GENETIC DIVERSITY OF *Streptococcus infantarius subsp. infantarius* ISOLATED FROM HUMAN STOOL, RAW AND FERMENTED MILK FROM ISIOLO CENTRAL SUB COUNTY, KENYA

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2018

Genetic diversity of *Streptococcus infantarius subsp. infantarius* isolated from human stool, raw and fermented milk from Isiolo Central Sub County, Kenya

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A thesis submitted in partial fulfillment for the Degree of Master of Science in Molecular Biology and Bioinformatics in the Jomo Kenyatta University of Agriculture and Technology

2018

DECLARATION

This thesis is my original work and has not been presented for the award of a degree in any other University

Signature……. Date…….

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This thesis has been submitted for examination with our approval as the University Supervisors

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DEDICATION

This work is dedicated to my parent's, Mr Ernest Aliwa, Mrs Lillian Akech and Uncle Walter Ologi for their everlasting support.

ACKNOWLEDGEMENT

I acknowledge the support from my supervisors Dr. Jane Ngaira and Mr. Henry Kissinger Ochieng (Department of Biochemistry and Statistics and Actuarial Sciences respectively, Jomo Kenyatta University of Agriculture and Technology**).** I would also like to appreciate the support and contribution of Professor Wambui-Kogi Makau and Dr. Dasel Kaindi Mulwa (Department of Food Science Nutrition and Technology, University of Nairobi).

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ABBREVIATIONS AND ACRONYMS

AFDP African Fermented Dairy Products

ASALs Arid and Semi-Arid Lands

BP Base pair

CFU Colony Forming Unit

DNTPs Deoxynucleotide Triphosphates

DNA Deoxyribonucleic acid

ERC Ethical Review Committee

EFSA European Food Safety Authority)

FDA Food and Drug Administration

FDP Fermented Dairy Products

GRAS Generally Recognized as Safe

LAB Lactic Acid Bacteria

QPS Qualified Presumption for Safety

RT Room Temperature

SBSEC *Streptococcus bovis*/*Streptococcus equinus* complex

Sii *Streptococcus infantarius* subsp. *infantarius*

SPSS Statistical package for social sciences

PCR Polymerase Chain Reaction

ABSTRACT

Unlike *Streptococcus thermophilus* which is classified for food production and human consumption using the "generally recognized as safe" (GRAS, Food and Drug Administration (FDA) or the "qualified presumption of safety" (QPS), European Food Safety Authority (EFSA), *Streptococcus infantarius subsp. infantarius* (Sii) is associated with various human and animal infections such as endocarditis, bacteremia and colorectal cancer. Sii has been isolated as predominant species from fermented African dairy products in Kenya, with a prevalence rate of being 8.5 percent. Information on microbial load and genetic diversity of Sii isolates from consumers of traditionally fermented dairy products and human stool from consuming population is limited. A total of 121 Sii isolates were obtained from 47 samples from each three sample categories collected from Isiolo Central Sub-County. The Sii isolates were morphologically and biochemically identified and found to have similarity. Sii isolate count were least present in human stool sample with mean $\log_{10}$ Colony forming unit/ml (CFU/ml) of 1.11, whereas raw milk had the second highest Sii isolates count with a mean $\log_{10}$Cfu/ml 2.64. Lastly, fermented milk registered the highest Sii count with a mean $\log_{10}$Cfu/ml of 6.03. The total Sii count across the three samples category differed significantly ($p<0.05$). Subsequently, Sii isolate molecular identifications were conducted using SBSEC-specific16S rRNA gene PCR assay. 7 out of 121 Sii isolates displayed positive results for 16S rRNA gene, representing a prevalence of 5.8%. Positive control of Sii CJ18 was used to confirm the results, at the same time, negative control was used during the PCR. Genetic diversity determination Sii was achieved by subjecting isolates to rep-PCR and the gel image of the isolates analyzed using UPGMA clustering Algorithm to generate Dendogram. Conclusively, raw and fermented milk are major sources of Sii among consumers of the traditionally fermented milk and its products leading to the faecal carriage of Sii.

CHAPTER ONE

INTRODUCTION

1.1 Background Information

Streptococcus infantarius subsp. infantarius (Sii) belong to a group of *Streptococcus bovis/ Streptococcus equinux* complex (SBSEC) composed of human pathogenic species associated with infections and predominant lactic acid bacteria (LAB) in spontaneously fermented milk products. Spontaneously fermented milk products such as fermented camel milk (*suusac)* in Kenya and Somalia or *gariss* in Sudan are initiated through continuous utilization of vessels and back slopping but play a vital role in the diet of pastoral communities (Farah *et al*., 2007). However, the predominance Sii has previously been reported for several traditionally fermented milk products found among West and East African countries, such products include *suusac* (Jans *et al*., 2012c), *gariss* (Abdelgadir *et al*., 2008) as well as fermented cow milk products *roab* from Sudan (Hamza *et al*., 2009) and *fene* from Côte d'Ivoire (Wullsschleger, 2009). The predominant species in spontaneous fermentation contribute to shelf-life and quality of fermented food product (Jans *et al*., 2012c). Some SBSEC strains are ingested by millions of humans as part of their daily diet and might therefore be considered safe. SBSEC have been detected in Mexican Greek and Italian cheese (Diaz-Ruiz *et al*., 2003; Pacini *et al*., 2006), fermented Mexican maize drink and fermented Bangladesh milk; (Rashid *et al*., 2009; Renye *et al*., 2011). Its isolation as predominant species from fermented dairy products in Europe, Asia and Africa has increased over the past few years (Jans *et al*., 2013b).

Streptococci form a diverse genus of lactic acid bacteria (LAB) which includes many pathogenic species. However, many species are either potentially harmless commensals or opportunistic pathogens and at least one species, *Streptococcus thermophilus* is considered safe for use in food (Whiley and Hardie, 2009). *Streptococcus bovis/*

Streptococcus equinux complex (SBSEC) is a collective group of human and animal derived *streptococci* that are commensals, opportunistic pathogens or associates of food fermentation (Angulo *et al*., 2009).

There has been massive changes in classification of SBSEC but it currently consists of 7 sub-species namely: *Streptococcus gallolyticus subsp*. *gallolyticus, S. gallolyticus subsp. macedonicus, S. gallolyticus subsp*. *pasteurianus, Streptococcus infantarius subsp*. *infantarius, Streptococcus lutetiensis, Streptococcus alactolyticus* and *Streptococcus equinux* (Jans *et al*., 2014). SBSEC are causative agents of rumen acidosis and infective endocarditis in animals. A strong association has been established between bacteremia, infective endocarditis and colorectal cancer (Jan's *et al*., 2013b).

Interestingly, the genetic analysis of major African dairy Sii strain has revealed genetic decay and a lactose metabolism paralleling that of *Streptococcus thermophilus* (Jans *et al*., 2013a). There are reports of higher prevalence of SBEC in *suusac* samples and less noticeable numbers in fermented milk products collected in Western Africa countries (Jans *et al*., 2013b). Fermented dairy products in Western Africa and Eastern Africa Contained high prevalence of *Sii* between 6 and 8 $\log_{10}$ CFU/mL (Abdelgadir *et al*., 2008; Jans *et al*., 2012a; Wullschleger, 2009; Wullschleger *et al*., 2013). However, there exists little information on potential presence of *Sii* and other SBSEC members in stool in other African regions such Kenya. Some of the microbes contained in raw milk include *Streptococcus agalactiae, Lactococcus spp*. and *Enterococcus spp* isolated on M17 agar media (Jans *et al*., 2012c). Spontaneously fermented milk has been identified as major source of dairy adapted variants *Sii* with no other natural reservoir discovered yet.

Therefore, the consumption of milk containing high titers of *Sii* by children representing 30% of the population from the study countries (United Nations Department of Economic and Social Affairs Population Division, 2011) has to be analytically assessed

for any assumed risks associated with *Sii* (Jans *et al*., 2013b). A comparison of the prevalence of African variant Sii in raw and spontaneously fermented milk products from different animals and human stool needs to be established to separate food from human commensals and pathogenic strains.

1.2 Statement of the Problem

Fermented dairy products play an important role for prolonged shelf life, microbial safety as well as for the preparation of weaning food for children across Africa. Traditionally fermented milk products in Kenya, Somalia, Sudan, Mali, and Ivory Coast have been found to be dominated by Sii and potentially other members of SBSEC in high titers up to 10^8 viable cells per mL of fermented end product. However, unlike *Streptococcus thermophilus* which is classified for food production and human consumption using the "generally recognized as safe" (GRAS, Food and Drug Administration (FDA)) or the "qualified presumption of safety" (QPS), European Food Safety Authority (EFSA), Sii is associated with various human and animal infections such as endocarditis, bacteremia and colorectal cancer. Elucidation of the genetic diversity of presumptive Sii is crucial for food safety due to consumption of large quantities of the bacteria especially by children. There is limited information linking presumptive Sii strain in raw milk, traditionally fermented milk products, and human stool samples from consuming population to clearly separate human commensal and pathogenic strains. Hence, an immediate need determine genetic diversity and epidemiological link and possible public health risks associated with Sii in order to improve traditional milk fermentation in Kenya.

1.3 Justification

Pastoralists living in Isiolo (Gabra, Rendile, Turkana, Sakuye and Somali) are primarily camel keepers while the Borana and Samburu are traditional cattle owners. Milk and milk products in these areas serve as their main livelihood strategy. Harvesting and handling of raw camel milk and fermented milk products (*suusac*) is done with little

consideration to hygiene. Milk and milk products are prepared and stored in containers which are difficult to clean and are usually cleaned with limited amount of water. This reduces the market value and increases the risks of gastro-intestinal illness among the consumers. Among the pastoralist communities it is common thing to consume camel milk and fermented milk products without boiling or any form of treatment and this could be a major risk factor contributing to incidences of diarrhea and vomiting among pastoralists of Northern Kenya. The information obtained from this study will be used to educate the public on the safety and quality of traditionally fermented milk. For example, the public will be educated on the novel methods for milk fermentation and hygienic considerations during milk fermentation processes and storage.

1.4 Main Objective

To determine morphological characteristics and genetic diversity of Sii isolates from human stool, raw and fermented milk from Isiolo Central Sub-County, Kenya

1.4.1 Specific Objectives

1. To isolate and identify Sii in human stool, raw, and fermented milk using a suitable morphological and molecular approach
2. To determine genetic diversity of Sii

1.5 Research questions

1. What are the morphological and molecular characteristics of Sii in human stool, raw, and fermented milk?
2. Are the Sii isolates diverse in human stool, raw and fermented milk?

CHAPTER TWO

LITERATURE REVIEW

2.1 Taxonomy and prevalence of members of the *Streptococcus bovis/Streptococcus equinus* complex

Streptococcus infantarius subsp. infantarius (Sii) belong to a group of *Streptococcus bovis/ Streptococcus equinux* complex (SBSEC). *S. infantarius* has been classified in both *S. bovis* group and *S. salivarius* group. This is due to some strains being bile – esculin negative, a fact that delineate them from *S. bovis* group. DNA-DNA reassociation studies have been used to identify bile-esculin negative strains as *S. infantarius* .In some situations *S.* infantarius strains have been misidentified as *S. bovis* II/1 when Rapid Strep system is used (Facklam, 2002). The SBSEC is a large association of species previously categorized only as *Streptococcus bovis* including several biotype subclasses. *S. bovis* and other members of the SBSEC are commensal inhabitants of animal and human gastrointestinal tracts and establish a constant population in the rumen of cattle, sheep, goats between 10^5 and 10^7 cells per mL (Jans *et al*., 2013a). The SBSEC comprises the species *Streptococcus bovis* (*S. bovis* biotype II.1), *Streptococcus equinus* (*S. bovis* biotype II.1), *Streptococcus gallolyticus* subsp. *gallolyticus*(*S. bovis* biotype I), *Streptococcus gallolyticus* subsp. *pasteurianus* (*S. bovis* biotype II.2), *Streptococcus gallolyticus* subsp. *macedonicus*, *Streptococcus infantarius* subsp. *infantarius* (*S. bovis* biotype II.1), *Streptococcus infantarius* subsp. *coli* (= *Streptococcus lutetiensis, S. bovis* biotype II.1) and *Streptococcus alactolyticus* (Schlegel *et al*., 2003).

2.2 Pathogenicity and clinical relevance of members of the *Streptococcus bovis/Streptococcus equinus* complex

The updated differentiation of SBSEC in its different species is crucial for defined risk assessments and the correct association with infections and clinical symptoms (Kaindi

et al., 2012; Boleij *et al*., 2011) as well as the separation of species in the SBSEC group proclaimed safe for food fermentation (Maragkoudakis *et al*., 2009). SBSEC are classified as non-*enterococcal* group D *streptococci* which are considered an emerging cause of infective endocarditis in France, Spain and Italy. Members of the SBSEC are furthermore associated with bacteremia and sepsis. In addition, *S. gallolyticus* subsp. *gallolyticus* (*S. bovis* biotype I) has strong association with colorectal cancer, the ability to adhere to collagen surfaces, form biofilms and translocate across polarized epithelial Caco2-cell monolayer (Kaindi *et al*., 2018). Sii has displayed similar translocation abilities, but induced on the other hand a significant increase of interleukin-8 while biofilm formation was not improved (Boleij *et al*., 2012). In addition, several cases of neonatal sepsis have been associated with members of the SBSEC including *S .gallolyticus* subsp. *pasteurianus* (*S. bovis* biotype II.2) (Onoyama *et al*., 2009), but also *S. gallolyticus subsp*. *macedonicus*. Epidemiological studies has shown a clear association of infective endocarditis with rural residency in France (Corredoira *et al*., 2008; Giannitsioti *et al*., 2007). Due to this pathogenic background in the taxonomy of the SBSEC, none of its members are QPS-approved *(Leuschner et al*., 2010). Surprisingly, *S. bovis* was prior to its association with colorectal cancer suggested for use in dairy fermentations. Unfortunately, no data on safety evaluation or further taxonomic background on those strains is available.

Pathogenic traits and virulence factors are well studied in clinically relevant streptococci of groups A, B and the pneumococcal group. Virulence factors can be defined as factors that allow an organism to become established in a host or to maintain the disease state once an infection has been established. Major *streptococcal* virulence factors include among others hemolysins, hyaluronidase, proteases, capsules, pyrogenic exotoxins, M-protein, Emm-like protein, streptokinase or streptolysins (Chattopadhyay *et al*., 2007).

In contrast, defined virulence factors of the SBSEC are less known. The SBSEC of group D streptococci has only recently been taxonomically restructured into several

different species and subspecies with different infections associated. *S. gallolyticus subsp*. *gallolyticus* seems to have the highest association with human infections followed by *S. gallolyticus* subsp. *pasteurianus*. *S. lutetiensis* and *Sii* are less frequently isolated from cases of human bacteremia and endocarditis even though case reports exist (Wullschleger *et al*., 2013). Therefore, most studies on virulence factors have been performed on the clinically more relevant species *S. gallolyticus* subsp. *gallolyticus* and limited to adhesion factors to collagen surfaces (Oguntoyinbo *et al*., 2011; Boleij *et al*., 2009), induction of inflammatory responses by the host (Abdulamir *et al*., 2010; Boleij *et al*., 2011). Little is known about hem agglutination, proteases, toxin production, and protection mechanisms from the host immune system, resistance to gastric acid or survival and establishment in the human colon after ingestion from food. Unfortunately, recent taxonomic changes, which are not yet fully implemented in clinical diagnosis, render a clear species association and risk assessment difficult (Boleij *et al*., 2012; Romero *et al.*, 2011). Specific virulence factors of Sii are largely unknown.

Unexpectedly, the microbial communities of Fermented dairy products (FDP) in Kenya, Somalia, Sudan, Mali, and Ivory Coast of camel and cow origin have shown to be predominated by Sii and not *S. thermophiles* (Abdelgadir *et al*., 2008; Hamza *et al*., 2009; Jans *et al*., 2012; Wullschleger, 2009). Only a previous record of *S. bovis* in fermented milk from Tanzania is available (Hosea *et al*., 2014). Interestingly, members of the SBSEC have been reportedly isolated from fermented African cereal foods such isolates are *S. lutetiensis* and *S. gallolyticus* subsp. *macedonicus* in West African fermented cereal food (Oguntoyinbo *et al*., 2011). SBSEC have also been found in fermented products from other regions such as cheese and fermented maize in Mexico (Diaz-Ruiz *et al*., 2003; Renye *et al*., 2011), Slovakian cheese (Chebeňová-Turcovská *et al*., 2011), Greek cheese (Tsakalidou *et al*., 2008), Italian cheese (Pacini *et al*., 2006), French cheese, fermented *dahi* milk (Rashid *et al*., 2009). Whether this high prevalence of members of the SBSEC in dairy products of Southern European countries can be linked with the higher rate of infective endocarditis by SBSEC (Corredoira *et al*., 2008;

Giannitsioti *et al*., 2007) remains to be investigated. Certainly, the high prevalence of Sii in high titers in African FDP warrants further investigation to elucidate any potential health risks.

2.3 Microbial communities in dairy products and potential risks associated

Milk provides an excellent growth medium for many microorganisms. The initial level of microbial contamination is largely dependent on animal health and milking procedures. Bacteria often detected in raw milk include opportunistic and obligate pathogens such as *Enterococcus faecalis, Staphylococcus aureus, Streptococcus agalactiae, Bacillus cereus* or *Escherichia coli* (Özer, 2004). General risks are associated with the consumption of raw milk and raw milk products (Lejeune and Rajala-Schultz, 2009). The risk have been demonstrated by the presence of *Staph. aureus* and *Streptococcus agalactiae* in raw milk, especially in East Africa (Jan's *et al*., 2012; Njage, 2010; Younan and Bornstein, 2007). *S. agalactiae*, group B *streptococci*, is a major cause of neonatal sepsis and meningitis in the industrialized world as well as Africa (Berkley *et al*., 2005; Schuchat *et al*., 2001; Schuchat and Wenger, 1994; Tyrrell *et al*., 2000). Furthermore, *S. agalactiae* is one of the most frequent causes for mastitis in Africa and Europe (Almaw *et al*., 2008; Bradley, 2002; Getahun *et al*., 2008; Mdegela *et al*., 2004; Piepers *et al*., 2007; Younan *et al*., 2001; Younan and Bornstein, 2007). Interestingly, a hyper invasive neonatal group B *Streptococcus* has been determined to have evolved from a bovine ancestor (Bisharat *et al*., 2004). Other bacteria often associated with raw milk and raw milk products are *Staphylococcus aureus*, other *staphylococci*, *Enterobacteriaceae*, *Listeria monocytogenes* or *Bacillus cereus* (Barrell and Rowland, 1980; Kivaria *et al*., 2006; Oliver *et al*., 2005). Therefore, pasteurization or boiling of milk followed by cold storage are highly recommended in order to reduce potential foodborne pathogens. However, refrigeration or cold storage is often not available to most pastoral communities in Sub-Saharan Africa rendering boiling of milk susceptible to re-contamination (Boleij *et al*., 2009; Hetzel *et al*., 2004). Lactic acid fermentation of milk is a traditional method of preservation. LAB are low mol % G+C

content bacteria comprising the genera *Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Oenococcus, Pediococcus, Streptococcus, Tetragenococcus, Vagococcus* and *Weissella* (Ludwig *et al.*, 2009). Only certain species of LAB such as most *Lactobacillus* spp., *Lactococcus* spp. or *Streptococcus thermophilus* were classified for food production and human consumption using the "generally recognized as safe" (GRAS), Food and Drug Administration (FDA) or the "qualified presumption of safety" (QPS), European Food Safety Authority (EFSA) evaluation scheme. Other LAB is classified as opportunistic and obligate pathogens such as most *streptococci* and many *enterococci* due to the association with nosocomial infections or spread of antibiotic resistances.

The QPS approach qualifies microorganisms based on four pillars taxonomy, body of knowledge and familiarity, pathogenicity and application including also history of use, ecology, industrial applications and scientific literature (Chamba and Jamet, 2008). *Lactococcus lactis* subsp. *lactis, Lactobacillus delbrueckii* or *S. thermophilus* are examples of QPS-approved species for which only small safety assessments such as proof of absence of antibiotic resistances are required prior to application in food (Leuschner *et al*., 2010).*S. thermophilus* and *Lc. lactis* have been safely used in food fermentations. Furthermore, recent advances in genome sequencing revealed major gene decay and loss of function in *S. thermophilus* as a result of adaptation to the dairy environment. Interestingly, virulence factors of common *streptococci* ancestors can still be detected as pseudo genes or gene fragments in *S. thermophilus* (Bolotin *et al*., 2004; Hols *et al*., 2005). Horizontal transfer gene, gaining of gene and loss of gene seems to be a regular finding especially among organisms in proto-cooperation or co-evolution in similar niches *(Liu et al., 2009; Makarova et al*., 2006).

2.4 Fermented food products in Africa and their utilization as weaning foods

Fermented milk have a long tradition in Africa for general consumption and as weaning food (Motarjemi, 2002). This has made African continent probably the largest spectrum

with richest variety of lactic fermented foods (Almaw et al., 2008; Motarjemi, 2002; Holzapfel, 1997). Fermented African food are made from raw materials such as cassava '*attiéké'* in West Africa (Kastner, 2008), maize, sorghum or millet gruel for *ogi*or in combination with milk for *fura da nunu* in West Africa (Anukam and Reid, 2009), cow milk for *fènè* in Mali (Wullschleger, 2009) and *kulenaoto* in Kenya (Mathara *et al*., 2004), camel milk for *suusac* in Kenya and Somalia (Jans *et al*., 2012a) or *gariss* in Sudan (Abdelgadir *et al.*, 2008).

Fermented weaning foods are often prepared on a basis of wheat, rice, maize or other grains in combination with milk (Oluwafemi and Ibeh, 2011; Oyewole, 1997). Furthermore, fermented foods are promoted for improved nutritional composition through phytic acid reduction yielding better iron availability or the reduction of toxic compounds in the raw material (Motarjemi *et al*., 1996). Nevertheless, little research has been conducted on the epidemiology of diarrhea among infants and young children who consume fermented and non-fermented foods (Motarjemi, 2002). Unfortunately, the utilization of traditional fermented foods has been discouraged by health-care workers in developing countries for some time even though they represent possibly the economically most feasible production process at household level for enhanced microbial safety and preservation (Watson *et al*., 1996). The use and development of fermentation through optimized starter cultures for production at household level has been promoted as a practicable solution for the preservation of foods (Holzapfel, 1997; Holzapfel, 2002).

2.5 African *Streptococcus infantarius subsp. infantarius* variant and implications for its use in dairy fermentations

Sii has been isolated as predominant species from fermented African dairy products in Kenya, Somalia, Sudan, Mali and Ivory Coast (Abdelgadir *et al.*, 2008; Hamza *et al*., 2009; Jan's *et al*., 2012b; Wullschleger, 2009). Studies on functional and genomic analysis of Sii isolates have revealed an adapted lactose uptake metabolism highly

identical to that of the QPS-approved *S. thermophilus* (Jan's *et al*., 2013a). DNA sequence identity of the responsible genes has also revealed horizontal gene transfer between *S. thermophilus* and Sii in African dairy isolates. The studies on the identical genes and operon have revealed no presence in any available genome sequence of members of the SBSEC. Furthermore, genome analysis of the African Sii strain CJ18 has displayed additional dairy adaptations and loss of function events paralleling the evolutionary adaptation of *S. thermophilus* to the dairy niche (Jan's *et al*., 2013a). The African Sii variant has been therefore defined as strains carrying a partial additional *gal-lac* operon consisting at least of the genes *lacS* and *lacZ* and exhibiting phenotypic lactose/ galactose exchange as *S. thermophilus* (Jan's *et al*., 2012b).

Preliminary genome analysis has revealed a conserved genome structure between CJ18 and *Sii* ATCC BAA-102^{T} and several unique loci for both strains. These loci comprised mostly unknown hypothetical proteins of which a role in pathogenicity needs to be elucidated (Jan's *et al*., 2013b). Furthermore, CJ18 comprises an extended locus of capsular polysaccharides (CPS) generally associated in *streptococci* with protection from host immune response. However, exopolysaccharides (EPS) are related to CPS and are important texture developing factors in *S. thermophilus* (Bolotin *et al*., 2004; Broadbent *et al*., 2003).

Sii has not been approved by the generally recognized as safe (GRAS) or qualified presumption for safety (QPS) status for utilization and consumption in food products (Jan's *et al*., 2013b). This fact is not surprising considering the taxonomic position within the SBSEC and several implications of SBSEC and also Sii with human infections. Therefore, the ingestion of 10^{8} viable Sii cells per mL of fermented milk product has to been critically assessed due to the potential risk of infection. However, the preliminary indications from genome comparison for an evolution paralleling that of *S. thermophilus* to a safe dairy species need to be considered. The possible benefit and indigenous role of the African Sii variant as potential highly-adapted and competitive

fermentative bacterial group in traditional dairy fermentation from East to West Africa should not be overlooked. The contribution of Sii to a potentially increased food-safety, inhibition of pathogens and reduction of public health risk factors was demonstrated by the high prevalence of antimicrobial activity through bacteriocins-like substances against *Listeria aivanovii* (Jan's *et al*., 2012a).

Through assessment of the epidemiology, evolution and virulence of Sii and SBSEC in Africa, an important contribution to food safety and food quality can be achieved either by defining the health risks of the current situation or the establishment of the African Sii variant as safe indigenous African dairy fermentation species. The simple replacement of the currently predominating commercially available starter culture with Sii would rarely be feasible (Jan's *et al*., 2012c). Non-adapted foreign strains are likely to lead to difficulties such as not being competitive against the indigenous microbial communities, different texture, flavor and aroma production, acceptance by local communities and implicated costs for small households. Improved knowledge on the African Sii could help to secure and improve quality and safety of fermented African dairy products.

2.6 Rep-PCR Typing Methods for genetic diversity studies

This technique involves the use of primer $(GTG)_5$ among others ,and it has been used in studies that require determination of genetic diversity and discrimination isolates at the serotype levels. In epidemiological studies, the use of $(GTG)_5$ primer has been used to discriminate among the members of strains to be differentiated into different serotypes. This technique have the ability of the isolates reproducibility in one PCR run, the ability to discriminate strains amongst themselves , and the genetic diversity (stability) of the fingerprint are similar for the ERIC2 primer set and the $(GTG)_5$ primer, so both have the capability to discriminate different strains that exists within bacterial species. Investigation done on each taxa revealed bands that are specific to every group and this could be verified with numerical analysis (Gevers *et al.,* 2001). Studies have shown that

complexity of the $(GTG)_5$-PCR band pattern is not the same for all species , One strain will not always correlate to only one ERIC or $(GTG)_5$ fingerprint but that the fingerprint heterogeneity within a strain can be limited (Matsheka *et al*., 2006; Rasschaert *et al*., 2005**).** In different studies that involve genetic diversity, it has been noted that the taxonomic resolution of the $(GTG)_5$ rep-PCR technique is higher than that of protein profiling. The closely related species *Lactobacillus pentosus*, *Lactobacillus plantarum* and *Lactobacillus paraplantarum*, and the species *Lactobacillus alimentarius* and *Lactobacillus paralimentarius* cannot be differentiated using protein profiling. $(GTG)_5$ rep-PCR fingerprinting is highly recommended for identification and possibly for subspecies differentiation and highly valuable for screening a large number of strains(Gevers *et al.,* 2001

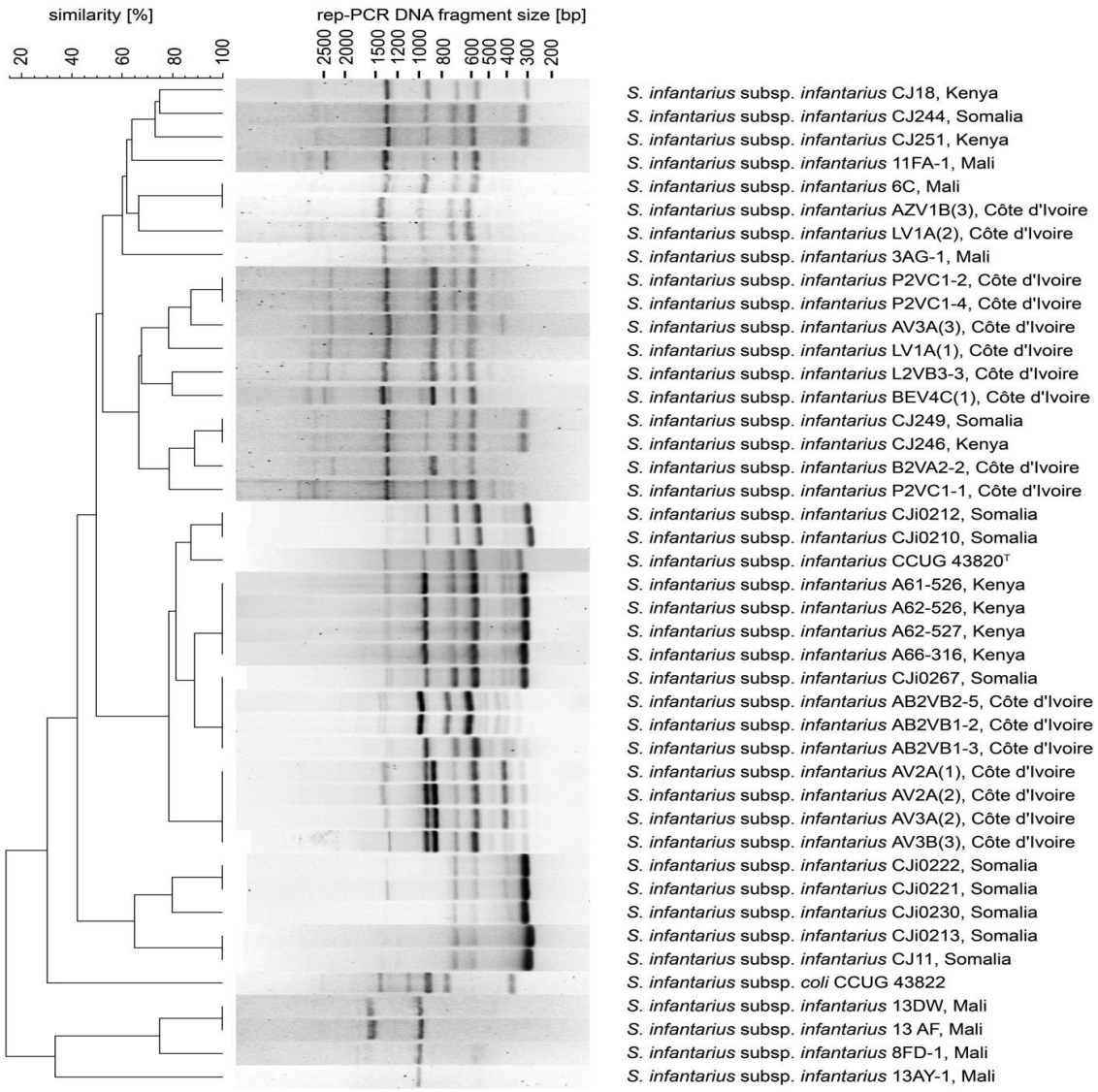

Figure 2.1 Dendogram Image showing different strains of Sii from East and West Africa (Jan's *et al*., 2013b)

2.7 16S rRNA gene for identification of genus *Streptococcus*

16S rRNA gene is among many molecular markers, it has gene with 1500 base pair that is responsible for coding catalytic RNA that forms part of the 30S ribosomal subunit, it has desirable attribute that makes it one the commonly used molecular marker. Functional consistency make 16S rRNA gene a reliable molecular marker and enables precise assessment of evolutionary related organisms. 16S rRNA is present in all

prokaryotic cells and has conserved and variable sequence region that evolves at different rates (Srinivasan *et al.,* 2015). Genetic approaches to the classification of bacteria are aimed at identifying a degree of relatedness between organisms to obtain a more-fundamental measure of the time elapsed since two organisms diverged from a common ancestor. The inner secrets of 16S rRNA gene, the signature have been used to differentiate species that are difficult to distinguish solely on the basis of 16S rRNA gene sequence. The objective of phylogenetic tree construction is to clearly define range of genetic variability that are found within the species and thereafter taking advantage of these for different strains identification. At the same time the use of restriction enzyme has been used to generate markers that can be used to differentiate very closely related species (Lal *et al*., 2011).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Setting

Isiolo Central Sub County found in Isiolo County was purposively selected as an ideal study site. Isiolo County borders Marsabit to the North, Garissa and Wajir to the South East and East respectively (Figure 3.1). It also borders Tana River and Meru County to the South and Laikipia and Samburu County to the West. Isiolo County covers an approximate area of 25,605 Km^2. Most of the county is a flat, low lying plain that receives an average rainfall of 580 mm with annual temperatures of 27^0C (Isiolo Smart Survey, 2017). The Kenyan new constitution dived Isiolo County into three sub-counties namely Isiolo Central Sub County, Garbatulla Sub County, and Merti Sub County. The study was carried out at Isiolo Central Sub County, which is divided into six sub-locations including Isiolo Central, Bulapesa, Kiwanjani, Burat, Wabera, and Ngaremara. Isiolo Sub Central Sub County is where the county capital is located (Isiolo Town). The main a number of ethnic groups found in Isiolo Sub Central Sub County include Samburu, Turkana, Meru, and Borana. The main economic activities include pastoralism which is the main livelihood, with other minor livelihoods in agropastoralism, formal employment, trade and charcoal. According to SNV (2010), in Isiolo milk production industry is majorly driven by camel milk production, the same study revealed that the county has 40,000 litres of milk being produced daily. In fact, about 45% of the milk produced is done by the herdsmen and about 23.7% is done by households headed by males. According to Mwangi *et al*. (2013), out of the 40,000 milk produced daily, only 5,000 litres of that is supplied to the main market in Nairobi Eastliegh area, the remaining is consumed within Isiolo Town and its surrounding.

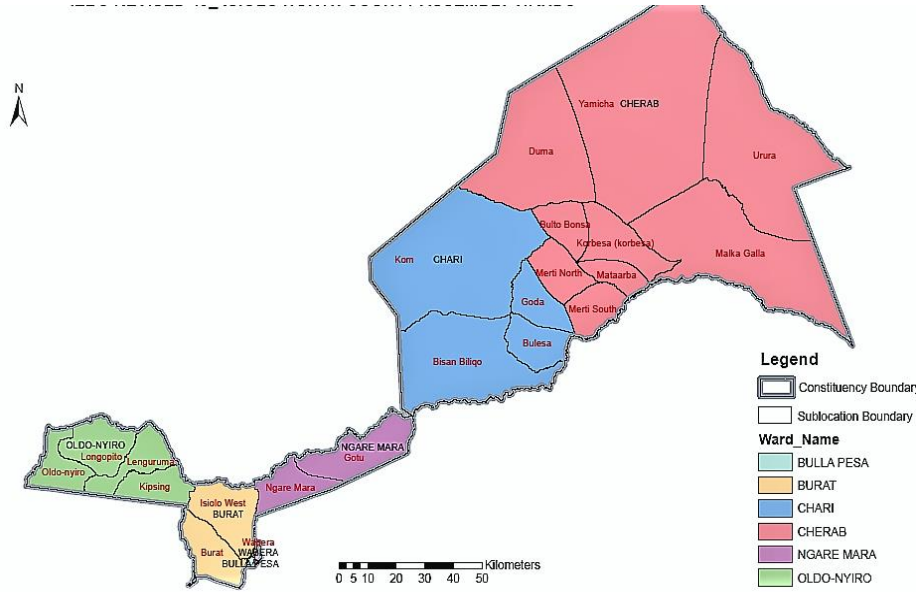

Figure 3.1: Map of Isiolo County (Isiolo Smart Survey, 2012).

3.2 Sample size determination

$$n = \frac{Z^2 * P(1-P)}{C^2}$$

Where:

Z= z value of 1.96 for 95% confidence interval

P= % of picking a choice expressed as a decimal.

C= Confidence interval expressed as a decimal.

n= $(1.96^2 \times 0.085 \times 0.915)/ (0.1^2)$

=30

Prevalence of 8.5% was used according to Teitelbaum and Triantafyllopoulou (2006).

3.3 Study Design

The study was conducted using a cross-sectional study design. The study participants were recruited from households consuming raw and fermented milk from camel, cow, and goat. Raw milk, fermented milk, and Stool samples were collected from randomly selected households in six Sub-locations within Isiolo Central Sub-County in the month of April 2016. The sample analyses using microbial, molecular, and bioinformatics techniques took place in the Molecular microbiology laboratory located in the University of Nairobi, Department of Food Science Nutrition and Technology. The research flow of activities commenced with recruitments of the respondents and stopped with both laboratory microbial and molecular analysis of the collected three samples category (Figure 3.2)

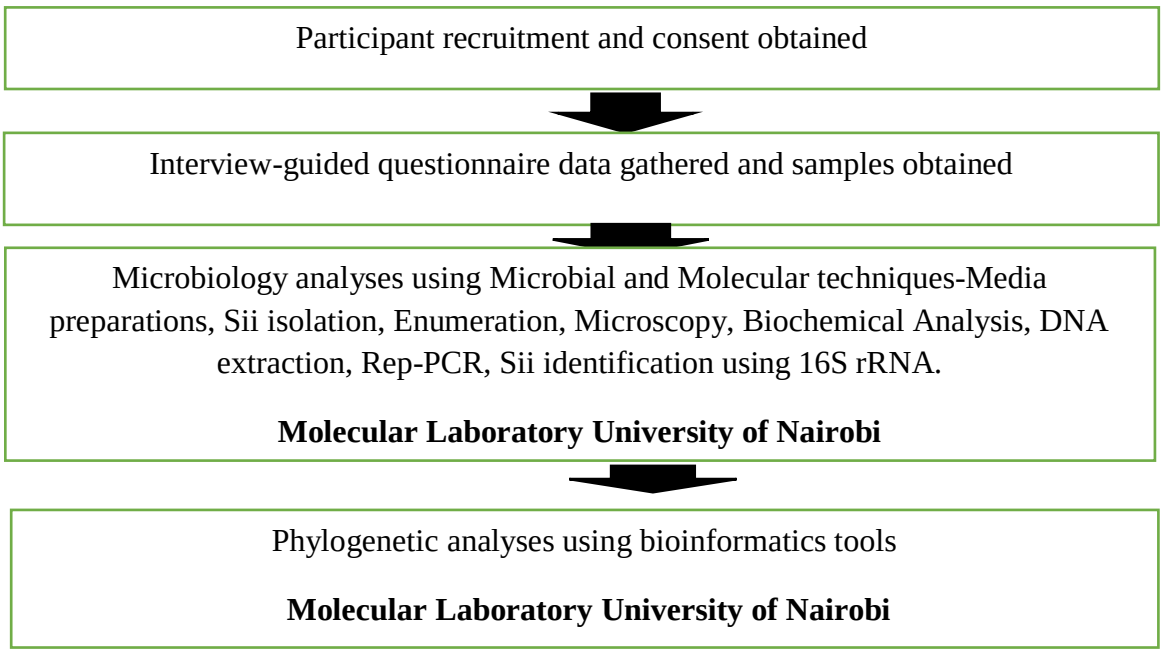

Figure 3.2: Sequence of activities for the study.

3.3.1 Study population recruitment

The study population consisted of persons above eighteen years, who are residence of Isiolo Central Sub County. Study participant within a household were randomly selected among all household members matching the inclusion and exclusion criteria. The study participants were asked to provide raw milk, fermented milk, and stool samples. Milk samples provided by the study participants were obtained from Camel, Cow and Goat, and the fermented milk samples were from traditional spontaneously fermented milk.

3.3.1.1 Inclusion Criteria

1. Household with participant eighteen years and above
2. Permanent residence of Isiolo Central Sub-County
3. Willingness of the study participant to consent and provide stool and milk samples.

3.3.1.2 Exclusion criteria

1. Household with no participant above eighteen years and above
2. Does not permanently reside in Isiolo Central Sub-County

3.3.2 Sampling procedure

The study was permitted by the ethical review committee of KNH and the University of Nairobi (Appendix 1).The sampling units used in this research were County, Sub-county, sub-locations, villages, and households as the smallest sampling units. Purposive sampling method was used to select Isiolo County and Isiolo Central Sub County. A two stage sampling methodology was employed. In the first stage 18 clusters were sampled using probability proportional to population size. A two stage multi cluster sampling was used to sample villages and households yielding 18 village clusters with 47 households. Population data was obtained from Kenya Bureau of Statistics (Census 2009) then triangulated with population data from the administrative leaders. The second stage involved obtaining an updated and complete list of households from village elder at the village level. 47 households were then selected using simple random sampling. All the households sampled were interviewed using the household questionnaire. Therefore, 47 households were targeted by the questionnaire and this yielded 47 samples for the three

sample category for laboratory analysis of Sii. Participant selection was based on the persons present in a household. Early morning hours was determined to be most successful for meeting mostly complete households. After informed consent was given, the trained enumerators administered the questionnaire in interview form and gave instructions on collection of stool, raw and fermented milk. All samples were transported to the storage freezer in cool boxes. Samples were frozen until analysis was done.

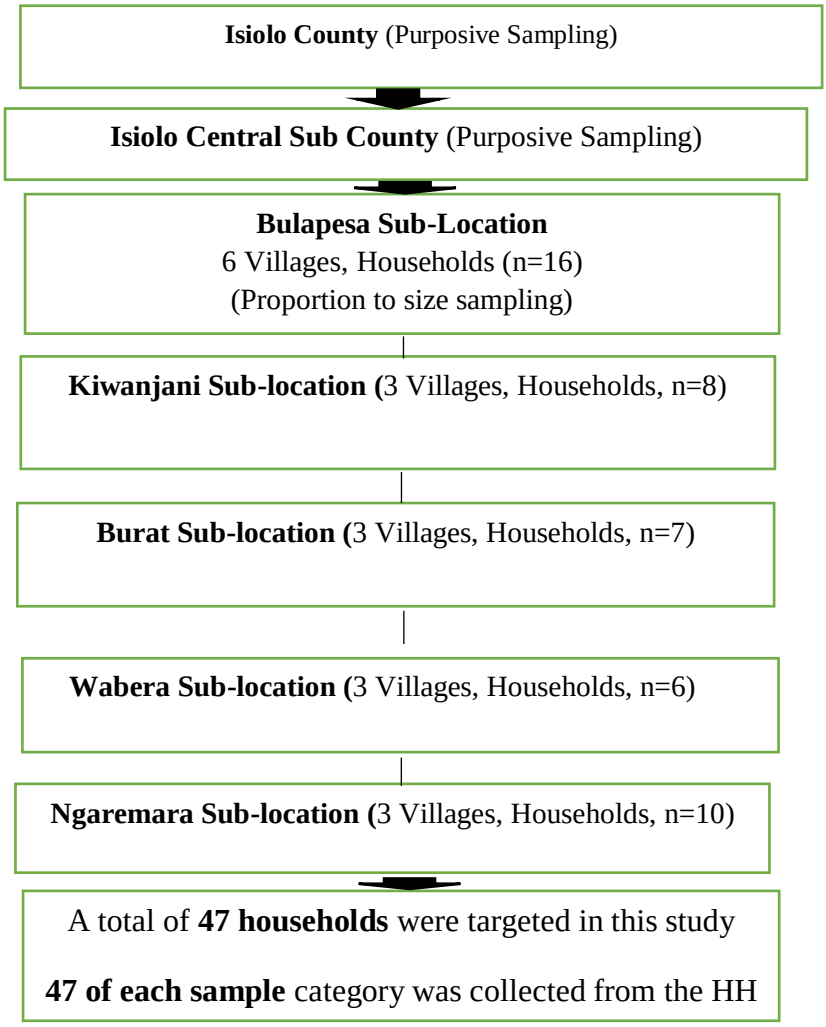

Figure 3. 3: Sampling scheme

3.3.3 Sample Collection

5-mL fermented milk and raw milk from camel, cow, and goat were collected from the respondents meeting the inclusion criteria from the randomly selected households. Milk samples were collected using sterile falcon tubes. At the same time, 2 g of stool was provided by the study respondents and kept in sterile stool container. Unique number identifier was used for each sample for the purpose of linking raw milk, fermented milk, stool samples and the household.

Samples collected in the field were stored in cool boxes filled with icepacks and transported to the Isiolo County Hospital laboratory and kept in freezer. Before analysis, the samples were kept at -20°C. A short repeated cycle of freezing and thawing was avoided.

3.4 Lab processing of samples

3.4.1 Media preparation and plating

Working was done near and below the flame of a Bunsen burner for sterility.1 g/l peptone from casein and 8.5 g/l NaCl in distilled H_2O (diluents) was used for all dilution series. It was autoclaved at 121°C for 15 minutes. 1 ml of samples was added to 9 ml of diluents to make an initial dilution of 10^{-1}. Duplicate serial dilution was prepared as per the (Table 3.1). 0.1 ml of dilutions 10^{-5} to 10^{-7} was transferred onto separate *Mitis salivarius* plates to yield final dilutions of 10^{-6}, 10^{-7} and 10^{-8}, the procedure was adopted from Jan's *et al*. (2013a). *Mitis salivarius* which is a selective media for Sii isolation was prepared according to the specifications on the bottle. 90 g was suspended in 1 liter of distilled water and sterilized for 15 minutes at 121 °C. It was cooled to 50 °C and 1% tellurite solution was added before pouring plates. The plates were left to dry and stored afterwards at 4°C protected from light and in airtight plastic bags.

Table 3.1: Sample product type and corresponding dilution requirements for plating and enumeration of Sii on Selective media (*Mitis salivarius* agar)

Product	Dilution series	Plate	No of agar plates
Raw fresh Milk	10^{-1} to 10^{-8}	10^{-1} to 10^{-2}	2
Fermented milk	10^{-1} to 10^{-8}	10^{-7} to 10^{-8}	2
Stool	10^{-1} to 10^{-8}	10^{-7} to 10^{-8}	2

3.4.2 Isolation and Enumeration of Sii from raw milk , fermented milk, and stool samples

Isolation was done from predominant colony morphologies on the plates. Isolation and purification of 3 colonies per colony morphology of the 3 most dominant morphologies was done for fermented milk, similar or identical colony morphologies from different dilutions were isolated for raw milk and stool samples. Each isolate was labeled with a unique number for traceability. Each isolate had a reference to the original product, description, dilution of the plate from where it was isolated, and CFU of the isolate on the plate. The highest dilution (example 10^{-8}) was used. Singly described predominant colony morphology was picked with a sterile inoculation loop and streaked to purify it onto a fresh sterile *Mitis* plate. This was named colony type 1. These procedures was repeated with a total of 3 colonies per colony morphology and continued with the 2nd and 3rd most predominant colony morphology of blue, blue slimy, black or black slimy colony types on plate1. The next lower dilution (10^{-6} = plate 2, 10^{-5} = plate 3) was checked for appearance of new colony morphology types which had been isolated from the previous plate1. All plates were then incubated aerobically at 37°C or 24 hours on *Mitis salivarius* agar. Purification status of each plate was done by checking if all colonies looked alike. A single colony was taken and streaked to purify it for a second time onto a fresh sterile *Mitis salivarius*. If contamination was detectable, the originally intended and described colony morphology was referred to, in order to further purify the correct colonies.

Colony morphology was described and recorded in terms of color, size and shape. Enumeration was done for colony forming units per colony morphology on each plate and the dilution factor on the plate noted.

3.4.3 Biochemical identification of Sii

3.4.3.1 Catalase activity

A single drop of 3% H_2O_2 was placed on a microscopy slide and emulsified with a single colony of Sii picked from *Mitis salivarius* media using sterile tooth pick. Metal or plating inoculation wire loop was avoided to ensure that no false positive result was recorded.

3.4.3.2 KOH Test

A drop of 3% KOH was placed onto a microscopy slide. With a sterile toothpick, a single purified colony of Sii was taken and rubbed into the 3% KOH drop.

3.4.4 Microscopy

For KOH negative isolates, a cover glass was placed directly onto the KOH drop and the isolate examined under a microscope and morphological characteristics noted.

3.4.5 Molecular identification and genetic diversity determination of Sii isolates from human stool, raw milk and fermented milk

3.4.5.1 DNA extraction and purification

Overnight bacterial broth culture was transferred into a sterile Eppendorf tube and centrifuged at 6000 rpm for 5 min thereafter supernatant was discarded. The pellet were re-suspended in 100 µl of solution of TE buffer and then mixed by vortexing. 1µl drop of 10 % SDS was added; besides addition of 10 µl of proteinase K. 10 µl of RNase was added to digest RNA. The tube content was then incubated at 50 º C for 1 hr. 400 µl of chloroform iso-amyl was then added to equal volume of the sample, and mixing was

done by gently inversion. The tube content was then centrifuged at 12,000 rpm for 5 minute, aqueous layer was transferred to clean eppendorf tube, and more dilution was done by adding 100 µl of TE buffer. 40 µl of 3M NaCl was added. Precipitation was achieved by adding 2.5 times of absolute ethanol to sample volume, followed by centrifuging at 12,000 rpm for 10 minute. 100 µl of 70% ethanol was added and again centrifuged at 12,000 rpm for 5 minutes and supernatant discarded the tube content was air dried in the oven to collect the pallet. The pellets were re-suspended in 100 µl TE buffer and stored at -20 ºC for further PCR procedures. DNA extraction method was adopted from (Goldenberg *et al*., 1995**).**

3.4.5.2 Molecular identification of Sii using 16S rRNA gene

All the 121 isolates were subjected to SBSEC-specific PCR procedure targeting the 16S rRNA gene for molecular identification. The DNA extracted from the three sample categories were analyzed using Sii SBSEC-specific 16S rRNA gene multiplex PCR assay targeting 16S rRNA gene, in which single reaction consisted of 5 primers (Table 3.2). The molecular identification was done by use of 16S rRNA identification method that was adopted from (Hutter *et al*., 2003). PCR thermo cycler (Bio rand), was used. PCR mixes were prepared by adding 2.0 µl DNA template to mix of 5.0 µl 10x reaction buffer, 10.0 µl 5x enhancer buffer, 1.0 µl dNTP, 0.25 µl Taq polymerase, and 0.4µl of each primer, topped up using 30.35 µl of DNAse free water to make a reaction volume of 50.0 µl. The PCR reagents used were imported from peQlab Germany. PCR was performed with initial start at 95°C for 5 minutes, followed by 35 cycles of 95°C for 1 minute, annealing temperature 60°C for 1 minute, 65°C for 3 min and final extension temperature 65°C for 8 min. Storage was at 4 °C waiting next process. 8 µl of PCR product were mixed with 2 µl loading dye the mixture was then loaded on 1.5 % agarose gel electrophoresis stained with 1µl of 10 mg/ml ethidium bromide in 1X TBE buffer (89 mM Tris HCL, 89 mM borate, 2 mM EDTA) the electrophoresis was run at 10 V for 1 hr. In all cases, 100 bp DNA ladder was used, a positive control was amplicons derived from DNA extract of already identified Sii strain known as CJ18 (Table 3.3),

and there was no amplicons added in the well in negative control. Gelmax UV illuminator (Fisher's scientific) was used to visualize stained gel. A single band was used to confirm the presence of Sii after the PCR assay.

Table 3.2 PCR primers for 16S rRNA

PCR Assay	Primer set	Sequence (5'-3')	Annealing Temperature (ºC)
16S rRNA	16S-SBSEC-1-fw	ATAACAGCATTTAACCCATGTTAG	
	16S-SBSEC-2-fw	CATAACAGTGTTTAACACATGTTAG	60 for 1 for all
	16S-SBSEC-3-fw	GCATAATAGTGTTTAACACATGTTAG	
	16S-SBSEC-4-fw	ATAACAGCTTTTGACACATGTTAG	
	16S-SBSEC-inf-rev	CTTTAAGAGATTTGCTTGCCG	
	Positive Control	Sii CJ 18 amplicons used	
	Negative control	No amplicons added in the well	

Adapted from (Jans *et al*., 2013)

Table 3.3: Reference and type strains including GenBank accession numbers used for primer design and controls

Strain Species	Strain and GenBank accession
Sii	CJ18 (HQ662525)

Adapted from (Jans *et al*., 2013)

3.4.5.3 Rep-PCR finger-printing to study genetic diversity within presumptive Sii isolates

Rep-PCR for DNA finger printing using $(GTG)_5$ primer was done the DNA of (n=7) positively identified presumptive Sii isolates and the (n=7) negative presumptive Sii isolates were all subjected to rep-PCR together with reference Sii strain CJ18. The rep-PCR method utilizes one primer with similar consensus sequences in the genomes of diverse bacteria to amplify portions of the genome .The PCR protocol was adopted from (Matsheka *et al*., 2005) with modification to the annealing temperature and extension temperature and extension time this ensured improved band visibility and quality. The sequence of the primer used was (5'-GTG GTG GTGGTGGTG -3'). PCR mixes were prepared by adding 2.0 µl of DNA template to mix of 5.0 µl 10x reaction buffer, 10.0 µl 5x enhancer buffer (solution p), 1.0µl dNTP, 0.25 µl Taq polymerase, 0.4µl primer, 1.0µl DMSO (1%), topped up using 30.35 µl of DNAse free water to make a reaction volume of 50.0 µl. The PCR reagents were imported from peQlab Germany while the primer was imported from Inqaba South Africa. PCR was performed with initial start at 95°C for 5 minutes, followed by 35 cycles of 95°C for 1 minute, annealing temperature 60°C for 1 minute, 72°C for 8min and final extension temperature 72°C for 8min. storage was at 4 °C for infinity.8 µl of PCR product was mixed with 2 µl loading dye the mixture will be loaded on 1.5 % agarose gel electrophoresis stained with 1µl of 10 mg/ml ethidium bromide in 1X TBE buffer (89mM Tris HCL, 89 mM borate, 2mM EDTA) the electrophoresis was run at 80 V for 1 hr. In all cases, 100 bp DNA ladder was used as molecular marker, a positive control

was amplicons derived from DNA extract of already identified Sii strain known as CJ18 (Table 3.3), and there was no amplicons added in the well in negative control. Gelmax UV illuminator (Fisher's scientific), was used to visualize stained gel image.

3.5 Data Analyses

The collected and recorded data of colony forming units were coded and analysed using Statistical Package for the Social Sciences (SPSS) version 20.Total microbial viable count were recorded in excel sheet and normalized by Log_{10}CFU/ml transformation. Analysis of Variance (ANOVA) was used to compare the mean variation of the three samples, this was presented inform of mean log10 CFU/ml and standard deviation for each sample. 2D gel images produced by rep-PCR was conveniently handled using the gel processing tools in BioNumerics Software under the application of bacterial community fingerprinting. Cluster analysis of the fingerprint was achieved using UPGMA clustering algorithm method which was performed to compare profiles with each other. However, since each peak in a community fingerprinting profile represented a phylotype, the typical analysis performed on the data was a band matching analysis.

3.6 Ethical Clearance

This study was approved by the Kenyatta National hospital/University of Nairobi, Ethics and Research Committee (KNH/UON) and the approval certificate obtained (Appendix 1). Moreover, informed consent was obtained from the respondents before the commencement of the interview and sample collections (Appendix 4).

CHAPTER FOUR

RESULTS

A total number of 47 study respondents participated in the study. All the questionnaires that were distributed to the study respondents were well filled and the data entered into an Excel sheet.

4.1 Demographic characteristics of study participants

Out of all 47 households from which each eligible member was recruited to be a study participant 13.4 % prepared traditionally fermented milk, and majority of the respondents who produced traditionally fermented milk were Male (57.2%). Majority of the respondents were below the age of 40 years. To determine the age difference between the gender, one factor t-test was performed to test for statistical significance, the result revealed that the mean age of men was higher at 38.2±13.4 years , whereas the mean age of female respondent was 37.1±2.4, but this was statistically insignificant with ($p=0.74$, df 54; $p>0.05$). Majority of the study respondents attained secondary education (41.6%), as compared to 7.5% of the respondents who did not attain formal education. Majority of the households sampled during the study had more than 6 members (47%), while single member occupying the household was least reported at (7.9%). The level of education was associated with residency. For example urban residence attained higher level of education as compared to residence of sub-locations found in rural areas (Chi-square test = 15.03, df 3; $p<0.05$).

Table 4.1: Demographic characteristics of study participants

Social demographic characteristics (n=47)	n(%)
Gender	
Male	27 (57.4)
Female	20 (42.6)
Educational Status	
Primary	16 (33.2)
Secondary	19 (41.6)
Post-secondary	8 (17.7)
No formal education	4 (7.5)
House Hold size	
Single	4 (7.9)
1-3	12(25.5)
4-6	9(19.6)
>6 individuals	22 (47.0)
Age (Years)	
>40	31(66.9)
< 40	16(33.1)
Traditional Fermentation of Milk	
Families that ferment milk	6(13.4)
Families that don't ferment milk	41(86.6)

*Sample size was based on n=47

4.2 Milk fermentation procedures and storage of the fermented milk products

4.2.1 Type of animal milk

Majority (58%) of the households consume raw milk from cow and the same is used for fermentation, while more than a quarter (26.3%) of the study respondents use camel milk, and less than a quarter (14.5%) use milk from goat. While about (1.2%), use milk mixture of the three. For the type of milk used in the traditional fermentation of milk (Figure 4.1).

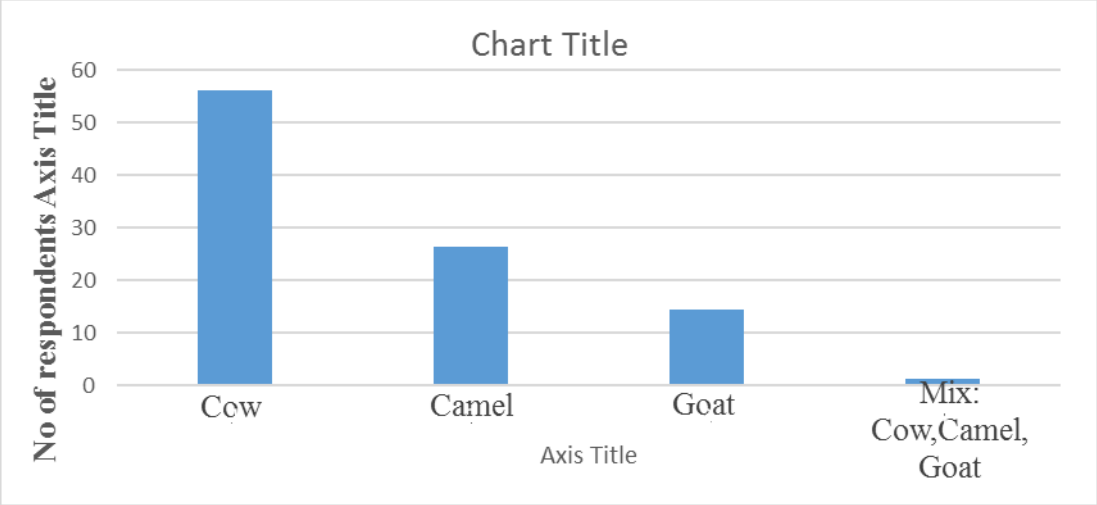

Figure 4.1: Type of milk used in the households for consumption and fermentation

4.2.2 Time taken for the fermentation process

The most common time taken for the for the milk fermentation according to the study respondents was the time 2-3 days and 4 -5 days (34.0 % and 38.2 %) respectively, while very few households respondents fermented milk in less than 6 hrs. (2.5 %) Table 4.2.

Table 4.2: Households fermentation period in the production of spontaneously fermented milk

Fermentation Period	n(%)
Less than 6 hrs.	1 (2.1)
Half a day (12 hrs.)	3 (6.4)
About 1 day (24hrs.)	4 (8.5)
For 2 to 3 days	16 (34.0)
For 4 to 5 days	18 (38.3)
For 7 days	5 (10.6)

4.2.3 Container used in storage of traditionally fermented milk

Majority (70%) of the study respondents use plastic containers for milk fermentation and storage, while (28%) use materials made from plants as milk fermentation containers and for its storage. Only (2%) of the study respondents use metallic materials for fermentation and storage (Figure 4.2).

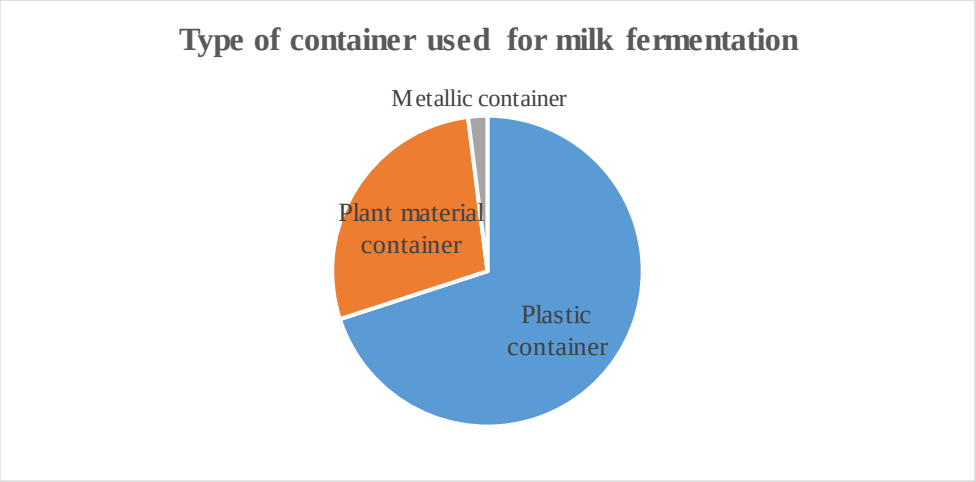

Figure 4.2: The percentage number of respondents using different container types for milk fermentation and storage.

4.3 The knowledge of traditional milk fermentation

The male respondents were more knowledgeable about the traditional milk fermentation process with a mean of 64.9±0.483, compared to the females who scored a mean of 50.8±0.369. However, there was no statistical significance difference on the knowledge of milk fermentation process based on the gender (p>0.05). The mean knowledge score about the milk fermentation process increased with increase in Age and level of education of the respondents. For example, the highest knowledge score was registered with the Age bracket 36-60 years and 26-35 years which almost had similar mean score of 56.9±0.202 and 56.3±0.351 respectively. However, the Age bracket of 18-25 years scored a low mean of 49.6±0.450. The mean score registered, however, were not affected by the age of the despondence having (p>0.05). The respondents with post

education primary education registered higher knowledge of milk fermentation process. Respondents with no formal education registered the least mean score ate 14.2±0.266 the same could be said with respondents with primary school education whose mean score was 35.9±0.653. In fact, the level of knowledge milk fermentation was affected by the level of education having (p<0.05).

Table 4.3: Mean Score knowledge of traditional milk fermentation

Demographic Variables		n=47	Mean± SD	P-Value
Gender				
	Male	27	64.9±0.483	0.077
	Female	20	50.8±0.369	
Age (Years)				
	18-25	11	49.6±0.450	0.707
	26-35	16	57.9±0.232	
	36-60	20	63.3±0.341	
Level of Education				
	Never attended school	2	14.2±0.266	0.044*
	Primary	10	35.9±0.653	
	Secondary	23	78.7±0.234	
	Tertiary	11	39.6±0.453	
	University	1	7.2±0.435	
Grand Mean			**68.4±0.567**	

***Mean difference significant at p< 0.05 level**

4.4 Morphological and biochemical characterization for identification of Sii

All the presumptive Sii isolates (n=121), shared some common characteristics in terms of morphology and growth as evidenced in (Plate1), but also exhibited diversity in terms of colony appearance on *Mitis salivarius* media. The Sii isolates were biochemically identified presumptive Sii isolates in terms of appearance on *Mitis salivarius* media dark blue light blue and blue slimy (Plate 3.1), under microscope all the isolates appeared cocci in shape. All the isolates did not have the catalase activity hence all were catalase negative and also KOH negative. In this study all the 121 isolates were identified as presumptive Sii based on the biochemical and morphological characteristics exhibited by the isolates and this was compared to the characteristics displayed by Sii Cj 18 reference strain grown under same conditions as the isolates.

Plate 3.1: Plate showing appearance of Sii on *Mitis Salivarius* media (Blue colonies)

4.5 Mean Microbial count in human stool, raw, and fermented milk

The mean microbial counts for Sii isolated from the 3 sample categories (human stool, raw, and fermented milk) that were collected from 5 different sub-locations are in Isiolo Central sub-county County are shown in (Table 4.1).

Table 4.4: The mean microbial counts for Sii isolates from fermented milk, raw milk, and human stool from different sub-locations

Sub-location	Sii (Cfu/ml) from human stool	Sii (Cfu/ml) from raw milk	Sii (Cfu/ml) from fermented milk
Bulapesa	13.91±9.16 a	316.6±95.24a	1874±18.21ab
Kiwanjani	14.92±8.89a	495.5±29.92a	1995±24.14ab
Burat	11.0±0.54 a	372.3±56.29a	3808±8.86ab
Ngaremara	12.26±5.86a	518.5±66.83a	4183±25.47ab
Wabera	15.0±14.14a	470.0±14.14a	4450±12.13ab
Grand mean log(Cfu/ml)	1.11	2.64	6.03 *P<0.001

1. Values are means of more than 10 determinations ± standard deviations.

2. Values with the same letters on the same column are not significantly different at p>0.05 level of significance.

Sii isolate count were least present in human stool sample with mean log_{10}Cfu/ml of 1.11, whereas raw milk had the second highest Sii isolates count with a mean log_{10}Cfu/ml 2.64. Lastly, fermented milk registered the highest Sii count with a mean log_{10} Cfu/ml of 6.03. The total Sii count across the three samples category differed significantly ($p<0.05$). Wabera had the highest mean Sii isolates count from human stool of 15.0 Cfu/ml while Burat had the lowest mean of Sii counts isolates from human stool of 11.0 Cfu/ml. Mean total count of Sii isolated from human stool sample across the five different sub-locations did not have significant difference ($p>0.05$) as indicated by the same letter (a). Wabera had the highest mean Sii isolates count from fermented milk of 4450 Cfu/ml while Bulapesa registered the least mean of Sii isolates counts rom fermented milk of 1874 Cfu/ml. Mean total count of Sii isolated from fermented milk sample across the five different sub-locations did not have significant difference ($p>0.05$) as indicated by the same letter. Ngaremara had the highest mean Sii isolates count from raw milk of 518.5 Cfu/ml while Bulapesa registered the least mean of Sii isolates counts rom raw milk of 316.6 Cfu/ml. Mean total count of Sii isolated from raw milk sample across the five different sub-locations did not have significant difference $p>0.05$ as indicated by the same letter.

4.6 16S rRNA identification of presumptive Sii isolates

A total of 121 of Sii isolates were obtained from stool, raw and fermented milk samples collected. All the 121 isolates were then subjected to SBSEC-specific PCR procedure targeting the 16S rRNA gene for molecular identification (Figure 4.3). To reduce the likelihood of false-positive identification of Sii and other members of the SBSEC a reference strain CJ18 was used as positive control and negative control was PCR mix with no DNA template. A single DNA fragment of sizes 500 bp was produced for the Sii isolate to be considered positive (Figure 4.3). 7 out of 121 Sii isolates were confirmed to be positive by producing 500 bp band and were similar to the band produced by positive reference strain (Sii CJ18). This represent prevalence of 5.8%.

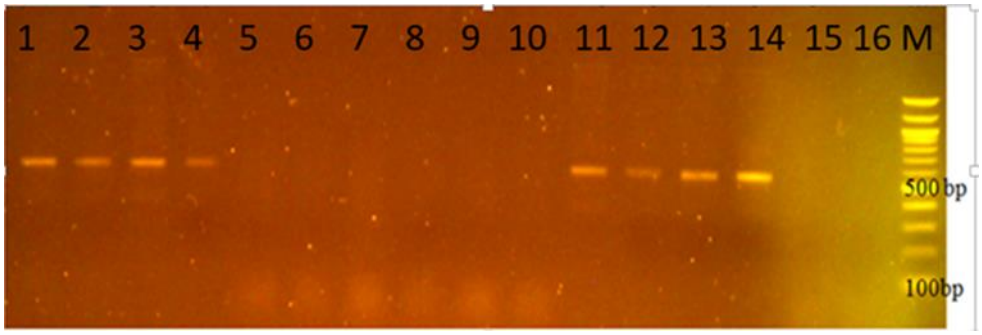

Figure 4.3: Gel image showing 16S rRNA gene

Wells: 1-(HS01STA), 2-(HS08STB), 3-(HS10STC), 4-(HS02FMA), 5-(HS05STB), 6-(HS07FMB), 7- (HS09FMA), 8- (HS12FMA), 9- (HS01RWA), 10-(Negative), 11-(HS08RWB), 12-(HS11RWC), 13-(HS16RWB), 14-(Positive control), 15-(HS15 RWA) 16-(HS06STA), and M-(1000 bps).

The samples coded as HS12FMA, HS09FMA, HS07FMA, HS02FMA, HS08RWB, HS11RWC, HS16RWB showed PCR product of 500 bp, hence positively identified as presumptive Sii having the 16S rRNA gene. While samples coded as HS05STB, HS07FMB, HS09FMA, HS12FMA, HS15 RWA, HS06STA were negative for 16S rRNA gene.

4.7 Rep-PCR finger-printing to study genetic diversity within Sii isolates

The (n=7) positively identified presumptive Sii isolates using 16S rRNA gene and the (n=7) negative presumptive Sii isolates were all subjected to rep-PCR together with reference Sii strain CJ18 (Table 3.3). The 16S rRNA gene negative presumptive Sii isolates were included in rep-PCR due to the fact that they could belong to a different clade in SBEC group of interest. All the samples were subjected to rep-PCR in order to determine genetic diversity that exists within the presumptive Sii strains isolated from the same sample and also by comparing isolated Sii strain of the three samples. All presumptive Sii isolates including type strain CJ18 displayed a subspecies-specific rep-PCR fingerprint patterns consisting of DNA fragments (Figure 4.4). 2D gel images produced by rep-PCR was conveniently handled using the gel processing tools in BioNumerics Software under the application of bacterial community fingerprinting.

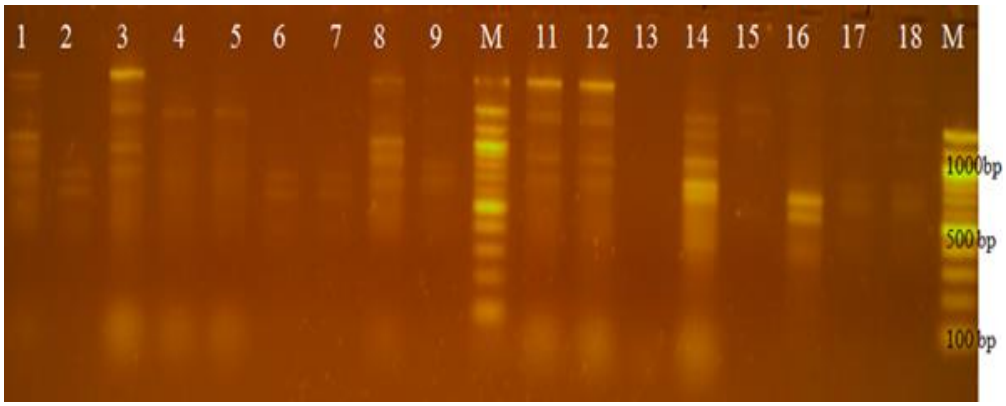

Figure 4.4: rep-PCR fingerprinting patterns of presumptive Sii strains isolated from stool, fermented milk and raw milk.

1-Positive control Sii strain CJ18-4 bands (700bp, 900bp, 1kb, 1.5 kb). **Sii isolates from raw milk:** 2(HS15 RWA)-2 bands (600 and 700bp), 3(HS16RWB)-4 bands (1.5 kb, 1.2 kb, 1 kb and 800bp), 4(HS14RWA)-1 band (1.2 kb), 5(HS11RWC), 6(HS08RWB)-2 bands (600 and 700bp), 7(HS01RWA). **Sii isolates from fermented milk**: 8(HS12FMA)-4 bands (700bp,900bp,1kb,1.5 kb), 9(HS09FMA)- 3 bands (600bp and 700bp and 1.5 kb) , 11(HS07FMB) -4 bands (1.5kb, 1.2kb, 900bp, 800bp), and 12(HS02FMA) **Strains isolated from Stool:** 14(HS05STB)-5 bands (600bp, 700bp, 900bp, 1.1kb and 1.2kb), 15(HS12STA)-2 bands (1.1kb and 1.2 kb), 16(HS10STC)-2 bands (500 and 600bp 600bp), 17(HS08STB)-2 bands (600 and 700bp), and 18(HS01STA) and 17(HS08STB)-2 bands (600 and 700bp). 10&19-ladder-1000bp. 13-Negative control.

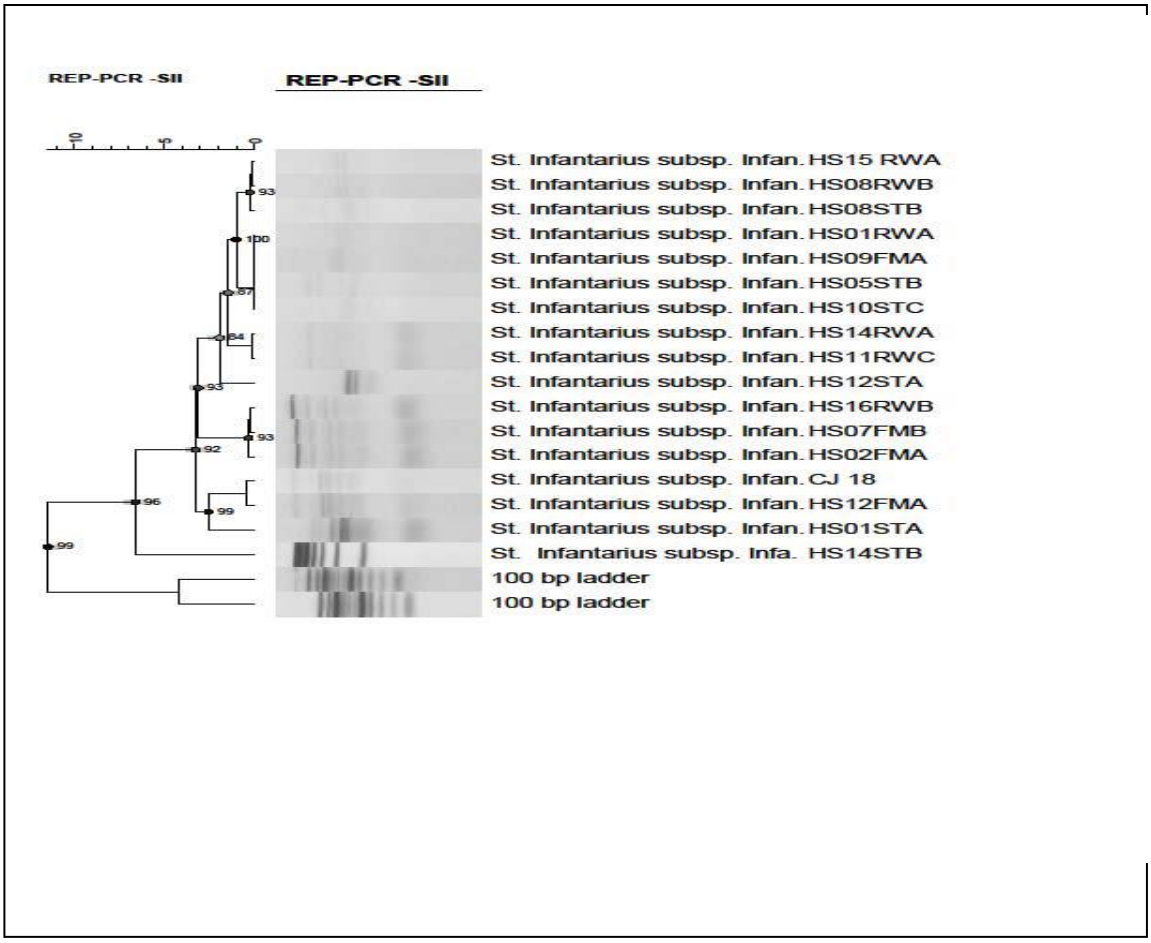

Figure 4.5: Dendogram of presumptive Sii strains generated from the rep-PCR fingerprint pattern using Gel Compar II version 6.6

CHAPTER FIVE

DISCUSSION

5.1 Demographic characteristics of study participants

Out of all 47 households from which each eligible member was recruited to be a study participant 13.4 % prepared traditionally fermented milk, and majority of the respondents who produced traditionally fermented milk were Male (57.2%). Majority of the respondents were below the age of 40 years. To determine the age difference between the gender, one factor t-test was performed to test for statistical significance, the result revealed that the mean age of men was higher at 38.2±13.4 years , whereas the mean age of female respondent was 37.1±2.4, but this was statistically insignificant with ($p=0.74$, df 54; $p>0.05$). Majority of the study respondents attained secondary education (41.6%), as compared to 7.5% of the respondents who did not attain formal education. Majority of the households sampled during the study had more than 6 members (47%), while single member occupying the household was least reported at (7.9%). The level of education was associated with residency. For example urban residence attained higher level of education as compared to residence of sub-locations found in rural areas (Chi-square test = 15.03, df 3; $p= 0.002$).

5.2 The knowledge of traditional milk fermentation

The male respondents were more knowledgeable about the traditional milk fermentation process with a mean of 64.9±0.483, compared to the females who scored a mean of 50.8±0.369. However, there was no statistical significance difference on the knowledge of milk fermentation process based on the gender ($p>0.05$). The mean knowledge score about the milk fermentation process increased with increase in Age and level of education of the respondents. For example, the highest knowledge score was registered with the Age bracket 36-60 years and 26-35 years which almost had similar mean score

of 56.9±0.202 and 56.3±0.351 respectively. However, the Age bracket of 18-25 years scored a low mean of 49.6±0.450. The mean score registered, however, were not affected by the age of the despondence having ($p>0.05$). The respondents with post education primary education registered higher knowledge of milk fermentation process. Respondents with no formal education registered the least mean score ate 14.2±0.266 the same could be said with respondents with primary school education whose mean score was 35.9±0.653. In fact, the level of knowledge milk fermentation was affected by the level of education having ($p<0.05$).

5.3 Morphological and biochemical characterization for identification of Sii

SBSEC members were isolated from West and East Africa sour milk and the isolates were unequivocally typed by semi-selective isolation, rep-PCR and 16S rRNA (Abdulamir *et al*., 2010; Chen *et al*., 2008; Gevers *et al*., 2001; Pacini *et al*., 2006; Papadelli *et al*., 2003). The isolation and identification of Sii requires three successive phases: (I) plating on selective medium, (II) Purification of distinctive colonies based on morphological characteristics on the *Mitis salivarius* selective media and presumptive Sii identification, and (III) confirmation with biochemical test. This method and protocol was adopted from. Colony growth Characteristics on the media such as colony color, shape was used for morphological identification (Plate 3.1). Furthermore, biochemical tests were used for isolate identification, the test employed in this study were Catalase and KOH tests. The Sii isolates were characterized for their morphology, their growth and their biochemical properties. All isolates (n=121) of presumptive Sii isolates shared a common characteristics of morphology and growth. They were KOH-negative, and catalase negative cocci occurring in pairs or short chains. The research findings on the morphological and biochemical characteristics were in line with findings from studies by (Jans *et al*., 2013; Schlegel *et al*., 2000), the findings and published results of the cited studies revealed gram positive cocci and catalase negative presumptive Sii isolates. The results of this study did not reveal any Sii diversity based on the color of the colony, microscopy, and biochemical tests.

5.4 Mean Microbial count in human stool, raw, and fermented milk

The three sample categories were analyzed for the microbial load of Sii. The findings from this study revealed that raw milk recorded 2.64 as the mean $\log_{10}$ CFU/ml (Table 4.4). The findings was in agreement with previous studies that have registered range of 2.6 to 6.7 mean $\log_{10}$ CFU/ml (Jans *et al*., 2012a). Fermented milk registered significant number of Sii isolate with a mean $\log_{10}$CFU/ml of 6.03, the results from this study were consistent with other current published studies indicating that traditionally fermented milk contains significant microbial load of Sii ranging between 6 to 8 log10 CFU/mL (Abdelgadir *et al*., 2008; Jans *et al*., 2012a; Wullschleger, 2009; Wullschleger *et al*., 2013). There was reported statistical significance difference between the three sample categories mean log10 CFU/ml ($p<0.001$). However, findings from this study revealed no evidence of association between specific animal and Sii. The findings were supported by other studies (Jans *et al*., 2012a; Wullschleger *et al*., 2009). The stool samples had Sii microbial load of $\log_{10}$ CFU/ml of 1.11. The reported mean of stool was low among the three sample category. The harsh environmental conditions within the gastrointestinal tract such as high acidic content could be a contributing factor to the reported low microbial load of Sii in the human stool. However, a study conducted by Kaindi *et al*. (2018), revealed 24% feacal carriage of Sii among the adults who consume informally traded fermented dairy products. Moreover, the same study Sii microbial load was more elevated among the individuals with colorectal cancer as compared to individuals with normal colon.

5.5 16S rRNA identification of presumptive Sii isolates

The identification of presumptive Sii isolates was achieved using the rep-PCR multiplex SBSEC-PCR assay targeting specifically the 16S rRNA gene of members of the SBSEC. In this study the results of multiplex SBSEC-PCR assay revealed that 7 out of 121 Sii isolates produced the desired 500bp band (Figure 4.3), this was 5.8% prevalence. The isolates that didn't produce the desired band segment could be other closely related

members of SBSEC. However, this findings were contrary to the 8.5 % findings by (Teitelbaum and Triantafyllopoulou, 2006). The difference between the two findings could be attributed to the fact that prevalence of 8.5% was reached after isolating Sii from fermented milk from camel milk alone, whereas, in this study the Sii was isolated from three sample category. At the same time, the studies were conducted in different geographical locations. The majority of the positive samples were obtained from the fermented and raw milk (4 and 3), respectively. This could be attributed to the fact that Sii has good adaptation in the environment offered by milk, especially fermented milk as compared to the human stool. Other research findings studies have revealed that for bacterial isolates the initial 500 bp sequence of the 16S rRNA gene provides enough identification and differentiation between strains due to the fact that the region shows more diversity per kilo base (Kattar *et al*., 2000). The other seven out of 15 that were negative could have been due to DNA degradation or belong to other members of SBEC group. The same findings were shared by a study conducted by Schlegel *et al*. (2000) within the SBEC group by carrying out comparison of morphological and genotypic characteristics of group D s*treptococcal* strains, mostly of human origin, confirms the heterogeneity of the *S. bovis S. equinus* complex (SBEC), and the description of a new species called *S. infantarius.*

5.6 Genetic diversity of Sii strains using rep-PCR

The rep-PCR yielded divergent pattern of bands indicating the genetic diversity that exists within presumptive Sii isolated from raw, fermented milk and stool (Figure 4.5). Different sized DNA fragment having divergent base pair size were generated. The results revealed different banding patterns produced by presumptive Sii isolates from the three sample categories. At the same time, there was different fingerprinting patterns when the Sii isolates from the three samples were compared (Figure 4.5). Therefore, this was a clear indicator of the genetic diversity that exists within presumptive Sii isolates. Rep-PCR based DNA fingerprinting has always been used to discriminate between closely related bacterial species and strains (Versalovic., *et al* 1994). The findings of this

study revealing different banding patterns is in great contrast with the study findings done and published by Abdelgadir *et al*. (2008), where he reported similar rep-PCR banding patterns for Sii isolates originating from the same geographical area (Western Africa and Eastern Africa). The difference in the findings could be due to the fact that the isolated presumptive Sii isolates were from three sample category collected from the same geographical region, contrary to the study conducted by Abdelgadir *et al*. (2008). Moreover, the difference in findings reported could be as result of the samples being collected from different study sites, for this study samples were collected from Isiolo central Sub-County while for the other study samples were obtained from Mandera County. Genetic diversity is a major pillar in species survival hence same species occupying different locations will have to adapt to different environmental condition that exists within the locality for survival, this is in line with the principal of natural selection, hence survival for the fittest. In this study we went further and isolated presumptive Sii isolates from stool sample and the fingerprinting patterns produced were compared with fingerprinting patterns produced by Sii isolates from fermented and raw milk. The study results revealed greater genetic diversity that existed between the presumptive Sii isolates from the three samples that was well demonstrated by the dendogram generated (Figure 4.5). The application consisting of background subtraction, spot removal, noise filtering, curve smoothing, normalization and band detection. The normalization procedure was thereby based on external reference patterns. Normalization made it possible to not only compare bands that were situated on the same gel, but also to compare bands on different gels with each other and this made it possible to build up a database of profiles over extended periods of time. Cluster analysis of the fingerprint was achieved using UPGMA clustering algorithm method which was performed to compare profiles with each other. However, since each peak in a community fingerprinting profile represented a phylotype, the typical analysis performed on the data was a band matching analysis (Figure 4.2). This was a clear evidence of adaptability of Sii as it occupies different environment that were provided by the three different samples in the study. The diversity that was exhibited among the

isolates reflects the genetic diversity that exists within. This could be as a result of pressure due to natural selection. This study reported lower strain diversity by Sii when comparing a cross the three samples category. This results was consistent with the findings by Wullschleger (2009), where he reported 10 out 79 and in his findings he highlighted strain diversity fluctuation within Sii found during first and second fermentation process as sampled during single season. Moreover, the findings regarding genetic strain diversity among the isolates from the three samples could be directly attributed to environment-dependent variation of micro biota diversity.

Conclusively, this study has established that fermented milk is major source Sii having high microbial load. With presence of Sii in considerable high titers, consumption of both raw and fermented milk acts as major sources of Sii and other SBSEC members among consumers of the milk and milk products translating to the stool carriage of the organisms. Therefore, traditionally fermented dairy products seem to be a major reservoir of dairy adapted African variants of Sii with no other natural reservoir yet. Consumption of large quantities of Sii and other members of SBSEC possess major health risk, based on the fact that they are associated with endocarditis, bacteremia, and colorectal cancer. This calls for public health awareness on issues such as milk handling and storage among the communities that consumes traditionally fermented milk. This study could not establish seasonal variation as major cause of variation in cell count, this is due to the fact that samples were collected only during one month of the year. Hence, this was one limitation of this study

.

This study is among the few studies that have attempted to study the genetic diversity of presumptive Sii strains collected from three sample category including human stool, raw milk, and home-made fermented milk in Isiolo Sub-county County, Kenya.

We recommend further deep evaluation in terms of desired genes and biotechnological properties that will be required to fully exploit the biotechnological potential of

presumptive Sii genetic diversity represented by these isolates, which could constitute novel starter culture. This is due to adaptability to process of fermentation. Moreover, longitudinal studies should be carried to determine genetic diversity as a result of seasonal variation which may give more insight.

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APPENDICES

Appendix 1: Ethical Review Committee Approval Letter

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15th October 2015

Prof. Wambui Kogi-Makau
Principal Investigator
Safe Dairy Project
Dept. of Food Science Nutrition and Technology
Faculty of Agriculture
University of Nairobi

Dear Prof. Makau

Research Proposal: Health Hazards caused by bacteria in African Traditionally Fermented Dairy products Erafrica: Safe Dairy Project (359/05/2015)

This is to inform you that the KNH/UoN-ethics & Research Committee (KNH/UoN-ERC) has reviewed and approved your above proposal. The approval period is 16th October 2015 – 16th October 2016.

This approval is subject to compliance with the following requirements:

a) Only approved documents (informed consents, study instruments, advertising materials etc) will be used.
b) All changes (amendments, deviations, violations etc) are submitted for review and approval by KNH-UoN ERC before implementation.
c) Death and life threatening problems and serious adverse events (SAEs) or unexpected adverse events whether related or unrelated to the study must be reported to the KNH-UoN ERC within 72 hours of notification.
d) Any changes, and appeal or all issues that may increase the risks or affect safety or welfare of study participants and others or affect the integrity of the research must be reported to KNH/UoN ERC within 72 hours.
e) Submission of a request for renewal of approval at least 60 days prior to expiry of the approval period. (Attach a comprehensive progress report to support the renewal)
f) Clearance for export of biological specimens must be obtained from KNH/UoN Ethics & Research Committee for each batch of shipment.
g) Submission of an executive summary report within 90 days upon completion of the study. This information will form part of the data base that will be consulted in future when processing related research studies so as to minimize chances of study duplication and/or plagiarism.

For more details consult the KNH/UoN ERC website http://www.erc.uonbi.ac.ke

"Protect to Discover"

Appendix 2: Questionnaire

ID of the participant (Initials of the enumerator plus 3 digits number code)

[_____]

Name of the Sub location: [_____]

Name of the Village: [_____]

No	**Inclusion Criteria**	Yes	No
1)	Age of the study respondent (18 years or older)		
2)	Give consent to take part in the study (signed consent form)		
	Respondent Socio-Demographic characteristics	Yes	NO
3)	Were you born in Kenya?		
4)	Are you residence of Isiolo county?		
5)	Are you permanent resident of Isiolo central sub county		
	Gender	Male	Female
6)	What is your gender?		
	Education	Yes	No
7)	Did you go to school?		
8)	If yes (Q7), please indicate your highest level of education completed 1- Nursery School	8[_]	

	2- Primary School 3- High School 4- College/Polytechnic 5- University 6- Adult Education 7- I don't know		
	Structure of your nuclear family		
9	**i)How many persons currently live in this household? (Indicate in the box)** ii)Number of persons aged 6-13 years living in this household iii)Number of persons below age of 5years living in this household	9 [ ] [ ] [ ]	
	Livestock kept by Households		
10	**Do you possess livestock in your household?** 1-yes: [ ] 2-No: [ ]		
11	**If yes in (Q10), please name type and indicate the possible number**		
12	Cow 1-yes: [__] 2-No: [_] 3-I don't know :[__]		
13	Camel 1-yes: [ ] 2-No: [ ] 3-I don't know :[ ]		
14	Goat 1-yes: [ ] 2-No: [ ] 3-I don't know :[ ]		
15	Sheep 1-yes: [ ] 2-No: [ ] 3-I don't know :[ ]		
16	Other_____ 1-yes: [ ] 2-No: [ ] 3-I don't know :[ ]		
	Section		
17	**Which type of raw milk do you use in this household?(Tick the answer in the box below)**		

	1-Whole milk yes: [__] 2-Powedered milk yes: [__] 3-Mix of whole milk and powdered milk [__]
	Methodology of fermented Milk production **Ask only if fermented milk is produced within the household, If not produced go to question.**
18	**Which animal Milk do you use when making fermented milk?** 1-Cow milk [ ] 5- Mixture of cow and goat milk [ ] 2-Goat milk [ ] 6- Mixture of cow and camel milk [ ] 3-Camel milk [ ] 7- Mixture of goat and camel milk [ ] 4-Sheep milk [ ] 8-Mixture of cow, goat, and camel milk[ ] 9-I don't know [ ]
19	**Do you boil milk before fermenting it?** 1-Yes: [ ] 2-No: [ ] 3-I don't know: [ ]
20	**How do you initiate the fermentation process?** 1-spontenous [ ] 2-addition of part of previous fermented milk [ ] 3-adition of yoghurt from commercial source [ ] 4- addition of mala from commercial source [ ] 5- continuous addition of fresh milk until fermentation [ ] 6- addition of starter culture from commercial source [ ]
21	**For how long do you do the fermentation?** 1-less than 6 hrs. [ ] 4- 2 to 3 days [ ] 2-half a day 12 hrs. [ ] 5- 4 to 5 days [ ] 3- about 1 day [ ] 6- 7 days [ ] 8-i don't know [ ]

22	**At what temperature do you carry out fermentation?** 1-I keep the fermentation container in the cold part of house,< 25ºC [_] 2-keep at ambient temperature 25-35 ºC [_] 3-warm place > 35 ºC [_] 4- I don't know [_]
23	**At what temperature do you store the final fermented product?** 1-I keep in the fridge < 10ºC [ ] 2-keep at ambient temperature 25-35 ºC [ ] 3-warm place > 35 ºC [ ] 4- I don't know [ ]
24	**The Fermentation milk container is made up of which material?** 1-plant material (wood, calabash) [ ] 3-metalic [ ] 2-plasitc [ ] 4- other [_____] 5-I don't know [ ]
	Collection of stool and milk samples
25	**Stool sample collected (2g) (mark appropriately)** 1-yes: [ ] 2-No: [ ]
26	**Stool sample I**D:[_____]
27	**Time of stool sample collection:** [ ] [ ] hr./ [ ] [ ] min
28	**Date of stool sample collection** : [ ] [ ] / [ ] [ ]/ 2016 (dd/mm/yy)
29	**Raw milk sample collected** 1-yes: [ ] 2-No: [ ]
30	**Raw milk sample ID**: [ ]
31	**Time of sample collection**: [ ] [ ] hr./ [ ] [ ] min
32	**Date of sample collection** : [ ] [ ] / [ ] [ ]/ 2016 (dd/mm/yy)
33	**Raw milk is from which type of animal?** 1-Cow milk [ ] 5- Mixture of cow and goat milk [ ] 2-Goat milk [ ] 6- Mixture of cow and camel milk [ ] 3-Camel milk [ ] 7- Mixture of goat and camel milk [ ]

4-Sheep milk [ ] 8-Mixture of cow, goat, and camel milk [ ]

9-I don't know [ ]

34 **Source?**

1-home made [ ]

2-bought at informal market (milk venders, hotel) [ ]

3-others [ ] 4- I don't know [ ]

35 **Fermented milk sample collected**

1-yes: [ ] 2-No: [ ]

36 **Fermented milk sample ID**: [ ]

31 **Time of sample collection**: [ ] [ ] hr./ [ ] [ ] min

32 **Date of sample collection** : [ ] [ ] / [ ] [ ]/ 2016 (dd/mm/yy)

33 **Fermented milk is from which type of animal?**

1-Cow milk [ ] 5- Mixture of cow and goat milk [ ]

2-Goat milk [ ] 6- Mixture of cow and camel milk [ ]

3-Camel milk [ ] 7- Mixture of goat and camel milk [ ]

4-Sheep milk [ ] 8-Mixture of cow, goat, and camel milk [ ]

9-I don't know [ ]

34 **Source of fermented milk?**

1-home made [ ]

2-bought at informal market (milk venders, hotel) [ ]

3-others [ ] 4- I don't know [ ]

Appendix 3: Informed Consent Form

Introduction and consent form for a study on household socio-demographic characteristics, knowledge on fermentation processes and handling of fermented milk products.

Introduction

Hello, my name (Enumerator) is ----------, and I am working collaboration with Benard Ochieng Aliwa MSc. Student from the Jomo Kenyatta University of Agriculture and Technology, Department of Biochemistry. I am conducting research that seek to determine the presence of Sii in homemade consumed raw, fermented and stool samples.

Purpose

The information you will provide will be only used assess the methods household uses in fermenting milk, consumption of the raw and fermented milk.

Confidentiality

The information that you will be provide during this study will be treated with a lot of confidentiality, the names of the respondent will not be documented. Moreover, the respondent have the right to withdraw from this study at any point in time. Therefore, your participation in this research is voluntary, and you are not forced to answer any question that in your opinion is deemed sensitive.

Study benefit

The findings from this study will be useful in ensuring safety of consumed raw and traditionally fermented milk for the community.

By giving consent to take part in the study, it indicate that you have understand what is expected of you and you are willing to voluntarily answer questions and in the end provide small amount of raw, fermented milk, and stool.

Signature of the study participant----------.

Signature of the interviewer----------.

Date --.