PROBIOTIC, STARTER CULTURE AND ANTIMICROBIAL PROPERTIES OF LACTIC ACID BACTERIA ISOLATED FROM UGANDAN TRADITIONAL GHEE

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DECLARATION

I, Immaculate Abbo, declare that this dissertation is my original piece of work and has not been submitted to any university or institution for the award of a degree and where other works have been included, it has been mentioned.

Student

Signed……

Immaculate Abbo

Date……

APPROVAL

This is to certify that the work presented herein is the students own, done by her under our supervision and guidance, and is now ready for submission to the Graduate Board of Kyambogo University.

Signed…….. Date…….

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Signed…… Date…….

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DEDICATION

This research is dedicated to the Almighty God who has generously provided for me throughout this journey and preserved my health during the inevitable movements I had to make to carry on with the research amidst the Covid-19 Pandemic.

This book is also dedicated to my beloved Father, Mr. John Martin Ochieng who has always been a positive role model. I'm grateful for his encouragement along this journey which gave me the strength to carry on and aim for the successful completion of my Masters.

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ABSTRACT

Ghee is one of the major fermented foods consumed in Uganda despite the fact that it is largely produced traditionally raising issues of quality control and assurance. This study investigated the bacterial diversity of traditional ghee obtained from different regions of Uganda in an effort to determine the technological properties of its dominant lactic acid bacterial (LAB) flora. A total of nine samples were purchased in original packaging, from Buganda (2), Bunyoro (2), Teso (2) & Ankole (2) regions including a control sample and subjected to microbial analysis using conventional and molecular protocols to obtain an overview of the microbial diversity of these different ghee varieties in order to focus on the dominant LAB flora.

Traditional ghee from Teso was contaminated with coliforms and *E. coli*, but the latter were not isolated which could be due to failure to collect these organisms from random colonies selected from the media plates during isolation. The species identified using 16S rDNA sequence analysis include *Enterococcus faecium* (22 isolates, 30%), *Lactobacillus plantarum* (20 isolates, 28%), *Lactobacillus rhamnosus* (11 isolates, 15%), *Enterococcus hirae* (11 isolates, 15%), *Enterococcus faecalis* (2 isolates, 3%) and *Bacillus cereus* (2 isolates, 3%), with *Lactobacillus* spp. being the dominant (31 isolates, 43%) genus detected in all samples. MEGA X analysis of the 16S rDNA PCR gel images gave separate clusters for each of *L. plantarum* and *L. rhamnosus*; each of the clusters was heterogeneous and contained isolates from different sub-regions of Uganda highlighting possible genetic relationship between these *Lactobacillus* isolates.

Since *L. rhamnosus* is preferred to *L. plantarum* for inclusion in fermented dairy products due to its probiotic properties, three (03) *L. rhamnosus* isolates were evaluated for potential probiotic properties, antimicrobial activity against *Staphylococcus aureus*, *Escherichia coli*, and *Salmonella enterica*, and growth and acid production profiles in milk, in order to establish their prospects for application as probiotics, bio-preservatives and/or starter cultures in milk and other fermented foods. Each of the tested isolates exhibited good acid tolerance at the exposure of 0.7% ox bile at 30ºC for 0, 3, 6 and 9 h and remained viable (3 log reduction) after 3 h of exposure at pH 3, did not produce biogenic amines and had high level of auto-aggregation at 24 h. However, all the isolates were sensitive to bile salts implying that they may not survive the

entire gut transit. On agar overlay assays, each of the *L. rhamnosus* isolates had antimicrobial activity against at all the tested indicator bacteria at 30^{o}C, 24 h and 37^{o}C, 24 h for LAB isolates and indicator bacterial strains, respectively. In the disc diffusion assays, only neutralized cell-free supernatants for each of the *Lactobacillus* isolates lost antimicrobial activity, implying that activity was due to acid production.

Subsequently, two *L. rhamnosus* isolates were studied further for growth kinetics and acid production prospects in UHT milk model to determine their starter culture potential. All the *L. rhamnosus* isolates had similar growth patterns in milk attaining 8-10 log cfu/ml at 48 h. However, the isolates had low acid production capacity leading to a pH decrease from 6.89-6.92 to 4.89-5.04 after 48 h of fermentation. Acid production was growth-dependent and maximum acidification occurred at exponential phase (8-24 h).

It was concluded that *L. rhamnosus* isolated from Ugandan traditional ghee does not have prospects for application as a starter culture due to its low acidification capability. However, these organisms have some favorable probiotic and antimicrobial properties which could be exploited commercially. Further research should investigate the aroma compounds which could be produced by these organisms in milk and other fermented foods when grown alone and in co-culture with *L. plantarum*, and/or yeasts and moulds which were also detected in the traditional Ugandan ghee. Further studies should focus on stabilization of the lactic acid bacterial isolates against bile.

CHAPTER ONE: INTRODUCTION

1.1 BACKGROUND

Fermentation is a potent tool for imparting longevity as well as transformation of milk into new products such as cheese, yoghurt and ghee (Gupta et al., 2013). Lactic acid bacteria have been and continue to be exploited by mankind to improve food supply mainly through improving the flavor, texture, shelf life and nutritional value of a wide array of food products (Hall et al., 2001). Fermented foods are popular for their social, religious, nutritional and therapeutic benefits (Aka, 2008). Ghee is a fat product exclusively obtained from cow milk, cream or butter, by means of heat processes which result in almost total removal of water and non-fat solids, with an especially developed flavour and physical structure.

Uganda is one of the highest milk-producing countries in East Africa accounting for 25% of total milk supplied among other East African Community (EAC) countries (Abdulsamad & Gereffi, 2016). Milk production increased to 2.51 billion litres in 2018 and the percentage of marketed milk stood at 80.2 % while the remaining portion was consumed by the farmers and their families (DDA Annual Performance Report, FY 2018/2019). The Dairy development Authority also reported that only 33% of the marketed milk is processed, leaving 67% to be sold in the raw form. Uganda has an operating dairy market built around a value chain that starts with rural producers (predominantly smallholder dairy farmers) and transporters or buyers. It is estimated that 89% of the farmers sell their milk as is to middlemen or processors. Of the remaining 11%, 9% process milk into ghee mainly for home consumption while 2% make other products particularly yoghurt (Katimbo et al., 2015).

Uganda is ranked in the 47th position globally among the ghee producing countries with an annual estimation of 22.84 metric tonnes of ghee representing 0.01% of the total global production (FAOSTAT, 2019). In Uganda, fermented ghee is referred to as *Amashita* amongst the *Banyankole* and *Bakiga* tribes of the western region. It is known as *Omuzigo* by the *Baganda* and *Amagita* by the *Batooro* and *Banyoro* tribes. Ghee is used in cooking to enhance flavour of food and is also used for cosmetic purposes by the aforementioned tribal groups (Ongol & Asano, 2009). It is a rich source of energy, fat soluble vitamins, essential fatty acids and other

growth-promoting factors (Lampert, 1975). The nutritional value of ghee per 100g is 99g of fat, which comprises 61.9g of saturated fat, 28.7g of monounsaturated fat, 4 g of trans fat, 3.7g of polyunsaturated fat, 1,447mg of Omega-3 fatty acids 2,247mg of Omega-6 fatty acids and 256mg of cholesterol. Non-fat nutrients include 3,069 IU of vitamin A (61% RDA), 2.8mg of vitamin E (14% RDA), 8.6µg of vitamin K (11% RDA) (USDA Nutrient Database, 2016). The characteristic flavor of ghee is its major criterion for acceptance and it is greatly influenced by the fermentation process. The flavor of ghee is due to a complex mixture of compounds such as lactones, which have a coconut like aroma, carbonyls (aldehydes and ketones) which have a sweet scent, free fatty acids (FFA), esters, alcohols and hydrocarbons (Achaya, 1997). These compounds are mainly formed as a result of microbial metabolism of various milk constituents (Sserunjogi et al., 1998). In addition to fermentation properties, the natural microorganisms in matured ghee could also exert probiotic/health effects (Kwak et al., 2014).

Production of ghee starts with boiling of fresh milk followed by addition of drops of pre-fermented milk commonly known as *Bongo* amongst the *Baganda* and *Enkamyo* amongst the *Bahima/Banyankole* tribes. This process is known as seeding and the pre-fermented milk is added to initiate fermentation. In Uganda, methods of ghee production have remained traditional. Drops of pre-fermented milk (Bongo) are introduced to serve as a source of fermentation microflora since they contain a mixed culture of lactic acid bacteria and other fermentative organisms (Arokiyamary & Sivakumaar, 2011). The milk is then allowed to ferment at room temperature for up to twelve hours and the butter is separated by churning in a gourd (Sempiira et al., 2017). The butter is washed to remove dirt and residual milk after which it is stored to ripen for at least one week. Ripening involves keeping the butter in small calabashes or well-closed containers at room temperature to allow native microorganisms to mediate reactions that yield the desirable aroma and colour of ghee (Ongol & Asano, 2009). Progress of fermentation is tracked by sniffing to determine changes in aroma. Upon completion of the ripening process, the butter is clarified by heating to reduce the moisture content and remove any contaminants to yield fermented ghee. The final product has a semisolid texture, a light yellow color and a characteristic nutty flavor (Aysegul & Issa, 2008).

Owing to the traditional methods of ghee production in Uganda, quality control and assurance is never guaranteed because the specific strain starter organisms are not known (Arokiyamary & Sivakumaar, 2011). In addition, the exact concentration of starter inoculum added to the fresh milk is not regulated and the ghee yield to be expected from a given amount of milk is also greatly unpredictable (Sempiira et al., 2017). Continual sniffing to track progress of fermentation during the ripening stage is not a sustainable method and may serve as a loophole for introduction of undesirable micro-organisms into the final product.

Moreover, consumers are increasingly demanding for natural and beneficial foods in order to improve their health and well-being. Probiotics play an important role in such a demand, and dairy foods are the most commonly used vehicles for such bacteria, represented predominantly by lactic acid bacteria. Probiotics are living bacteria in the human body that offer enhanced human health as well as production of antimicrobial agents against undesirable sensitive microorganisms (Kivanc et al., 2011).

Studies indicate that LAB from dairy products can be isolated, dried and used as starter cultures in fermentation processes in dairy and baking technology among other food processing technologies (Denkova et al., 2014; Menteş et al., 2007). This could reduce fermentation failure and consequently improve final product quality, predictability and reliability as well as shortening the fermentation process, due to introduction of competitive microorganisms in fermentation media (Leroy & De Vuyst, 2004).

Defining the probiotic potential, possible antimicrobial properties and other useful properties of the indigenous microbiota in traditional fermented ghee is a vital step towards starter culture development. For instance, LAB have been reported to exhibit antimicrobial effects against pathogenic bacteria (Gong et al., 2010; Kivanc et al., 2011; Rzepkowska et al., 2016; Saarela et al., 2002). Therefore, utilization of LAB isolated from matured ghee as antimicrobial agents in other fermented food products could lead to their improved safety and extended shelf life (Denkova et al., 2014; Georgieva et al., 2014; Menteş, et al., 2007). Some of the LAB isolates from ghee could also be applied as probiotics in food fermentations for purposes of enhanced human health. For instance, studies have indicated LAB strains such as *Lactobacillus plantarum*,

Lactobacillus rhamnosus, *Lactobacillus brevis*, *Pediococcus pentosaceus* and *Weissella* spp. can survive acidic pH and bile salts in the gut to serve as probiotics in the body (Hamon et al., 2014; Rzepkowska et al., 2016; Stojanovski et al., 2013; Verónica et al., 2016). Isolates with prospects for use in the manufacture of other fermented products in a commercial context could be considered as novel cultures to reduce the often-unpredictable product quality inconsistencies. The aims of this study were to determine the diversity, technological properties and potential probiotic properties of the dominant lactic acid bacterial flora involved in fermentation of traditional ghee.

1.2 PROBLEM STATEMENT

The production of ghee in Uganda is currently dependent on wild fermentation and the time it takes to accomplish the process varies widely amongst different producers and regions. The methods used in ghee production are traditional and little is known about the specific microorganisms responsible for the production of each of desirable flavor components of the final product (Ongol & Asano, 2009). Isolation and identification of lactic acid bacteria for use as starter cultures could shorten the fermentation process and reduce the risk of fermentation failure (Leroy & De Vuyst, 2004). Therefore, there is a need for build-up of information on the diversity and characteristics of indigenous LAB in traditionally fermented ghee in Uganda in order to screen biotypes with desirable technological properties, and characterize the strains for reliable fermentation of traditional ghee. Lack of sufficient information about properties of LAB species responsible for production of this widely appreciated fermented dairy product (ghee) impedes further developments and improvements of the dairy fermentation industry. The purpose of this study was to determine the diversity and technological properties of the dominant lactic acid bacterial flora involved in fermentation of traditional ghee.

1.3 JUSTIFICATION

In spite of a variety of indigenous natural fermented food products in Uganda, there has been limited efforts for starter culture and probiotic development despite studies elsewhere indicating the role of LAB as probiotics and starter cultures in fermented foods (Georgieva et al., 2014). Ghee being one of the major fermented foods whose production process is still largely traditional in Uganda would be a primary source of lactic acid bacteria which normally dominate the microflora of these products where they serve as starter cultures, probiotics and antimicrobial agents (Denkova et al., 2014: Menteş et al., 2007). However, these efforts entail obtaining a reliable description of the physiologically active microbial communities in the product in order to understand the role played by each of the microbial LAB species in the fermentation technology (Ongol & Asano, 2009). Due to continuous changes in trends, it is possible that traditional fermentation would become less preferred thus risking loss of knowledge in production of traditional products such as ghee in Uganda. Therefore, the diversity of LAB species which contribute to the desirable properties of ghee must be determined and characterized and the strains with high probiotic potential identified and prospected for

commercial fermentations due to their incremental health benefits in the human body (Vandenplas et al., 2014). The data obtained could be used to determine the right proportions of the different LAB strains for use in commercial production of ghee where predictability of processes is important.

1.4 OBJECTIVES

1.4.1 General Objective

To assess the diversity, technological properties and probiotic potential of the dominant lactic acid bacterial flora of traditional ghee produced in different regions of Uganda.

1.4.2 Specific Objectives

1. To determine the microbial populations in traditional ghee obtained from different regions of Uganda.
2. To determine the potential probiotic properties (acid and bile salt tolerance, production of biogenic amines and auto aggregation) of the dominant LAB species isolated from the Ugandan traditional ghee.
3. To determine the antimicrobial properties of the dominant LAB isolates for potential application against potentially pathogenic microbes in food.
4. To determine the starter culture properties (growth characteristics and acid production capability) of the dominant LAB species isolated from the Ugandan traditional ghee.

1.5 HYPOTHESES

1. There is no difference in the microbial populations of lactic acid bacteria in traditional ghee obtained from different regions of Uganda.
2. There is no difference in potential probiotic properties of the dominant LAB species in traditional ghee obtained from different regions of Uganda.
3. There is no difference in antimicrobial properties of the dominant LAB species in traditional ghee obtained from different regions of Uganda.
4. There is no difference in growth and acid production properties of the dominant LAB species in traditional ghee obtained from different regions of Uganda.

CHAPTER TWO: LITERATURE REVIEW

2.1 Milk Production and Consumption in Uganda

Milk is a translucent white liquid produced by the mammary glands of mammals (Bowen & Lawrence, 2005). Milk is a non-cellular, almost complete food with no waste and ready to be consumed, it is derived from human breast, cow, goat, camel, sheep and buffaloes (Kilgour, 2005). From the beginning of history, human beings have used milk of other mammals as a food source because it was recognized that the milk of some of the domesticated mammal was equally as satisfying in meeting the physiological demands for energy and nutrients as the human milk (Korger et al., 1999). In Uganda, milk is widely consumed especially in the western region of the country and is generally used as a weaning food for infants. Uganda's milk production was estimated at 2.5 billion liters by close of the financial year 2017/2018 and this accounts for 25% of total milk supplied amongst the East African Community (Abdulsamad & Gereffi, 2016; Achan, 2018). In Uganda, milk is mainly produced and consumed in the western and central regions (171.6 and 127.5 liters per household per year, respectively) while the eastern and northern regions have the lowest production and consumption of milk in the country 98.3 and 26.7 liters per household per year, respectively (IFPRI Land management survey, 2002).

2.2 Nutrient Composition of Milk

Milk is a complex mixture of proteins, carbohydrates, vitamins, minerals and other constituents dispersed in water (Harding, 1999). On the basis of its protein content, milk is generally regarded as "nature's most nearly perfect food" owing to its rich protein profile containing more essential amino acids than any other natural food (Ogilvie, 1986). In addition, milk is an important source of mineral substances, especially calcium, phosphorus, sodium, potassium, chloride, iodine, magnesium, and small amounts of iron (Al-Wabel, 2008). Of these minerals, calcium and phosphorus constitute a larger fraction in milk; both are needed for bone growth and for proper development of newborns (Al-Wabel, 2008). Umar Dandare, Ezeonwumelu, and Gusau (2014) reported moisture content of milk in the range of 81-83%, ash content 0.73- 0.97%, crude protein in the range of 5.7-6.4%, lactose 4.2- 5.40% and lipid content 5.96%-6.8%. Cow milk is the most universal raw material for processing of dairy products for improved sensory and technological

properties resulting in the broadest spectrum of manufactured dairy products such as ghee, yoghurt, fermented milk, ice cream among others.

2.3 Natural Components in Milk

Lactoferrin, lactoperoxidase and xanthine oxidase are naturally present in milk and have some specific properties that are beneficial for the shelf life and quality of dairy products. These components are nonimmune antimicrobial proteins that have been investigated by several researchers (Grappin & Beuvier, 1997; Pakkanen & Aalto, 1997; Schanbacher et al., 1998).

2.4 Fermented Dairy Products

Fermented milk products have beneficial hypotensive, hypo-cholesterolemic and antimicrobial effects (Ohsawa et al., 2015; Shiby and Mishra, 2013), they constitute an important part of human nutrition (Adolfsson et al., 2004). Examples of fermented dairy products are discussed below.

Cheese is a generally high-quality fermented dairy product with high energy value and high fat, protein, calcium and vitamin B content (Ansorena & Astiasaran, 2016). During cheese production, milk, rennet, starter culture, and proteases and peptidases from secondary microbial flora are used to break down casein and produce bioactive compounds that are responsible for a wide range of biological activities (López-Expósito et al., 2017). Cheese's vitamin and mineral content together with bioactive peptides (antihypertensive, antioxidant, opioid, anti-proliferative and antimicrobial peptides and conjugated linoleic acids (CLA)) are mainly responsible for its effects in preventing and treating diseases (Hur et al., 2016). Cheese's anti-carcinogenic characteristics originate from CLA and sphingolipids it contains (Walther et al., 2008). CLA also helps to fight obesity by reducing energy intake, increasing energy expenditure, modulating lipid metabolism and changing skeletal muscle metabolism (Kim et al., 2016). In addition to its anti-carcinogenic and anti-obesity characteristics, one research study suggested that cheese enriched with CLA may have positive effects on many atherosclerotic biomarkers (Sofi et al., 2010).

Yoghurt, the most well-known food containing probiotics, is defined as a coagulated dairy product that is formed by lactic acid fermentation using *Lactobacillus bulgaricus* and *Streptococcus thermophilus* (Eales et al., 2015). While it has the same micronutrient composition

as milk, yoghurt contains more protein, vitamin B12 and B2, calcium, magnesium, potassium and zinc (Wang et al., 2013). During fermentation of milk to produce yoghurt, folate is synthesized; as well as the protein and CLA content, shelf life, protein digestibility, and calcium absorption all increase (Adolfsson et al., 2004). Biologically active peptides are also produced (Ivey et al., 2015).

Koumiss is slightly alcoholic fermented beverage traditionally made from unpasteurized mare's milk (Rong et al., 2015; Yao et al., 2017). Koumiss originated from the nomads of Asia and it is still commonly consumed in west and central Asian nations such as Kazakhstan, Mongolia, Kyrgyzstan, and Russia (Abdel-Salam et al., 2010; Uniacke-Lowe., 2011). Its microflora contains lactic acid bacteria (*Lactobacillus delbrueckii* subsp. *bulgaricus* and *Lactobacillus acidophilus*), lactose-fermenting yeast (*Saccharomyces* spp. *Kluyveromyces marxianus* var. *marxianus* and *Candida koumiss*), non-lactose-fermenting yeast (*Saccharomyces cartilaginous*), and non-carbohydrate-fermenting yeast (*Mycoderma* spp.) (Wszolek et al., 2006). The main microorganisms in koumiss are lactic acid bacteria that transform lactose to lactic acid, and yeasts that transform sugars to carbon dioxide and ethyl alcohol. Koumiss undergoes two main fermentations, namely lactic acid fermentation and alcohol fermentation (Chen et al., 2014), and these changes produce a distinctive sour, alcoholic flavour (Choi, 2016; Lv and Wang, 2009; Zhang and Zhang, 2012). This beverage usually contains about 2% alcohol, 0.5–1.5% lactic acid, 2–4% lactose and 2% fat (Mu et al., 2012; Sun et al., 2009). In addition to this content, it is rich in vitamins C, A, E, D, B1, B2, B12 and trace elements and antibiotics (Abdel-Salam et al., 2010). Koumiss was first used by the Mongolian people to treat diseases such as tuberculosis, ulcers, and hepatitis (Wu et al., 2009). Modern studies on koumiss have shown positive effects on the kidneys, liver, endocrine glands, blood formation, and the digestive, nervous, immune and cardiovascular systems in addition to healing effects on disorders such as anemia, avitaminosis, gastric and intestinal diseases (Mu et al., 2012; Rong et al., 2015; Sari et al., 2014). Consequently, koumiss is regarded as complete food with many health benefits (Zhang and Zhang, 2012).

Kefir is an ancient fermented milk drink with a sour, acidic, and mildly alcoholic taste and has a creamy consistency. It originated in the Caucasus (Rai, Sanjukta & Jeyaram, 2017) and is

produced by the acid-alcoholic fermentation of milk by microorganisms found in kefir grains (Kesenkas et al., 2017). Acid-alcoholic fermentation is mediated by a combination of various yeasts, acetic acid, and lactic acid bacterial strains (Adam et al., 2004). The potential health benefits of kefir are attributed to the complex microbiota created by these various microorganisms and fermentation metabolites (Bourrie et al., 2016). Because kefir has pleasing organoleptic characteristics in addition to anti-hypertensive, anticarcinogenic, hypocholesterolemic, anti-inflammatory, antimutagenic, anti-allergenic, anti-bacterial, anti-diabetic, antioxidant, and probiotic effects, it has become a focus of interest in recent years (Guzel-Seydim et al., 2011; Leite et al., 2013; Nielsen et al., 2014; Rosa et al., 2017). Regular consumption of kefir is also beneficial to intestinal health and the immune system. It alleviates symptoms of lactose intolerance by regulating serum glucose levels (Ahmed et al., 2013). A recent study by Gamba et al. (2016) has shown that kefir has anti-fungal properties by inhibiting the growth of *Aspergillus flavus*. In addition, the health benefits of bioactive compounds formed during the production of kefir have recently attracted further research attention (Adiloglu et al., 2013; Kesenkas et al., 2017).

2.5 Benefits of Consuming Fermented Dairy Products

Sour milk has enhanced sensory characteristics and prolonged shelf-life due to metabolites (organic acids, alcohol and carbon dioxide) that inhibit growth of pathogenic organisms in fermented dairy products (Vasiljevic & Shah, 2007). Raw cow's milk has all 8 essential amino acids in varying amounts, depending on stage of lactation, about 80% of the proteins in milk are caseins which are reasonably heat stable and easy to digest. People with lactose intolerance for one reason or another no longer make the enzyme lactase and so cannot digest milk sugar. This leads to some unsavory symptoms. However, fermented milk products such as yoghurt or sour raw milk, with its lactose-digesting lactobacilli, may allow people who traditionally have avoided milk to give it a trial due to the breakdown of lactose to lactic acid (Sarkiyayi & Shehu, 2011). Some of the resulting beneficial effects on human health (Jeong et al., 2015) include modification of gut microbiota and prevention and treatment of inflammatory bowel disease (Saez-Lara et al., 2015), in addition to anti-carcinogenic and hypo-cholesterolemic effects (Kapila et al., 2007). Furthermore, the conversion of the milk sugar lactose into lactic acid is one of the major changes that occurs during lactic acid bacteria induced milk fermentation (Adam et

al., 2004; Ansorena & Astiasara, 2016), and this also provides health benefits by alleviating abdominal pains and diarrhea in individuals with lactose intolerance (Ceapa et al., 2013). Fermented dairy foods, therefore, provide a variety of health benefits, such as modulating gut microbiota and immune response and lowering a person's risk of hypertension, diabetes, and high cholesterol (Linares et al., 2017).

2.6 Definition of Ghee

Ghee is a clarified butter of Indian origin commonly prepared from cow's milk or mixed milk (Sserunjogi, et al., 1998). According to the Food Safety and Standards Regulations of 2011, ghee is defined as a pure clarified fat derived solely from milk or curd or from cream to which no coloring matter or preservative has been added. The codex Standard for milk fat products on the other hand defines ghee as a product exclusively obtained from milk, cream or butter, by means of processes which result in almost total removal of water and non‐fat solids, with an especially developed flavor and physical structure. In ancient India, ghee was the preferred cooking medium and it was considered to be the healthiest source of edible fat, with many beneficial properties. In Uganda, ghee is mainly manufactured from cow's milk and it is used in cooking to enhance flavour of food and also for cosmetic purposes. Fermented ghee is also referred to as *Amashita* amongst the *Banyankole* and *Bakiga* tribes, *Omuzigo* by the *Baganda* and *Amagita* by the *Batooro* and *Banyoro* tribes.

2.6.1 Composition of Ghee

Ghee consists of a combination of saturated (approximately 65%), unsaturated (approximately 10%) and monounsaturated fat (approximately 25%). The saturated fat is primarily made from the easy-to-digest short chain fatty acids (89%). Both saturated and unsaturated fats are required in a healthy diet. Ghee also contains carotenoids which impart a yellowish colour to the product (Achaya, 1997). Ghee contains fat soluble vitamins A, D, E and K, small amounts of essential fatty acids, arachidonic acid and linoleic acid. Since the indigenous technology of ghee manufacture is quite simple, the gross composition of the various ghee products is likely to vary. The ranges in chemical composition of ghee are: 99-99.5% milk fat, 1-3% free fatty acids, 3.2-7.4 mg/g of carotene, 19-34 IU/g of vitamin A, 302-362 mg of cholesterol/100 g, 26-48 mg/g of tocopherol and less than 0.5% moisture (USDA Nutrient Database, 2016). The nutritional value

of ghee per 100 g is 99.5 g of fat, which comprises 61.9 g of saturated fat, 28.7 g of monounsaturated fat, 4 g of trans fat, 3.7 g of polyunsaturated fat, 1447 mg of Omega-3 fatty acids 2,247 mg of Omega-6 fatty acids and 256 mg of cholesterol. Non-fat nutrients include 3069 IU of vitamin A (61% RDA, 2.8 mg of vitamin E (14% RDA), 8.6 µg of vitamin K (11% RDA) (USDA Nutrient Database, 2016).

2.6.2 Global Production of Ghee

A considerable amount of ghee is consumed in many parts of the world particularly in India with an annual production amounts of 800,000 t/year (Aneja and Murthi, 1991; Sserunjogi, Abrahamsen and Narvhus, 1998). In Assyria, household consumption of ghee (*meshho*) is estimated at 60 kg of ghee every year (Abdalla, 1994). In Sudan, in the mid-1980s, a total annual consumption of ghee (*samin*) was estimated at 4500 t/year in the Khartoum province alone (Hamid, 1993). The consumption figures for most other regions of the world, where ghee is consumed are not readily available.

2.6.3 Products Related to Ghee

There are a variety of ghee related products such as *Samna*, *Meshho*, Ethiopian indigenous ghee, *Samin* and *Samuli*. *Samna* is a traditional salted butter consumed in Egypt (Abou-Donia and El-Agamy, 1993). It is mainly produced by heating the salted butter at 50-60^{o}C while stirring. The added salt precipitates the proteins in the ghee hence increasing the dry matter content of the ghee as well as enhancing its taste.

Meshho is an Assyrian non-perishable salted milkfat made by heat clarification of an indigenous butter locally known as *Zebdo* (Abdalla, 1994). *Zebdo* is obtained by churning yoghurt which is made by the addition of a yoghurt culture to boiled milk which has been cooled to about 40^{o}C. The butter is also salted and then boiled to precipitate the proteins. The fat phase is further boiled with a piece of bread until the bread becomes crispy and light brown in colour.

Ethiopian ghee is made from soured milk (Bekele & Kassaye, 1987). The soured milk is churned to butter in a smoked *Gorfa* (container woven from asparagus root fibres). Some buttermilk left in the *Gorfa* from a previous batch serves as an inoculum for the new batch. The butter is then melted in a saucepan, usually made of clay, and heated over a slow fire. Some herbs are added to flavor the product. After most of the moisture has evaporated and the ghee has been clarified, a

handful of maize, sorghum or other cereal flour is added, together with some clean fresh grass and a pinch of salt. The ghee is then decanted and kept in a container with a tight lid.

Samin is a Sudanese milk fat locally known as *Dihin or Dihn* (Hamid, 1993). It is made by fermenting milk in a gourd that has been smoked with wood from selected trees or in a container made of skin. If a gourd is used, a starter from the previous batch is added to initiate the fermentation. When a sufficient amount of butter has been collected over a few days, it is heat clarified to *Samin*. Small flat pieces of dough made from sorghum or millet are added to help clarify the product during heating. *Samin* has a characteristic pleasant flavor but if the boiling process is incomplete, it develops rancid flavor in a short time.

Samuli is a Ugandan butter made by heat clarification of a local milk fat known as *Mashita* (Sserunjogi et al., 1998). It is mainly produced by the Bahima tribe in Uganda. *Mashita* is usually made by the churning of raw fermented milk in smoked gourds to which a starter from the previous batch has been added. The churning is locally done by rocking it back and forth while held on the lap and then ripened in small gourds for 2 to 4 weeks. The *Mashita* may be washed again during the ripening period to keep it free from off-flavours resulting mainly from the putrefaction of milk proteins

2.6.4 Utilization of Ghee

The different modes of utilization of ghee have been documented from different parts of the world. In Uganda, ghee is mainly used for culinary purposes such as frying and dressing for various foods (Katimbo et al., 2015). In India, ghee is considered as a sacred article and used in religious rites (Mortensen, 2011). Ghee is heavily utilized in *Ayurveda* for numerous medical applications, including the treatment of allergy, skin, and respiratory diseases. Proper digestion, absorption, and delivery to a target organ system are crucial in obtaining the maximum benefits from any therapeutic formulation; the lipophilic action of ghee facilitates transportation of molecules to a target organ and final delivery inside the cell since the cell membrane also contains lipid (Sharma, 1990). Other uses of ghee of minor significance include ladies' hair dressing, body massage of athletes and in the formulation of indigenous pharmaceutical drugs. Many Indian medicinal preparations are made by cooking herbs into ghee. Ghee is an excellent vehicle for transporting herbs to the deeper tissue layers of the body (Lad, 1998).

2.6.4.1 Ghee as a Food

Ghee is a rapid source of energy compared with other vegetable oils. Studies have shown that absorption of ghee occurs rapidly than other vegetable fats since the lower chain fatty acids of ghee are quickly absorbed and metabolized (Nhavi & Patwardhan, 1946; Basu & Nath, 1946). Ghee improves the digestibility of proteins and improves the absorption of minerals resulting in improved growth rate. Mineral absorption from diet increases with ghee consumption. An increase in retention of calcium up to 45% and phosphorous up to 57% has been observed upon consumption of cow ghee (Steggerda & Mitchell, 1951). Ghee is a source of fat which is the storehouse of energy in the body and forms an integral part of all body cells. The fat layer beneath the skin helps in maintaining the body temperature. Delicate internal organs and some bony projections are protected against injury by thick cushioning of fatty tissues. Increased consumption of fermented dairy foods is associated with reduced low-density lipoprotein (LDL) cholesterol that would otherwise have bad effects on one's cardiovascular health (Huth and Park, 2012). Ghee is produced mainly for direct consumption and as an ingredient of food preparations including sweets and also as a frying medium for food preparations like curries, soups, vegetables, nuts, etc. It is also used in confectionary items where it is used to enhance their flavor profile.

2.6.5 Health Benefits of Ghee

The health benefits of ghee are categorized as, those that are obtained from consuming ghee as a food and those that are obtained by using ghee as a medicine. Ghee is a carrier of fat-soluble vitamins, A, D, E and K, which are needed by the body in small quantities but the body cannot make for itself. These vitamins perform many essential functions. Similarly, the essential fatty acids, which cannot be synthesized in the body, are also supplied by ghee. Milk fat components such as conjugated linoleic acid, sphingomyelin, butyric acid, ether lipids and β-carotene which have anticarcinogenic properties are also found in ghee (AkalIn & Tokusoglu, 2003; Khanal & Olson, 2004).

2.7 Methods of Ghee Production

Methods of ghee manufacture vary with respect to the material used (milk, cream, butter), the intermediate treatment of raw materials, and the handling of the semi-finished or fully formed

ghee. Four methods of ghee making are known: the indigenous milk butter (MB) process, the direct cream (DC) method, the cream butter (CB) method, and the prestratification (PS) method. Ghee may also be made from fresh cream using the CB, DC or PS methods but such ghee lacks the characteristic flavor.

In the MB method, also known as the desi method, sour whole raw milk is churned to butter. The butter is then converted to ghee (Munro et al., 1992; Podmore, 1994). The MB method, as applied in the homes in India, involves the souring of raw milk in earthenware vessels which have been used previously as a milk container, and which contain an inoculum of bacteria within pores of the wall. After addition of more milk over successive days, the fermented milk is churned to butter. The butter is then boiled in an open pan to allow evaporation of the water without charring the proteins. The ghee is transferred while hot and stored in earthen vessels (Munro et al., 1992).

In the DC, which is a technologically improved method, fresh cream, cultured cream or washed cream may be used (Rajorhia, 1993). This process omits the butter production step as the cream is directly converted to ghee. The limitations of the DC method include the long heating time required to remove the moisture and the production of a highly caramelized flavor in the ghee due to the presence of a high content of SNF in the cream during heating.
The CB method is a three-step process of ghee manufacture whereby milk is separated into cream, which is then churned into butter or butter granules which are then processed into ghee (Van den Berg, 1988).

The PS method which is also known as the clarified butter method' "induced-stratification method" or the 'stratification method' is particularly suitable if large quantities of butter are available (Van den Berg, 1988). The method as described by Rajorhia (1993), involves melting butter at 80-85^{o}C for 30 min to separate it into the top layer composed of floating denatured protein particles and impurities probably suspended by entrapped air bubbles, the middle layer of almost clear fat, and the bottom layer of buttermilk serum. The buttermilk is drained off and the fat layer is heated together with the top layer to a temperature of 110^{o}C to remove more moisture and develop flavor. The removal of buttermilk eliminates the need for prolonged heating for

evaporation of the moisture. The PS method has been reported to save fuel consumption, time and labour, and to produce ghee with low FFA levels and acidity. This method is further reported to produce ghee with a mild flavour (Rajorhia, 1993). Ghee made by the PS method has a relatively high-water content, but still believed to be too low to support microbial growth. Hence, its rapid deterioration is attributed to oxidative reactions (Van den Berg, 1988).

2.7.1 Ghee Production in Uganda

Ghee is made from the milk of a cow that has been lactating for at least one month. Ghee production in Uganda is still greatly traditional and the ghee-making practices amongst the different cattle keeping communities differ in how the milk is handled before churning to separate the butterfat. The fresh milk first goes through stages of either boiling or separation, followed by addition of drops of pre-fermented milk (*Bongo*). This process is known as seeding and *Bongo* is added to initiate fermentation. Milk and/or cream are then allowed to ferment for up to twelve hours and butter is separated by churning in a gourd (Sempiira et al., 2017). Churning is the process of shaking/mixing whole milk or cream to coagulate the fat into large chunks thereby forming butter. The butter formation process occurs in three phases and the process takes between 30-60 minutes depending on the speed, gourd size and milk quantity. The first ten minutes (phase one), involve vigorous shaking whilst removing the lid of the gourd to let out the pressure that has forms inside. In phase two, shaking of the gourd is slightly reduced since butter forms in the process and covers the utensil surface with small droplets that finally coagulate to form bigger pieces. The last stage (phase three) is associated with slower rotation of the gourd to allow the bigger pieces of butter fat to coagulate into single larger pieces, which enhances separation of butter from the milk residue. The milk residue is poured out of the gourd through a sieve, separating it from the butter fat (Sempiira et al., 2017).

The butter separated from the milk residue is washed to remove dirt and residual milk. Washing involves rinsing small portions of butter at a time, with clean water while pressing in between palms to squeeze out any milk left. The butter is then stored to ripen for at least one week. Ripening involves keeping the butter in small calabashes or well-closed containers at room temperature to allow native microorganisms to mediate reactions that yield the desirable aroma and colour (Ongol & Asano, 2009). The butter is then clarified by heating to reduce the moisture

content and remove contaminants to yield ghee. The shelf life for ghee is about one year as reported by Sempiira et al., 2017.

2.8 Antimicrobial Activity of the Microflora of Fermented Dairy Products

Food choices are now not only driven by the taste and nutritional value, but also influenced by the safety and storability (Georgieva et al., 2014). This has led to increased research interest in lactic acid bacteria since they are capable of acting as both probiotics and they produce bioactive compounds, which act as antimicrobial agents (Saarela et al., 2002). For instance, the lactic acid produced by LAB lead to reduced intracellular pH thus inhibiting vital cell functions of undesirable sensitive microorganisms (Kivanc et al., 2011). Therefore, the acidic pH of lactic acid fermented foods offers protection against food-borne pathogens such as *Escherichia coli, Salmonella typhimurium, Listeria monocytogenes, Salmonella enteritidis* hence improving the safety and shelf life of food products (Gong et al., 2010; Rzepkowska et al., 2016; Simova et al., 2009). Other antimicrobial compounds produced by lactic acid bacteria include ethanol, formic acid, fatty acids, hydrogen peroxide and bacteriocins (De Vuyst & Leroy, 2007). However, the antimicrobial effects of LAB are more prominent against Gram-positive bacteria than the Gram-negatives (Rzepkowska et al., 2016).

2.9 Starter Culture Properties of Dairy Microflora

Studies indicated that lactic acid bacteria such as *Lactobacillus plantarum* and *Lactobacillus fermentum* can retain the ability to produce antimicrobial agents even after lyophilization (Georgieva et al., 2014). This means that *Lactobacillus* strains can be freeze dried and used as active dry starter and probiotic cultures in food production. For example, they could be used as starter cultures in the production of bakery products against *Bacillus cereus* which causes ropiness in bread and other bakery products (Denkova et al., 2014; Menteş et al., 2007). Therefore, the use of starter culture strains of lactic acid bacteria with inhibitory effects against *Staphylococcus aureus*, *Escherichia coli* and other undesirable microorganisms may provide a safe alternative in food preservation.

2.10 Lactic Acid Bacteria as Probiotics

In order to offer health benefits in the body, probiotic bacteria must survive the physical and chemical stresses along the intestinal tract such as low pH of the gastric acids and bile salts (Jensen et al., 2012; Vandenplas et al., 2014). Studies have shown that some of the lactic acid bacteria for instance *L. plantarum*, *Lactobacillus brevis* and *Pediococcus pentosaceus* can survive these stresses to serve as probiotics in the body (Hamon et al., 2014; Rzepkowska et al., 2016; Verónica et al., 2016; Vries et al., 2006). LAB as probiotic bacteria have been reported to exhibit high enzymatic activities of β-galactosidase, leucine arylamidase, naphtol-AS-BI-phosphohydrolase and valine arylamidase but with low ability to produce α-chymotrypsin and low activities of esterase, esterase lipase, and lipase thus they are weakly lipolytic (Rzepkowska et al., 2016; Stojanovski et al., 2013).

2.11 Evaluation of probiotic and technological properties of LAB

Earlier studies have documented different methods for evaluation of the technological and probiotic potential of LAB. For instance, the probiotic properties of the LAB were determined by Byakika by evaluating the acid and bile salt tolerance, production of biogenic amines and auto aggregation of the dominant LAB (Bao, Zhang, Zhang, Liu, Wang and Dong, 2010; Byakika et al., 2019). Acid and bile salt tolerance have been evaluated by culturing the isolates in MRS broth for 24 h at 30^{o}C. The culture was diluted to 6 log cfu/ml and spiked in 10 ml of MRS broth acidified to pH 3 using concentrated HCl. The inoculated test tube was incubated at 30ºC and cell counts enumerated at 0, 1, 2 and 3 h of incubation (Tambekar & Bhutada, 2010; Bao et al., 2010).

For bile salt tolerance has been document by subjecting the microbial samples to of MRS broth (pH=7.8) containing 0.7% ox bile. The inoculated broth is usually incubated at 30ºC and cell counts are enumerated at 0, 3, 6 and 9 h of incubation by spread plating serial dilutions of the sample on MRS agar (Tambekar & Bhutada, 2010; Guo, Kim, Nam, Park, & Kim (2010). Frengova and collegues valuated the biogenic amines production by growing the bacterial samples on decarboxylation medium containing 3 g/L yeast extract, 1 g/L glucose and 0.016 g/L bromocresol purple and 5 g/L of corresponding amino acids. The amino acids used included L-histidine, L-tyrosine, L-lysine, L-phenylalanine, L-arginine and L-ornithine. The pH of the

medium was adjusted to 6.1 using 1 M NaOH. Then, 1 ml of sterile paraffin was overlaid on the inoculated tube to create anaerobic conditions. Decarboxylation medium without added amino acids was used as a negative control (Frengova, Simova, Beskova, & Simov, 2000).

Auto aggregation has been determined by culturing the LAB isolates in MRS broth for 24 h at 30^{o}C (~7 log cfu/ml). The culture was gently homogenized and 4 ml pipetted into a clean 50 ml universal tube. The tube was vortexed for 10 s and left to stand at 25°C for 24 h. Then, 0.1 ml of the upper phase was separately taken at 5 and 24 h intervals, and added to a tube containing 3.9 ml of quarter strength ringer's solution and its absorbance read at 600 nm. Percentage auto-aggregation was calculated as [(A0–A1)/A0] x 100, where A0 is absorbance at 0 h and A1 is absorbance at 5 or 24 h (Doyle & Rosenberg, 1995; Canzi, Guglielmetti, Mora, Tamagnini, & Parini, 2005).

Screening for antimicrobial activity of the LAB isolates has been documented by the E-test method (Abbiodisk) using De Man Rogosa-Sharpe agar (MRS) for *Lactobacillus* spp. and *Enterococcus faecium* (Lin, Yu, Jang, & Tsen, 2007; Florez, Ammor, Mayo, van Hoek, Aarts & Huys, 2008). For each of the LAB isolates, 10-fold dilutions of the culture grown at 30^{o}C, 24 h were performed to obtain 10-50 colonies on a plate. The colonies were overlaid with 0.7% BHI agar (Oxoid, UK), seeded with 10^5 cfu/ml of the indicator bacterial strain. The plates were allowed to solidify at room temperature for 10 min, incubated at 37^{o}C, 24 h and then examined for distinct colonies surrounded by a clear halo. Antimicrobial activity has also been documented using the paper disc diffusion assay (Mugampoza et al., 2020). Each of the LAB isolates were grown in 10 ml MRS broth at 30^{o}C, 24 h. The culture was centrifuged (8000 rpm, 15 min) at 4^{o}C to obtain the cell-free supernatant (CFS), which was used to screen the isolates for the possible mechanisms of antimicrobial activity. The CFS were sterilized by membrane filtration and stored at 4^{o}C until use. The indicator bacterial strains were then grown in 10 ml BHI broth (Oxoid) at 37^{o}C, 24 h. Petri dishes were filled with ~10 ml of BHI agar (Oxoid) were overlaid with 10 ml of 0.7% BHI agar (Oxoid) seeded with 10^5 cfu/ml of the indicator bacterial strain.

CHAPTER THREE: METHODOLOGY

3.1 Sampling and Sample Handling

A total of nine samples (Table 1) were used in the study. Eight (08) samples of fully ripened traditional ghee (~2 weeks) were purchased in original packaging, from local markets in different districts of Uganda representing different regions of the country i.e; the central, eastern, mid-western and south western regions. The samples representing the central region (Buganda) were collected from Kiko market in Mukono & Mulukoola market in Luweero, Mid-Western (Bunyoro) samples were picked from Masindi central market and a dairy in the town, South western (Ankole) samples were picked from Mbarara central market & Kizungu market while the samples representing the eastern region (Teso) were drawn from Soroti central market & Kenya market. A commercial reference ghee sample was bought from Sameer Livestock and Agriculture Limited. Each of the samples was placed in a sterile stomacher bag, labelled and transported to Makerere University School of Food Science, Nutrition and Bioengineering Microbiology laboratory under ice. Samples were stored at 4^{o}C in a refrigerator until analysis within 8-24 h.

Table 1 Ghee samples used in the study

Ghee sample	Region	No. of samples
Amashita	Ankole	02
Omuzigo	Buganda	02
Amagita	Bunyoro	02
Akinyet	Teso	02
Commercial	Industry	01
Total		09

3.2 Microbial Analysis

The samples were microbially investigated to obtain an overview of the diversity of the microflora in the different ghee varieties in order to focus on the dominant microorganisms.

3.2.1 Culture Media

M17, de Man Rogosa Sharpe (MRS), violet red bile lactose (VRBL), KF *Streptococcus*, potato dextrose and nutrient agar were used for selective enumeration of lactococci, total lactic acid bacteria, total coliforms/ *E. coli*, streptococci, yeasts and molds, and total viable counts, respectively. All media were obtained from Oxoid (United Kingdom), prepared according to manufacturer's instructions and stored in a cold room at 4^{o}C until use.

3.2.2 Sample Preparation and Enumeration of Viable Counts

The ghee sample (25 g) was weighed into a stomacher bag, diluted in 225 ml of sterile peptone water and homogenized using a stomacher machine at 230 rpm for 2 min to obtain a sample with dilution factor 10^{-1}. Further 10-fold dilutions were prepared up to 10^{-9}. Samples (0.1 ml) of each dilution were transferred in duplicate on the various media plates and carefully spread on the media using an L-shaped disposable spreader. All plates were incubated under appropriate conditions of temperature, aeration, and incubation time (Table 2).

Table 2 Incubation conditions for various inoculated media

Medium	Temperature (^{o}C)	Time	Environment	Target
Nutrient agar	30	48 h	Aerobic	Total plate counts
M17 agar	30	48 h	Aerobic	*Lactococcus*
KF *Streptococcus* agar	30	48 h	Aerobic	*Enterococcus*
VRBL agar	30/44.5	24 h	Aerobic	Coliforms/ *E. coli*
MRS agar	30	48 h	Anaerobic	Total LAB
Potato dextrose agar	25	3-5 days	Aerobic	Yeasts & molds

After incubation, plates with colonies in the range 30-300 were enumerated using a colony counter (Stuart Scientific) and the viable count was derived using the following formula:

CFU/g = count x 1/dilution factor x 1/sample volume plated

3.2.3 Microbial Purification and Isolation

Three colonies with different morphologies (colour, shape, size and texture) were randomly selected from each countable plate giving a total of 123 isolates. These isolates were streaked twice on respective selective media for purification. The isolates were stored in brain heart infusion (BHI, Oxoid) broth with the addition of 20% glycerol at -25^{o}C until further analysis.

3.2.4 Presumptive Identification Based on Biochemical Characteristics

3.2.4.1 Gram Staining

Gram staining was performed according to the method described by Mugampoza et al. (2020). A drop of peptone water was placed on a glass slide in which a microbial colony was dispersed to form a smear which was then spread on the glass slide using a sterile wire loop. The smear was air dried for approx. 15 min at room temperature and heat fixed near a Bunsen flame. The smear was irrigated with the primary stain crystal violet for 1 min, and then washed it off with tap water. Lugol's iodine was then applied for 30 s and excess iodine was washed off with tap water. Absolute ethanol was applied for 1 min and the excess washed off with tap water. Finally, the smear was counter stained with the safranin for 30 s, the excess washed off with tap water and the slide allowed to air-dry for about 15 min at room temperature. Gram reaction and cell shape were examined using a light microscope at X1000. *Escherichia coli* and *Staphylococcus aureus* (Table 3) were used as the Gram-negative and Gram-positive controls, respectively.

3.2.4.2 Catalase Test

A single colony from a 24 h plate culture was transferred to a clean slide using a wire loop. Then, a drop of 30% hydrogen peroxide was added to the slide using a plastic dropper and observed for bubbling. Bubbling was taken as positive catalase test while no bubbling was considered as negative. *Pseudomonas flourescens* and *Lactobacillus plantarum* (Table 3) were used as positive and negative controls, respectively.

3.2.4.3 Oxidase Test

Production of cytochrome oxidase enzyme was detected using an oxidase identification strip. One end of the strip paper was rolled on the colonies from a 24 h plate culture and left to stand for 1 min. A color change from pink to purplish-black was considered as the positive oxidase

test. *Pseudomonas flourescens* and *Lactobacillus plantarum* (Table 3) were used as oxidase positive and negative controls, respectively.

Table 3 Bacterial strains used as test controls

Control strain	Test	Source of strain
Staphylococcus aureus	Gram-positive	Mak, Food Science
E. coli	Gram-negative	"
Ps. fluorescens	Catalase and oxidase positive	"
L. plantarum	Catalase, oxidase negative	"

3.3 Confirmation of Bacterial Species Using Polymerase Chain Reaction

3.3.1 DNA Extraction

Template DNA for amplification of the 16S rRNA gene was extracted using the cetyl trimethyl ammonium bromide (CTAB) method as described by Mugampoza et al. (2020). Each of the bacterial isolates was grown in 10 ml of the respective selective media broth (Table 2) for 24 h at 30-37^{o}C. Cells from 1 ml of the culture were harvested by centrifugation at 8000 rpm for 5 min. The cell pellet was resuspended and washed twice by spinning at 8000 rpm at 4^{o}C with 1 ml ice cold TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5).

Then, 30 µl of 20% sodium dodecyl sulphate (SDS) solution was added followed by 100 µl of CTAB solution. The mixture was incubated at 65ºC for 10 min and 967 µl of 24:1 chloroform: isoamyl alcohol added. The solution was mixed thoroughly and centrifuged at 13,000 rpm for 5 min. The upper phase supernatant (500 µl) was transferred into a clean microcentrifuge tube, 500 µl of ice-cold isopropanol added and gently mixed for 1 min to precipitate the DNA. The DNA was recovered by centrifugation at 13,000 rpm for 5 min at 4^{o}C. Then, the DNA pellet was washed twice in 500 µl of 70% absolute ethanol, air dried for 30 min at room temperature, re-suspended in 100 µl TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5) and stored at -25^{o}C in a freezer until use.

3.3.2 Measurement of DNA Concentration

The concentration of DNA in the extract was measured using a NanoDrop spectrophotometer. Sample carryover between successive measurements was minimized by wiping the sample from the pedestals using a lens cleaning tissue. TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5) was used as the blank. The DNA extract was diluted with TE buffer to a working concentration of approx. 100 ng/µl. A 2 µl aliquot of the diluted DNA extract was used for confirmation of presence of DNA in the extract by electrophoresis on a 1% agarose gel prepared with 1X TAE buffer [40 mM Tris base, 20 mM glacial acetic acid, 1 mM EDTA, 0.2 µg/ml Ethidium bromide, pH 8]. TAE (1X) was used as the running buffer for approx. 30 min at 90 V.

3.3.3 Amplification of the 16S rRNA Gene

Primers V3F and V3R (Table 4; Mugampoza et al., 2020) were used to amplify the variable V3 region of the 16S rRNA gene giving a PCR product of 200 bp. The reaction mixture (final volume, 50 µl) contained 5 µl of 10X PCR buffer (10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl, pH 8.3); 2.5 mM deoxynucleotide triphosphates; 0.2 pmol/µl (each) forward and reverse primers; 1.25 U of *Taq* DNA polymerase; and 1 µl of template DNA. The sample was amplified in a PCR thermocycler as follows: DNA denaturation for 5 min at 94^{o}C followed by a touchdown PCR performed as follows: initial annealing temperature 66°C, and this decreased 1°C every cycle for 10 cycles; finally, 20 cycles were performed at 56°C. The extension for each cycle was carried out at 72°C, 3 min, while the final extension was at 72°C, 10 min.

Table 4 Primers and their target sequences in the PCR reactions

Primer name	Sequences	Gene location	Target
V3F	5'-CCTACGGGAGGCAGCAG-3'	341-357	V3 region of 16S rRNA (forward primer)
V3R	5'-ATTACCGCGGCTGCTCG-3'	518-534	V3 region of 16S rRNA (reverse primer)

Source: Mugampoza et al. (2020)

3.3.4 Gel Electrophoresis of PCR Amplicons

Each of the PCR amplicon (10 µl) was mixed with 2 µl of 6X loading dye and run on a 2% agarose gel containing 0.2 µg/ml ethidium bromide in 1X TAE buffer at 75 V, 2 h. A 100 bp DNA ladder was used as the molecular size marker. The gel was visualized on a UV transilluminator, and images recorded with Quantity One Gel Doc software.

3.3.5 Sequencing and Database Search

Samples (40 µl) containing the PCR products (72 in total) were labelled and transported to Macrogen Meibergdreef 31, Amsterdam (Netherlands) for purification and sequencing. In order to establish the closest known relatives of the PCR sequences, database searches were performed using the basic local alignment search tool (BLAST). Similarity variables (% ID and e-value) of the most closely related species were retrieved from NCBI gene bank.

3.3.6 Cluster Analysis

16S rDNA PCR gel images were processed using MEGA X software to generate the dendrogram. Genetic clustering (evolutionary history) was inferred using the un-weighted pair group method with arithmetic averages (UPGMA). The evolutionary distances were computed using the Maximum Composite Likelihood method involving 72 nucleotide sequences.

3.4 Determination of Probiotic Properties of the Ghee LAB Isolates

Probiotic properties (acid and bile salt tolerance, production of biogenic amines and auto aggregation) of the dominant LAB isolates were determined according to the method of Byakika et al. (2019).

3.4.1 Acid and Bile Salt Tolerance

The LAB isolates were separately grown in MRS broth for 24 h at 30ºC. The culture was diluted to 6 log cfu/ml and spiked in 10 ml of MRS broth acidified to pH 3 using concentrated HCl. The inoculated test tube was incubated at 30ºC and cell counts enumerated at 0, 1, 2 and 3 h of incubation as in section 3.2.2.

For bile salt tolerance, 1 ml of the overnight MRS broth culture was transferred into 10 ml of MRS broth (pH=7.8) containing 0.7% ox bile. The inoculated broth was incubated at 30ºC and cell counts enumerated at 0, 3, 6 and 9 h of incubation by spread plating serial dilutions of the sample on MRS agar. All plates were incubated at 30 ºC for 48 h and viable counts enumerated as in section 3.2.2.

3.4.2 Production of Biogenic Amines

Production of biogenic amines was assayed on decarboxylation medium containing 3 g/L yeast extract, 1 g/L glucose and 0.016 g/L bromocresol purple and 5 g/L of corresponding amino acids. The amino acids used included L-histidine, L-tyrosine, L-lysine, L-phenylalanine, L-arginine and L-ornithine. The pH of the medium was adjusted to 6.1 using 1 M NaOH. The medium was autoclaved at 121°C for 15 min and 10 ml separately inoculated with 0.1 ml of each LAB isolate. Then, 1 ml of sterile paraffin was overlaid on the inoculated tube to create anaerobic conditions. Decarboxylation medium without added amino acids was used as a negative control. All tubes were incubated at 30°C for 5 days and decarboxylase activity indicated by a deep purple coloration of the medium.

3.4.3 Determination of Auto Aggregation

Each of the LAB isolates was cultured in MRS broth for 24 h at 30ºC (~7 log cfu/ml). The culture was gently homogenized and 4 ml pipetted into a clean 50 ml universal tube. The tube was vortexed for 10 s and left to stand at 25 °C for 24 h. Then, 0.1 ml of the upper phase was separately taken at 5 and 24 h intervals, and added to a tube containing 3.9 ml of quarter strength ringer's solution and its absorbance read at 600 nm. Percentage auto-aggregation was calculated as $[(A_0–A_1)/A_0] \times 100$, where A_0 is absorbance at 0 h and A_1 is absorbance at 5 or 24 h.

3.5 Determination of Antimicrobial Activity of the Isolates

This was done using the plate agar overlay and paper disc diffusion methods as described by Mugampoza et al. (2020).

3.5.1 Antimicrobial Activity Using the Agar Overlay Method

Screening for antimicrobial activity of the LAB isolates was conducted against selected indicator bacterial strains (Table 5). For each of the LAB isolates, 10-fold dilutions of the culture grown at 30^{o}C, 24 h was performed to obtain 10-50 colonies on a plate. The colonies were overlaid with 0.7% BHI agar (Oxoid), seeded with 10^5 cfu/ml of the indicator bacterial strain. The plates were allowed to solidify at room temperature for 10 min, incubated at 37^{o}C, 24 h and then examined for distinct colonies surrounded by a clear halo.

Table 5 Bacterial strains used for testing the antimicrobial activity of LAB isolates

Indicator strain	Source
Staphylococcus aureus	Makerere University, Food Science
Salmonella enterica	"
Escherichia coli ATCC 25922	"

3.5.2 Antimicrobial Activity Using the Paper Disc Diffusion Assay

The protocol of Mugampoza et al. (2020) was followed. Each of the LAB isolates was grown in 10 ml MRS broth at 30^{o}C, 24 h. The culture was centrifuged (8000 rpm, 15 min) at 4^{o}C to obtain the cell-free supernatant (CFS), which was used to screen the isolates for the possible mechanisms of antimicrobial activity. The CFS was sterilized by membrane filtration and stored at 4^{o}C until use. The indicator bacterial strains (Table 5) were grown in 10 ml BHI broth (Oxoid) at 37^{o}C, 24 h. Petri dishes filled with ~10 ml of BHI agar (Oxoid) were overlaid with 10 ml of 0.7% BHI agar (Oxoid) seeded with 10^5 cfu/ml of the indicator bacterial strain.

The agar overlay was allowed to set at room temperature for 15 min. An antibiotic filter paper disc (Whatman, 13 mm) was soaked in the supernatant for 20 min. The disc was applied to the seeded plate, allowed to set at room temperature (15 min) and incubated at 37^{o}C, 24 h. The diameter (mm) of the zone of inhibition was measured with a meter ruler as the distance from the edge of the paper disc to the edge of the clear zone. Clear zones extending up to 11 mm or more were considered as positive result.

In order to determine whether or not antimicrobial activity was due to presence of organic acids or bacteriocins produced by the ghee LAB isolates, the experiment was repeated using CFS that was neutralized by adjusting its pH to 6.5 using 1 M NaOH (CFS-N) and CFS treated with 1mg/ml proteinase K and incubated at 37^{o}C, 3 h (CFS-P). Each of the treated CFS was filter sterilized prior for use in the experiment as described in section 3.5.2. Antibiotic filter paper disc soaked in sterile MRS broth was used as the negative control.

3.6 Determination of Starter Culture Properties

3.6.1 Growth Kinetics

Two ghee LAB isolates (*L. rhamnosus* spp.) were separately spiked into 50 ml of sterile cows' UHT milk (Jesa brand) in a 100 ml sterile conical flask to attain an initial cell count of ~3 $\log_{10}$ CFU/ml. The flasks were incubated at 30^{o}C. Aliquots (0.1 ml) were withdrawn at different time intervals, transferred into 1.5 ml Eppendorf tubes and used for enumeration of viable counts on MRS agar as described in section 3.2.2. The experiment was conducted in duplicate. Un-inoculated milk (50 ml) incubated under similar conditions was used as the control.

3.6.2 Acid Production Capacity

3.6.2.1 pH Measurement

Two milli-liter aliquots taken at different time intervals (section 3.6.1) were analyzed for pH using a digital pH meter. The pH meter probe was calibrated using standard buffers (pH 4 and 7) prior to measuring sample pH. Sample pH was measured by inserting the tip of the probe into the sample for approx. 2 min until a stable reading was registered on the pH meter scale.

3.6.2.2 Percent Titratable Acidity

Percent titratable acidity (TTA) of the samples taken at different time intervals (section 3.6.1) was determined by titrating 10 ml of the sample against 0.1 M Sodium hydroxide solution using phenolphthalein indicator.

Thus, % TTA = titre volume x 10

3.7 Statistical Analysis

All microbial counts were converted to log cfu/ml. Means, standard deviations and analysis of variance (ANOVA) were computed using SPSS software. Means were separated using the least significant difference (LSD) test at $p<0.05$.

CHAPTER FOUR: RESULTS AND DISCUSSION

4.1 Diversity of the Microbial Populations in Ugandan Traditional Ghee

4.1.1 Microbial Counts

Generally, microbial counts of traditional ghee obtained from different regions of Uganda were not significantly ($p>0.05$) different from each other (Table 6). Ghee from Bunyoro had the highest total plate counts (7.83 log cfu/g) while the commercial sample had the lowest (4.85 log cfu/g) (Table 6). Total coliforms and *Escherichia coli* were only detected in ghee from Teso. Ghee from Bunyoro had the highest total lactic acid bacteria (LAB) and *lactococci* while that from Buganda had the lowest for each. *Enterococci* were highest in traditional ghee from Bunyoro (2.16 log cfu/g), followed by that from Ankole (2.04 log cfu/g) Teso (1.47 log cfu/g), and were undetected in ghee samples from Buganda. Yeast & moulds were highest in traditional ghee from Bunyoro and lowest in that from Teso while they were undetected in the commercial ghee sample. Commercial ghee had the lowest level of almost all the tested parameters implying that it was not highly contaminated due to proper hygienic handling compared to the traditional ghee.

Table 6 Microbial counts (log cfu/g) of traditional ghee (n=9) obtained from different regions of Uganda

	TPC	Total coliforms	*E. coli*	LAB	*Lactococcus*	Enterococci	Yeasts & Moulds
Bunyoro	7.83^{a}	0.00^{a}	0.00^{a}	7.58^{a}	7.27^{a}	2.16^{a}	6.04^{a}
Ankole	7.15^{ab}	0.00^{a}	0.00^{a}	6.45^{ab}	6.32^{a}	2.04^{a}	6.48^{a}
Teso	4.81^{b}	1.59^{a}	0.97^{a}	3.91^{b}	3.82^{a}	1.47^{a}	1.47^{ab}
Buganda	6.15^{ab}	0.00^{a}	0.00^{a}	5.69^{ab}	5.37^{a}	0.00^{a}	3.47^{ab}
Commercial	4.85^{b}	0.00^{a}	0.00^{a}	0.00^{c}	0.00^{b}	0.00^{a}	0.00^{b}

Values are means of three independent samples from each region. Values in columns with similar superscript letters are not significantly different ($p>0.05$). 0.00: not detected, Limit of detection = 1 log cfu/g

The low level of total plate counts in commercial and traditional ghee from Teso could be due to better observance of hygiene in handling of raw materials and/or adequate thermal processing of milk used in ghee processing (Wangalwa et al., 2016). The major sources of microbial contamination during milk processing include the milking area & containers, cow dung, unhygienic processing and handling of processed dairy products (Wangalwa et al., 2016). These factors could be controlled by proper treatment of animals and providing enough withdraw period as recommended by veterinary personnel, hygienic milking and handling of milk and milk products (Hayes and Boor, 2001; Mufandaedza et al., 2006). The commonest examples of spoilage and pathogenic microorganisms in milk and fermented dairy products include *Escherichia coli*, *Salmonella enteritidis, Listeria monocytogenes* and *Staphylococcus aureus* (Kanak and Yilmaz, 2019). As coliforms and *E. coli* are indicators of faecal contamination, the presence of these organisms in the ghee samples from Teso creates a need to establish the specific pathogenic organisms present in these samples. The absence of coliform bacteria in all other ghee samples could be attributed to either adequate thermal processing or production of antimicrobial agents such as lactic acid and hydrogen peroxide by the inherent LAB species (Schlegelova et al., 2003), that might have inhibited the growth of these organisms in the ghee.

The major study on microbial communities present in Ugandan ghee was conducted by Ongol and Asano (2009). These authors reported that the product is mainly dominated by yeasts and lactic acid bacteria (LAB) including *Lactobacillus paracasei, L. helveticus, L. plantarum* and *L. perolens* constituting 37.3%, 10.1%, 8.1% and 7.7%, respectively. These authors also reported that *Acetobacter aceti*, *Ac. lovaniensis, Ac. orientalis* and *Ac. pasteurianus* are the main species of acetic acid bacteria (AAB) identified in the ghee whereas *Bifidobacterium* sp., *Enterococcus faecium, L. brevis, L. helveticus, L. acetotolerans, Lactobacillus* sp., *Lactococcus raffinolactis, Lactococcus* sp. and *Streptococcus salivarius* are other strains of LAB in the product.

The fermentation process plays a role in eliminating some of these organisms due to production of antimicrobial metabolites such as lactic acid and bacteriocins by the fermentative LAB (Azhari, 2010), which extends the shelf-life of fermented dairy products as well as preserving their nutritional value (Zacharof & Lovitt, 2012). During ripening, LAB protect fermented food products against spoilage microorganisms and enhance their organoleptic attributes (Liu et al.,

2011; Zacharof & Lovitt, 2012). In ghee, the most detected yeast species include *Brettanomyces custersianus, Candida silvae, Geotrichum candidum, Issatchenkia occidentalis, Issatchenkia orientalis, Kluyveromyces marxianus, Saccharomyces cerevisiae,* and *Trichosporon asahii* whose metabolic products contribute to the flavor of the fermented ghee (Ongol & Asano, 2009). LAB and yeasts participate in the fermentation process (Narvhus & Gadaga, 2003). These organisms may be inherent in raw milk or may enter the product as a result of cross contamination from calabashes used in the process of ghee making and ripening (Mukisa & Kiwanuka, 2018). The flavor of fermented dairy products is mainly determined by the secondary non-starter microorganisms involved in their ripening. The major steps in ghee flavor development by LAB include metabolism of lactose, lactate and citrate, lipolysis that liberates free fatty acids, and proteolysis of casein followed by amino acid catabolism (McSweeney & Sousa-Gallagher, 2000). It is these metabolites that mostly contribute to the flavor and antimicrobial properties of ghee and other fermented dairy products (Mukisa & Kiwanuka, 2018).

Given that the commercial ghee sample in this study had the lowest microbial load, this suggests that the method of processing and level of equipment and process hygiene could be the main factors influencing the microbial composition of ghee (Wangalwa et al., 2016), especially for coliforms, LAB, as well as yeasts and moulds as presented in Table 6. However, strict hygiene in fermented dairy products may remove microflora that contribute to their sensory quality attributes. For instance, it was reported that the desirable flavor in traditional ghee is mostly attributed to the diversity of microorganisms such as LAB and fungi that participate in the biosynthesis of the flavors (Sserunjogi, et al., 1998). *Enterococci* have not been considered as "generally recognized as safe" (GRAS) substances (Giraffa, Carminati & Neviani, 1997) and have been associated with negative effects such as potential pathogens associated with endocarditis (Aguirre & Collins, 1993).

Thus, the presence of *enterococci* such as *E. faecalis, E. faecium,* and *Streptococcus bovis* in dairy products, in large numbers (up to 10^7 to 10^8 cfu/g) has been associated with poor hygiene during milk production and processing (Tsakalidou, Manolopoulou, Tsilibari, Georgalaki, & Kalantzopoulos, 1993). Therefore, levels of enterococci in fermented dairy products has been

suggested to be an indicator of poor hygiene (Gatti, Borio, Fornasari, & Neviani. 1993). However, some enterococci, mainly *E. faecium*, have been adopted as starter cultures in cheese making (Dahlberg & Kosikowsky, 1948; Thunell, & Sandine, 1985), as commercial silage inoculants (Seale et al., 1986; Giraffa, Picchioni, Neviani, & Carminati, 1995) and as probiotics (Fernandes, Shahani, & Amer, 1987; Fuller, 1989). Given the above results, there was a need to identify the species of the different microbial groups enumerated in the different ghee samples.

4.1.2 Presumptive Identification of the Bacterial Isolates

Overall, 123 microorganisms were isolated as described in section 3.2.3 from the different culture media of which 76 isolates (61.8%) were bacteria and the rest were yeasts. Only bacterial isolates were studied further and results of their conventional and molecular identification are presented in sections 4.2.2-4.2.3.

The bacterial isolates were examined for colony characteristics, catalase and oxidase reactions, Gram stain and microscopic cell shape, and categorized into four groups (Table 7). *Lactobacillus* and *clostridium* which were presumptively identified as the dominant (50%) bacterial isolates. These isolates formed cream, round, shiny and smooth colonies on M17 and MRS agar. They were Gram positive rods, catalase and oxidase negative and presumptively identified as *Lactobacillus* or *Clostridium* spp. according to the scheme given in www.bacteriainphotos.com. *Streptococcus* and *Enterococcus* isolates formed red, round and shiny colonies on KF *Streptococcus* agar. They were found to be Gram-positive, catalase and oxidase-negative cocci and presumptively identified as *Streptococcus or Enterococcus*. *Bacillus, Listeria* and *Corynebacterium* were Gram-positive rods, catalase positive and oxidase negative. Group 4 comprised of only one isolate which was found to be Gram-positive rods, catalase and oxidase positive. This isolate could not be presumptively identified based on these characteristics.

Table 7 Presumptive identification of bacteria isolated from Ugandan traditional ghee based conventional microbiology methods

Group	Catalase	Oxidase	Gram stain	Cell shape	Number (%) of isolates	Presumptive identification
1	-	-	+	rods	38 (50)	*Lactobacillus, Clostridium*
2	-	-	+	cocci	21 (27.6)	*Streptococcus, Enterococcus*
3	+	-	+	rods	16 (21.05)	*Bacillus, Listeria, Corynebacterium*
4	+	+	+	rods	1 (1.32)	Not identified

While results from section 4.2.1 indicated that traditional ghee from Teso was contaminated with coliforms and *E. coli*, the latter were not isolated as evidenced from the results in Table 7. This could be due to failure to collect these organisms from random colonies selected from the media plates during isolation. Overall, the results of this study revealed that Ugandan traditional ghee was dominated by *Lactobacillus* and *Streptococcus* spp. These organisms were mostly detected in ghee from Bonyoro. *Bacillus, Listeria* and *Corynebacterium* spp. were mostly detected in ghee from Buganda.

The dominance of lactic acid bacteria in Ugandan traditional ghee is, therefore, indicative of their potential contribution to the product's fermentative, preservative and probiotic properties. The presence of *Enterococcus* in fermented dairy products made from pasteurized milk is undesirable as these organisms have potential to cause spoilage problems and could be attributed to poor hygienic conditions during ghee processing (Gasser, 1994; Van Kessel, Karns, Gorski, McCluskey & Perdue, 2004). The presence of *enterococci* from natural milk starter cultures could also be explained by their thermophilic and heat resistant properties especially species belonging to *Streptococcus thermophilus* and *Enterococcus* spp. (Manzano, Citterio, Rondinini, & Bertoldi, 1993). Strains of *Enterococci* such as *E. faecium* and *E. faecalis* have been reported to produce bacteriocins against other microorganisms which enable then survive in fermented dairy products (Brock, Peacher, & Pierson, 1963; Gálvez, Maqueda, Valdivia, Quesada & Montoya, 1986). Studies have been reported about the formation of biogenic amines by *E. faecalis* and *E. faecium*; these organisms have also been reported to cause food poisoning in addition to antibiotic resistance (Aguirre, & Collins, 1993; Gálvez, Maqueda, Valdivia, Quesada & Montoya, 1986; Garg, & Mital, 1991).

The data obtained by conventional methods could not discriminate the different bacterial genera identified in each group. Therefore, 16S rDNA sequence analysis was performed to (1) obtain reliable identification of the genera in each group and, (2) establish the different bacterial species in each of the identified genera and the results are presented in section 4.2.3.

4.1.3 Molecular Identification of the Bacteria Isolated from Ugandan Traditional Ghee

Of the 76 isolates obtained in Table 6, only 72 were recovered for further identification; the results of PCR analysis are shown in Fig. 1.

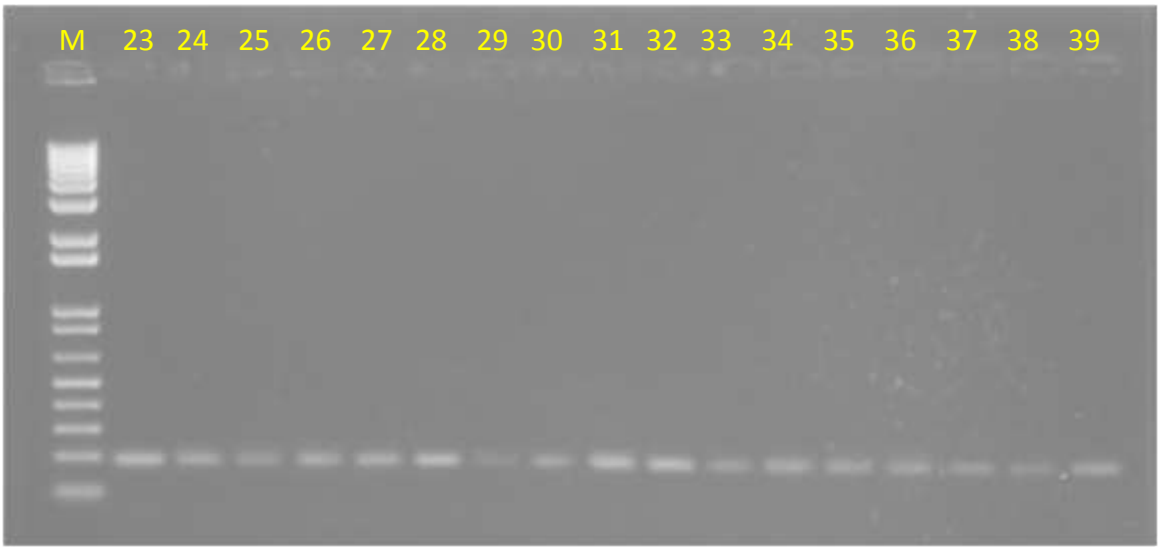

Figure 1 Gel image showing PCR amplification of 16S rDNA gene of DNA extracted from the bacterial isolates obtained from Ugandan traditional ghee. Samples were run on 2% agarose gel in 1X TAE buffer for 2 h at 75 V

M – 100 bp ladder

23 to 39 – some of the ghee isolates presumptively identified as LAB

A 200 bp band (PCR amplicon) was obtained for each of the 72 tested isolates (Fig. 1) implying accuracy of the primers to amplify the target region of the gene. After PCR, each of the remaining samples containing the PCR amplicons was purified and sequenced. The sequences were processed using the BLAST programme to retrieve the closest known relatives of the isolates in the National Centre for Biotechnology Information (NCBI) database, and the results are shown in Table 8.

Table 8 Summary of BLAST results of the sequences obtained from PCR amplification of 16S rDNA gene of the bacteria isolated from Ugandan traditional ghee

Isolate	Gene accession	Close relative of	Number of isolates	Proportion (%)	E-value	%ID
Group 1	MN493726	*Enterococcus faecium*	22	30.6	9e -71	100
Group 2	MT645592	*Lactobacillus plantarum*	20	27.8	1e -72	100
Group 3	MN341130	*Lactobacillus rhamnosus*	11	15.3	2e -73	100
Group 4	MN341098	*Enterococcus hirae*	11	15.3	2e -72	100
Group 5	MN493753	*Enterococcus faecalis*	02	2.8	2e -71	100
Group 6	MH997515	*Bacillus cereus*	02	2.8	2e -72	100

BLAST database searches gave identifiable sequences for 68 of the 72 isolates with percent identities ranging from 89.3 to 100% (Appendix I). Only two isolates gave percent identity matches lower than the threshold value of 95%. There were no matching sequences for four isolates implying that these could be new bacterial strains whose DNA sequences are not yet uploaded in public library databases, and this is an area that needs further investigation.

Six (06) bacterial species were obtained by BLAST. *Enterococcus faecium* (30.6%) and *Lactobacillus plantarum* (27.8%) were the dominant species; both species were detected in ghee samples from all sub-regions under study. *Lactobacillus rhamnosus* and *Enterococcus hirae* occurred in same proportions (15.3%). Presence of *Bacillus cereus* (2.8%) and *Enterococcus* spp. in Ugandan traditional ghee is undesirable due to their pathogenicity (Giraffa et al., 1997; Bhardwaj, Malik, & Chauhan, 2008; Eglezos, Huang, Dykes, & Fegan, 2010).

Overall, results of molecular identification were in agreement with those obtained by conventional microbiology methods (Sections 4.2.1-4.2.2). For instance, lactic acid bacteria and *Enterococci* were found to be the dominantly enumerated microorganisms (section 4.2.1) and presumptive identification studies (section 4.2.2) which is similar with the data obtained by PCR. Molecular microbiology methods are considered to be more accurate than conventional methods. Therefore, the presence of *Clostridium* spp., *Streptococcus* spp., *Listeria* spp. and *Corynebacterium* spp. in Ugandan traditional ghee (section 4.2.2) was ruled out (Table 7). The isolate which could not be presumptively identified by conventional methods could also not be identifiable by PCR suggesting this could be a new bacterial species.

4.1.4 Cluster Analysis

In order to establish the level of genetic relatedness of the dominant LAB species, cluster analysis was performed on the 31 *Lactobacillus* isolates identified by PCR as *Lactobacillus plantarum* and *Lactobacillus rhamnosus*. The dendrogram which was derived using the average linkage method (UPGMA) of the PCR sequences produced two main clusters comprising of *L. plantarum* (cluster A) and *L. rhamnosus* (cluster B) (Fig. 2).

Majority of *L. plantarum* isolated from ghee which was obtained from Central Kampala Market (CKM, Buganda) and Central Mulukoola Market (CMM, Buganda) were clustered together.

Similarly, cluster B largely comprised of *L. rhamnosus* isolated from ghee which was obtained from Mbarara Kizungu Market (MbKM, Ankole) and Mbarara Central Market (MbCM, Ankole) suggesting genetic relatedness. Overall, each of the two clusters was homogeneous and contained isolates from different sub-regions of the study area highlighting possible genetic relationship between the *Lactobacillus* isolates in ghee samples obtained from different areas in Uganda.

In cluster A, isolates 11 CKM and 15 CKM had 100% identity and so were considered to be identical. A similar conclusion was made for isolates 22 CKM and 31 MbCM in cluster B. As expected, the dendrogram aligned *L. plantarum* separate from *L. rhamnosus* because they belong to different species with different genetic composition. This indicates high level of discrimination (precision) of molecular methods and suggests low level of genetic homology of these two LAB species.

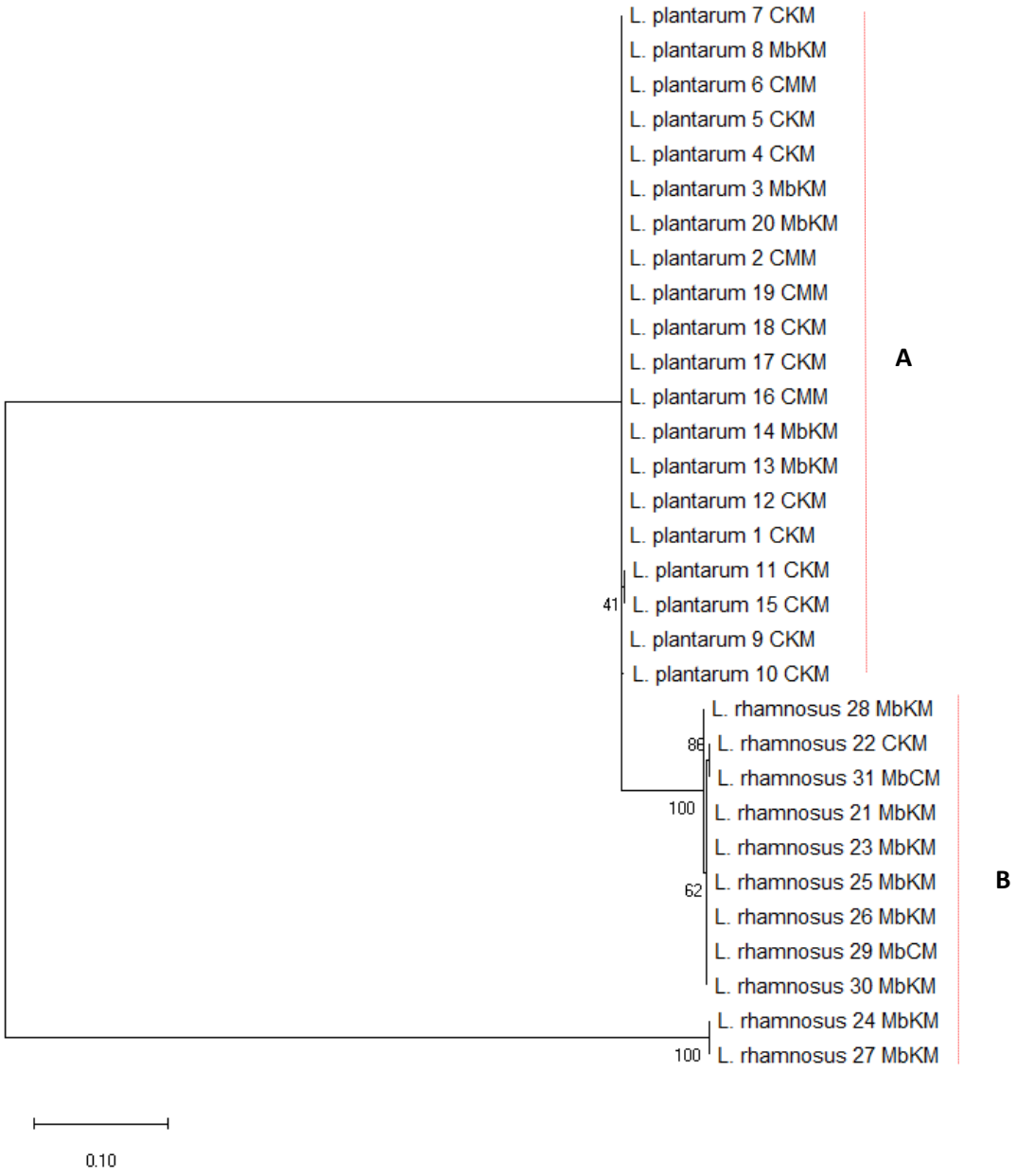

Figure 2 Cluster analysis of *Lactobacillus* spp. isolated from Ugandan traditional ghee. Clustering was based on UPGMA method. Isolates were obtained from: CKM (Central Kampala Market, Buganda), CMM (Central Mulukoola Market, Buganda), MbKM (Mbarara Kizungu Market, Ankole), MbCM (Mbarara Central Market, Ankole).

Lactobacillus rhamnosus have been previously isolated from breast milk and milk products where they inherently harbor to offer protection to infants against pathogenic microbes (Bautista-Gallego et al., 2013; Kozak et al., 2015; Martín et al., 2003). *Lactobacillus* is known to be bile salt and phenol tolerant, salt tolerant (2.5% to 5% NaCl concentration), produces antimicrobial agents, has DPPH scavenging ability and acid tolerant with survival rates above 81% and 90% after 3 h exposure to pH 2 and pH 3, respectively, which enables it to survive in fermented dairy products (Kaewiad, Kaewnopparat, & Kaewnopparat, 2015). The ability of *Lactobacillus rhamnosus* to survive under low pH is an important attribute towards their survival in fermented dairy products (Mandal, Jariwala, & Bagchi, 2016). Their ability to tolerate phenols indicates that they can resist bacteriostatic effects of phenol which is produced as an aromatic byproduct during production of fermented dairy products (Palaniswamy & Govindaswamy, 2016). Their survival in foods has also been associated with production of antimicrobial agents which enables them to compete with pathogenic bacteria in food products (Collado et al., 2006; Tulumoglu et al., 2013). However, *L. rhamnosus* has been reported to have antibiotic resistance against antibiotics such as streptomycin, ampicillin, gentamicin, kanamycin, penicillin, cephalotoxin, and ciprofloxacin which pauses serious public health concerns (Maragkoudakis et al., 2006). The ability of *L. rhamnosus* to adhere to Caco-2 cells is also an important criterion for the selection of these probiotic lactic acid bacteria since probiotic potential provides beneficial effects such as immune system modulation and exclusion of pathogenic microbes (Lee et al., 2003; Schiffrin et al., 1995).

Lactobacillus plantarum on the other side, is a versatile lactic acid bacterium, that is encountered in a range of environmental niches including dairy products such as cheese (Baruzzi et al., Morea, Matarante, & Cocconcelli, 2000; Manolopoulou et al., 2003; Ercolini, Hill, & Dodd, 2003). A variety of *L. plantarum* strains are presently marketed as starter culture adjuncts and/or probiotics (Ercolini et al., 2003; Randazzo et al., 2004; Spano et al., 2004). Different studies have reported probiotic effects of *L. plantarum* in the human body such as reduction in LDL cholesterol (11.7%) and fibrinogen (21.0%), improved natural immune response, up to six times and reduction in carriage of faecal *Enterobacteriaceae* (Cunningham-Rundles et al., 2000; Naruszewicz et al., 2003). However, *L. plantarum* has been associated with spoilage of foods, such as meat, wine and orange juice (Alwazeer, Cachon, & Divies, 2002; Beneduce et al., 2004).

L. plantarum have also been associated with infections such as in-vitro coagulation of blood by aggregation of human platelets causing blood clotting (Harty et al., 1994). Viability and fermentative ability are important parameters for evaluation of prospects for application of a microbial strain as a probiotic and/or starter culture (Widyastuti et al., 2014). Moreover, *L. rhamnosus* is preferred to *L. plantarum* for inclusion in fermented dairy products due to its probiotic properties (Gill et al., 2001). *L. plantarum* are regarded as secondary microbiota in fermented foods (Marco et al., 2017). In the next part of the study, *in vitro* methods were used to ascertain whether or not, *L. rhamnosus* strains isolated from Ugandan traditional ghee have prospects for application as (1) starter cultures in fermented dairy products on the basis of their growth and acid production capability, (2) probiotic properties and, (3) application as bio-preservatives due to their antimicrobial properties. Thus, three genetically distinct *L. rhamnosus* isolates 24 MbKM, 27 MbKM and 31 MbCM) were selected from cluster B (Fig. 2) and evaluated for probiotic, antimicrobial, growth and acid production properties in milk as described in sections 3.4-3.6, the results of which are presented in sections 4.3 – 4.5.

4.2 Probiotic Properties

4.2.1 Acid and Bile Salt Tolerance

Generally, there were significant ($p<0.05$) differences in the tolerance of between *L. rhamnosus* isolates to pH and bile salts (Fig. 3). Fig. 3 shows the results of acid (Fig. 3A) and bile salt (Fig. 3B) tolerance of the three *L. rhamnosus* isolates 24 MbKM, 27 MbKM and 31 MbCM at 30^{o}C in MRS broth acidified to pH 3 and 7.8 simulating the pH conditions of the stomach and small intestine, respectively.

The isolates had significant differences ($p<0.05$) sensitivities to acid treatment leading significant decrease ($p<0.05$) in viable counts (Fig. 3A). After 3 h, *L. rhamnosus* 27 MbKM had the highest number of surviving colonies (3 log reduction) implying highest acid tolerance while 24 MbKM had the lowest number of surviving colonies (5 log reduction) implying lowest acid tolerance (Fig. 3A). All isolates exhibited high acid tolerance and remained viable after 3 h of exposure with *L. rhamnosus* 27 MbKM showing the highest resistance to low pH indicating its potential to survive gastric acids. However, the three isolates were found to be sensitive to bile salts and were unable to grow in the medium throughout the experiment (Fig. 3B). These results imply that *L.*

rhamnosus isolated from Ugandan traditional ghee could survive stomach conditions but may not tolerate bile salts in the small intestine.

It is possible that *L. rhamnosus* were extremely bile salt stressed so that they could not be counted on the selective MRS medium. Succi et al., (2005) reported that bile salt stressed *L. rhamnosus* can only be possibly enumerated after 168 h of incubation on MRS agar, owing to slow recovery from stress conditions. Similar to the above study, the ability of *L. rhamnosus* strains isolated from Ugandan traditional ghee to survive high amounts of bile salts and low pH was tested in vitro utilizing MRS broth. Thus, there is a need to assess this important prerequisite of probiotic interest in vivo in order to ascertain the real capacity of these *Lactobacillus* strains to survive transit through the gastro-intestinal tract. However, the results obtained in the present work allows to admit that ingestion of high amounts of *L. rhamonosus* results in gastric acid survival and enables them to reach the small intestines where they could be inhibited by bile salts.

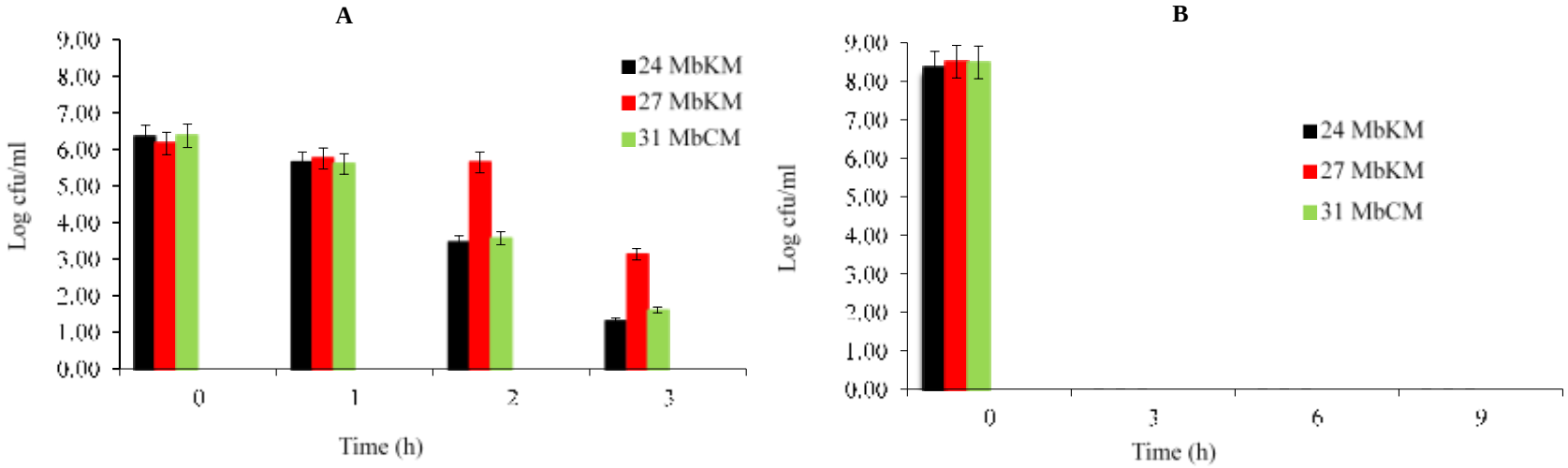

Figure 3 Acid (**A**) and bile salt (**B**) tolerance of *L. rhamnosus* isolated from Ugandan traditional ghee. *L. rhamnosus* isolates: 24 MbKM from Mbarara Kizungu Market (black font), 27 MbKM from Mbarara Kizungu Market (red font), and 31 MbCM from Mbarara Central Market (green font). Enumeration was performed on MRS agar at 30ºC. Values are means of three independent determinations and error bars are ± standard errors of the means, $p = 0.05$.

4.2.2 Production of Biogenic Amines

Results from this study (Table 9) demonstrated that *L. rhamnosus* isolated from Ugandan traditional ghee did not produce biogenic amines from majority of the tested amino acids. However, two of the three *Lactobacillus* isolates (24 MbKM and 27 MbKM) produced putrescine from L-ornithine suggesting that production of this amine could be strain-dependent rather than being related to the entire *Lactobacillus rhamnosus* species.

Table 9 Biogenic amine production by *L. rhamnosus* isolated from Ugandan traditional ghee

Amino acid	Biogenic amine	*L. rhamnosus* 24 MbKM	*L. rhamnosus* 27 MbKM	*L. rhamnosus* 31 MbCM
L-Histidine	Histamine	Negative	Negative	Negative
L-Tyrosine	Tyramine	Negative	Negative	Negative
L-Lysine	Cadaverine	Negative	Negative	Negative
L-Phenylalanine	Phenylethylamine	Negative	Negative	Negative
L-Ornithine	Putrescine	**Positive**	**Positive**	Negative
L-arginine	Agmatine	Negative	Negative	Negative

Biogenic amines are organic bases with low molecular weight and are synthesized by microbial, vegetable and animal metabolisms. In fermented foods, biogenic amines are formed by enzymatic decarboxylation of amino acids (Garai et al., 2007). These compounds constitute a potential public health concern due to their physiological and toxicological effects if ingested in large amounts or when the natural detoxification process of the body is inhibited (Garai et al., 2007). It is also important to note that biogenic amines are thermostable hence the need to control their presence in food in order to ensure high levels of food quality and safety. Amongst the known biogenic amines, histamine is the most commonly observed in food intoxication cases and it is one of the toxins targeted by the Food and Drug Administration (FDA) and European Food Safety Authority (EFSA). Histamine is a mediator of several allergic disorders and the common symptoms of histamine poisoning are due to the effects it has on the different bodily systems (cardiovascular, gastrointestinal, respiratory, etc.) producing low blood pressure, skin

irritation, headaches, edemas and rashes (Bardócz, 1995; Kalaˇc, 2013). However, histamine is not the only biogenic amine involved in food poisoning (Pegg, 2013). Other amines, such as putrescine and cadaverine, are also associated with food borne illness, although both seem to have much lower pharmacological activity on their own but enhance the toxicity of histamine and decrease the catabolism of this amine when they interact with amine oxidases, thus hindering histamine detoxification and favoring its absorption (Halász et al., 1994; Ruiz-Capillas & Jiménez-Colmenero, 2004).

Although lactic acid bacteria are Generally Regarded as Safe (GRAS) organisms, genetic studies have revealed that many strains harbor genes or operons coding for decarboxylating enzymes or other pathways implicated in biogenic amine biosynthesis. *L. rhamnosus* 24 MbKM and *L. rhamnosus* 27 MbKM have proved this phenomenon by converting L-ornithine into putrescine. Therefore, *L. rhamnosus* 31 MbCM could be recommended for application in food fermentations and/or as a probiotic since it did not produce biogenic amines from all the tested amino acids.

4.2.3 Auto Aggregation

The results of auto aggregation of *L. rhamnosus* isolated from Ugandan traditional ghee measured at 5 and 24 h are shown in Table 10. After 5 h, *L. rhmnosus* 31 MbCM showed significantly higher (72%) auto aggregation than 24 MbKM and 27 MbKM ($P<0.05$). After 24 h, all *lactobacillus* isolates showed 100% auto aggregation, implying that these they have good probiotic prospects on the basis of their possible ability to adhere to the gut epithelial cells.

Adhesion to epithelial cells is an important prerequisite for colonization by probiotic bacterial strains in the gastrointestinal tract, preventing their elimination from the body in order to outcompete the undesirable microflora (Kos et al., 2003). Due to difficulties involved in studying these effects *in vivo* (in humans), auto aggregation is considered a suitable *in vitro* model which is used for preliminary screening of potentially adherent microbial strains to intestinal epithelial cells (Rajoka et al., 2017). Strains that exhibit high auto aggregation tendencies are considered to have the physicochemical characteristics on the cell surface such as hydrophobicity, which promote adhesion of the bacteria to different surfaces (Rajoka et al., 2017).

Table 10 Percent auto aggregation of *L. rhamnosus* isolated from Ugandan traditional ghee at different time intervals

L. rhamnosus isolate	5 h	24 h
24 MbKM	62.63±0.00[b]	100± 0.0[a]
27 MbKM	64.22±1.15[b]	100± 0.0[a]
31 MbCM	72.69±0.59[a]	100± 0.0[a]

Values are means of three independent determinations ±standard errors of the means, $p = 0.05$

Thus, *Lactobacillus* isolates present the potential as probiotic organisms and could modulate the immune system and protect the host against pathogenic microorganisms (Kaewiad et al., 2015). However, the contribution of an organism as a probiotic in the body also depends on its ability to survive bile salts at concentrations that mimic the bile salt concentrations in the small intestines. However, all isolates obtained from this study could not survive the bile salt conditions indicating poor proliferation ability through the gut transit. Similar results were also reported by Mandal et al. (2016).

On the other hand, each of the three *Lactobacillus* isolates had similar and maximum auto aggregation tendency of 100% at 24 h indicating suitability of these isolates to be used as probiotics for humans on the basis of their possible ability to adhere to the gut epithelial cells. The findings of this study are in agreement with earlier studies which reported high auto aggregation tendency of 100% at 24 h indicates bacterial suitability as a probiotic (Palaniswamy & Govindaswamy, 2016; Rajoka, et al., 2017).

4.3 Antimicrobial properties of *L. rhamnosus* Isolates by Agar Overlay and Paper Disc Diffusion Methods

Each of the *L. rhamnosus* isolates formed an array of clear halo zones on the agar plates due to lysis of the indicator bacteria (Appendix II) highlighting positive antimicrobial activity against each of the tested indicator bacterial species (Table 11). Therefore, the study revealed that both

Gram-positive and Gram-negative bacterial species were inhibited by each of the *L. rhamnosus* isolates, irrespective of the cell shape of the indicator bacterial species.

Table 11 Inhibitory activity of *L. rhamnosus* isolates against the selected indicator bacteria

	L. rhamnosus isolates		
Indicator bacteria	24 MbKM	27 MbKM	31 MbCM
Staphylococcus aureus	+	+	+
E. coli ATCC 25922	+	+	+
Salmonella enterica	+	+	+

Activity was based on the presence (+) or absence (-) of a clear zone (halo) around the colonies of *L. rhamnosus* isolates. *L. rhamnosus* isolates: 24 MbKM from Mbarara Kizungu Market, 27 MbKM from Mbarara Kizungu Market, and 31 MbCM from Mbarara Central Market.

In order to determine whether or not antimicrobial activity of the different *L. rhamnosus* isolates was due acid and/or bacteriocin production, the experiment was repeated using antibiotic paper discs soaked in cell-free supernatants (CFS) and CFS treated to remove organic acids (CFS-N) and the proteinaceous bacteriocins (CFS-P). The untreated cell-free supernatants (CFS) had variable effects on each of the tested indicator bacteria. *E. coli* was the most sensitive and was inhibited by CFS obtained from each of the *L. rhamnosus* isolates (Table 12). Both of *Staphylococcus aureus* and *Salmonella enterica* were only inhibited by CFS obtained from *L. rhamnosus* 24 MbKM and 31 MbCM. None of these two indicator bacteria was inhibited by the CFS from *L. rhamnosus* 27 MbKM implying weaker antimicrobial activity of the latter *Lactobacillus* isolate. Since Gram-positive and Gram-negative bacterial species did not show antimicrobial sensitivity to CFS obtained from *L. rhamnosus* 27 MbKM, this implies that antimicrobial sensitivity does not depend on the cell-wall properties of the indicator bacteria.

Each of the three *L. rhamnosus* isolates was evaluated for antimicrobial activity against *Staphylococcus aureus, E. coli* and *Salmonella enterica*. The latter indicator bacteria are some examples of major food spoilage and pathogenic microorganisms. Inclusion of Gram-negative (*E. coli* and *Salmonella*, rod-shaped) and Gram-positive (*Staphylococcus*, cocci) species was

aimed to establish whether there could be variation in antimicrobial sensitivity due to differences in cell-wall properties of these two bacterial groups.

Table 12 Inhibitory activity of cell-free supernatants and treated cell-free supernatants of *L. rhamnosus* isolates on selected indicator bacteria

Type of CFS	Indicator bacteria	*L. rhamnosus* isolates		
		24 MbKM	27 MbKM	31 MbCM
CFS	*Staphylococcus aureus*	+	-	+
	E. coli ATCC 25922	+	+	+
	Salmonella enterica	+	-	+
CFS-N (pH 6.5)	*Staphylococcus aureus*	-	-	-
	E. coli ATCC 25922	-	-	-
	Salmonella enterica	-	-	-
CFS-P	*Staphylococcus aureus*	+	-	+
	E. coli ATCC 25922	+	+	+
	Salmonella enterica	+	-	+

1) CFS, cell-free supernatant without any treatment; CFS-N, cell-free supernatant neutralized to pH 6.5 using 1 M NaOH solution; CFS-P, cell-free supernatant treated with 1mg/ml proteinase K and incubated at 37°C for 3h.
2) (-) indicates that the diameter of the clear zone of inhibition was less than 11 mm
3) (+) indicates that the diameter of the clear zone of inhibition was greater than 11 mm.
4) *L. rhamnosus* isolates: 24 MbKM from Mbarara Kizungu Market, 27 MbKM from Mbarara Kizungu Market, and 31 MbCM from Mbarara Central Market.

Each of the cell-free supernatants treated to remove organic acids (CFS-N) lost antimicrobial activity and formed small clear halo zones of less than 11 mm (Table 12), implying that activity was due to production of organic acids which was subsequently lost after removal of the acids produced by the *Lactobacillus* isolates. In comparison, the CFS treated to remove bacteriocins (CFS-P) exerted variable effects with majority of the indicator bacteria showing sensitivity to these *Lactobacillus* CFS (Table 12). Similar to the results of the untreated CFS, *Staphylococcus*

aureus and *Salmonella enterica* could not be inhibited by the CFS-P obtained from *L. rhamnosus* 27 MbKM. Overall, these results confirmed that antimicrobial activity of the *L. rhamnosus* isolated from Ugandan traditional ghee was mainly due to production of organic acids. However, for the case of *L. rhamnosus* 27 MbKM, there is a need to investigate reasons responsible for low sensitivity to its untreated CFS and CFS treated to remove bacteriocins against *Staphylococcus aureus* and *Salmonella enterica.*

Production of antimicrobial compounds is one of the main mechanisms by which probiotic microorganisms compete against pathogenic microbes for their survival in the GIT and be able to offer the probiotic benefits to the host (Collado et al., 2006; Rajoka, et al., 2017). In this study, all the *L. rhamnosus* isolates had strong antimicrobial activity against each of the tested indicator bacteria in the agar overlay assays. In the CFS assays, it was confirmed that antimicrobial activity against each of the indicator bacterial species was due to acid production which is similar to the results of Siragusa et al. (2007). Different bacteria express antimicrobial properties in different ways. For instance, some bacteria produce ropy like polysaccharides used in biofilm formation against environmental stresses for colonization of new habitats for instance. *L. rhamnosus* has been reported for its ability to form biofilms in vitro (Martín, Soberón, Camino, & Suárez, 2008; Jones & Versalovic, 2009; Degeest, Janssens, & De Vuyst, 2001; Minervini et al., 2010). Other mechanisms include production of acids, bacteriocins, CO_2 and hydrogen peroxide among others.

4.4 Starter Culture Properties: Growth Kinetics and Acid Production Capacity

As there were no major variations in the results of probiotic and antimicrobial properties among the three *L. rhamnosus* isolates, only two of these isolates (27 MbKM and 31 MbCM) were studied further for growth kinetics and acid production capacity prospects. These isolates were selected from different clusters of the dendrogram (Fig. 2). The data for growth kinetics, pH and percent titratable acidity changes of the two *L. rhamnosus* isolates and the UHT milk control are shown in Fig. 4. UHT milk was used as a growth medium because it is sterile, has high pH, high nutritive value and high-water activity all of which would support microbial growth. Moreover, commercial ghee could not be used as a growth medium because it was found to contain some viable counts as shown in Table 6.

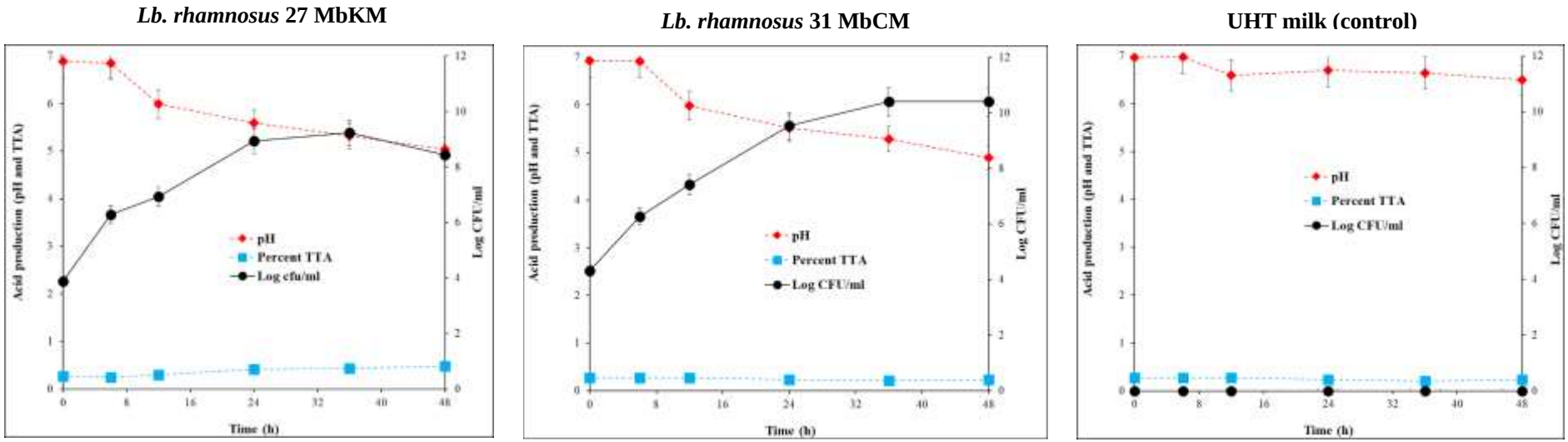

Figure 4 Growth kinetics, percent titratable acidity and pH changes of *L. rhamnosus* isolated from Ugandan traditional ghee. Solid line (black circle) log cfu/ml; broken line: (red diamond) pH changes, (blue square) percent titratable acidity. *L. rhamnosus* isolates: 27 MbKM from Mbarara Kizungu Market and 31 MbCM from Mbarara Central Market. Values are means of two independent determinations and error bars are ± standard errors of the means, $p = 0.05$.

Each of the *Lactobacillus* isolates caused significant pH decrease at the end of the experiment ($p<0.05$). At 48 h, the lowest pH (4.89) was observed from *L. rhamnosus* 27 MbKM, whereas the highest (5.04) was detected from 31 MbCM (Fig. 4). The reason for the poor fermentation of milk could be related to the poor utilization related to sugar fermentation abilities by *L. rhamnosus.* The observed pH reduction could be attributed to breakdown of lactose in milk into lactic and other organic acids (Eklund, 1989).

Percent titratable acidity (TTA) remained relatively constant for all treatments (Fig. 4) highlighting the high buffering capacity of the milk medium (Manini et al., 2016). The different proteins in milk can increase its buffering capacity thus resisting change in the acidity content. However, *L. rhamnosus* 31 MbCM caused a higher %TTA increase (0.32 to 0.68) than *L. rhamnosus* 27 MbKM (0.27 to 0.48) ($p<0.05$). This can be attributed to the fact that *L. rhamnosus* 31 MbCM had higher viable counts in its medium. The larger bacterial population of this isolate caused a much more vigorous carbohydrate metabolism of sugars, resulting in a higher production of organic acids which in turn increased the total titratable acidity (Schmidt et al., 1996).

The two *L. rhamnosus* isolates generally had similar growth patterns in UHT milk (Fig. 4). At the end of the experiment (48 h), *L. rhamnosus* 31 MbCM had significantly higher viable counts (10.41 log cfu/ml) than *L. rhamnosus* 27 MbKM (8.43 log cfu/ml) ($p<0.05$). All the tested parameters remained relatively constant in the milk control.

These results implied that *L. rhamnosus* isolated from Ugandan traditional ghee could cause significant acidification of milk leading to pH decline, probably due to production of organic acids as evidenced from the results of the neutralized cell-free supernatant (CFS-N) in Table 12.

CHAPTER FIVE: CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

This study has characterized the dominant lactic acid bacterial flora of Ugandan traditional ghee on the basis of their probiotic, starter culture and antimicrobial properties. Data obtained by conventional and molecular microbiology protocols were generally congruent. The ghee samples obtained from different regions of Uganda were dominated by *Lactobacillus* and notably *L. plantarum* and *L. rhamnosus*. Both of these LAB species were isolated from ghee in all regions and largely co-existed with *Enterococcus* spp. As all the detected microorganisms are fortuitous in ghee, their occurrence in the product could be as a result of contamination or survival in milk upon pasteurization in the case of the commercial sample.

Results obtained from the *in vitro* assays employed to assess some of the probiotic properties of *L. rhamnosus* showed that the isolates did not produce biogenic amines, and exhibited good auto-aggregation and acid tolerance which are desirable properties. However, they were sensitive to bile salts implying that they may not survive transit of the entire gastro intestinal tract passage.

Each of the tested *L. rhamnosus* isolates exhibited good growth capabilities with good acid production and remained viable in milk for 48 h probably. Acid production by each isolate was growth-dependent with maximum acidification rates occurring during exponential growth phase (8-24 h). On the basis of these findings, it was concluded that *L. rhamnosus* isolated from Ugandan traditional ghee does not have prospects for application as a starter culture due to its slow acidification capability.

Since majority of lactic acid bacteria produce lactic and other organic acids, it was not surprising that all the tested *L. rhamnosus* isolates inhibited the Gram-negative and Gram-positive indicator bacteria due to acid production. However, inhibition due to bacteriocin production was not demonstrated from this study probably due to a small scope of indicator bacteria tested and since bacteriocins are known to inhibit closely related members to the producer organism. This is an area that needs further investigation. Based on these results, it could be recommended that *L.*

rhamnosus from Ugandan traditional ghee could be applied as a bio-preservative in fermented foods whose acid content due to lactic acid production is a desirable quality attribute.

5.2 Recommendations

L. rhamnosus isolated from Ugandan traditional ghee have some favorable probiotic and antimicrobial properties which could be exploited commercially.

5.3 Further work

Further research should investigate the aroma compounds produced by these organisms in milk and other fermented food matrices, when grown alone and in co-culture with other LAB species (such as *L. plantarum*) and/or yeasts and moulds which also dominate food fermentation systems. This could explain why traditional ghee which has been shown in this study to contain different microbial populations, has superior taste than commercial ghee which has been shown to be microbially cleaner.

Future studies should screen these *Lactobacillus* isolates for gut survival against lysozyme, ability to adhere to Caco-2 cells which is indicative of their immune system modulation and exclusion of pathogenic microbes (Lee et al., 2003; Schiffrin et al., 1995).

Further research should also probe their co-aggregation, cell surface hydrophobicity and congregation properties which are associated with the adhesion and protection characteristics of the intestinal tract.

More work is also required to study the phenol tolerance of these organisms as these bactericidal compounds are by-products of aromatic amino acid metabolism in the gut (Del Re, Sgorbati, Miglioli, & Palenzona, 2000; Palaniswamy & Govindaswamy, 2016).

REFERENCES

Abdalla, M. (1994) Milk in the rural culture of contemporary Assyrians in the Middle-East. In Milk and Milk Products from Medieval to Modern ¹imes, ed. P. Lysaght. Canongate Press, Edinburgh, pp. 27 -39.

Abdel-Salam, A. M., Al-Dekheil, A., Babkr, A., Farahna, M., & Mousa H. M. (2010). High Fiber Probiotic Fermented Mare's Milk Reduces the Toxic Effects of Mercury in Rats. *North American Journal of Medical Sciences*, *2*(12):569-575.

Abdulsamad, A., & Gereffi, G. (2016). East Africa Dairy Value Chains: Firm Capabilities to Expand Regional Trade. Technical Report.

Abou-Donia, S. A. and El-Agamy, S. I. (1993) Ghee. In Encyclopaedia of Food Science, Food ¹echnology and Nutrition, Vol. 6, eds R. Macrae, R. K. Robinson and M. J. Sadler. Academic Press Ltd, London, pp. 3992 -3994.

Achan, J. (2018, June 28). Increased Milk Production With Low Consumption. *New Vision*. Retrieved from https://www.newvision.co.ug/news/

Achaya, K.T. (1997). *Lipid Technologies and Applications,* Page 22. Routledge.

Adam, A. C., Rubio-Texeira, M., & Polaina, J. (2004). Lactose: The Milk Sugar from a Biotechnological Perspective. *Critical Reviews in Food Science and Nutrition, 44*(7-8):553-557.

Adiloglu, A., Gönülateş, N., Işler, M., & Senol, A. (2013). The Effect of Kefir Consumption on Human Immune System: A Cytokine Study. *Mikrobiyoloji Bülteni, 47*(2):273-281.

Adolfsson, O., Meydani S. N., & Russell R. M. (2004). Yogurt and Gut Function. *The American Journal of Clinical Nutrition, 80*(2):245-256.

Aguirre, M., & Collins, M. D. (1993). Lactic Acid Bacteria and Human Clinical Infection. *Journal of Applied Bacteriology, 75*(2):95-107.

Ahmed, Z., Wang, Y., Ahmad, A., Khan, S. T., Nisa, M., Ahmad, H., & Afreen, A. (2013). Kefir and Health: A Contemporary Perspective. *Critical Reviews in Food Science and Nutrition 53*(5):422-434.

Aka, Solange. (2008). Evaluation of Organic Acids and Sugars Contents During the Production of "Tchapalo", a Traditional Sorghum Beer in Cote D'ivoire. *Journal of Food Technology*, 6:189-195.

AkalIn, A. S., & Tokusoglu, O. (2003). A Potential Anticarcinogenic Agent: Conjugated Linoleic Acid (CLA). *Pakistan Journal of Nutrition*, *2*(2):109-110.

Al-Wabel, Professor Dr. Naser. (2008). Mineral Contents of Milk of Cattle, Camels, Goats and Sheep in the Central Region of Saudi Arabia. *Asian Journal of Biochemistry*. 3. 373-375.

Alwazeer, D., Cachon, R., & Divies, C. (2002). Behavior of *Lactobacillus plantarum* and *Saccharomyces cerevisiae* in Fresh and Thermally Processed Orange Juice. *Journal of Food Protection*, *65*(10):1586-1589.

Aneja, R. P. and Murthi, T. N. (1991) Beneficial effects of ghee. Nature 350(28), 280.

Ansorena, D., & Astiasaran I. (2016). Fermented Foods: Composition and Health Effects. *Encyclopedia of Food and Health*, Pages 649-655. Academic Press.

Arokiyamary, A., & Sivakumaar, P. K., 2011. Microbiological and Biochemical Characteristics of Tradition Dairy Products: Identification of Dominant Lactobacillus. *International Journal of Pharmaceutical & Biological Archives*, *2*(4):1196-1201.

Aysegul Kirazci & Issa Javidipour, 2008. Some chemical and microbiological properties of ghee produced in Eastern Anatolia. *International Journal of Dairy Technology*, *61*(3), 300-306.

Azhari, Ali. (2010). Beneficial Role of Lactic Acid Bacteria in Food Preservation and Human Health: A Review. *Research Journal of Microbiology*, *5*(12):1213-1221.

Bao, Y., Zhang, Y., Zhang, Y., Liu, Y., Wang, S., Dong, X. and Zhang, H. (2010). Screening of potential probiotic properties of Lactobacillus fermentum isolated from traditional dairy products. Food Control, 21, 695–701. doi:10.1016/j.foodcont.2009.10.010.

Baruzzi, F., Morea, M., Matarante, A., & Cocconcelli, P. S. (2000). Changes in the Lactobacillus Community during Ricotta Forte Cheese Natural Fermentation. *Journal of Applied Microbiology*, *89*(5):807-814.

Bardócz, S. (1995). Polyamines in Food and Their Consequences for Food Quality and Human Health. *Trends in Food Science and Technology*, 6:341-346.

Basu, K. P., & Nath, H. P. (1946). The Rate of Absorption of Different Fats and Oils. *Indian Journal of Medical Research*, 34:19-25.

Bautista-Gallego, J., Arroyo-López, F., Rantsiou, K., Jiménez-Díaz, R., Garrido Fernández, A., & Cocolin, L. (2013). Screening of Lactic Acid Bacteria Isolated from Fermented Table Olives with Probiotic Potential. *Food Research International*, DOI:10.1016/j.foodres.2012.10.004

Bekele, E. and Kassaye, T. (1987) Traditional Borana milk processing efficient use of subtle factors needs further research work. International ¸ivestock Centre for Africa (I¸CA) Newsletter 6(4), 4-5.

Beneduce, L., Spano, G., Vernile, A., Tarantino, D., & Massa, S. (2004). Molecular Characterization of Lactic Acid Populations Associated with Wine Spoilage. *Journal of Basic Microbiology, 44*(1):10-16.

Benz, Z. & Zimmermann, U. (1980). Pulse-Length Dependence of the Electrical Breakdown in Lipid Bilayer Membranes, *Biochimica et Biophysica Acta, 597*(3):637-642.

Bhardwaj, A., Malik, R., & Chauhan, P. (2008). Functional and Safety Aspects of Enterococci in Dairy Foods. *Indian Journal of Microbiology, 48*(3):317-325.

Bourrie B. C. T., Willing B. P. & Cotter P. D. (2016). The Microbiota and Health Promoting Characteristics of the Fermented Beverage Kefir. *Frontiers in Microbiology*, 7:647.

Bowen, W., & Lawrence, R. (2005). Comparison of the Cariogenicity of Cola, Honey, Cow Milk, Human Milk, and Sucrose. *Pediatrics, 116*(4):921-926.

Brock, T. D., Peacher, B., & Pierson, D. (1963). Survey of the Bacteriocins of Enterococci. *Journal of Bacteriology, 86*(4):702-707.

Buick, R.K. & Damoglou, A.P. (1989). Effect of Modified Atmosphere Packaging on the Microbial Development and Visible Shelf Life of Mayonnaise-Based Vegetable Salad. *Journal of the Science of Food and Agriculture*, *46*(3):339-347.

Byakika, S., Mukisa, I. M., Mugabi, R., & Muyanja, C. (2019). Antimicrobial Activity of Lactic Acid Bacteria Starters against Acid Tolerant, Antibiotic Resistant, and Potentially Virulent *E. coli* Isolated from a Fermented Sorghum-Millet Beverage. *International Journal of Microbiology*, DOI: 10.1155/2019/2013539. ECollection.

Canzi, E., Guglielmetti, S., Mora, D., Tamagnini, I., & Parini, C. (2005). Conditions affecting cell surface properties of human intestinal bifidobacteria. Antonie Van Leeuwenhoek, 88, 207–219. doi:10.1007/s10482-005-6501-3.

Ceapa, C., Wopereis, H., Rezaïki, L., Kleerebezem, M., Knol, J. & Oozeer, R. (2013). Influence of Fermented Milk Products, Prebiotics and Probiotics on Microbiota Composition and Health. *Best Practice & Research: Clinical Gastroenterology*, *27*(1):139-155.

Cheftel, J. C. (1995). High-Pressure Microbial Inactivation and Food Preservation. *Food Science and Technology International*, *1*(2-3):75-80.

Chen, Y., Liu, W., Xue, J., Yang, J., Chen, X., Shao, Y., Kwok, L., Bilige, M., Mang, L., Zhang, H. (2014). Angiotensin-Converting Enzyme Inhibitory Activity of Lactobacillus Helveticus Strains from Traditional Fermented Dairy Foods and Antihypertensive Effect of Fermented Milk of Strain H9. *Journal of Dairy Science*, *97*(11):6680-6692.

Choi, Suk-Ho. (2016). Characterization of Airag Collected in Ulaanbaatar, Mongolia with Emphasis on Isolated Lactic Acid Bacteria. *Journal of Animal Science and Technology*, *58*(1):10.

Collado, M. C., Gueimonde, M., Hernández, M., Sanz, Y., & Salminen, S. (2006). Adhesion of Selected Bifidobacterium Strains to Human Intestinal Mucus and the Role of Adhesion in Enteropathogen Exclusion. *Journal of Food Protection*, 68:2672-2678.

Cunningham-Rundles, S., Ahrné, S., Bengmark, S., Johann-Liang, R., Marshall, F., Metakis, L., Califano, C., Dunn, A. M., Grassey, C., Hinds, G., & Cervia, J. (2000). Probiotics and Immune Response. *The American Journal of Gastroenterology*, *95*(1):22-25.

Dahlberg, A. C., & Kosikowsky, F.V. (1948). The Development of Flavor in American Cheddar Cheese Made from Pasteurized Milk with *Streptococcus faecalis* Starter1. *Journal of Dairy Science*, *31*(4):275-284.

Dairy Development Authority Annual Performance Report, Financial Year 2018/2019

De Vuyst, L., & Leroy, F. (2007). Bacteriocins from Lactic Acid Bacteria: Production, Purification, and Food Applications. *Journal of Molecular Microbiology and Biotechnology, 13*(4):194-199.

Degeest, B., Janssens, B., & De Vuyst, L. (2001). Exopolysaccharide (EPS) Biosynthesis by *Lactobacillus Sakei* 0-1: Production Kinetics, Enzyme Activities and EPS Yields. *Journal of Applied Microbiology, 91*(3):470-477.

Del Re, B., Sgorbati, B., Miglioli, M., & Palenzona, D. (2000). Adhesion, Autoaggregation and Hydrophobicity of 13 Strains of *Bifidobacterium longum*. *Letters in Applied Microbiology, 31*(6):438-442.

Denkova, R., Ilieva, S., Denkova, Z., Georgieva, L., & Krastanov, A. (2014). Examination of the Technological Properties of Newly Isolated Strains of the Genus Lactobacillus and Possibilities for their Application in the Composition of Starters. *Biotechnology & Biotechnological Equipment, 28*(3):487-494.

Doyle, R. J., & Rosenberg, M. (1995). Measurement of microbial adhesion to hydrophobic substrata. Methods in Enzymology, 253, 542–550. Doi: 10.1016/S0076-6879(95)53046-0.

Dunn, Joseph. (1996). Pulsed Light and Pulsed Electric Field for Foods and Eggs. *Poultry Sciience, 75*(6):1133-1136.

Eales, J., Lenoir-Wijnkoop, I., King, S., Wood, H., Kok, F. J., Shamir, R., Prentice, A., Edwards, M., Glanville, J., & Atkinson, R. L. (2015). Is Consuming Yoghurt Associated With Weight Management Outcomes? Results from a Systematic Review. *International Journal of Obesity, 40*(5):731-746.

Eglezos, S., Huang, B., Dykes, G., & Fegan, N. (2010). The Prevalence and Concentration of *Bacillus cereus* in Retail Food Products in Brisbane, Australia. *Foodborne Pathogens and Disease, 7*(7):867-870.

Eie, T., Larsen, H., Sørheim, O., Pettersen, M., Hansen, A. Å., Wold, J., Naterstad, K., & Mielnik, M. (2007). New Technologies for Extending Shelf Life. *Italian Journal of Food Science, 19*(2):127-152.

Eklund, T. (1989). Organic Acids and Esters. In *Mechanisms of Action of Food Preservation Procedures*, Pages 196-200. Elsevier Applied Sciences.

Ercolini, D., Hill, P., & Dodd, C. (2003). Bacterial Community Structure and Location in Stilton Cheese. *Applied and Environmental Microbiology, 69*(6):3540-3548.

Farr, D. (1990). High Pressure Technology in the Food Industry. *Trends in Food Science and Technology, 1*(1):14-16.

Fernandes, C. F., Shahani, K. M., & Amer M. A. (1987). Therapeutic Role of Dietary Lactobacilli and Lactobacillic Fermented Dairy Products. *FEMS Microbiology Letters, 46*(3):343-356.

Florez, A.B, Ammor, M.S, Mayo, B., van Hoek, A.H., Aarts, H.J., Huys, G. (2008). Antimicrobial susceptibility of Lactobacillus, Bifidobacterium, Lactococcus and Streptococcus spp. Int J Antimicrob Agents; 31:484-6.

Food Safety and Standards (Licensing and Registration of Food Businesses) Regulation, 2011. Food Safety and Standards Authority of India.

Frengova, G.I., Simova, E.D., Beskova, D.M. & Simov, Z.I. (2002). Exopolysaccharides produced by lactic acid bacteria of kefir grains. Z Naturforsch 57:805–10.

Fuller, R. (1989). Probiotics in Man and Animals. *Journal of Applied Bacteriology*, *66*(5):365-78.

Gálvez, A., Maqueda, M., Valdivia, E., Quesada, A., & Montoya, E. (1986). Characterization and Partial Purification of a Broad Spectrum Antibiotic AS-48 Produced by *Streptococcus faecalis*. *Canadian Journal of Microbiology, 32*(10):765-771.

Gamba, R., Caro, C., Martínez, O., Moretti, A., Antoni, G., & Leon, A. (2016). Antifungal Effect of Kefir Fermented Milk and Shelf Life Improvement of Corn Arepas. *International Journal of Food Microbiology*, 235:85-92.

Garai, G., Duen, M. T., Irastorza, A., & Moreno-Arribas, M. V. (2007). Biogenic Amine Production by Lactic Acid Bacteria Isolated from Cider. *Letters in Applied Microbiology*, 45(5):473-478.

Garg, S. K., & Mital, B. K. (1991). Enterococci in Milk and Milk Products. *Critical Reviews in Microbiology*, *18*(1):15-45.

Gasser, F. (1994). Safety of Lactic Acid Bacteria and Their Occurrence in Human Clinical Infections (Opportunistic Bacteria, Various Infections). *The International System for Agricultural Science and Technology*, 92(1):45-67.

Gatti, M., Borio, F., Fornasari, E. & Neviani, E. (1993) Enterococci in Italian Cheeses. *Latte*, 18:392-397.

Georgieva, R., Yocheva, L., Tserovska, L., Zhelezova, G., Stefanova, N., Atanasova, A., Danguleva, A., Ivanova, G., Karapetkov, N., Rumyan, N., & Karaivanova, E. (2014). Antimicrobial Activity and Antibiotic Susceptibility of Lactobacillus and Bifidobacterium spp. Intended for Use as Starter And Probiotic Cultures. *Biotechnology & Biotechnological Equipment*, *29*(1):84-91.

Gill, H. S., Shu, Q., Lin, H., Rutherfurd, K. J., & Cross, M. L. (2001). Protection against Translocating *Salmonella typhimurium* Infection in Mice by Feeding the Immuno-Enhancing Probiotic *Lactobacillus rhamnosus* Strain HN001. *Medical Microbiology & Immunology*, *190*(3):97-104.

Giraffa, G., Carminati, D., & Neviani, E. (1997). Enterococci Isolated from Dairy Products: A Review of Risks and Potential Technological Use. *Journal of Food Protection*, *60*(6):732-738.

Giraffa, G., Picchioni, N., Neviani, E., & Carminati, D. (1995) Production and Stability of an *Enterococcus faecium* Bacteriocin during Taleggio Cheese Making and Ripening. *Food Microbiology*, 12:301-307.

Gong, H., Meng, X., & Wang, H. (2010). Plantaricin MG Active Against Gram-Negative Bacteria Produced by Lactobacillus Plantarum KLDS1.0391 Isolated from "Jiaoke", A Traditional Fermented Cream from China. *Food Control*, *21*(1):89-96.

Grappin, R., & Beuvier, E. (1997). Possible Implications of Milk Pasteurization on the Manufacture and Sensory Quality of Ripened Cheese, *International Dairy Journal*, *7*(12):751-761.

Guo, X.H., Kim, J.M., Nam, H.M., Park, S.Y., & Kim, J.M. (2010). Screening lactic acid bacteria from swine origins for multistrain probiotics based on in vitro functional properties. Anaerobe, 16, 321–326. doi:10.1016/j.anaerobe.2010.03.006.

Gupta, A., Sharma, N., & Handa, S. (2013). A Comprehensive Study of Different Traditional Fermented Foods/Beverages of Himachal Pradesh to Evaluate their Nutrition Impact on Health and Rich Biodiversity of Fermenting Microorganisms. *International Journal of Research in Applied, Natural and Social Sciences*, 1:19-28.

Guzel-Seydim, Z. B., Kok-Tas, T., Greene, A. K. & Seydim A. C. (2011). Functional Properties of Kefir. *Critical Reviews in Food Science and Nutrition, 51*(3):261-268.

Halász, A., Baráth, Á., Simon-Sarkadi, L. & Holzapfel, W. (1994). Biogenic Amines and Their Production by Microorganisms in Food. *Trends in Food Science and Technology*, 5:42-49.

Hall P. A., Ledenbach L., & Flowers R. S. (2001). Acid Producing Micro-organisms. Compendium of Methods for the Microbiological Examination of Foods, 4:201–207.

Hamid, A. D. (1993) ¹he Indigenous Fermented Foods of the Sudan: A Study in African Food and Nutrition. CAB International, Wallinford, UK.

Hamid, A. D. (1993). The Indigenous Fermented Foods of the Sudan: A Study in African Food and Nutrition. CAB International, Wallinford, UK.

Hamon, E., Horvatovich, P., Marchioni, E., Werner, D., & Ennahar, S. (2014). Investigation of Potential Markers of Acid Resistance in Lactobacillus Plantarum by Comparative Proteomics. *Journal of Applied Microbiology, 116*(1):134-144.

Harding, F. (1999). Milk Quality. A Chapman and Hall Food Science Book. 1st Edition. Aspen Publishers Inc., Gaithersburg, Maryland, 23-50.

Harty, D. W., Oakey, H. J., Patrikakis, M., Hume, E. B., & Knox, K. W. (1994). Pathogenic Potential of Lactobacilli. *International Journal of Food Microbiology, 24*(1-2):179-189.

Hayes, M.C. & Boor, K. (2001). Raw Milk and Fluid Milk Products. *Applied Dairy Microbiology*, 2nd Edition, Pages 59-76. Marcel Dekker.

Hintlian, C. B., & Hotchkiss, J. H. (1987). Comparative Growth of Spoilage and Pathogenic Organisms on Modified Atmosphere-Packed Cooked Beef. *Journal of Food Protection, 50*(3):218-223.

Hotchkiss, J.H. and Chen, J.H. (1996) Microbiological effects of the direct addition of CO_2 to pasteurized milk, J. Dairy Sci., 79 (suppl. 1), 87.

Hur, S., Kim, H., Bahk, Y. Y., & Park, Y. (2016). Overview of Conjugated Linoleic Acid Formation and Accumulation in Animal Products. *Livestock Science*, 195:105–11.

Huth, P., & Park, K. (2012). Influence of Dairy Product and Milk Fat Consumption on Cardiovascular Disease Risk: A Review of the Evidence. *Advances in Nutrition*, 3:266-285.

Ivey, K., Hodgson, J., Kerr, D., Thompson, P., Stojceski, B., & Prince, R. (2014). The Effect of Yoghurt and Its Probiotics on Blood Pressure and Serum Lipid Profile; A Randomised Controlled Trial. *Nutrition, Metabolism and Cardiovascular Diseases, 25*(1):46-51.

Jensen, H., Grimmer, S., Naterstad, K., & Axelsson, L. (2012). In Vitro Testing of Commercial and Potential Probiotic Lactic Acid Bacteria. *International Journal of Food Microbiology, 153*(1-2), 216-222.

Jeong, J., Lee, C., & Chung, D. (2015). Probiotic Lactic Acid Bacteria and Skin Health. *Critical Reviews in Food Science and Nutrition, 56*(14):2331-2337.

Jones, S. E., & Versalovic, J. (2009). Probiotic *Lactobacillus reuteri* Biofilms Produce Antimicrobial and Anti-Inflammatory Factors. *BMC Microbiology, 9*(9):35.

Kaewiad, K., Kaewnopparat, S., & Kaewnopparat, N. (2014). In Vitro Comparison of Probiotic Properties of *Lactobacillus fermentum* SK54 Isolated from New Born Baby with *Lactobacillus rhamnosus* GG ATCC 53103. *Advanced Materials Research*, 1060:215-218.

Kalaˇc, P. (2014). Health Effects and Occurrence of Dietary Polyamines: A Review for the Period 2005 to Mid-2013. *Food Chemistry*, 161:27-39.

Kanak, E. K., & Yilmaz, S. Ö. (2019). Maldi-Tof Mass Spectrometry for the Identification And Detection of Antimicrobial Activity of Lactic Acid Bacteria Isolated from Local Cheeses. *Journal of Food Science and Technology, 39*(2):462-469.

Kapila, S., Sinha, P. R., & Singh, S. (2007). Influence of Feeding Fermented Milk and Non-Fermented Milk Containing Lactobacillus Casei on Immune Response in Mice. *Food and Agricultural Immunology, 18*(1):75-82.

Katimbo, A., Sempiira, E., Mugisa, J. & Kisaalita, W. (2015). Ghee Consumption in Uganda. *Livestock Research for Rural Development, 27*(7), Article 131.

Kesenkas, H., Gursoy O., & Ozbas, H. (2017). Kefir. *Fermented Foods in Health and Disease Prevention,* Chapter 14, Pages. 339-361. Academic Press.

Khanal, R. C. & Olson, K. C. (2004). Factors Affecting Conjugated Linoleic Acid Content in Milk, Meat and Egg: A Review. *Pakistan Journal of Nutrition, 3*(2):82-98.

Kilgour, O. F. G. (2005). Mastering Nutrition (3rd edition). McMillan Publishing Limited New York, 61-63.

Kim, J., Kim, Y., Kim, Y. & Park, Y. (2016). Conjugated Linoleic Acid: Potential Health Benefits as a Functional Food Ingredient. *Annual Review of Food Science and Technology*, 7:221-244.

Kivanc, M., Yilmaz, M. & Cakir, E. (2011). Isolation and Identification of Lactic Acid Bacteria from Boza, and Their Microbial Activity against Several Reporter Strains. *Turkish Journal of Biology, 35*(3):313-324.

Knorr, D., Geulen M., Grahl, T., & Sitzmann, W. (1994). Food Application of High Electric Field Pulses, *Trends Food Science and Technology, 5*(3):71-75.

Kos, B., Suskovic, J., Vukovic, S., Simpraga, M., Frece, J. & Matos, S. (2003). Adhesion And Aggregation Ability of Probiotic Strain *Lactobacillus acidophilus* M92. *Journal of Applied Microbiology,* 94:981-987.

Kozak, K., Charbonneau, D., Sanozky-Dawes, R., & Klaenhammer, T. (2015). Characterization of Bacterial Isolates from the Microbiota of Mothers' Breast Milk and Their Infants. *Gut Microbes, 6*(6):341-351.

Kroger M., Kurmann J. A., & Rasic J. L. (1999). Fermented Milk-Past Present and Future. *Journal of Food Technology*, 43:98-99.

Kwak, D., Jun, D. W., Seo, J., Chung, W., Park, S., Lee, K., Saeed, W., Lee, H., Lee, O., Yoon, B. & Choi, H. (2014). Short-Term Probiotic Therapy Alleviates Small Intestinal Bacterial Overgrowth, But Does Not Improve Intestinal Permeability in Chronic Liver Disease. *European Journal of Gastroenterology & Hepatology*, *26*(12):1353–1359.

Lad, V., (1998). The Complete Book of Ayurvedic Home Remedies. New York: Harmony Books.

Lampert, L. M. (1975). *Modern Dairy Products — Composition, Food Value, Processing, Chemistry, Bacteriology, Testing, Imitation Dairy Products*. Chemical Publishing Company.

Lee, Y., Puong, K., Ouwehand, A., & Salminen, S. (2003). Displacement of Bacterial Pathogens from Mucus and CaCO-2 Cell Surface by Lactobacilli. *Journal of medical microbiology, 52*(10)925-930.

Leite, A. M. O., Miguel, M. A. L., Peixoto, R. S., Rosado, A. S., Silva, J. T., & Paschoalin, V. M. F. (2013). Microbiological, Technological and Therapeutic Properties of Kefir: A Natural Probiotic Beverage. *Brazilian Journal of Microbiology 44*(2):341-349.

Leroy, F., & De Vuyst, L. (2004). Lactic Acid Bacteria as Functional Starter Cultures for the Food Fermentation Industry. *Trends in Food Science & Technology, 15*(2):67-78.

Lin, W.H., Yu, B., Jang, S.H., & Tsen, H.Y. (2007). Different probiotic properties for Lactobacillus fermentum strains isolated from swine and poultry. Anaerobe, 13, 107–113. doi:10.1016/j. anaerobe.2007.04.006.

Linares, D. M., Gomez, C., Renes, E., Fresno, J. M., Tornadijo, M. E., Ross, R. P. & Stanton, C. (2017). Lactic Acid Bacteria and Bifidobacteria with Potential to Design Natural Biofunctional Health-Promoting Dairy Foods. *Frontiers in Microbiology*, 8:846.

Liu, S., Han, Y., & Zhou, Z. (2011). Lactic Acid Bacteria in Traditional Fermented Chinese Foods, *Food Research International*, *44*(3):643-651.

López-Expósito, I., Miralles, B., Amigo, L., & Hernández-Ledesma, B. (2017). Health Effects of Cheese Components with a Focus on Bioactive Peptides. *Fermented Foods in Health and Disease Prevention*, Pages 239-273. Academic Press.

Lv, Jia- ping & Wang, Li- Min. (2009). Bioactive Components in Kefir and Koumiss. *Bioactive Components in Milk and Dairy Products*, Wiley-Blackwell, Pages 251-262.

Mandal, H., Jariwala, R., & Bagchi, T. (2016). Isolation and Characterization of Lactobacilli from Human Faeces and Indigenous Fermented Foods for Their Potential Application as Probiotics. *Canadian Journal of Microbiology, 62*(4):349-59.

Manini, F., Casiraghi, M. C., Poutanen, K., Brasca, M., Erba, D., & Plumed-Ferrer, C. (2016). Characterization of Lactic Acid Bacteria Isolated from Wheat Bran Sourdough. *LWT - Food Science and Technology, 66*:275-283.

Manolopoulou, E., Sarantinopoulos, P., Zoidou, E., Aktypis, A., Moschopoulou, E., Kandarakis, I., & Anifantakis, E. (2003). Evolution of Microbial Populations during Traditional Feta Cheese Manufacture and Ripening. *International Journal of Food Microbiology, 82*(2):153-161.

Manzano, M., Citterio, B., Rondinini, G., & Bertoldi, M. (1993). Microbiological Aspects of Natural Starter in Montasio Cheese Making. *International System for Agricultural Science and Technology*, 42(1):163-170.

Maragkoudakis, P., Zoumpopoulou, G., Miaris, C., Kalantzopoulos, G., Pot, B., & Tsakalidou, E. (2006). Probiotic Potential of Lactobacillus Strains Isolated from Dairy Products. *International Dairy Journal, 16*(3):189-199.

Marco, M. L., Heeney, D., Binda, S., Cifelli, C. J., Cotter, P. D., Foligné, B., Gänzle, M., Kort, R., Pasin, G., Pihlanto, A., Smid, E. J., & Hutkins, R. (2017). Health Benefits of Fermented Foods: Microbiota and Beyond. *Current Opinion in Biotechnology,* 44:94-102.

Martín, R., Langa, S., Reviriego, C., Jimínez, E., Marín, M. L., Xaus, J., Fernández, L., & Rodríguez, J. M. (2003). Human Milk is a Source of Lactic Acid Bacteria for the Infant Gut. *The Journal of Pediatrics, 143*(6):754-758.

Martín, R., Soberón, N., Vaneechoutte, M., Flórez, A., Vázquez, F., & Suárez, J. (2008). Characterization of Indigenous Vaginal Lactobacilli from Healthy Women as Probiotic Candidates. *International Microbiology*, 11:262-266.

McSweeney, P., & Sousa-Gallagher, M. J. (2000). Biochemical Pathways for the Production of Flavour Compounds in Cheeses During Ripening: A Review. *Dairy Science and Technology, 80*(3):293-324.

Menteş, Ö., Ercan, R., & Akçelik, M. (2007). Inhibitor Activities of Two Lactobacillus Strains, Isolated from Sourdough, Against Rope-Forming Bacillus Strains. *Food Control, 18*(4):359-363.

Messens, W., Estepar-Garcia, J., Dewettinck, K., & Huyghebaert, A. (1999). Proteolysis of High-Pressure-Treated *Gouda* Cheese. *International Dairy Journal, 9*(11):775-782.

Minervini, F., De Angelis, M., Di Cagno, R., Pinto, D., Siragusa, S., Rizzello, C., & Gobbetti, M. (2010). Robustness of *Lactobacillus plantarum* Starters during Daily Propagation of Wheat Flour Sourdough Type I. *Food Microbiology, 27*(7):897-908.

Mohr, C. M., Engelgau, D. E., Leeper, S. A., & Charboneau, B. L. (1989). Membrane Technology Overview, In *Membrane Applications and Research in Food Processing*, Noyes Data Co., Park Ridge, NJ, Page 15–39.

Mortensen, B. K. (2011). Butter and Other Milk Fat Products | Anhydrous Milk Fat/Butter Oil and Ghee. *Encyclopedia of Dairy Sciences*, Page 515-521.

Mu, Z., Yang, X., & Hongli, Y. (2012). Detection and Identification of Wild Yeast in Koumiss. *Food Microbiology, 31*(2):301-308.

Mufandaedza, J., Viljoen, B., Feresu, S., & Gadaga, H. (2006). Antimicrobial Properties of Lactic Acid Bacteria and Yeast-LAB Cultures Isolated from Traditional Fermented Milk against Pathogenic *Escherichia coli* And *Salmonella enteritidis* Strains. *International Journal of Food Microbiology, 108*(1):147-152.

Mugampoza, D., Gkatzionis, K., Swift, B., Rees, C., & Dodd, C. (2020). Diversity of *Lactobacillus* Species of Stilton Cheese Relates to Site of Isolation. *Frontiers in Microbiology*, *11*:904.

Mukisa, I., & Kiwanuka, B. (2017). Traditional Processing, Composition, Microbial Quality and Sensory Characteristics of Eshabwe (Ghee Sauce). *International Journal of Dairy Technology, 71*(1):149-157.

Munro, D. S., Cant, P. A. E, Mac Gibbon, A. K. H., Illingworth, D., Kennett, A. and Main, A. J. (1992) Concentrated milkfat products. In ¹he ¹echnology of Dairy products, ed. R. Early. Blackie and Sons Ltd, Glasgow, pp. 117-145.

Naruszewicz, M., Johansson, M., Zapolska-Downar, D., & Bukowska, H. (2003). Effect of *Lactobacillus plantarum* 299v on Cardiovascular Disease Risk Factor in Smokers. *The American Journal of Clinical Nutrition, 76*(6):1249-1255.

Narvhus, J. A., & Gadaga, T. H. (2003). The Role of Interaction between Yeasts and Lactic Acid Bacteria in African Fermented Milks: A Review. *International Journal of Food Microbiology, 86*(1-2):51-60.

Nhavi N. G. & Patwardhan V. N. (1946). The Absorption of Fats from the Human Intestine. *Indian Journal of Medical Research,* 34:49-58.

Nickerson, S.C. (1999). Milk production: factors affecting milk composition. In: Milk quality, Aspan, H.F. (Ed.). 1st Edition. Chapman and Hall, Glasgow, Scotland, UK. Pages: 3-23.

Nielsen, B., Gurakan G. C., & Unlu G. (2014). Kefir: A Multifaceted Fermented Dairy Product. *Probiotics and Antimicrobial Proteins, 6*(3-4):123-135.

Nutrition Data for Butter Oil, Anhydrous (ghee) per 100 gram reference amount". US Department of Agriculture National Nutrient Database. May 2016.

Ogilvie, T. H. (1986). Dairy Cattle: Principles, Practices, Problems, Profits. Third Edition. *The Canadian Veterinary Journal, 27*(2):101.

Ohsawa, K., Uchida, N., Ohki, K., Nakamura, Y., & Yokogoshi, H. (2015). *Lactobacillus helveticus*–Fermented Milk Improves Learning and Memory in Mice. *Nutritional Neuroscience, 18*(5):232-240.

Ongol, M. P., & Asano, K. (2009). Main Microorganisms Involved in the Fermentation of Ugandan Ghee. *International Journal of Food Microbiology*, *133*(3):286-291.

Pakkanen, R., & Aalto, J. (1997). Growth Factors and Antimicrobial Factors of Bovine Colostrum: A Review. *International Dairy Journal,* 7(5):285-297.

Palaniswamy, S. K., & Govindaswamy V. (2016). In-Vitro Probiotic Characteristics Assessment of Feruloyl Esterase and Glutamate Decarboxylase Producing *Lactobacillus* Spp. Isolated From Traditional Fermented Millet Porridge (Kambu Koozh), *LWT-Food Science and Technology*, 68:208-216.

Pegg, A. E (2013). Toxicity of Polyamines and their Metabolic Products. *Chemical Research in Toxicology*, 26:1782–1800.

Rai, A. K., Sanjukta, S., & Jeyaram, K. (2017). Production of Angiotensin I Converting Enzyme Inhibitory (ACE-I) Peptides During Milk Fermentation and their Role in Reducing Hypertension. *Critical Reviews in Food Science and Nutrition, 57*(13):2789-2800.

Rajoka, R. M. S., Mehwish, H. M., Siddiq, M., Haobin, Z., Zhu, J., Yan, L., Shao, D., Xu, X., & Shi, J. (2017). Identification, Characterization, and Probiotic Potential of Lactobacillus Rhamnosus Isolated from Human Milk. *LWT-Food Science and Technology*, 84:271-280.

Rajorhia, G. S. (1993) Ghee. In Encyclopaedia of Food Science, Food ¹echnology and Nutrition, Vol. 4, eds R. Macrae, R. K. Robinson and M. J. Sadler. Academic Press Ltd, London, pp. 2186-2192.

Randazzo, C., Restuccia, C., Romano, A., & Caggia, C. (2004). Lactobacillus Casei, Dominant Species in Naturally Fermented Sicilian Green Olives. *International Journal of Food Microbiology*, 90(1):9-14.

Rong, J., Zheng, H., Liu, M., Hu, X., Wang, T., Zhang, X., Jin, F. & Wang, L. (2015). Probiotic and Anti-Inflammatory Attributes of an Isolate Lactobacillus helveticus NS8 from Mongolian Fermented Koumiss. *BMC Microbiology*, *15*(1):196.

Rosa, D. D., Dias, M. M., Grzeskowiak, L. M., Reis, S., Conceicao, L. L. & Peluzio, M. G. (2017). Milk Kefir: Nutritional, Microbiological and Health Benefits. *Nutrition Research Reviews, 30*(1):1-15.

Rosenberg, Moshe. (1995). Current and Future Applications for Membrane Processes in the Dairy Industry. *Trends Food Science and Technology, 6*(1):12-19.

Routray, W., & Mishra, H. N. (2011). Scientific and Technical Aspects of Yogurt Aroma and Taste: A Review. *Comprehensive Reviews in Food Science and Food Safety*, 10(4):208-220.

Ruiz-Capillas, C. & Jiménez-Colmenero, F. (2004). Biogenic Amines in Meat and Meat Products. *Critical Reviews in Food Science and Nutrition*, 44:489-499.

Rzepkowska, A., Zielińska, D., Ołdak, A. & Kołożyn-Krajewska, D. (2016). Safety Assessment and Antimicrobial Properties of the Lactic Acid Bacteria Strains Isolated From Polish Raw Fermented Meat Products. *International Journal of Food Properties, 20*(11):2736-2747.

Saarela, M., Lähteenmäki, L., Crittenden, R., Salminen, S., & Mattila-Sandholm, T. (2002). Gut Bacteria and Health Foods - The European Perspective. *International Journal of Food Microbiology, 78*(1-2):99-117.

Saez-Lara, M., Gomez-Llorente, C., Plaza-Díaz, J. & Gil, Á. (2015). The Role of Probiotic Lactic Acid Bacteria and Bifidobacteria in the Prevention and Treatment of Inflammatory Bowel Disease and Other Related Diseases: A Systematic Review of Randomized Human Clinical Trials. *BioMed Research International*, 3:15.

Sari, E. K., Bakir, B., Aydin, B. D. & Sozmen, M. The Effects of Kefir, Koumiss, Yogurt and Commercial Probiotic Formulations on PPARα and PPAR-β/δ Expressions in Mouse Kidney. *Biotechnic & Histochemistry, 89*(4):287-295.

Sarkiyayi S. & Shehu M. (2011). Effects of Boiling and Fermentation on the Nutrient Composition of Cow Milk in Kaduna Metropolis. *Research Journal of Chemical Sciences, 1*(7):81-84.

Satin, M. (1996). Food Irradiation and Pasteurization, in *Food Irradiation*: A Guidebook, (2nd edition) Lancaster, Page: 1-41.

Schanbacher, F. L., Talhouk, R., Murray, F. A., Gherman, L. I., & Willett, L. B. (1998). Milk-Borne Bioactive Peptides. *International Dairy Journal, 8*(3):393-403.

Schiffrin, E.J., Rochat, F., Link-Amster, H., Aeschlimann, J.M., & Donnet-Hughes, A. (1995). Immunomodulation of Human Blood Cells Following the Ingestion of Lactic Acid Bacteria. *Journal of Dairy Science*, 78:491-497.

Schlegelova, J., Brychta, J., Klimova, E., Napravnikova, E., & Babak, V. (2003). The Prevalence of and Resistance to Antimicrobial Agents of Bacillus Cereus Isolates from Foodstuffs. *Veterinární Medicína*, 48:331-338.

Schmidt, Karen A., Stupar, J., Shirley, John E., & Adapa, S. (1996). Factors Affecting Titratable Acidity in Raw Milk, *Kansas Agricultural Experiment Station Research Reports*, Vol. 0: Iss. 2.

Seale, D. R., Henderson, A. R., Pettersson, K. O., & Lowe, J. F. (1986). The Effect of Addition of Sugar and Inoculation with Two Commercial Inoculants on the Fermentation of Lucerne Silage in Laboratory Silos. *Grass and Forage Science, 41*(1):61-70.

Sempiira, J. E., Katimbo, A., Mugisa, D. J., & Kisaalita, W. S., 2017. Ghee-Making in the Cattle Corridor of Uganda. *African Journal of Food, Agriculture, Nutrition and Development, 17*(1):11771-11786.

Sharma H. M., (1990) Butter Oil (Ghee) – Myths and Facts. *Indian Journal of Clinical Practice*, 1:31-32

Shiby, V. K., & Mishra. H. N., (2013). Fermented Milks and Milk Products as Functional Foods—A Review. *Critical Reviews in Food Science and Nutrition 53*(5):482-496.

Simova, E. D., Beshkova, D. B., & Dimitrov, Z. (2009). Characterization and Antimicrobial Spectrum of Bacteriocins Produced by Lactic Acid Bacteria Isolated from Traditional Bulgarian Dairy Products. *Journal of Applied Microbiology*, *106*(2):692-701.

Siragusa, S., De-Angelis, M., Di-Cagno, R., Rizzelo, C. G., Coda, R., & Gobbetti, M. (2007). Synthesis of γ-Aminobutyric Acid by Lactic Acid Bacteria Isolated from a Variety of Italian Cheeses. *Applied and Environmental Microbiology*, *73*(22):7283-7290.

Sofi, F., Buccioni, A., Cesari, F., Gori, A. M., Minieri, S., Mannini, L., Casini, A., Gensini, G. F., Abbate, R., & Antongiovanni, M. (2010). Effects of a Dairy Product (Pecorino Cheese) Naturally Rich in Cis-9, Trans-11 Conjugated Linoleic Acid on Lipid, Inflammatory and Haemorheological Variables: A Dietary Intervention Study. *Nutrition, Metabolism and Cardiovascular Diseases, 20*(2):117-124.

Spano, G., Chieppa, G., Beneduce. L., & Massa, S. (2004). Expression Analysis of Putative *arcA*, *arcB* and *arcC* Genes Partially Cloned from *Lactobacillus plantarum* isolated from Wine. *Journal of Applied Microbiology*, *96*(1):185-193.

Sserunjogi M. L., Abrahamsen, R. K. and Narvhusa, J. (1998). A Review Paper: Current Knowledge of Ghee and Related Products. Int. Dairy Journal 8 (1998) 677-688.

Sserunjogi, M., Abrahamsen, R., & Narvhus, J. (1998). A Review Paper: Current Knowledge of Ghee and Related Products. *International Dairy Journal*, 8:677-688.

Staal, P. (1986). IDF and the Development of Milk Pasteurization. Bulletin, International Dairy Federation, 200:4-8.

Steggerda, F. R. & Mitchell, H. H. (1951). The Calcium Balance of Adult Human Subjects on High- and Low-Fat (Butter) Diets: One Figure. *The Journal of Nutrition, 45*(2):201-211.

Stojanovski, S., Kirilov, N. & Ivanova, I. (2013). API ZYM Enzymatic Profile of Lactic Acid Bacteria Isolated from Traditional Bulgarian Meat Product "Lukanka". *Bulgarian Journal of Agricultural Science, 19*(2):86-89.

Succi, M., Tremonte, P., Reale, A., Sorrentino, E., Grazia, L., Pacifico, S. and Coppola, R. (2005). Bile salt and acid tolerance of *Lactobacillus rhamnosus* strains isolated from Parmigiano Reggiano cheese. *FEMS Microbiology Letters* 244, 129-137.

Tambekar, D. H., & Bhutada, S. A. (2010). An evaluation of probiotic potential of Lactobacillus sp. from milk of domestic animals and commercial available probiotic preparations in prevention of enteric bacterial infections. Recent Research in Science and Technology, 2, 82–88.

Thakur, B. R., & Singh, R. K. (1995). Combination Process in Food Irradiation: A Review. *Trends in Food Science and Technology, 6*(1):7-11.

Thunell, R. K., & Sandine, W. E. (1985). Types of Starter Cultures. In *Bacterial Starter Cultures for Foods*. Pages, 127-132. CRC Press.

Tsakalidou, E., Manolopoulou, E., Tsilibari, V., Georgalaki, M., & Kalantzopoulos, G. (1993). Esterolytic Activities of Enterococcus Durans and Enterococcus Faecium Strains Isolated from Greek Cheese. *Netherlands Milk and Dairy Journal, 47*(3):145-150.

Tsong, T.Y. (1990). On Electroporation of Cell Membranes and Some Related Phenomena. *Bioelectrochemistry and Bioenergetics, 24*(3):271-295.

Tulumoglu, S., Yuksekdag, Z., Beyatli, Y., Simsek, Ö., Cinar, B., & Tok, E. (2013). Probiotic Properties of Lactobacilli Species Isolated from Children's Feces. *Anaerobe*, 24:36-42.

Umar Dandare, S., Ezeonwumelu, I., & Gusau, A. (2014). Comparative analysis of nutrient composition of milk from different breeds of cows. *European Journal of Applied Engineering and Scientific Research, 3*(2):33-36.

Uniacke-Lowe, T. (2011). Koumiss. *Encyclopedia of Dairy Sciences,* 2:512-517.

Valente, B. A. & Taehyun, J. (2003). Emerging Processing and Preservation Technologies for Milk and Dairy Products. Food science and food technology, 8, 393-403.

Van den Berg, J. C. T. (1988) Dairy ¹echnology in the ¹ropics. Pudoc, Wageningen, Netherlands.

Van Kessel, J. A., Karns, J. S., Gorski, L., Mccluskey, B. & Perdue, M. L. (2004). Prevalence of Salmonellae, *Listeria monocytogenes*, and Fecal Coliforms in Bulk Tank Milk on US Dairies. *Journal of Dairy Science, 87*(9):2822-2830.

Vandenplas, Y., Huys, G. & Daube, G. (2014). Probiotics: An Update. *Jornal de Pediatria, 91*(1):6-21.

Vasiljevic, T. and Shah, N. P. (2007). Fermented milk: Health benefits beyond probiotic effect. *Handbook of Food Products Manufacturing*, pp.99-115

Verónica, F., Andrea, Q., Jorge, R. & Suárez, V. (2016). Functional Properties of Lactobacillus Plantarum Strains: A Study In Vitro of Heat Stress Influence. *Food Microbiology*, 54:154-161.

Vries, M., Vaughan, E., Kleerebezem, M., & De Vos, W. (2006). Lactobacillus plantarum - Survival, Functional and Potential Probiotic Properties in the Human Intestinal Tract. *International Dairy Journal, 16*(9):1018-1028.

Walstra, P., Geurts, T. J., Noomen, A., Jellema, A., & Van Boekel, M. A. J. S. (1999). Heat Treatment, in Dairy Technology: Principles of Milk Properties and Processes, Marcel Dekker, Page: 189-240.

Walther, B., Schmid, A., Sieber, R., & Wehrmuller, K. (2008). Cheese in Nutrition and Health. *Dairy Science and Technology, 88*(4):389-405.

Wang, H., K. A. Livingston, C. S. Fox, J. B. Meigs, and P. F. Jacques. (2013). Yogurt consumption is associated with better diet quality and metabolic profile in American men and women. Nutrition Research 33 (1):18–26.

Wangalwa, R., Tolo, C., Kagoro, G., & Matofari, J. (2016). Assessment of On-Farm Milk Handling Practices in Mbarara District Southwestern Uganda. *African Journal of Dairy Farming and Milk Production, 3*:141-148.

Widyastuti, Y., Rohmatussolihat, R. & Febrisiantosa, A. (2014). The Role of Lactic Acid Bacteria in Milk Fermentation. *Food and Nutrition Sciences, 5*(4):435-442.

Wszolek, M., Teahan, B., Guldager, H. S. & Tamime, A.Y. (2007). Production of Kefir, Koumiss and other Related Products. *Fermented Milks,* Pages 174-216.

Wu, R., Wang, L., Wang, J., Li, H., Menghe, B., Wu, J., Guo, M., & Zhang, H. (2009). Isolation and Preliminary Probiotic Selection of Lactobacilli from Koumiss in Inner Mongolia. *Journal of Basic Microbiology, 49*(3):318-326.

Yam, K. L., & Lee, D. S. (1995). Design of Modified Atmosphere Packaging for Fresh Produce. In *Active Food Packaging*, Pages. 55-73. Chapman & Hall.

Yao, G., Yu, J., Hou, Q., Hui, W., Liu, W., Kwok, L. Y., Menghe, B., Sun, T., Zhang, H., & Zhang, W. (2017). A perspective study of koumiss microbiome by metagenomics analysis based on single-cell amplification technique. *Frontiers in Microbiology*, 8.

Yin, Y., Zhang, Q. H., & Sastry, S. K. (1997.) High Voltage Pulsed Electric Field Treatment Chambers for the Preservation of Liquid Food Products, U.S. Patent No. 5,690,978.

Zacharof, M. P. & Lovitt, R. W. (2012). Bacteriocins Produced by Lactic Acid Bacteria. *APCBEE Procedia*, 2:50-56.

Zhang, Q., Barbosa-Cánovas, G.V. & Swanson, B.G. (1995). Engineering Aspects of Pulsed Electric Field Pasteurization. *Journal of Food Engineering, 25*(2):261-281.

Zhang, Wenyi & Zhang, Heping. (2012). Fermentation and Koumiss. *Handbook of Animal-Based Fermented Food and Beverage Technology*, CRC Press, Pages 165-172.

APPENDICES

Appendix I: BLAST results for the 72 bacteria isolated from Ugandan traditional ghee

S/No	CLOSEST RELATIVE	% ID	E-VALUE	GENE ACCESSION NO.
1.	Enterococcus hirae	100	2e -72	MN341098
2.	Enterococcus faecium	100	4e -75	KT626391
3	Enterococcus faecium	89.33	3e -15	KT626391
4.	Enterococcus hirae	95.70	7e -32	MN341098
5.	Enterococcus hirae	98.99	9e -41	MN341098
6.	Enterococcus faecium	99.01	7e -42	KT626391
7.	Enterococcus faecalis	98.73	8e -71	KX648537
8.	Enterococcus hirae	97.75	1e -33	MN341098
9.	Enterococcus hirae	94.74	5e -58	MN341098
10	Enterococcus faecalis	100	2e -71	MN493753
11.	**No Search Results- No Significant Similarity Found**			
12.	Enterococcus faecium	98.19	7e -73	LC035103
13.	Enterococcus hirae	91.13	2e -37	MN341098
14.	Enterococcus faecium	100	2e -70	KY569502
15.	Enterococcus faecium	100	2e -71	KT626392
16.	Enterococcus faecium	99.37	8e -72	KT626391
17.	Enterococcus faecium	100	3e -74	KT626391
18.	Enterococcus hirae	100	7e -71	MN341099
19.	Enterococcus faecium	100	2e -70	KY569500
20.	Lactobacillus rhamnosus	100	2e -73	MN341130
21.	Enterococcus faecium	100	3e -70	KY569502
22.	Enterococcus faecium	100	7e -71	MN341099
23.	Enterococcus faecium	100	2e- 71	KM921922
24.	**No Search Results- No Significant Similarity Found**			
25.	Enterococcus faecium	98.77	1e -73	MT000128
26.	Lactobacillus rhamnosus	92.62	2e -51	MN341176
27.	Lactobacillus rhamnosus	99.36	3e -71	MN341130
28.	Lactobacillus plantarum	100	1e -72	MT645592
29.	Enterococcus faecium	100	1e -72	KT626392
30.	Lactobacillus plantarum	100	2e -72	MT645597
31.	Lactobacillus rhamnosus	99.36	5e -72	AF375897
32.	Enterococcus faecium	100	9e -75	KT626391
33.	Enterococcus hirae	100	2e -71	MN341099
34.	Enterococcus faecium	100	4e -73	KT626391
35.	Bacillus cereus	100	2e -72	MH997515
36.	Bacillus cereus	97.39	3e -65	MH997530
37.	Lactobacillus rhamnosus	100	4e -73	MN341130
38.	Enterococcus faecium	100	1e -73	KT626391
39.	Lactobacillus plantarum	100	6e -72	MT645592
40.	Lactobacillus rhamnosus	100	2e -72	KU510246
41.	Enterococcus faecium	96.73	1e -63	MN493726

42.	Lactobacillus plantarum	100	2e -72	MT645594
43.	Enterococcus faecium	99.37	5e -73	KM921921
44.	Lactobacillus rhamnosus	98.14	7e -71	KM457453
45.	**No Search Results- No Significant Similarity Found**			
46.	Enterococcus faecium	98.69	5e -68	MT000128
47.	Lactobacillus plantarum	100	8e -72	MT645597
48.	Enterococcus faecium	98.74	2e - 71	KT626391
49.	Lactobacillus plantarum	100	2e -72	MT645592
50.	Lactobacillus plantarum	100	2e -72	MT645592
51.	Lactobacillus rhamnosus	93.33	3e -59	KT820080
52.	Lactobacillus plantarum	100	1e- 72	MT645607
53.	Lactobacillus plantarum	100	1e- 72	MT645597
54.	Enterococcus hirae	100	2e -71	MN341099
55.	Lactobacillus rhamnosus	99.38	1e -74	KU510245
56.	Enterococcus faecium	100	5e -72	MT000128
57.	Lactobacillus plantarum	100	4e -74	KT626385
58.	Lactobacillus plantarum	100	2e -71	KJ775808
59.	Enterococcus hirae	100	7e -71	MN341099
60.	Enterococcus hirae	100	2e -71	MN341099
61.	Lactobacillus plantarum	100	5e -72	MT645597
62.	Lactobacillus plantarum	100	5e -72	MT645592
63.	Lactobacillus plantarum	100	5e -72	MT645592
64.	Lactobacillus rhamnosus	100	6e -74	KT626387
65.	Lactobacillus plantarum	99.36	2e -71	MT434011
66.	Lactobacillus plantarum	100	2e -72	MT645592
67.	Lactobacillus plantarum	100	6e -72	MT645607
68.	Lactobacillus plantarum	98.73	2e -70	MT434011
69.	Lactobacillus plantarum	100	5e -73	MT434011
70.	Lactobacillus rhamnosus	96.49	9e -16	MN341147
71.	Lactobacillus plantarum	100	6e -72	MT645597
72.	Clostridium neonatale	93.55	4e -14	

Appendix II Antimicrobial activity of *L. rhamnosus* 31 MbCM against *E. coli* ATCC 25922. Antagonism is demonstrated by the presence of clear halo zones in different sections of the plate.