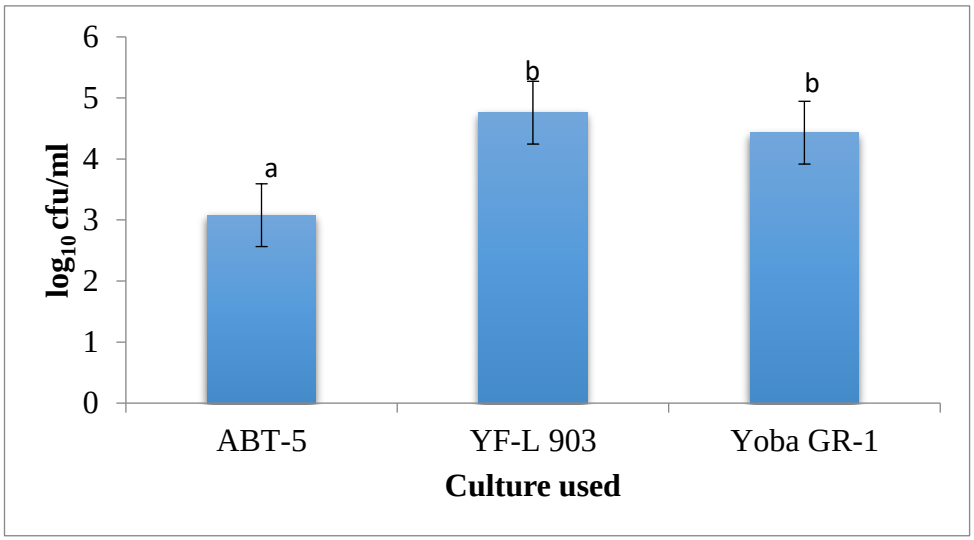

Figure 4.1: Initial inoculation colony forming uinit (cfu) counts for cowpea milk inoculated with probiotic cultures.

YF-L 903 (*Streptococcus thermophilus* and *Lactobacillus* subs *bulgaricus*), ABT-5 (*Lactobacillus acidophilus* La-5, *Bifidobacterium animalis* Bp-12 and *Streptococcus thermophilus*) and Yoba fiti (*Lactobacillus rhamnosus* GR-1 and *Streptococcus thermophilus)*

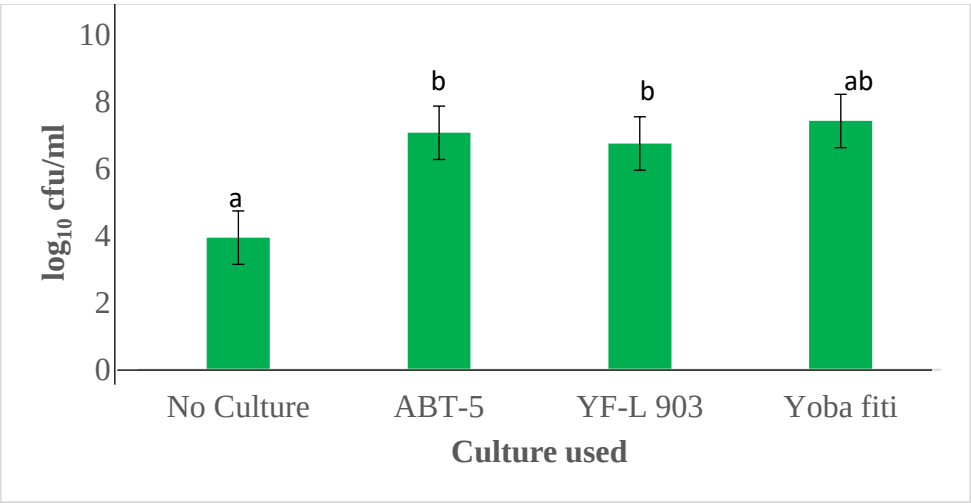

Figure 4.2: Probiotic bacteria counts after 14 hours fermentation at 45˚C. Values are averages of three independent fermentations and bars are standard deviations of averages.

No culture = unfermented cowpea milk; YF-L 903 (*Streptococcus thermophilus* and *Lactobacillus* subs *bulgaricus*), ABT-5 (*Lactobacillus acidophilus* La-5, *Bifidobacterium animalis* Bp-12 and *Streptococcus thermophilus*) and Yoba fiti (*Lactobacillus rhamnosus* GR-1 and *Streptococcus thermophilus)*

After fermentation, the bacterial counts ranged from $\log_{10}$ 3.9±0.7 cfu/ml to $\log_{10}$ 7.4±0.8 cfu/ml (Figure 4.2). Cowpea milk fermented with the three cultures, ABT-5, YF-L 903 and Yoba fiti, gave colony counts greater than $\log_{10}$ 6.0 cfu/ml (Figure 4.2), the recommended bacterial population for a healthy probiotic product (Anino *et al.,* 2019; Niyibituronsa *et al.,* 2019). Mani-López and Palou, (2014) reported LAB counts in cow's milk fermented with YF-L 903 and ABT-5 cultures to be within the range of $\log_{10}$ 7.2 cfu/ml to $\log_{10}$ 9.2 cfu/ml. Results of the present study show that it is possible to attain final populations of these microorganisms in cowpea milk that are comparable to cow's milk. The increase in log cfu was highest for ABT-5 (~ 4 log units) followed by Yoba fiti and least in YF-L 903 (~ 2 log units), and these values were within the range recently reported for the growth of various lactic acid bacteria in soymilk (Niyibituronsa *et al.,* 2019). The control culture was also found to have considerable growth of lactic acid bacteria, attaining a final count of $\log_{10}$ 4.0 cfu/ml when incubated at the fermentation temperature for 24 hours. The species involved were however not characterized.

4.2 Lactic Acid Bacteria survival determination during storage

Survival rate of the bacteria was monitored over 28 days under refrigeration (4˚C). There was an initial increase in colony forming units in the first two weeks of storage, followed by a slight decline thereafter (Figure 4.3). Decline in population of probiotic bacteria could be attributed to reduced metabolic activity of the bacteria because of the long and cold storage temperatures and also post acidification. Bacterial population in the control sample with no culture increased from $\log_{10}$ 3.94 cfu/ml to $\log_{10}$ 6.31 cfu/ml after 14 days of storage (Figure 4.3). Thereafter the number reduced to $\log_{10}$ 4.11 cfu/ml on the 28th day of storage. In sample with Yoba fiti (containing *L rhamnosus* GR-1 and *S. thermophilus*), there was an increase in growth of LAB from $\log_{10}$ 7.43 cfu/ml to Log_{10} 8.05 cfu/ml after 14 days of storage. On the 28th day, the number decreased to $\log_{10}$ 7.42 cfu/ml. This is similar to the previously reported survival of *Lactobacillus rhamnosus* GG in various leguminous porridges (Petruláková and Valík, 2015).

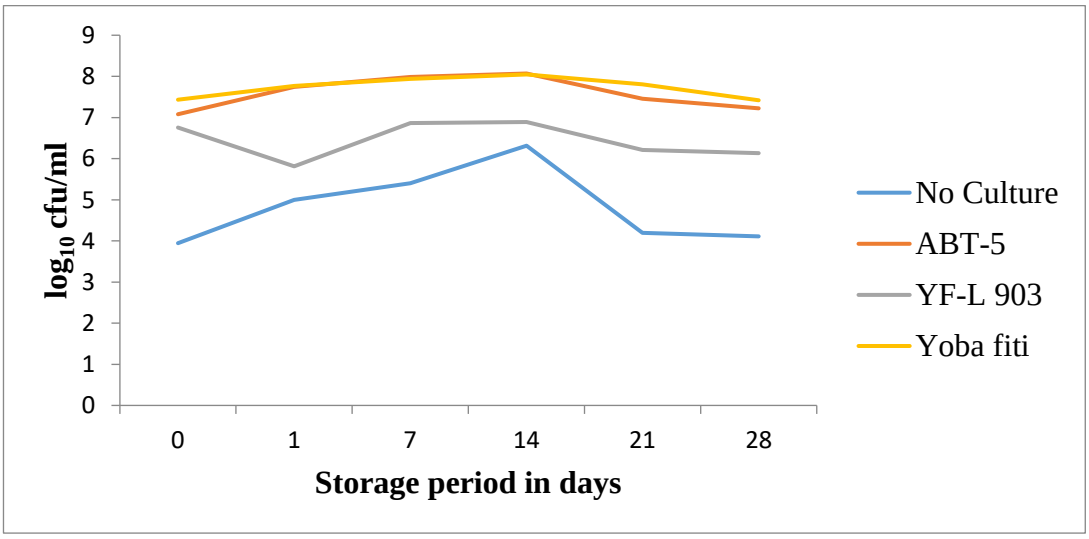

Figure 4.3: Shows survival of lactic acid bacteria during storage (4˚C) of the fermented cowpea milk. Values = mean of triplicates (log10 cfu/ml).

*No culture = unfermented cowpea milk; YF-L 903 (*Streptococcus thermophilus* and *Lactobacillus* subs *bulgaricus*), ABT-5 (*Lactobacillus acidophilus* La-5, *Bifidobacterium animalis* Bp-12 and *Streptococcus thermophilus*) and Yoba fiti (*Lactobacillus rhamnosus* GR-1 and *Streptococcus thermophilus)*

LAB growth for sample with YF-L 903 increased from $\log_{10}$ 6.76 cfu/ml to $\log_{10}$ 6.89 cfu/ml after 14 days of storage and a slight decrease in growth ($\log_{10}$ 6.13 cfu/ml) was observed on the 28th day. ABT-5 culture registered LAB growth of $\log_{10}$ 8.07 cfu/ml after 14 days of storage and a slight decrease in growth ($\log_{10}$ 7.22 cfu/ml) after 28 days of storage (Figure 4.3).

For consumers to have confidence in a product considered probiotic, the product must demonstrate survival of the probiotic bacteria during storage (Kort *et al.,* 2015). Suitable probiotic strains for product manufacture are those that can survive and maintain their stability during food production and storage (Petruláková and Valík, 2015).

In this present study, viable cells of probiotic bacteria remained at the levels greater than $\log_{10}$ 6 cfu/ml, which are the recommended levels for a probiotic product (Uusitupa *et al.,* 2020) during 28 day storage at 4˚C. Several studies have reported reduced number of viable cells of probiotic bacteria during storage. In yoghurt fermented with *L. acidophilus*, a loss of $\log_{10}$ 2 cfu/ml was observed after 28 days of storage (Ejtahed *et al.,* 2011; Anino *et al.,* 2019). In cow milk fermented with *L. acidophilus*, a loss of $\log_{10}$ 1.5 cfu/ml was observed after 28 days storage at 4˚C (Mani-López *et al.,* 2014) and $\log_{10}$ 2 cfu/ml loss in plain yoghurt fermented with *L. reuteri.*

Yazdi *et al.,* (2017) reported that decline in probiotic bacteria population could be attributed to post acidification. In this study also, the decline in viability was noted to be strain dependent and culture type. The bacteria that grew in the control sample underwent a sharper decline after the 14th day of storage (Figure 4.3).

4.3 Change in pH over time during cowpea milk fermentation

During incubation of cowpea milk (control) or cowpea milk inoculated with the different starter cultures, pH was monitored over a 14-hour period as an indicator of the progress of fermentation. As illustrated in Figure 4.4, the control sample exhibited some albeit minimal decrease in pH, consistent with a small extent of natural fermentation. On the other hand, samples fermented with the three starter cultures

underwent a rapid pH decline from pH 7 to pH 5 within the first 6 hours, followed by a slower decrease to between pH 4.5 and 4.8.

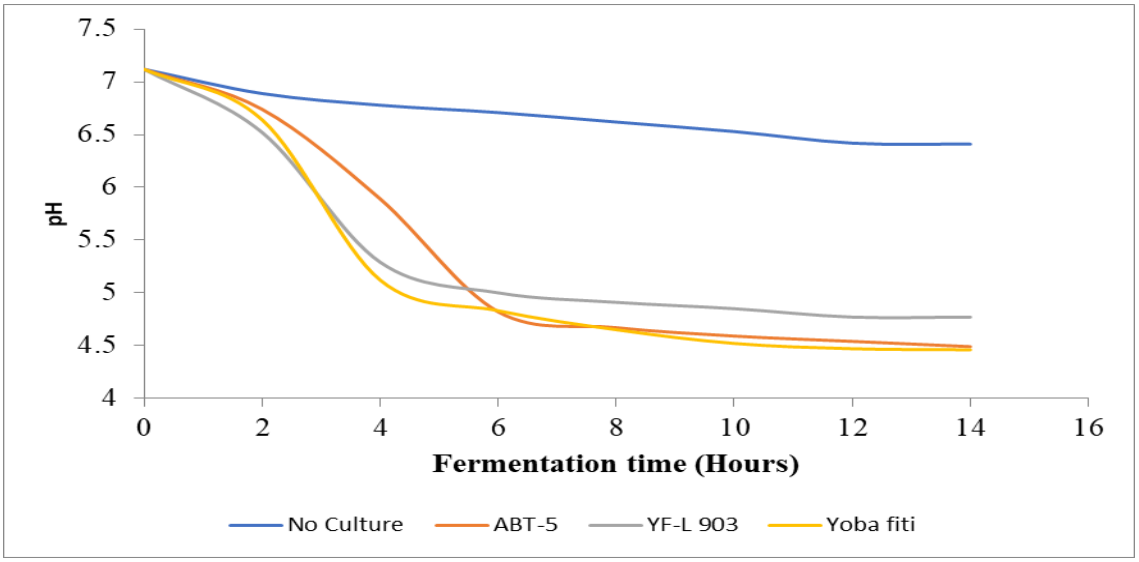

Figure 4.4: Presents changes in pH over time during fermentation of cowpea milk.

Values are means of three independent fermentations.

* YF-L 903 (*Streptococcus thermophilus* and *Lactobacillus* subs bulgaricus), ABT-5 (*Lactobacillus acidophilus* La-5, *Bifidobacterium animalis* Bp-12 and Streptococcus *thermophilus*) and Yoba fiti (*Lactobacillus rhamnosus* GR-1 and *Streptococcus thermophilus),* No culture = unfermented cowpea milk

There was significant decrease in pH during fermentation for all samples (Figure 4.4). This is due to acidification by the fermenting bacteria. Lactic acid bacteria have been reported to degrade carbohydrates resulting in acidification through formation of lactic acid (Nwabugo *et al.,* 2016; Martínez *et al.,* 2016). Martínez *et al.,* (2016) reported reduction in pH in soymilk during fermentation with Lactic Acid Bacteria from 6.5 to 4.5 after 12 hours. Acidity in fermented legumes has been reported to reduce the incidence of diarrhea among infants (Ejtahed *et al*., 2011). The lactic acid and other short-chain organic acids produced from fermentation of sugars contributes to the sour taste of fermented food products by decreasing the pH (Lee *et al.,* 2010).

4.4 Proximate composition of fermented cowpea milk

Apart from knowing the growth and survival of probiotic microorganisms in cowpea milk, it is also of interest to know the nutritional composition of the milk. In this study, the proximate and mineral compositions were determined in unfermented cowpea milk and cowpea milk fermented by the different lactic acid bacteria cultures.

Table 4.1: Proximate composition of fermented cowpea milk

Sample	% Composition					
	Moisture	**Crude fat**	**Crude fibre**	**Ash**	**Protein**	**Carbohydrate**
Yoba fiti	92.3±0.06^{a}	0.5±0.02^{de}	ND	0.2±0.01^{a}	1.7±0.01^{a}	5.3±0.01^{a}
ABT-5	92.5±0.15^{b}	0.3±0.01^{a}	ND	0.2±0.20^{a}	1.5±0.01^{a}	5.2±0.01^{a}
YF-L 903	92.8±0.12^{b}	0.4±0.01^{b}	ND	0.2±0.01^{a}	1.6±0.01^{a}	5.1±0.02^{a}
NC	92.5±0.01^{b}	0.4±0.02^{b}	ND	0.2±0.01^{a}	1.5±0.01^{a}	5.5±0.01^{a}

Values are mean±standard deviations of triplicates. Values with different letter superscript in the same column are significantly different at ($p<0.05$) based on Bonferroni tests.

*****NC (No culture) = unfermented cowpea milk; YF-L 903 (*Streptococcus thermophilus* and *Lactobacillus* subs *bulgaricus*), ABT-5 (*Lactobacillus acidophilus* La-5, *Bifidobacterium animalis* Bp-12 and *Streptococcus thermophilus*) and Yoba fiti (*Lactobacillus rhamnosus* GR-1 and *Streptococcus thermophilus);* ND = Not detected

As shown in Table 4.1, the moisture content ranged between 92.3 and 92.8%. The moisture content of the cowpea and its fermented products in the current study is slightly higher than the earlier research done by Anino *et al.,* (2019) who reported moisture content of range 71.53 – 91.22%. Crude fibre was not detected, and this differs from results obtained by Alain *et al.*, (2013) who reported a crude fibre content of 0.02 – 0.09%. Dehulling has been previously reported to greatly reduce crude fibre in legumes (Anino *et al.,* 2019). The difference obtained can be attributed to loss through adherence to the cheese cloth used for filtration. Crude fat in the ABT-5

fermented product (0.3%) and Yoba fiti-fermented product (0.5%) were significantly different ($p<0.05$). The percentage lipid content obtained for cowpea milk and its fermented products in this study was consistent and in agreement with other researchers (Adenike, 2016; Inobeme *et al.,* 2014), but slightly differs from the findings of Ojokoh *et al.,* (2013) that found higher lipid content in fermented cowpea milk in the range of 0.55-1.34%.

The ash content in this present study is 0.2%, with no significant change after fermentation (Table 4.1). This falls within the range reported by Awika *et al.,* (2011), who reported ash content of cowpea milk to be within the range 0.21 – 1.09%. There was no change in ash content during fermentation and this is contrary to study findings reported by Day and Morawicki, (2018). They reported an increase in as content after fermentation and attributed it to loss of dry matter during fermentation as microorganisms degrade carbohydrates and proteins.

The percentage protein content ranged between 1.5- 1.7% (Table 4.1). This was slightly lower than the protein content reported by Inobeme *et al.,* (2014) who reported a percentage protein content of 1.9-2.5. Effect of fermentation on proteins has remained inconsistent in various studies. Several studies have reported protein increase after fermentation of leguminous grains (Anino *et al.,* 2019; Nkhata *et al.,* 2018; Niyibituronsa *et al.,* 2019; Pranoto *et al.,* 2013), while others have reported decrease in proteins and some amino acids after fermentation (Osman, 2011; Pranoto *et al.,* 2013) and they attributed this to the fact that fermenting microorganisms also use amino acids which could lower the protein content and quality of fermented foods. An insignificant decrease ($P>0.05$) was observed for total carbohydrate content after fermentation, with YFL-903 culture leading to the highest decrease of 7.1%.

Fermentation activates starch-hydrolysing enzymes such as α-amylase and maltase which degrade starch into malto-dextrins and simple sugars respectively (Nkhata *et al.,* 2018). The glucose released during fermentation is a preferred substrate for microorganisms and could partly explain the decrease in carbohydrates (Osman, 2011). This study reported an insignificant decrease in total carbohydrate after

fermentation. This could be attributable to the use of sugars as substrates, by the microorganisms, during fermentation (Osman, 2011).

Table 4.2: Mineral composition of unfermented and fermented cowpea milk

Sample	Mineral elements (mg/100g)			
	Zinc (Zn)	Calcium (Ca)	Iron (Fe)	Magnesium (Mg)
NC	0.07±0.01[a]	0.02±0.01[b]	0.49±0.07[a]	17.62±0.07[a]
ABT-5	0.13±0.02[a]	0.01±0.01[b]	0.34±0.03[a]	20.62±0.34[a]
Yoba fiti	0.16±0.01[a]	0.01±0.01[b]	0.38±0.04[a]	20.35±0.11[a]
YF-L 903	0.13±0.01[a]	0.01±0.01[b]	0.32±0.01[a]	18.18±0.14[a]

Values are mean±standard deviations of triplicates. Values with different letter superscript in the same column are significantly different at ($p<0.05$) based on Bonferroni tests.

*****NC (No culture) = unfermented cowpea milk; YF-L 903 (*Streptococcus thermophilus* and *Lactobacillus* subs *bulgaricus*), ABT-5 (*Lactobacillus acidophilus* La-5, *Bifidobacterium animalis* Bp-12 and *Streptococcus thermophilus*) and Yoba fiti (*Lactobacillus rhamnosus* GR-1 and *Streptococcus thermophilus)*

Table 4.2 below shows the mineral composition of the fermented cowpeas. These results were in line with a study by Difo *et al.*, (2014) who reported over 90% decrease in calcium and 50% reduction in iron after fermentation of cowpea flour. The results are also consistent with study by Alain *et al.*, (2013) who reported zinc content (7.33-13.74 mg/100g) and magnesium content (16.53-22.84 mg/100g).

Mamiro *et al.*, (2011) reported iron content of cowpeas in the range of 9.9 - 23.8 mg/kg; calcium (320 - 1112.9 mg/kg) and zinc (17.1 - 32.2 mg/kg). These were slightly higher than the results in present study.

4.5 Effects of fermentation with starter cultures on cowpea oligosaccharide content

Table 4.3 shows the content of the oligosaccharides stachyose, raffinose and verbascose in unfermented cowpea milk and cowpea milk fermented with the different

starter cultures. Stachyose and raffinose were detected in raw cowpeas, unfermented cowpea milk and fermented cowpea milk while verbascose was not detected in any of the samples. In all cases, stachyose content was higher than raffinose. Retention times of raffinose, stachyose and verbascose were 3.6 minutes, 4.3 minutes and 5.8 minutes, respectively (Appendix VI).

Table 4.3: Oligosaccharide concentrations in raw cowpea (RC), unfermented cowpea milk (UM) and cowpea milk fermented with different starter cultures

Sample ID	Oligosaccharide concentrations (g/100g)			
	Stachyose	Raffinose	Verbascose	Total oligosaccharide (Raffinose + Stachyose)
RC	13.9±0.2^{c}	2.2±0.1^{f}	ND	16.1
NC	8.8±0.1^{a}	0.6±0.0^{e}	ND	9.4
ABT-5	0.4±0.0^{b}	ND	ND	0.4
Yoba fiti	7.0±0.1^{ab}	ND	ND	7.0
YF-L 903	2.6±0.0^{bd}	0.2±0.0^{e}	ND	2.8

Values are mean±standard deviations of triplicates. Values with different letter superscript in the same column are significantly different at ($p<0.05$) based on Bonferroni tests.

***** RC = raw cowpeas, NC (No culture) = unfermented cowpea milk; YF-L 903 (*Streptococcus thermophilus* and *Lactobacillus* subs *bulgaricus*), ABT-5 (*Lactobacillus acidophilus* La-5, *Bifidobacterium animalis* Bp-12 and *Streptococcus thermophilus*) and Yoba fiti (*Lactobacillus rhamnosus* GR-1 and *Streptococcus thermophilus)*; ND = Not Detected

Sreerama *et al.,* (2012) found higher contents of the three oligosaccharides than presented in Table 4 for raw cowpeas, but a similar trend of stachyose (17.8 mg/g) >raffinose (10.3 mg/g) >verbascose (3.6 mg/g) in cowpea flour. Unfermented cowpea milk (NC) had lower content of stachyose and raffinose than the raw cowpea (RC) (Table 4.3). Since the cowpeas were soaked for 12 hours and dehulled prior to milk extraction, the lower oligosaccharide content in the NC is attributable to their leaching into the soaking water and removal with the hulls. Such oligosaccharide-reducing

effect of legume soaking and dehulling has been previously reported (Anino *et al.*, 2019). Fermentation of cowpea milk further reduced the oligosaccharide contents, and the extent of reduction of each sugar depended on the culture used (Table 4.3).

The Yoba fiti culture depleted raffinose but only minimally reduced the stachyose content form 8.8 mg/g in the unfermented cowpea milk to 7.0 mg/g (20% reduction). On the other hand, fermentation with YF-L 903 did not deplete raffinose, but reduced stachyose by 70%. ABT-5 also depleted raffinose and reduced stachyose by 59%. In terms of combined stachyose and raffinose reduction, YF-L 903 was the most effective (70% reduction), followed by ABT-5 (62%) and Yoba fiti (26%). Thus, probiotic microorganisms in YF-L 903 and ABT-5 might be more effective in reducing oligosaccharide-dependent flatulence than Yoba fiti. Nevertheless, because soaking and dehulling also contribute to oligosaccharide reduction, cowpea milk fermented with GT had less than half the oligosaccharides in raw cowpea (Table 4.3).

Liu *et al.*, (2006) found that *L. rhamnosus* strains 6013, 6013+DH_1 and 6013+GH_4 completely metabolized raffinose in soybean within six hours and strain-dependently reduced the stachyose content by between 50 to 70%. *Streptococcus thermophilus* was found to be more efficient than *Lactobacillus acidophilus* in the metabolism of these oligosaccharides, and combining either of these with *Bifidobacterium* increased the oligosaccharide reduction (Niyibituronsa *et al.*, 2019). Thus, the different combinations of bacteria in the starter cultures contribute to differences in the extent of oligosaccharide reductions. The strains tested were mixtures of *S. thermophilus* and *L. rhamnosus gr-1*(Yoba fiti), *S. thermophilus* and *L. bulgaricus* (YF-L 903) and *S. thermophilus*, *L. acidophilus* and *Bifidobacteria* (ABT-5). Thus, the different extents of their reduction of stachyose and raffinose depended on the unique combinations of different organisms and perhaps other factors in the cowpea such as varietal differences.

4.6 Production of short chain fatty acids during cowpea fermentation with starter cultures

Table 4.4 shows the short chain fatty acid content of nonfermented cowpea milk and cowpea milk fermented with different starter cultures. Butyric acid, valeric acid and

isovaleric acid were not detected in the unfermented sample (NC) which was incubated under same conditions as the samples fermented with starter cultures. However, this sample was found to contain a small quantity of propionic acid (50 ppm), which can be attributed to natural fermentation.

Table 4.4: Concentration (parts per million) of short-chain fatty acids in unfermented cowpea (NC) and cowpea fermented with cultures ABT-5, Yoba fiti and YF-L 903.

Treatment	Propionic acid	Butyric acid	Valeric acid	Isovaleric acid
NC	50 ± 6^{a}	ND	ND	ND
ABT-5	510 ± 8^{b}	40 ± 1^{a}	20 ± 1^{c}	ND
Yoba fiti	220 ± 17^{c}	40 ± 1^{a}	10 ± 1^{c}	70 ± 1^{b}
YF-L 903	2430 ± 19^{d}	50 ± 2^{a}	60 ± 1^{d}	40 ± 1^{b}

Values are mean±standard deviations of triplicates. Values with different letter superscript in the same column are significantly different at ($p<0.05$) based on Bonferroni tests.

***** NC (No culture) = unfermented cowpea milk; YF-L 903 (*Streptococcus thermophilus* and *Lactobacillus* subs *bulgaricus*), ABT-5 (*Lactobacillus acidophilus* La-5, *Bifidobacterium animalis* Bp-12 and *Streptococcus thermophilus*) and Yoba fiti (*Lactobacillus rhamnosus* GR-1 and *Streptococcus thermophilus)*; ND = Not Detected

The ABT-5 culture produced small quantities of all the short chain fatty acids except isovaleric acid, with valeric acid being the least produced (20 ppm) and propionic acid the most produced (510 ppm). Yoba fiti culture produced small quantities of all the fatty acids, ranging from 10 ppm of valeric acid to 220 ppm of propionic acid. The YF-L 903 culture also produced all the fatty acids, and remarkably high levels of propionic acid (2430 ppm), which was significantly different from the other cultures (Table 4.4).

Propionic acid is one of the generally recognized as safe (GRAS) food preservatives (Eş *et al.,* 2017) and its presence at these levels is expected to contribute to the preservation of the fermented cowpea milk. It not only inhibits spoilage fungi and bacteria but also pathogenic ones such as *Salmonella* (Haque *et al.,* 2012). Thus, it

may contribute to reducing gastrointestinal infections. Other benefits of propionic acid include lowering fatty acid content in the liver and plasma, reducing food intake and thus potentially preventing obesity, and improves tissue insulin sensitivity (Abubakr *et al.,* 2012). Thus, a significantly higher propionic acid production by YF-L 903 culture indicates that it has nutraceutical potential in cowpea milk. YF-L 903 culture also had significantly higher production of valeric acid than the other cultures. Yoba fiti culture had the highest production of isovaleric, though this was not significantly different from YF-L 903, the only other culture which produced this acid. Unlike propionic acid, the concentration of butyric acid produced by the three cultures was not significantly different.

Interestingly, both ABT-5 and Yoba fiti contain bacteria that are clearly regarded as probiotic, namely *Lactobacillus acidophilus, Bifidobacteria,* and *Lactobacillus rhamnosus,* while the probiotic potential of *Streptococcus thermophilus* is still debated (Yang and Zhang, 2009). Thus, the current study shows that *S. thermophilus* or *L. bulgaricus* have potential probiotic properties and are therefore suitable in fermentation of cowpea milk for health benefits.

4.7 Sensory evaluation

Sensory evaluation helps in making predictions on customer acceptance and preferences of a new product. It is also important during upgrade of an existing product due to changes in market trends (Ibrahim *et al.,* 2014). In the present study, sensory evaluation was done for fermented cowpea milk to test consumer acceptability and preference. Rating was done for appearance, aroma, taste, texture and overall acceptability on a nine-point hedonic scale where 1 = like extremely, 5 = neither like nor dislike and 9 = dislike extremely (Fidelis *et al.*, 2014).

Table 4.5: Sensory evaluation of fermented cowpea milk

Sample	Aroma	Taste	Texture	Appearance	Overall acceptability
E_i	4.9 ± 1.4^{a}	4.8 ± 1.4^{c}	4.1 ± 1.4^{d}	4.0 ± 1.5^{e}	5.1 ± 0.9^{g}
E_{ii}	4.9 ± 1.6^{a}	4.9 ± 1.9^{c}	4.3 ± 0.8^{d}	4.0 ± 1.8^{ef}	4.9 ± 1.1^{g}
F_i	4.6 ± 1.6^{a}	5.1 ± 2.0^{c}	4.4 ± 1.0^{d}	4.1 ± 1.7^{e}	5.3 ± 0.2^{g}
F_{ii}	4.8 ± 1.3^{a}	4.9 ± 1.6^{c}	4.2 ± 1.3^{d}	3.7 ± 1.3^{ef}	5.0 ± 1.0^{g}
G_i	5.1 ± 1.4^{b}	5.0 ± 1.6^{c}	4.1 ± 1.1^{d}	3.9 ± 1.4^{ef}	5.1 ± 1.2^{g}
G_{ii}	5.1 ± 1.5^{b}	5.3 ± 1.9^{c}	4.4 ± 1.0^{d}	4.5 ± 1.7^{e}	5.3 ± 1.3^{g}
H_i	4.8 ± 1.5^{ab}	5.0 ± 1.9^{c}	4.4 ± 1.0^{d}	4.2 ± 1.7^{e}	4.9 ± 0.4^{g}
H_{ii}	5.0 ± 1.4^{ab}	5.1 ± 1.4^{c}	4.2 ± 1.3^{d}	4.0 ± 1.5^{e}	4.7 ± 1.2^{g}

* E_i = ABT-5 Vanilla, E_{ii} = ABT-5 Strawberry, F_i = YF-L 903 Vanilla, F_{ii} = YF-L 903 Strawberry, * H_i = Yoba GR-1 Vanilla, H_{ii} = Yoba GR-1 Strawberry, G_i = No culture vanilla, G_{ii} = No culture Strawberry

Values are mean±standard deviations of triplicates. Values with different letter superscript in the same column are significantly different at ($p<0.05$) based on Bonferroni tests.

Table 4.5 shows that no significant differences were observed for sensory attributes of taste, texture and overall acceptability in all the samples. However, aroma ($p=0.02$) and appearance ($p=0.01$) had significant differences among the samples. Turgut and Cakmakci, (2018) reported that storage time and probiotic strains affect sensory properties of yoghurts. They also reported that addition of *L. acidophilus* and *B. bifidum* to yoghurts significantly ($P<0.05$) affects the taste of yoghurt, but not odor, texture and general acceptability. Faria *et al.*, (2011) stated that presence of probiotics especially *Bifidobacterium* genus in the yoghurt can contribute to rejection of the product due to excess acidity and more viscous texture. Overall acceptability score of the product ranged between 4.7 – 5.3 indicating dislike to like slightly. Therefore, despite the potential benefits, more work should be done to improve the sensory and overall acceptability of the fermented cowpea milk.

CHAPTER FIVE

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

Study demonstrated suitability of probiotic bacteria in the fermentation of cowpea milk. Cowpea milk fermentation with the three starter cultures ABT-5, YF-L 903 and Yoba fiti, resulted in colony counts of lactic acid bacteria greater than $\log_{10}$ 6.0 cfu/ml. Therefore, this fermented product can be considered a healthy probiotic product because even after 28 days of storage and 4˚C, the lactic acid bacteria viability still remained at a level that is acceptable as a probiotic product.

The Yoba fiti culture depleted raffinose but only reduced the stachyose content by 20%. On the other hand, fermentation with YF-L 903 did not deplete raffinose, but reduced stachyose by 70%. ABT-5 also depleted raffinose and reduced stachyose by 59%. In terms of combined stachyose and raffinose reduction, YF-L 903 was the most effective (70% reduction), followed by ABT-5 (62%) and Yoba fiti (26%). Thus, probiotic microorganisms in YF-L 903 and ABT-5 might be more effective in reducing oligosaccharide-dependent flatulence than Yoba fiti.

All the three cultures led to production of short chain fatty acids after fermentation. However, fermentation with ABT-5 culture did not result into formation of isovaleric acid. The YF-L 903 culture produced remarkably high levels of propionic acid (2430 ppm). The three cultures contain bacteria regarded as probiotic, namely *Lactobacillus acidophilus, Bifidobacteria, Lactobacillus rhamnosus*, *Streptococcus thermophilus* and *L. bulgaricus.* Thus, significantly higher production of the short chain fatty acids by the cultures indicates nutraceutical potential in cowpea milk.

Overall acceptability score of the product ranged between 4.7 – 5.3 indicating dislike to like slightly. Therefore, despite the potential benefits, more work should be done to improve the sensory and overall acceptability of the fermented cowpea milk.

5.2 Recommendation

The study recommends that the potential health benefits of probiotic bacteria in the three cultures, ABT-5, YF-L 903 and Yoba fiti, should be determined through *in vivo* studies. This shall give more evidence on health benefits of probiotics on human health.

Potential of the probiotic bacteria in the three cultures in reducing flatulence-causing oligosaccharides in cowpea milk should be investigated through *in vivo* studies to give more evidence on their benefits to human health.

The government through Ministry of Health should enlighten the public on the health benefits of probiotic products and lower taxes for such products to make them more affordable and available to general public. This will help in reducing the burden of treatment of the NCDs in the long run.

Policy makers in the education sector can incorporate these healthy probiotic foods like fermented cowpea milk into the school feeding programmes so as to make the products available to the school going children to help in prevention of development of NCDs.

More work should be done to improve the sensory acceptability of the products in terms of taste, aroma and appearance to increase general consumer acceptability.

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APPENDICES

Appendix I: Sensory evaluation consent form

SENSORY PANELIST CONSENT FORM

Sensory evaluation of fermented cowpea milk

Thank you for your willingness to potentially participate in a sensory evaluation project at the Department of Human Nutrition Sciences, Jomo Kenyatta University of Agriculture and Technology.

Date of Participation: ……

Voluntary Nature of Participation: I understand that participation in this project is completely voluntary and I do not have to participate in this sensory project if I do not agree to participate hence I can withdraw my participation at any time.

Risks to the individual: I understand that I will evaluate different varieties of soybean grains using descriptive sensory evaluation. I note that people who are allergic to soybeans should avoid these products.

Medical Liability: I understand that no financial compensation will be paid to me in connection with any physical injury or injury in the unlikely event of physical injury or illness as a direct or indirect result of my participation in this sensory project.

Confidentiality: participants are not required to reveal any confidential information. All responses to questions will be treated in a confidential manner. Responses to sensory questions via the evaluation form are tracked using numbers only. These numbers are not in any way related to the participant's name.

If you have any questions about this sensory project, contact Aduol Kevin. Department of Human Nutrition Sciences, Jomo Kenyatta University of Agriculture and Technology 0725823259

I HAVE READ THIS CONSENT FORM AND I AM PREPARED TO PARTICIPATE IN THIS PROJECT.

Participant's Signature …….Date……

Participant's Name……. *(Optional)*

Sensory Panel Leader Signature……Date……..

Appendix II: Consumer acceptability sheet

WELCOME TO THIS FERMENTED COWPEA MILK TASTING SESSION

JOMO KENYATTA UNIVERSITY OF AGRICULTURE AND TECHNOLOGY

Age: ……

Gender: ……...

Tray Number: …….

Instructions

You are provided with four (3) samples of cowpea yoghurt. Please taste the samples in the order presented from left to right. Take a sip of water before you start tasting and in between tasting the different samples. Indicate your liking or disliking by placing a check mark at the relevant bar on the scale provided for each attribute.

Sample Number												
Scale	**A**	**S**	**T1**	**T2**	**A**	**S**	**T1**	**T2**	**A**	**S**	**T1**	**T2**
Like extremely												
Like very much												
Like moderately												
Like slightly												
Neither like nor dislike												
Dislike slightly												
Dislike moderately												
Dislike very much												
Dislike extremely												

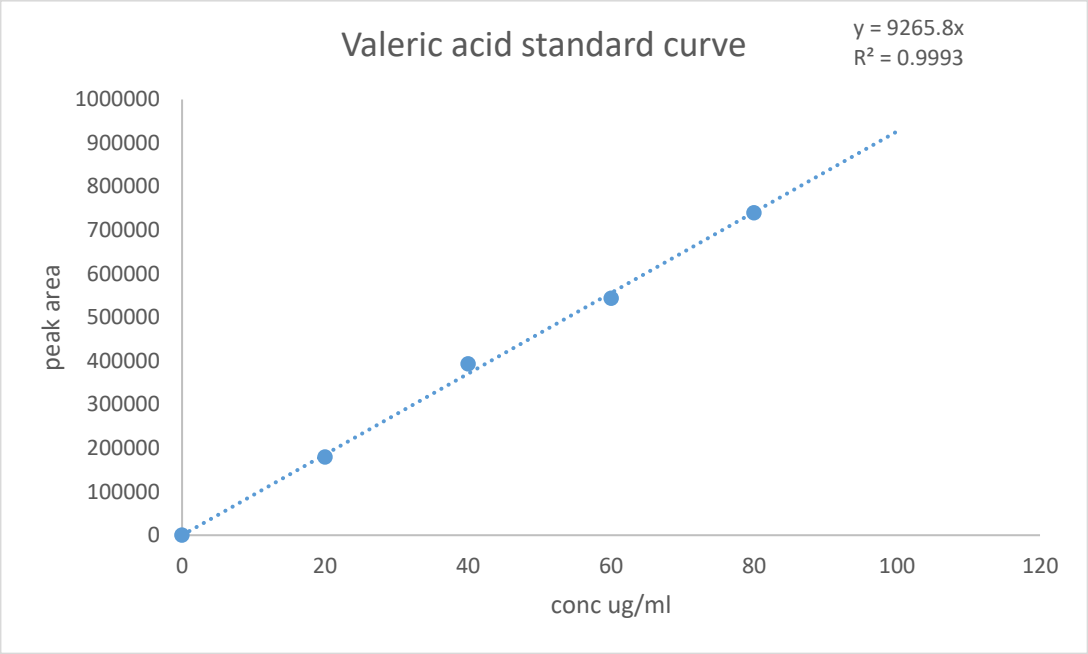
KEY: A - Appearance. S – Smell. T1 - Taste. T2 - Texture

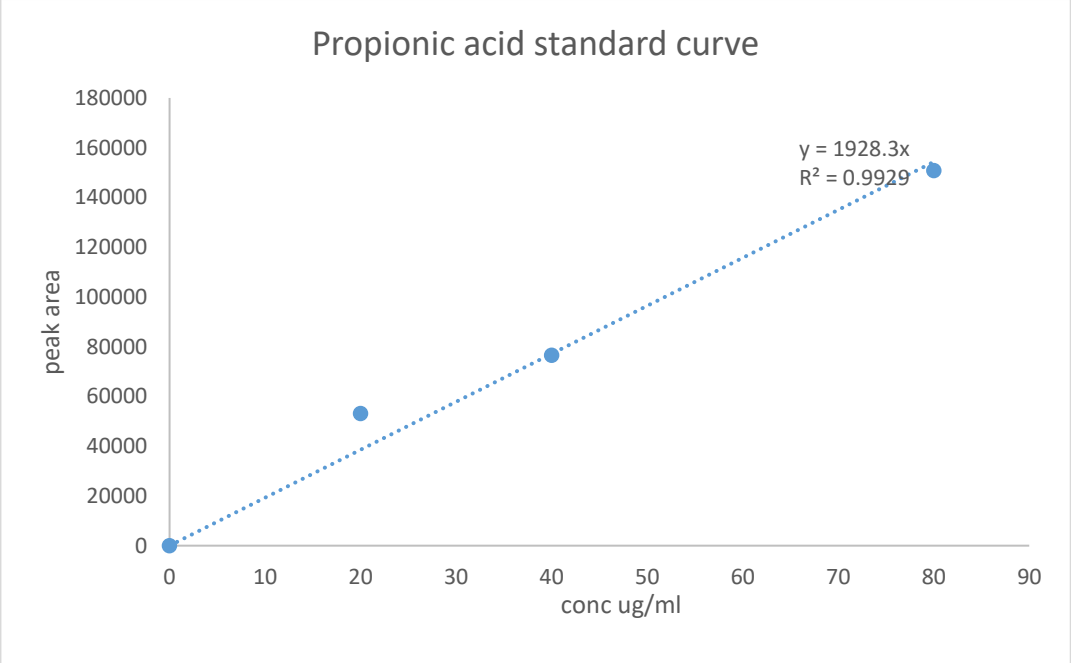
Comments:

……

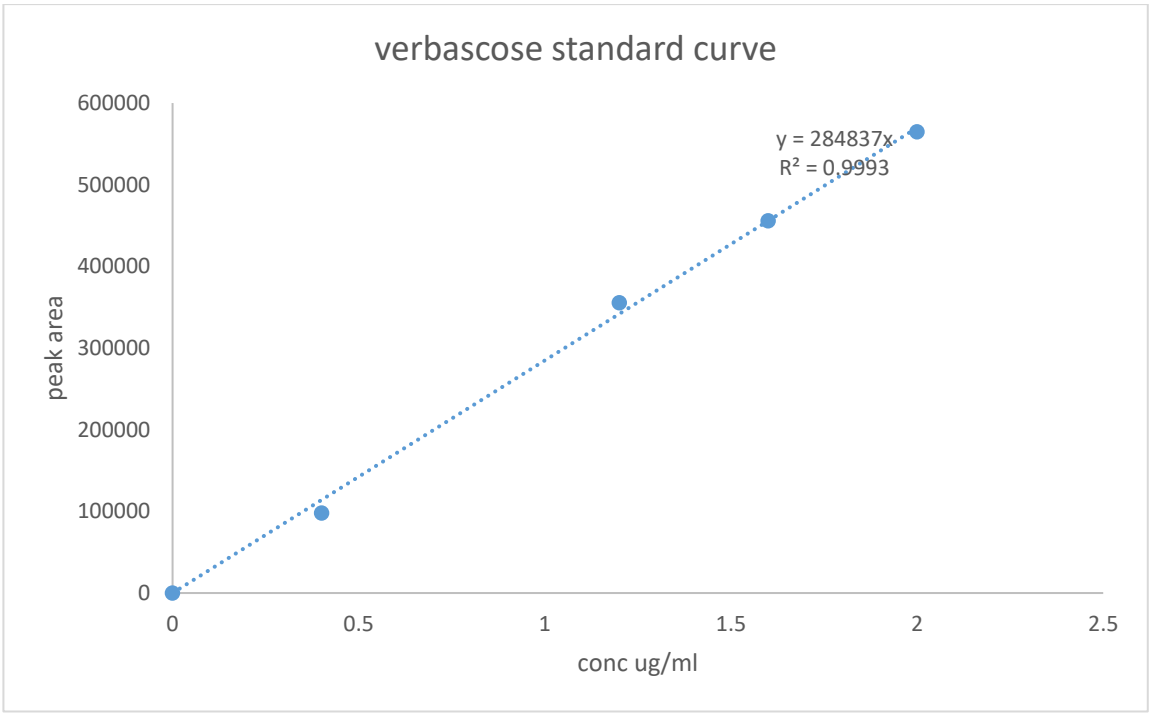
……

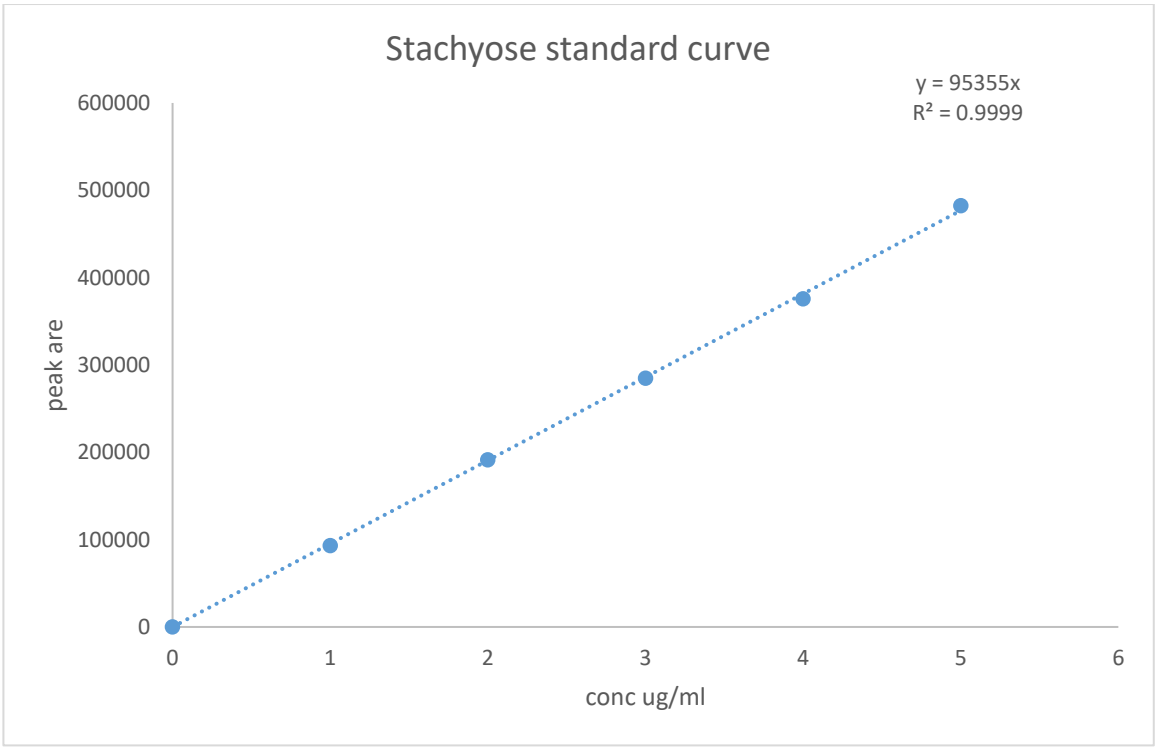
Appendix III: Standard curves for organic acids



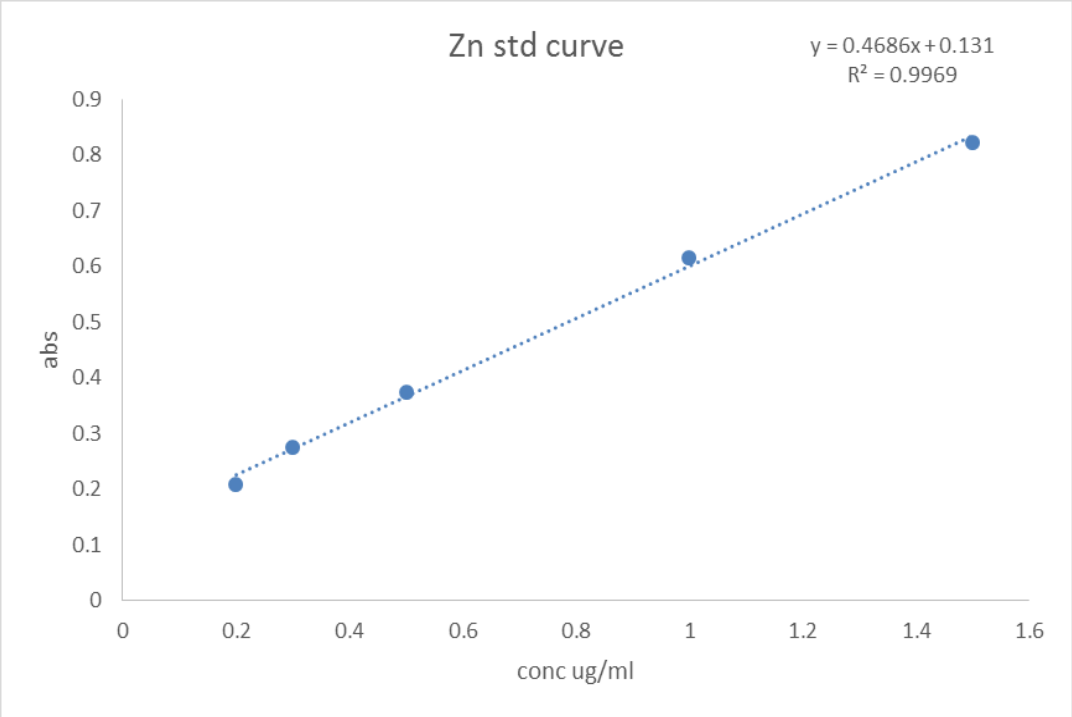


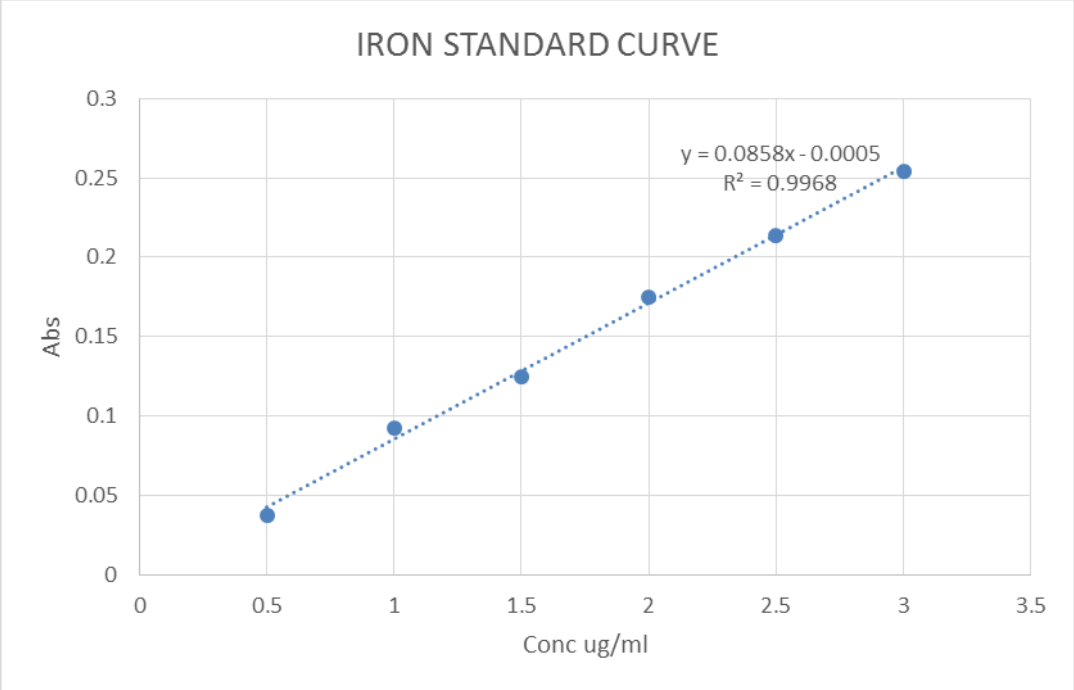
Appendix IV: Standard curves for oligosaccharides



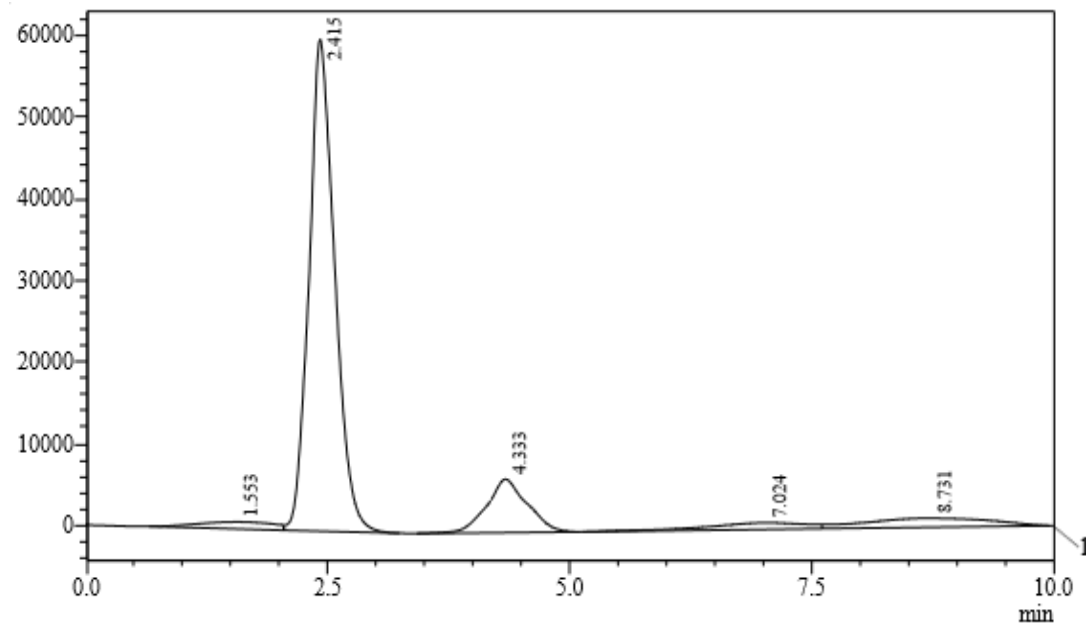


Appendix V: Standard curves for minerals

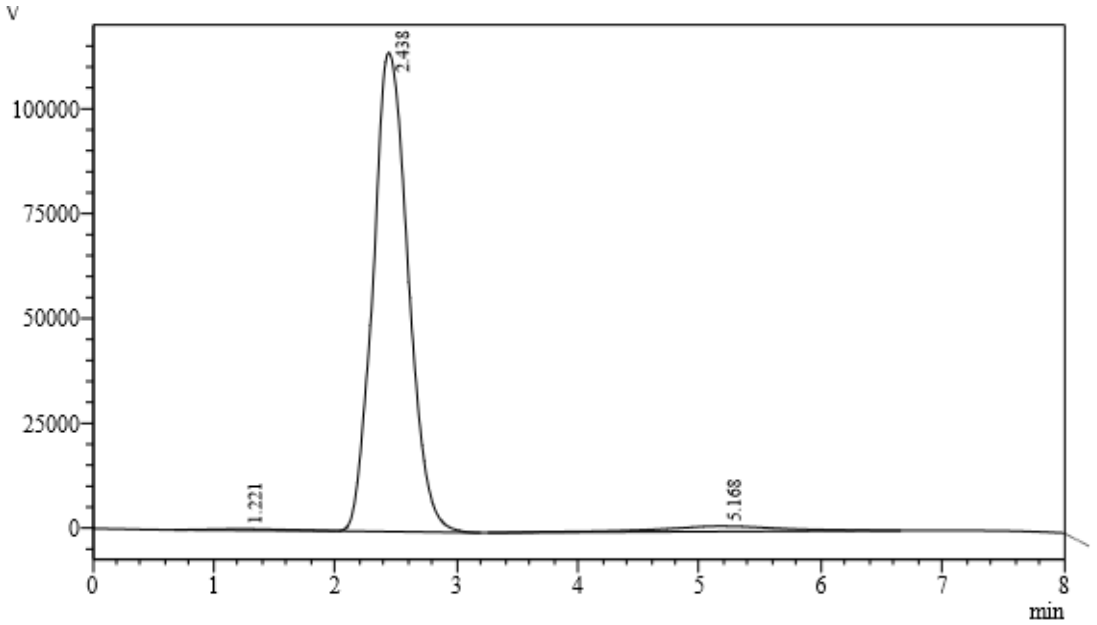




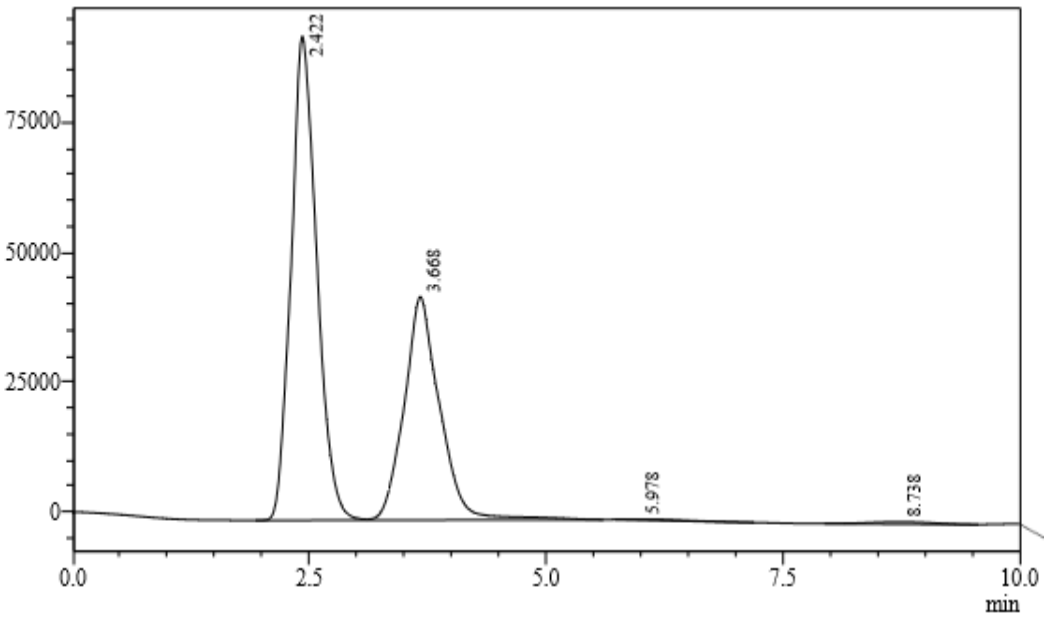
Appendix VI: High Performance Liquid Chromatography (HPLC) chromatograms of oligosaccharides



- HPLC chromatogram of stachyose

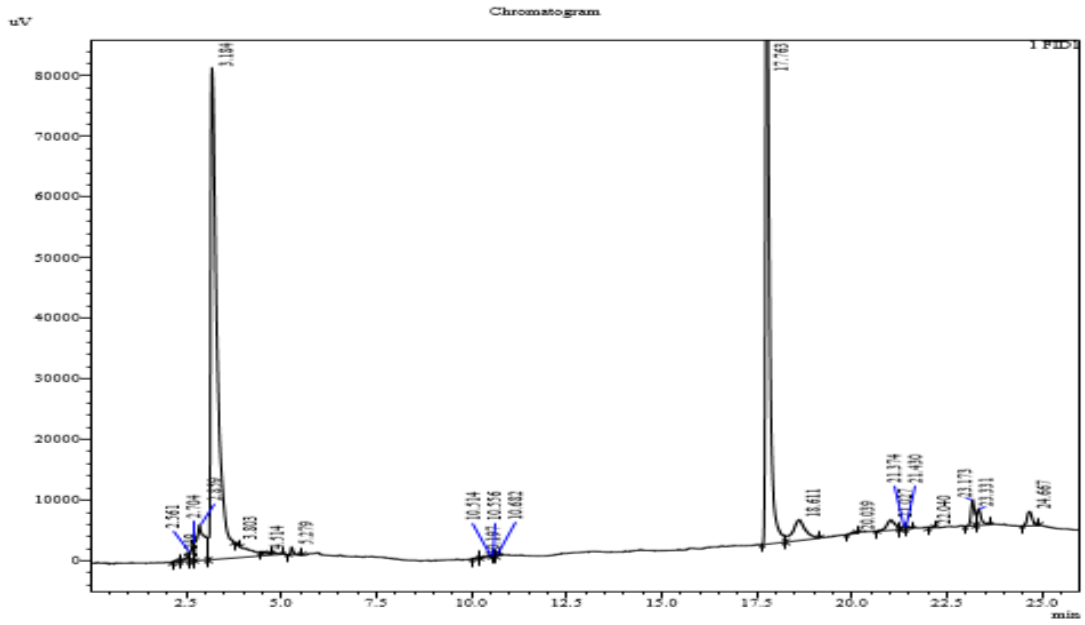


- HPLC chromatogram of verbascose

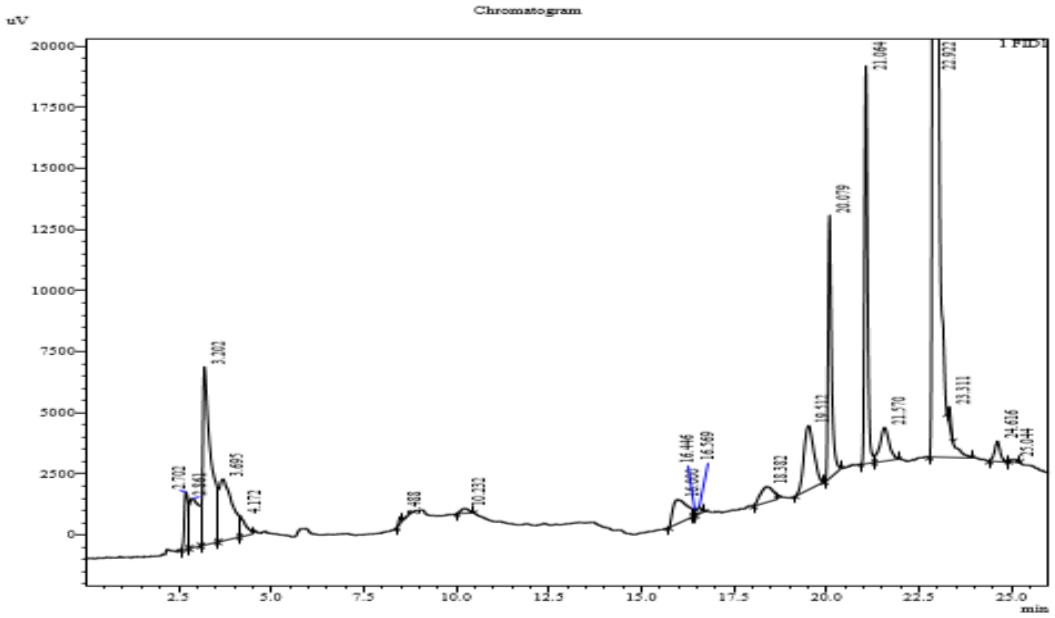


- HPLC chromatogram of raffinose

Appendix VII: Chromatograms of short-chain fatty acids

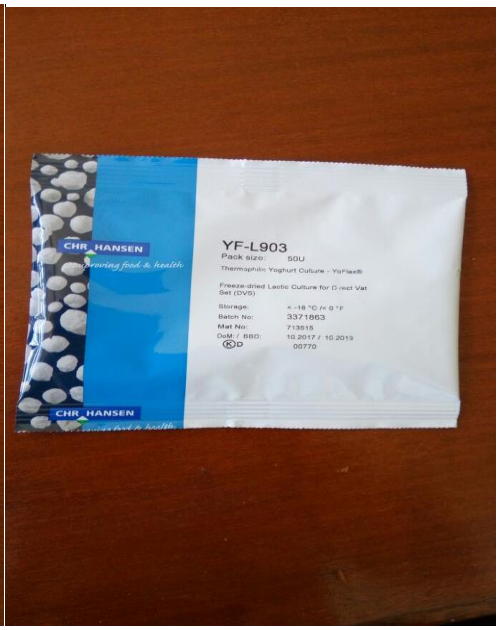


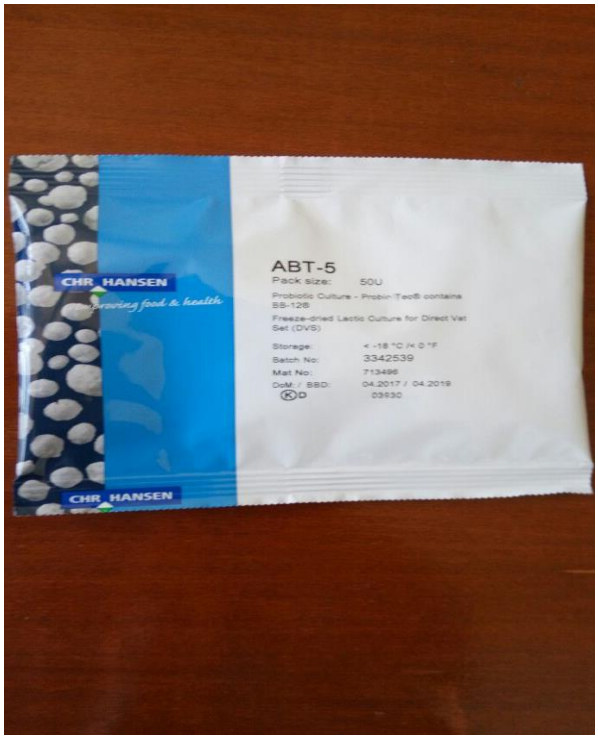
A. Standard propionic 100 ppm



B. Standard valeric acid 100ppm

Appendix VIII: Cultures used for fermentation





Appendix IX: Cowpea milk

A: Extracted cowpea milk

B: The researcher extracting the cowpea milk