Cow Milk Fermentation trials with *Streptococcus infantarius* subsp*. infantarius* and its effect on the growth of selected pathogenic bacteria

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A Research Dissertation Submitted in Partial Fulfillment for Degree of Master of Science in Food Safety and Quality of the University of Nairobi
Department of Food Science, Nutrition and Technology

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I declare that this dissertation is my original work and has not been presented for an award of degree in any other university

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DEDICATION

I dedicate this work to my loving parents; Johnson Nyagemi Nyangoya and Martha Moraa Nyangoya and my siblings (Skirther, Stephen and Brenda). Your love, prayers and financial support were instrumental towards this achievement.

LIST OF ABBREVIATIONS

ATR	Acid Tolerance Response
BLIS	Bacteriocin like Inhibitory Substances
CFU	Colony Forming Units
FDP	Fermented Dairy products
GRAS	Generally Recognized as Safe
LAB	Lactic Acid Bacteria
pH	Potential Hydrogen ions
QPS	Qualified Presumptive for Safety
SBSEC	*Streptococcus bovis/Streptococcus equinus complex*
Sii	*Streptococcus infantarius* subsp. *infantarius*

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GENERAL ABSTRACT

Streptococcus infantarius subsp. *infantarius (Sii*) is a lactic acid bacterium predominant in most traditional African fermented dairy products such as *suusac, fene* and *gariss*. Most lactic acid bacteria (LAB) have antagonistic activity against food spoilage microorganisms and pathogenic groups such as *Listeriamonocytogenes, Staphylococcus aureus*, *Clostridium species* (spp) and *Bacillus* spp. The aim of this study was to identify the optimum fermentation conditions of *Sii* that leads to fast pH drop to inhibit common human pathogens.

A factorial experimental design was used to conduct fermentation trials of local raw and pasteurized milk. Raw/pasteurized cow milk were divided into equal aliquots of 100 mL which were inoculated with separate starter cultures of *Sii* CJ 18 and *Sii* CCUG 43820T at the following inoculation rates; 0% (control), 5%, 10% and 15% v/v. Incubations were done at 20, 30, 37 and 45^{o}C in parallel. pH and titratable acidity were measured at 0 h, 3 h, 6 h and 9 h. The antibacterial properties *Sii* strains against *Staphylococcus aureus*, *Listeria monocytogenes*, *Escherichia coli* and *Salmonella enterica* subsp. *enterica* were done using disc diffusion and competition assays methods. Disc diffusion was done as follows;culture pathogens *S. aureus* ATCC 25923, *E. coli* ATCC 25922, *Salmonella* ATCC 722569 and *L. monocytogenes* ATCC 7644 were grown aerobically on TSA (Oxiod) at 37^{o}C for 24 h and suspended in 0.85% normal sterile water. A lawn of each indicator strain was made by spreading cell suspension over the surface of Muller Hinton agar plates (Oxiod) with sterile cotton swabs and allowed to dry.

Sterile discs were soaked with *Sii* strains cell-free supernatants for 5 minutes, excess carefully removed, placed on the surface of Muller Hinton agar plates and allowed to dry for 10 minutes. After incubation at 37^{o}C for 48h, the plates were observed for a zone of inhibition around the discs. Inhibition patterns of African *Sii* strains isolates CJ18 and CCUG 43820T against selected pathogenic bacteria were done by competition assay using local raw and pasteurized milk. Each type of cow milk were divided into equal aliquots of 100 mL, spiked with known CFU/mL of each pathogenic bacterium, inoculated with 15% v/v *Sii* CJ 18 and *Sii* CCUG 43820T starter cultures independently and incubated at 37^{o}C. Appropriate double serial dilutions were plated in duplicate onto appropriate selective or semi-selective growth medium after every 3 h and enumerated. Enumeration of *E. coli*was done by surface plating 0.1 ml of each sample on brilliance *E. coli* coliform agar (Oxiod).*Salmonella* was enumerated by pour plating onto Violet Red Bile Agar (Merck) and plates incubated at 37^{o}C for 24 h. *Staphylococcus aureus* was enumerated by surface plating onto Baird Parker Agar media (Oxoid) and plates incubated at 37 ^{o}C for 24 h. *L. monocytogenes* was surface plated in *Listeria* semi-selective agar (Oxiod).

Fermentation was faster in pasteurized milk than raw milk for both *Sii* CJ 18 and *Sii* CCUG 43820T. The target pH of below 4.5 was attained at 45^{o}C, sixth hour at 10% inoculation rate by CCUG 43820T pH(4.36±0.00) while CJ 18 attained a pH of 4.55±0.00 at 45^{o}C, 6th h, 15% inoculation rate. The highest percent lactic acid formed by CCUG 43820T was 1.29±0.02% at 45^{o}C, after nine hours with 15%

inoculation, while CJ 18 attained 0.82±0.02% lactic acid under same conditions. In disc diffusion assay, the growth of *Staphylococcus aureus* and *Listeria monocytogenes* were inhibited. In competition assays, Growth of pathogens increased within 9 h of fermentation in control samples whereas growth in decreased after 3 h of fermentation in samples inoculated with *Sii* strains within 9 h. The growth was higher in raw milk than pasteurized milk. In conclusion, African dairy *Sii* isolates could be potentially used as African-specific native starter cultures that will result to the fast drop in pH to inhibit human pathogens resulting in an improved and safe traditional fermented product. Safety assessment such as resistance to antibiotics, acid tolerance, viability, safety, and organoleptic properties should be carried out.

CHAPTER 1

INTRODUCTION

1.1 BACKGROUND

Milk is an excellent medium for growth of many microorganisms (Hadrya *et al*., 2012). The initial microbial load is dependent on the milking procedures and animal health (Jans *et al*., 2012a). Bacteria pathogens that are often isolated from raw milk include *Staphylococcus aureus, Escherichia coli, Streptococcus agalactiae* or *enterococcus faecalis*(Jans *et al*., 2012a). Other bacteria associated with raw milk and milk products include *Enterobacteriaceae*, *Listeria monocytogenes* or *Bacillus cereus*(Jans *et al*., 2012a). Therefore, there is a general risk associated with consuming raw milk and milk products. Pasteurization and other heat treatment procedures, followed by cold storage can greatly reduce potential pathogens. However, refrigeration and other cold storage procedures are often not available in sub-Saharan Africa rendering the heat-treated milk susceptible to recontamination (Hetzel *et al*., 2004).

Lactic acid fermentation has a long tradition in Africa as a method of preservation(Rhee *et al*., 2011). Lactic acid bacteria play a crucial role in acidification, flavor and aroma development during fermentation (Heller, 2001). The genera of Lactic acid bacteria involved in fermentation include; *Lactococcus,*

Leuconostoc, Streptococcus, Pediococcus, Weissella, Oenococcus, Enterococcus and *Carnobacterium* (Gemechu, 2015).

Streptococcus infantarius subsp. *infantarius* is a lactic acid bacterium belonging to Lancefield group D *Streptococcus bovis*/*Streptococcus equinus complex* (SBSEC). SBSEC comprises of *Streptococcus (S). bovis*, *S. equinus, Streptococcus lutetiensis*, *Sii, Streptococcus macedonicus, Streptococcus gallolyticus* subsp. *gallolyticus*, *Streptococcus gallolyticus* subsp. *pasteurianus* and *Streptococcus alactolyticus*(Jans et al., 2015).

A high prevalence of up to 10^8 CFU/mL of a novel dairy *Sii* was reported in African fermented dairy products such as *suusac, fene* and *gariss* in researches carried out in Kenya, Somalia, Mali and Code d'Ivoire (Jans *et al*., 2013a). African dairy *Sii* regularly produced bacteriocin inhibitory substance (BLIS) against other fermentative bacteria and *Listeria* spp. potentially enhancing food safety. The high prevalence suggests the vital role it plays in fermentation and possible benefit of being highly adapted and competitive bacterial group. Functional analysis and comparative genomicsshow an adapted lactose uptake metabolism similar to that of Western Quality presumption of safety QPS-approved *Streptococcus thermophilus. Sii* shows similar genome decay as *S. thermophilus* but at less advanced (10-19%) state probably due to short history in dairy fermentations (Jans*et al*., 2013a). In this study, African dairy *Sii* was screened for its potential

application as an adapted African-specific starter culture for enhancement of food safety previously not studied.

1.2 Problem statement

In Africa, fermented dairy products such as *fene* in Mali and *kule naoto* in Kenya from cow milk, *suusac* from camel milk in Kenya and Somalia have a long tradition for general nutrition. They are however, often contaminated with foodborne pathogens such as, *Staphylococcus aureus*, other *Staphylococci*, *Enterobacteriaceae, Listeria monocytogenes* or *Bacillus cereus*. This is possibly due to uncontrolled and unhygienic fermentation leading to low acidification, reduced inhibition capabilities and thus increased health risks.

1.3 Justification

Fermented dairy products play an important role in prolonging the shelf life and microbiological safety through production of organic acids, other antimicrobial compounds and contribute to a healthy nutritional basis. Among the *Streptococcal* spp., only *S. thermophilus* is generally recognized as safe GRAS for use in fermented food products despite its close genetic relationship with SBSEC (Hols *et al*., 2005; Leuschner *et al*., 2010). *S. thermophilus* was accepted for use in dairy environment because of loss or inactivation of virulence factors, genome reduction and long history of use despite its close genetic relationship to the SBSEC by QPS (Hols *et al*.,

2005). Other members of SBSEC show the same evolutionary characteristics similar to *S. thermophilus.* For instance, *Streptococcus macedonicus* ACA-DC 198 has genome reduction that is comparable to *S. thermophilus*. *Sii* displays an adaptation to the dairy environment that is similar to that of *S. thermophilus* (Papadimitriou *et al*., 2012; Jans *et al*., 2012b; Bolotin *et al*., 2004).

Functional and genomic analysis of African dairy *Sii* done by (Jans *et al*., 2012b) indicate an adapted lactose uptake metabolism highly identical to that of *S. thermophilus.* DNA sequence identity of the responsible gene indicates a horizontal gene transfer between *S. thermophilus* and African dairy *Sii* isolates. Furthermore, genome analysis of the African *Sii* strain CJ18 displays additional dairy adaptations similar to that of *S. thermophilus* to dairy environment (Jans *et al*., 2012b). Non-adapted foreign strains such as *S. thermophilus* are not competitive against indigenous microbial communities, and produce texture, flavour and aroma that are less acceptance by local communities especially in camel milk. Improved knowledge on the African *Sii* could help secure and improve the quality and safety of fermented African dairy products as important nutritive food through inhibition of well-known pathogens, reducing health risks for infants and children who have a weak immune system while milk is a key weaning food.

Fermented foods can be feasibly produced at home-scale level to provide a nutritional security for children and households. Therefore, proper knowledge of traditional fermentation and food safety is crucial for the well-being of consumers,

and there is need to study the potential application of new novel starter cultures to meet the current short falls in fermented milk quality and safety in African Arid and semi-Arid regions.

In addition, the scientific demonstration of the innocuity of the African *Sii* may help in securing valuable traditional products and improve safety of the products through the inhibition of pathogens such as *S. typhimurium, E. coli, S. aureus* or *L. monocytogenes*, which are associated with human infections

1.4 Objectives

1.4.1 Main objective

To determine the fermentative and inhibitory ability of African *Sii*against well-known pathogens- *S. typhimurium, L. monocytogenes, E. coli* and *S. aureus* during fermentation of cow milk

1.4.2 Specific objectives

1. To determine the fermentative ability of African dairy *Streptococcus infantarius* subsp. *infantarius* strains (CJ 18 and CCUG 42830T) in cow milk
2. To assess the inhibitory effect of *Sii* CJ18 and *Sii*CCUG 43820T strains on *E. coli, S. aureus, S. typhimurium*and *L. monocytogenes*during fermentation of cow milk

1.5 Hypotheses

1. African dairy *Sii* can be utilized for highly competitive sustainable dairy fermentation
2. African dairy *Sii* can be utilized to inhibit food-borne pathogens.

CHAPTER 2

2 LITERATURE REVIEW

2.1 Utilization of fermented foods in Africa

Fermented foods and beverages serve as a major component of diet in the world (Savadogo *et al*., 2004). Milk fermentation is a preservation process and occurs through conversion of lactose into lactic acid with the use of lactic acid bacteria and other microorganisms. The process also leads to development of aromatic substances, improved digestibility of proteins, development of sugar polymers, vitamins and useful enzymes (Shori and Baba, 2012). Some lactic acid bacteria produce antimicrobial substances and bacteriocins that inhibit growth of pathogenic and spoilage microorganisms (Hernández *et al*., 2005). Lactic acid development leads to development of an acidic environment consequently lowering the pH that suppresses the growth of undesirable bacteria (Ross *et al*., 2005).

Lactic acid bacteria like African dairy *Sii* has been reported to produce inhibitory substances against other pathogenic bacteria such as *Listeria* spp. *S. aureus* or *E. coli*. They also play an important role in food preservation and are considered an extremely effective tool for food safety applications (Fraga *et al*., 2013; Jans *et al*., 2012c).

During early stages of fermentation, the growth certain groups of microorganisms are high compared to others when the levels of lactic acid is still low. Such microorganisms can adapt to acidic conditions. An example of such is *Escherichia coli* O157:H7 and has the ability to survive low acid levels. This present threat to consumers of fermented milk and milk products since *E. coli* has low infective dose of 10 cells (Tamime and McNulty, 1999)

2.2 Use of starter cultures and their potential benefits in dairy fermentation

Starter cultures are microorganisms that are inoculated in the food to overcome and dominate the existing flora and develop intended desirable properties in the final product. The desirable changes targeted include enhanced preservation, reduced food safety risks, enhanced sensory attributes and improved nutritional value (Robinson, 2005). Addition of starter culture ensures that the processing is done in timed and repeated schedule while maintaining the quality and consistency in the final product. Dairy cultures ferment lactose to lactic acid, generates flavor compounds and textural properties in the product.

Traditional fermentation occurs because of inherent microflora in food and through backslopping (Holzapfel, 2002). Dominant group involved in fermentation are the lactic acid bacteria (Rhee *et al*., 2011). There are four genera of lactic acid bacteria used in the manufacture of fermented dairy products; *Lactococcus, Lactobacillus, Streptococcus* and *Leuconostoc* (Heller, 2001; Panesar, 2011a). They

are accepted for use in foods because they are considered safe (GRAS) for human consumption (Panesar, 2011b). *S. thermophilus* is the only member among the *Streptococci* considered safe for application in dairy fermentation.

Despite other members of SBSEC being pathogenic, virulence factors have not been reported in African *Sii* isolates (Jans *et al*., 2013a). Lack of reports on virulence factors of *Sii* and its ability to ferment lactose in a similar way to *S. thermophilus* suggests a possible application of this strain in developing African novel dairy product with *Streptococcus infantarius* subsp. *infantarius* as the starter culture.

2.3 Taxonomy and prevalence of SBSEC members

The *Streptococcus bovis/streptococcus equinus complex* (SBSEC) consists of an extensive group of species categorized as *streptococcus bovis* including several biotype subclasses (Jans *et al*., 2015). The members of SBSEC are commensal inhabitants of human and animal gastrointestinal tracts where they establish a constant population in the rumen of the sheep, cattle, goats between 10^5 and 10^7 cells per mL (Jans *et al*., 2013b).

Sii is a member (SBSEC) (Jans *et al*., 2013a; Lanie *et al*., 2007). It is commonly found in the GIT of both humans and animals (Abdelgadir *et al*., 2008). It also found in abundance in several spontaneously fermented traditional African dairy products

such as *Suusac*(Hols *et al*., 2005; Jans *et al*., 2012c) and other forms of fermented products such as *pozol,* a Mexican fermented maize beverage (Ferretti *et al*., 2001).

Only *S. thermophilus* among the streptococcal species is generally recognized as safe (GRAS) for use in fermented food products (Motarjemi *et al*., 1993). Despite its close relationship to pathogens (Bolotin *et al*., 2004).*S. thermophilus* was accepted for use in dairy products based on its long history of use in dairy fermentations and inactivation of virulence factors (Bolotin *et al*., 2004). Other members of SBSEC show similar gene decay and evolution in same way to that of *S. thermophilus* e.g. *S. macedonicus* ACA-DC 198, isolated from Greek cheese hence potential of other members of the SBSEC to be adapted to dairy environment (Jans *et al*., 2015). This could also suggest that certain members of the SBSEC could have vital contributions to dairy fermentations (Jans *et al*., 2012c). *Sii* has the same gene decay and loss of function that is similar to that of *S. thermophilus*(Jans *et al*., 2013a). African strains of *Sii* display a lactose fermentation pattern paralleling that of *S. thermophilus*(Tettelin *et al*., 2002). The predominance of African variant *Sii* in dairy environment makes it to be highly adapted. Gal-lac operon in *Sii* is responsible for predominance of bacteriocin producers in the African variant *Sii* a feature that is absent in other members of SBSEC (Rusniok *et al*., 2010; Tettelin *et al*., 2002).

2.4 Implications of African *Sii* in its use in dairy fermentation

Sii is a member of SBSEC is a predominant bacterium in spontaneously fermented milk products in Africa (Abdelgadir *et al*., 2008; Jans *et al*., 2013b, 2015). It plays an important role in spontaneous dairy fermentation with up to 10^8 CFU/mL in the final product (Jans *et al*., 2012c)

A study carried out by Jans *et al*., (2013a) indicated that CJ18, a strain of *Sii* contains several unique regions when compared to *Sii* ATCC BAA-102^T (isolated from human stool) associated with several human infections such as bacteremia, endocarditis and colorectal cancer. Their functional and genomic analysis revealed adapted lactose uptake metabolism that is identical to that of GRAS approved *S. thermophilus* (Jans *et al*., 2012b). It also displayed loss of functional events paralleling the evolutionary adaptation of *S. thermophilus* to dairy environment (Jans *et al*., 2012c). Hence, the role of *Sii* CJ18 in dairy fermentations for possible adaptations in dairy environment needs to be investigated. This will also shed light on the strains predominance in fermented African milk and milk products.

2.5 Microflora of fermented foods

Lactic acid fermentation to prolong the shelf life of food has existed for a long period of time (Djadouni and Kihal, 2012). The usefulness of LAB is derived mainly from their safe metabolic activity. LAB utilizes available sugar to produce organic acids and other metabolites while growing in foods. Their natural existence in foods and long history of use contributed to their acceptance for human

consumption (Picard *et al*., 2005). Fermented foods contain several kinds of microorganisms such as yeast and the mold (Heller, 2001). Many a times mixtures of cultures originating from a natural flora of the raw materials are involved in most of the food fermentation process (Holzapfel, 2002). For large production of fermented foods specific starter culture are used under controlled conditions to give a high-preferred quality of finished product in terms of texture, taste and aroma (Panesar, 2011b).

Fermentation results to a wide variety of microbial populations in fermented foods such as *Streptococcus lactis, Lactobacillus casei, Streptococcus diacetylactis, Lactobacillus lactis, Leuconostoc cremoris, Lactobacillus bulgaricus* and *Lactobacillus acidophilus* (Lore *et al*., 2005). The bacterial microflora play a vital role in producing acid, texture taste and aroma of dairy and other fermented products (Leroy and De Vuyst, 2004).

2.6 The role of fermented foods in inhibiting other microorganisms

The ability of fermented foods to inhibit undesirable microorganisms is determined by assessing the antimicrobial activity against selected test microorganism and/ or their ability to improve the microbial quality of that particular food (García *et al*., 2010; Paul Ross *et al*., 2002). The total viable counts of *C. botulinum* and *L. monocytogenes* reduces with time during fermentation process (Paul Ross *et al*., 2002). Pathogenic *E. coli* survive under low pH for longer period compared other

common food-borne pathogens (Gadaga *et al*., 2004). *Enterococcus faecalis*, *S. aureus* and *B. cereus* are susceptible to bacteriocins extracted from fermented milk products (Savadogo *et al*., 2004). Fermented foods are usually considered safe because of the low pH that that results from production of lactic acid, production of antimicrobial compounds, low molecular metabolites such organic acids, diacetyl, ethanol, hydrogen peroxide and lacto peroxides that inhibit food-borne pathogens and spoilage bacteria (Savadogo *et al*., 2004)

The inhibitive substances produced during lactic acid fermentation act differently on pathogenic reference indicators. The gram-positive bacteria are more sensitive to bacteriocin than gram-negative bacteria (Savadogo *et al*., 2004). The particular nature of gram-negative bacteria makes them resistance to bacteriocin compared to gram-positive bacteria (Savadogo *et al*., 2006). Being gram-positive bacteria, lactic acid bacteria produce bacteriocins that have a broad spectrum of activity (Leroy and De Vuyst, 2004). The efficacy of the bacteriocin against a given bacteria also depend on the strain of the microorganism (Vuyst and Leroy, 2007). This due to the fact that, some strains have receiving sites of immunoprotein and thus easily hurt by inhibitive factor while other strains lack such sites hence more resistant such bacteriocin (Savadogo *et al*., 2006).

2.7 Use of starter cultures

Improved technologies have resulted to development of lactic acid bacteria (LAB) starter cultures (Leroy and De Vuyst, 2004). Use of starter cultures results for fermentation under controlled conditions give a product of desired quality such texture, taste, flavour, aroma and improved microbial quality (Platteeuw *et al*., 1996). The improved microbial quality is due to fast pH drop that control the growth of undesirable competing microorganism (Djadouni and Kihal, 2012). This result to improved storage period by producing antimicrobial substance such as bacteriocins (García *et al*., 2010). The most commonly used bacteriocin as bio preservative in food is nisin (Hernández *et al*., 2005). It is produced by *Lactococcus lactis* and the only recognized as safe for use in dairy products (Delves-Broughton *et al*., 1996). Because of changing lifestyle and eating habits, food is often contaminated with a variety of food-pathogens especially, *L. monocytogenes*, *S. typhimurium*, MRSA (Lynch *et al*., 2009). This results to people increased use of antibiotics resulting to the microorganisms developing resistant to antibiotics (Saikia, 2008). Thus, there has been continued interest in developing probiotics with the ability to produce bacteriocins that will inhibit food-borne pathogens in order to enhance the quality and safety of other fermented milk products (Buyong *et al*., 1998).

The potential of bacteriocin-producing starters is the most widely studied especially on lactococci, enterococci and lactobacilli (Vuyst and Leroy, 2007).

Bacteriocins have been used successfully in milk products such as cheddar cheese using *Lactococcus* as a starter culture (produces nisin) to control *Listeria monocytogenes*, *Clostridium sporogenes* and *Staphylococcus aureus* (Zottola *et al*., 1994). Sulzer and Busse, (1991) carried out research on the ability of *lactococci, enterococci* and *lactobacilli* alone and in combination with other commercial starters against *Listeria monocytogenes* in Camembert cheese. They found out the strains were more effective when they are used alone than when in combination with other commercial cultures. *L. monocytogenes*was also suppressed more if contamination took place in early stages of ripening of camembert cheese.

The safety of fermented food is improved when starter cultures are used because they produce enough lactic acid from lactose that inhibits the growth of pathogenic microorganisms. An effective starter culture is one that produces sufficient acid in early stages of fermentation for fast pH drop to inhibit pathogenic and spoilage microorganisms and maintains low pH during storage. The efficacy of a starter culture is influenced by among other things, the initial level of contamination of product, sanitation, overall hygiene the activity of the starter culture itself (Motarjemi *et al*., 1993). It also depends on the type test microorganism in question, the ability of food to maintain the conditions for pathogen growth and the amount of undissociated acid produced. The undissociated acid is the active form of lactic acid. The undissociated acid interferes with the metabolic of bacterial

pathogens by diffusing and reducing the intracellular pH. During lactic acid fermentation, it is desired that the pH decrease to below four. At this pH,growth of most undesirable microorganisms is suppressed.

In spontaneous fermentation, the pathogens are likely to dominate because the initial pH is very low to suppress their growth. Use of starter culture results to fast pH drop hence control the level of microorganisms before infectious dose is achieved(Holzapfel, 1997). Traditional natural fermentation process leads to variation in the pH of the fermented product, hence undesirable quality attributes. For instance, *Escherichia coli* and *Listeria monocytogenes* survived in Zimbabwean naturally fermented milk. The pathogens were inhibited when the milk was fermented under controlled conditions using a starter culture (Dalu JB and SB 1996) due to a rapid decrease in pH.

2.8 Food-borne pathogens in traditionally fermented dairy products

2.8.1 Listeria monocytogenes

Genus *Listeria* is grouped into *Listeria ivanovii*, *Listeria innocua*, *Listeria welshimeri*, *Listeria seeligeri, Listeria grayi* and *Listeria monocytogenes* (Ryser and Marth, 2007). Previous reviews have however been updated by a recent taxonomic review of the genus by (Ryser and Marth, 2007). Of all the above species, only *Listeria monocytogenes*is linked with causing human and animal illness. The illness associated with *L. monocytogenes* in animals and humans include sepsis, meningitis

and even abortion. *L. ivanovii* and *L. seeligeri*are thought to cause listeriosis in humans but in very rare occasions. *L. monocytogenes* occurs universally in food because of its ability to survive in extreme conditions (Ryser and Marth, 2007). This therefore is a potential route of transmission *L. monocytogenes*.

The universal occurrence and the ability to withstand extreme environmental stress such as low temperature, low pH, ability to adapt to host immune system and high salt concentration of up 10% makes *Listeria monocytogenes* one the most vital food-borne pathogen (Cosentino *et al*., 2012). A wide variety of products have been associated with *Listeria* contamination such as milk and milk products, various meats and meat products such as beef, pork, fermented sausages, fresh produce such as radishes, cabbage, seafood and fish products (Cosentino *et al*., 2012; Gandhi and Chikindas, 2007). These characteristics give it the ability to contaminate food products especially the refrigerated and minimally processed. Foodborne listeriosis poses a serious health hazard when it occurs in newborns, pregnant women, persons with weakened immune system (Mead *et al*., 2006).

2.8.2 Staphylococcus aureus

Staphylococcus aureus is a facultative anaerobic gram-positive coccus, non-motile, catalase and coagulase positive of the micrococcaceae family(Bhatia and Zahoor, 2007). The foodstuffs commonly associated with *S. aureus* are milk and milk products especially the raw milk (Jackson *et al*., 2012). It is the leading cause of

gastroenteritis in humans and bovine mastitis in cattle worldwide. It is however, considered as a low risk compared with other milk associated pathogens such as *Salmonella* sp., *Listeria monocytogenes* and *Escherichia coli* because of its low mortality rate (Arques *et al*., 2015).In addition, the levels of enterotoxigenic staphylococci in dairy products are low. It is also a frequent contaminant of foodstuffs where some strains produce staphylococcal enterotoxins(Arques *et al*., 2015).. The symptoms of food-borne illness due to *S. aureus* include abdominal cramps, nausea, vomiting and diarrhoea, which reduce after 12 to 72 h(Arques *et al*., 2015).. The reported dairy outbreaks of Staphylococcal intoxication are due to temperature abuse above 10◦C and poor starter culture activity during fermentation (Cretenet *et al*., 2011).Staphylococcal enterotoxins are considered a potential biological threat because of their stability at high temperatures (100°C for 1 h) and the ability to incapacitate individuals for several days to two weeks.

2.8.3 Escherichia coli O157:H7

There are many serotypes of *E. coli*. Shiga-toxin producing *Escherichia coli* O157:H7 (STEC) is of high virulence. It can cause disease at a dose of 5–50 cells. They are regularly isolated from dairy products. The number of cases of severe disease caused by STEC in dairy products has remained quite low probably due to the compliance with good hygienic practices at the farm level (Arques*et al*., 2015). The sporadic cases are due to ingestion of milk and dairy products(Arques *et al*., 2015). The main reservoirs of STEC are ruminants, contaminating milk through subclinical

mastitis or faecal routes and the bacteria can persist in milking equipment. While severe cases of bloody diarrhoea or haemolytic uremic syndrome caused by STEC are uncommon, they do affect mostly vulnerable groups such as young children and elderly people.

2.8.4 *Salmonella enterica* serovar typhimurium

Salmonella is an important human health problem of economic significance in animal and humans(Arques *et al.*, 2015). It is an inhabitant in the environment and in the gastrointestinal tract of domestic and wild animals. *Salmonella* is the leading cause disease among human pathogens with 108,614 confirmed cases of salmonellosis in the European Union in 2009 (Arques *et al*., 2015). The foods associated with food-borne infection by *Salmonella* are dairy products, meat, and eggs. Food-borne infection by contaminated milk and dairy products *Salmonella* is due to inadequate pasteurization and post-process contamination. Pasteurization together with controlled fermentation appear to pose no significant health risk of *Salmonella* infection as seen in cases of manufacture of dairy products such as cheeses.

2.9 Knowledge gaps

Raw dairy products can be a vehicle for infection and foodborne diseases with bacterial pathogens such as *Staph. aureus, S. agalactiae* or *Listeria.* This represents a clear health risk for consumers and especially children and infants. Predominant

Sii was shown to have a high prevalence of antimicrobial activity against *Listeria* spp. The extent, range and mechanisms of antimicrobial activity against species other than *Listeria* are not known. Given that food-associated strains can be clearly delineated from human commensal and pathogens, the technological development of food-grade *Sii* to inhibit food-borne pathogens need to be elucidated through characterization of the antimicrobial compounds and competitive assays. This would result in a reduced health risk for consumers of traditional fermented foods through securing and applying this traditional process in an improved form.

CHAPTER 3

3 Fermentative trials of *Streptococcus infantarius* subsp. *infantarius* strains CJ 18 and CCUG 43820T under different incubation temperatures

Abstract

Fermentation serve a key role in inhibiting pathogens and spoilage microorganisms, mainly by acidification and production of antimicrobial compounds, thereby ensuring safety and preservation of food without refrigeration and contributing a healthy nutritional basis (Ashenafi, 2006). The fermentation ability of *Streptococcus infantarius* subsp. *infantarius* has not been documented and therefore two *Sii* strains (CJ 18 and CCUG 43820T) were assed in this chapter. A factorial experimental design was used to conduct fermentation trials using raw and pasteurized cow milk. Raw/pasteurized cow milk was divided into equal aliquots of 100 mL which were inoculated with separate starter cultures of *Sii* CJ 18 and *Sii* CCUG 43820T at the following inoculation rates; 0% (control), 5%, 10% and 15% v/v. Incubations were at 20, 30, 37 and 45^{o}C in parallel. pH and titratable acidity were measured at 0 h, 3 h, 6 h and 9 h. Fermentation was faster in pasteurized milk than raw milk in both *Sii* CJ 18 and *Sii* CCUG 43820T. *Sii* CCUG 43820T resulted to faster pH drop than *Sii* CJ 18. In pasteurized milk, the target pH of below 4.5 was attained after six hours at 45^{o}C, and 10% inoculation rate by CCUG 43820T pH (4.36±0.00) while CJ 18 attained a pH of 4.55±0.00 but at 15%

inoculation rate. In raw milk, the lowest pH attained was 4.55±00 at 45^{o}C, 15% inoculation rate by CCUG 43820T while CJ 18 attained a pH of 4.67±0.23 under the conditions after nine hours. In pasteurized milk the highest percent lactic acid formed by CCUG 43820T was 1.29±0.02% at 45^{o}C, 15% inoculation ,while CJ 18 attained 0.82±0.02% lactic acid under same conditions after nine hours of fermentation. In raw milk, the highest percent lactic acid attained by CCUG 43820T was 0.70±0.02% at 45^{o}C, 15% inoculation rate, while CJ 18 attained 0.61±0.01% lactic acid under same conditions after nine hours of fermentation. In conclusion, *Sii* strains CJ 18 and CCUG 43820T can be potentially applied in dairy industry as starter cultures that results to faster pH drop and conversion of sugars to organic acids at thermophilic range of temperature. However, further fermentation trials and overall acceptability of products should be done at community level.

3.1 Introduction

Fermented dairy products are processed from milk using specific cultures through fermentation of lactose especially by lactic acid bacteria (LAB) (Özer and Kirmaci, 2010). LAB is a group of gram-positive, non-spore forming, cocci or rods, which produce lactic acid as the major product during fermentation of lactose. This group includes *Lactobacillus*, *Lactococcus*, *Streptococcus* and *Leuconostoc* species (Gemechu, 2015).

In Kenya, there are diverse traditionally fermented milk products that belong to different communities. Kalenjin community have *murski,* Kisii *amabere amaruranu*, Masaai *kule naoto* while Somali have *Suusac* (Mathara *et al*., 2004). *Suusac* is prepared by spontaneous fermentation of raw camel milk that without heat treatment (Lore *et al*., 2005). The milk is left in smoke-treated gourds or plastic containers for two days to naturally ferment (Lore *et al*., 2005; Panesar, 2011b). In some cases, camel raw milk is added to small amount of *Suusac* through backslopping. Spontaneous fermentation takes advantage of autochthonous lactic acid bacteria in the milk (Mathara *et al*., 2008). However, since spontaneous fermentation is done under uncontrolled conditions, it often results in products that that are white, less viscous, distinctive flavor with astringent taste and in most instances the product quality is not consistent (Lore *et al*., 2005).

Starter cultures are deliberately added into milk in a controlled fermentation process to give consistent quality and shelf-stable products of desired sensorial properties (Hati *et al*., 2013). Fermentation of milk has a long history of being used as an attainable method of extending the shelf life of milk and generating flavor (Hati *et al*., 2013). This is achieved through rapid acidification of the raw material through the production of organic acids, mainly lactic acid thereby lowering the pH and subsequently inhibiting the growth of pathogenic and spoilage bacteria (Caplice and Fitzgerald, 1999; Leroy and De Vuyst, 2004). In addition, production of acetic acid, ethanol, aroma compounds, bacteriocins, exopolysaccharides and several enzymes is also important (De Vuyst, 2000; Tamime, 2008). In this way, shelf life and microbial safety are enhanced, texture is improved and it also contributes to the pleasant sensory profile of the end product (Hati *et al*., 2013).

Fermentation also improves the safety of milk by producing antimicrobial substances such as organic acids and bacteriocins that suppress the growth of pathogens such as *E. coli* and *S.aureus* (Fraga *et al*., 2013; Yang *et al*., 2012). There is an increasing interest in studying of traditional fermentation to gain deeper understanding among food microbiologists, with particular interest on the identity and functional properties of the microorganisms involved (Magalhães *et al*., 2011). Research studies have been carried out to isolate, identify and characterize the microorganisms involved in producing traditionally fermented African dairy

products in West and East Africa (Jans *et al*., 2013b). However, fermentation trials of such novel strains have not been conducted (Jans *et al*., 2013a). The objective of this study was to test the fermentation ability of *Sii* CJ 18 and *Sii* CCUG 43820T with the goal of adapting these novel strains in development of suitable starter cultures for the purposes of standardizing the quality and scale up production of traditionally fermented dairy products.

3.2 Materials and Methodology

3.2.1 Sample collection

Fermentation inoculum for this study was formulated with sterile UHT milk. The inhibition potential of *Sii* against selected pathogens was assayed using fermentation trials at Microbiology laboratory of Department of Food Science, Nutrition and Technology. Cow milk was procured from the Department's Pilot plant, which obtains milk from the University Veterinary Farm Kanyariri located in Ndumbuini, Kiambu County.

3.2.2 Experimental study design

3.2.2.1 Preparation of Sii starter cultures

A first overnight culture of *Sii* CJ 18 and *Sii* CCUG 43820T strains were prepared in M17 broth. This M17 broth was used to inoculate UHT milk at 1% v/v and incubated overnight to provide inoculum for fermentation.

3.2.2.2 Fermentation trials

A factorial experimental design was used to conduct fermentation trials of raw and pasteurized cow milk. Each milk typewas divided into equal aliquots of 100 mL. The test organisms were *Sii* CJ 18 and *Sii* CCUG 43820T, which were inoculated separately in the following inoculation rates; 0% (control), 5%, 10%, and 15% v/v. Incubations were at 20, 30, 37 and 45^{o}C in parallel. pH and titratable acidity were measured at 0 h, 3 h, 6 h and 9 h.

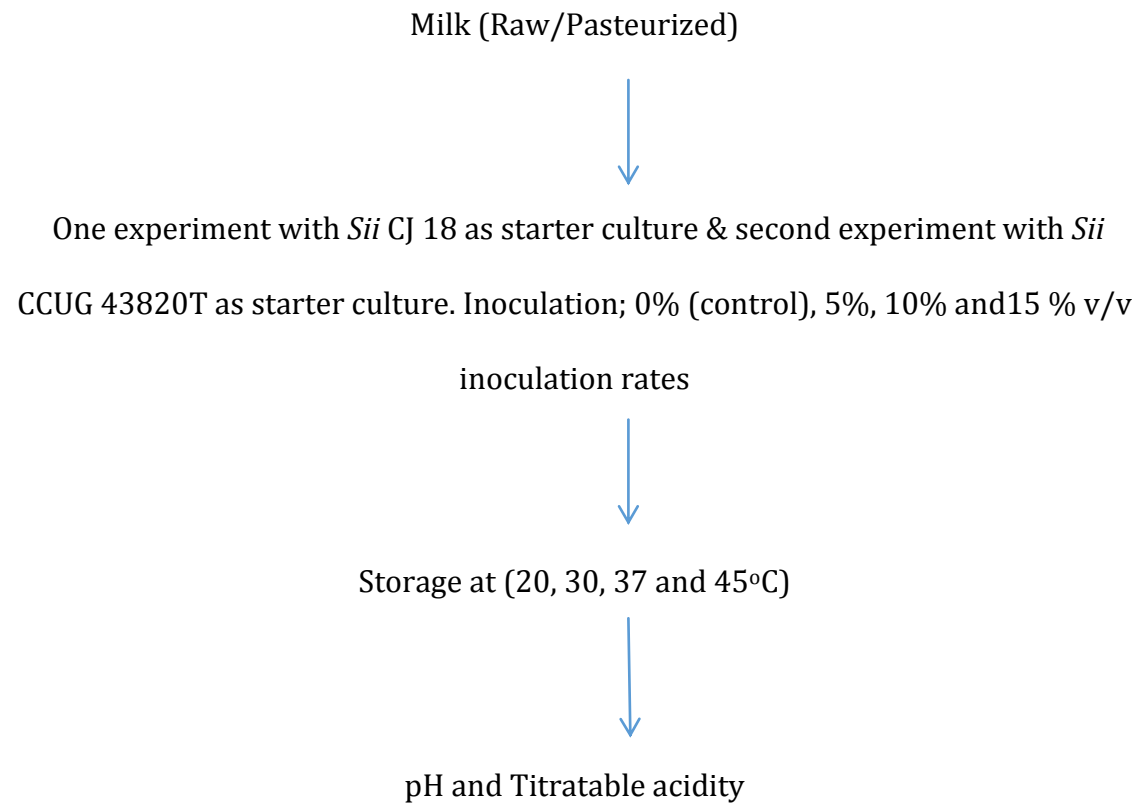

Figure 1: Inoculation with cultures, incubation, and assay of pH and acidity during fermentation period.

3.2.3 Analytical Methods

3.2.3.1 Determination of lactic acid concentration in fermented milk samples

Acidity of fermented milk was determined according to the AOAC method number 947.05. Nine milliliters of the milk was pipetted into a flask followed by addition of 3 drops of phenolphthalein indicator. It was titrated against 0.1N NaOH until end point was achieved. Volume of 0.1N NaOH used to neutralize the lactic acid was used to determine percent lactic acid in fermented milk.

Percent lactic acid was calculated as follows:-

$$= \frac{(\text{ml of NaOH used})(\text{conc NaOH})(0.09 \text{ this is milli equivalent weight of lactic acid})}{\text{Weight of Sample}}$$

3.2.3.2 Determination of pH of milk samples

The pH of milk sample during fermentation was determined using a multi-parameter analyzer model peqMETER 1.14 that was calibrated before pH measurement using standard pH 4.0 and 7.0 by pouring 15mL of buffer solutions into a 50 mL beaker at ambient temperature and dipping a probe into beaker and carrying out electrode calibration sequence. The procedure was repeated for each buffer. The electrode meter of the digital pH meter was dipped in well-stirred 15 mL milk sample in 50 mL beaker. The readings on the digital meter were then recorded.

3.2.3.3 Statistical analysis

The data obtained from fermentation trial was entered in Microsoft Excel. Statistical analysis was carried using SPSS to determine statistical difference in pH drop and percent titratable acid production against time intervals during the storage period. One-way analysis of variance was carried out to compare means of pH drop and percent lactic acid during storage periods for *Sii* C J18 and *Sii* CCUG 43820T for raw and pasteurized cow milk.

3.3 Results

3.3.1 Fermentation of pasteurized milk with *Sii* CJ 18 and *Sii* CCUG 43820T starter culture

The effect of different temperatures on acid production by *Sii* strains (CJ 18 and CCUG 43820T) pasteurized milkis showninTable 1. The rate of acidification was faster in milk inoculated with CCUG 43820T than CJ 18 (Table 1). Milk inoculated with CCUG 43820T attained a final acidity of 1.29 ± 0.02% at 45^{o}C, 15% inoculation rate after 9 h of storage while CJ 18 attained 0.81 ± 0.01% at the same conditions. The rate of acidification was lowest in the control by both *Sii* strains in all incubation temperatures.

Table 1: Percent titratable acidity produced by *Sii* strains CJ 18 and CCUG 43820T in Pasteurized milk during fermentation

Temperature	Incubation time (h)	% lactic acid							
		CJ 18				CCUG 43820T			
		Inoculation rate							
		Control	5%	10%	15%	Control	5%	10%	15%
20°c	0	0.12 ± 0.01 a	0.15 ± 0.01 a	0.20 ± 0.01 a	0.24 ± 0.01 a	0.11 ± 0.01a	0.17 ± 0.01a	0.20 ± 0.01a	0.23 ± 0.01 a
	3	0.12 ± 0.01 a	0.18 ± 0.01 b	0.21 ± 0.01 a	0.24 ± 0.01 a	0.14 ± 0.01ab	0.17 ± 0.01a	0.21 ± 0.01a	0.25 ± 0.01 a
	6	0.15 ± 0.00 b	0.23 ± 0.01 c	0.27 ± 0.00 b	0.32 ± 0.01 b	0.14 ± 0.01b	0.22 ± 0.01b	0.26 ± 0.02b	0.32 ± 0.01 b
	9	0.20 ± 0.01 c	0.28 ± 0.01 d	0.38 ± 0.02 c	0.41 ± 0.02 c	0.18 ± 0.02c	0.26 ± 0.00c	0.31 ± 0.01c	0.37 ± 0.02 c
30°c	0	0.12 ± 0.01 a	0.18 ± 0.01 a	0.20 ± 0.02 a	0.24 ± 0.01 a	0.13 ± 0.01 a	0.18 ± 0.02 a	0.20 ± 0.02 a	0.23 ± 0.02 a
	3	0.14 ± 0.01 b	0.22 ± 0.01 b	0.24 ± 0.01 b	0.30 ± 0.01 b	0.15 ± 0.01 a	0.20 ± 0.01 a	0.24 ± 0.01 b	0.32 ± 0.01 b
	6	0.18 ± 0.00 c	0.39 ± 0.01 c	0.47 ± 0.01 c	0.31 ± 0.01 c	0.19 ± 0.01 a	0.38 ± 0.01 b	0.48 ± 0.01 c	0.51 ± 0.01 c
	9	0.45 ± 0.01 d	0.53 ± 0.01 d	0.58 ± 0.02 d	0.62 ± 0.02 d	0.45 ± 0.01 b	0.51 ± 0.01 c	0.60 ± 0.01 d	0.62 ± 0.01 d
37°c	0	0.13 ± 0.01 a	0.18 ± 0.01 a	0.19 ± 0.01 a	0.24 ± 0.01 a	0.13 ± 0.01 a	0.18 ± 0.02 a	0.20 ± 0.02 a	0.30 ± 0.02 a
	3	0.14 ± 0.00 a	0.23 ± 0.02 b	0.29 ± 0.01 b	0.33 ± 0.01 b	0.13 ± 0.02 a	0.33 ± 0.02 b	0.24 ± 0.01 b	0.37 ± 0.01 b
	6	0.20 ± 0.01 b	0.51 ± 0.01 c	0.57 ± 0.01 c	0.57 ± 0.01 c	0.22 ± 0.01 a	0.62 ± 0.01 c	0.48 ± 0.01 c	0.70 ± 0.01 c
	9	0.49 ± 0.01 c	0.58 ± 0.01 d	0.61 ± 0.01 d	0.67 ± 0.02 d	0.45 ± 0.01 b	0.69 ± 0.01 d	0.60 ± 0.01 d	0.75 ± 0.01 d
45°c	0	0.12 ± 0.01 a	0.17 ± 0.01a	0.19 ± 0.00 a	0.26 ± 0.00 a	0.13 ± 0.01 a	0.17 ± 0.01 a	0.20 ± 0.02 a	0.24 ± 0.02 a
	3	0.14 ± 0.01 a	0.26 ± 0.01b	0.38 ± 0.01 b	0.45 ± 0.02 b	0.14 ± 0.01 a	0.25 ± 0.01 b	0.33 ± 0.01 b	0.34 ± 0.01 b
	6	0.14 ± 0.01 a	0.43 ± 0.01c	0.73 ± 0.01 c	0.78 ± 0.01 c	0.13 ± 0.01 a	0.47 ± 0.01 c	0.50 ± 0.01 c	0.71 ± 0.01 c
	9	0.29 ± 0.02 b	0.47 ± 0.02d	0.81 ± 0.01 d	0.82 ± 0.02 d	0.33 ± 0.02 b	0.63 ± 0.02 d	0.99 ± 0.01 d	1.29 ± 0.02 d

[a]Each value is a mean of three replicate experiments with three titrations each.

[b]Data with a different letter within a column is significantly different ($p<0.05$).

[c]Time is the incubation time in hours.

The pH in pasteurized milk dropped with fermentation time, inoculation rates and storage period (Table 2). There was a slow drop in pH in un-inoculated milk during the storage period. ANOVA showed a highly significant difference at all inoculation rates in all incubation times from 30^{o}C - 45^{o}C, between different storage periods ($P<0.001$). In pasteurized milk, the desirable pH of below 4.5 was attained after six hours at 45^{o}C, and 10% inoculation rate by CCUG 43820T (pH 4.36 ± 0.00) while CJ 18 attained it (4.55 ± 0.00) at 15% inoculation rate. The lowest pH attained was 4.14 ± 0.000; at 45^{o}C, 15% inoculation after 9 hours of fermentation at 45^{o}C, 15% inoculation rate by *Sii* CJ 18 while *Sii* CCUG 43820T attained a pH of 4.05 ± 0.000 under same conditions.

Table 2: Effect of different temperatures and inoculation rates on pH drop by *Sii* strains CJ 18 and CCUG 43820T in pasteurized milk during fermentation

Temperature	Incubation time (h)	pH values							
		CJ 18				CCUG 43820T			
		Inoculation rate							
		Control	5%	10%	15%	Control	5%	10%	15%
20°c	0	6.64 ± 0.011 a	6.33 ± 0.017 a	6.13 ± 0.029 a	5.93 ± 0.023 a	6.68 ± 0.006 a	6.39 ± 0.000 a	6.11 ± 0.012 a	5.94 ± 0.006 a
	3	6.60 ± 0.000 b	6.30 ± 0.005 a	6.02 ± 0.035 b	5.83 ± 0.017 b	6.64 ± 0.010 b	5.72 ± 0.023 b	5.40 ± 0.023 b	5.11 ± 0.023 b
	6	6.43 ± 0.000 c	6.10 ± 0.010 b	5.91 ± 0.010 c	5.73 ± 0.000 c	6.47 ± 0.000 c	6.09 ± 0.012 c	5.90 ± 0.000 c	5.78 ± 0.000 c
	9	6.05 ± 0.012 d	5.63 ± 0.027 c	5.51 ± 0.012 d	5.41 ± 0.006 d	6.01 ± 0.012 d	5.60 ± 0.006 d	5.53 ± 0.000 d	5.41 ± 0.000 d
30°c	0	6.81 ± 0.023 a	6.55 ± 0.000 a	6.43 ± 0.000 a	6.35 ± 0.000 a	6.81 ± 0.023 a	6.51 ± 0.000 a	6.28 ± 0.012 a	6.19 ± 0.000 a
	3	6.72 ± 0.000 a	6.23 ± 0.000 b	5.98 ± 0.000 b	5.73 ± 0.000 b	6.72 ± 0.000 b	6.38 ± 0.023 b	5.98 ± 0.000 b	5.82 ± 0.000 b
	6	6.23 ± 0.075 b	5.32 ± 0.045 c	5.01 ± 0.023 c	5.09 ± 0.046 c	6.26 ± 0.046 c	5.34 ± 0.029 c	5.19 ± 0.046 c	5.11 ± 0.046 c
	9	4.83 ± 0.006 c	4.72 ± 0.023 d	4.63 ± 0.000 d	4.60 ± 0.006 d	4.95 ± 0.000 d	4.71± 0.000 d	4.67 ± 0.000 d	4.63 ± 0.000 d
37°c	0	6.81 ± 0.023 a	6.55 ± 0.000 a	6.47 ± 0.000 a	6.23 ± 0.000 a	6.81 ± 0.023 a	6.52 ± 0.023 a	6.43 ± 0.000 a	6.31 ± 0.000 a
	3	6.76 ± 0.006 b	6.15 ± 0.000 b	5.90 ± 0.000 b	5.73 ± 0.000 b	6.80 ± 0.000 b	6.23 ± 0.000 b	5.82 ± 0.000 b	5.69 ± 0.000 b
	6	6.03 ± 0.023 c	4.92 ± 0.023 c	4.85 ± 0.029 c	4.80 ± 0.023 c	6.18 ± 0.046 c	5.04 ± 0.040 c	4.84 ± 0.061 c	4.85 ± 0.029 c
	9	5.55 ± 0.021 d	4.72 ± 0.023 d	4.70 ± 0.023 d	4.64 ± 0.023 d	5.69 ± 0.040 c	4.76 ± 0.023 d	4.68 ± 0.023 d	4.66 ± 0.017 d
45°c	0	6.64 ± 0.012 a	6.34 ± 0.017 a	6.13 ± 0.029 a	5.94 ± 0.006 a	6.66 ± 0.029 a	6.38 ± 0.023 a	6.14 ± 0.017 a	5.94 ± 0.000 a
	3	6.62 ± 0.021 b	5.79 ± 0.026 b	5.53 ± 0.040 b	5.19 ± 0.023 b	6.67 ± 0.023 a	5.72 ± 0.023 b	5.40 ± 0.023 b	5.11 ± 0.023 b
	6	6.72 ± 0.000 b	4.99 ± 0.000 c	4.77 ± 0.000 c	4.55 ± 0.000 c	6.68 ± 0.000 a	5.00 ± 0.000 c	4.36 ± 0.000 c	4.32 ± 0.000 c
	9	5.45 ± 0.000 c	4.71 ± 0.000 d	4.18 ± 0.000 d	4.05 ± 0.000 d	5.32 ± 0.000 b	4.26 ± 0.000 d	4.22 ± 0.000 d	4.14 ± 0.000 d

[a]Each value is a mean of three replicates of pH meter readings.
[b]Data with a different letter within a column is significantly different ($p<0.05$).
[c]Time is the incubation time in hours.

3.3.2 Fermentation of raw milk with *Sii* CJ 18 and *Sii* CCUG 43820T starter cultures

The fermentation pattern of raw milk with *Sii* strains was similar but at reduced rate. Percent titratable acid production increased with incubation temperature, storage period and inoculation rates (Table 3). CCUG 43820T resulted to faster titratable acidity than CJ 18. CCUG 43820T attained an acidity of 0.70 ± 0.02%, at 45^{o}C, 15% inoculation rate, after 9 h of storage while CJ 18 developed an acidity of 0.61 ± 0.01% under same conditions. There was a significant difference in acid development in all inoculation rates from 30^{o}C in inoculated milk throughout the storage period ($p < 0.001$).

Table 3: percent titratable acidity produced by *Sii* strains CJ 18 and CCUG 43820T in raw milk during fermentation

tempe rature	time (h)	percent lactic acid							
		CJ 18				CCUG 43820T			
		Inoculation rate							
		Control	5%	10%	15%	Control	5%	10%	15%
20[o]c	0	0.15 ± 0.01 a	0.18 ± 0.01 a	0.20 ± 0.01 a	0.22 ± 0.02 a	0.15 ± 0.01a	0.19 ± 0.01 a	0.20 ± 0.01 a	0.23 ± 0.01 a
	3	0.14 ± 0.01 a	0.19 ± 0.01 a	0.23 ± 0.01 a	0.26 ± 0.01 a	0.16 ± 0.01a	0.21 ± 0.01 a	0.23 ± 0.01 a	0.26 ± 0.01 a
	6	0.16 ± 0.01 a	0.25 ± 0.02 a	0.30 ± 0.01 b	0.38 ± 0.01 b	0.15 ± 0.01ab	0.21 ± 0.02 a	0.30 ± 0.02 b	0.31 ± 0.02 b
	9	0.19 ± 0.02 b	0.38 ± 0.01 b	0.41 ± 0.00 c	0.49 ± 0.01 c	0.18 ± 0.01b	0.32 ± 0.01 b	0.36 ± 0.01 c	0.44 ± 0.02 c
30[o]c	0	0.16 ± 0.01 a	0.17 ± 0.00 a	0.18 ± 0.01 a	0.21 ± 0.01 a	0.12 ± 0.01 a	0.18 ± 0.02 a	0.20 ± 0.02 a	0.23 ± 0.02 a
	3	0.16 ± 0.00 a	0.20 ± 0.01 b	0.23 ± 0.01 b	0.26 ± 0.01 b	0.19 ± 0.01 b	0.20 ± 0.01 a	0.24 ± 0.01 b	0.32 ± 0.01 b
	6	0.21 ± 0.01 b	0.31 ± 0.01 c	0.35 ± 0.01 c	0.35 ± 0.01 c	0.21 ± 0.02 b	0.38 ± 0.01 b	0.48 ± 0.01 c	0.51 ± 0.01 c
	9	0.34 ± 0.01 c	0.59 ± 0.01 d	0.63 ± 0.01 d	0.69 ± 0.02 d	0.33 ± 0.00 d	0.51 ± 0.01 c	0.60 ± 0.01 d	0.62 ± 0.01 d
37[o]c	0	0.14 ± 0.01 a	0.18 ± 0.01 a	0.19 ± 0.40 a	0.22 ± 0.01 a	0.12 ± 0.01 a	0.18 ± 0.01 a	0.44 ± 0.01 a	0.22 ± 0.01 a
	3	0.18 ± 0.01 b	0.25 ± 0.01 b	0.27 ± 0.01 b	0.30 ± 0.01 b	0.16 ± 0.01 b	0.23 ± 0.02 b	0.27 ± 0.01 a	0.31 ± 0.01 b
	6	0.24 ± 0.01 c	0.39 ± 0.01 c	0.42 ± 0.01 c	0.43 ± 0.01 c	0.27 ± 0.01 c	0.38 ± 0.01 c	0.43 ± 0.00 a	0.46 ± 0.01 c
	9	0.34 ± 0.01 d	0.62 ± 0.01 d	0.63 ± 0.01 d	0.69 ± 0.01 d	0.37 ± 0.01 d	0.64 ± 0.01 d	0.68 ± 0.01 a	0.70 ± 0.02 d
45[o]c	0	0.15 ± 0.02 a	0.18 ± 0.01 a	0.18 ± 0.01 a	0.22 ± 0.01 a	0.15 ± 0.01 a	0.18 ± 0.01 a	0.19 ± 0.01 a	0.22 ± 0.01 a
	3	0.14 ± 0.01 a	0.23 ± 0.01 b	0.23 ± 0.01 b	0.32 ± 0.01 b	0.15 ± 0.01 a	0.24 ± 0.01 b	0.25 ± 0.01 b	0.32 ± 0.01 b
	6	0.16 ± 0.01 a	0.41 ± 0.01 c	0.42 ± 0.01 c	0.50 ± 0.01 c	0.17 ± 0.02 b	0.42 ± 0.01 c	0.45 ± 0.02 c	0.50 ± 0.01 c
	9	0.21 ± 0.01 b	0.46 ± 0.01 d	0.53 ± 0.02 d	0.61 ± 0.01 d	0.21 ± 0.01 c	0.62 ± 0.01 d	0.66 ± 0.01 d	0.70 ± 0.02 d

[a]Each value is a mean of three replicate experiments with three titrations each.

[b]Data with a different letter within a column is significantly different ($p<0.05$).

[c]Time is the incubation time in hours.

In raw milk, fermentation was similar with pasteurized milk but at reduced rate. The pH dropped with incubation temperature, storage periods and inoculation rates (Table 4). The rate of pH drop was faster in milk inoculated with *Sii* CCUG 43820T than *Sii* CJ 18. The lowest pH attained was 4.55 ± 0.000 by both *Sii* CCUG 43820T and *Sii* CJ 18. pH drop was rapid in 37^{o}C and 45^{o}C. There was a significant difference in all inoculation rates, in all temperatures, from the sixth hour of storage ($P<0.001$).

Table 4: Effect of different temperatures and inoculation rates on pH drop by *Sii* strains CJ 18 and CCUG 43820T in raw milk during fermentation

Temperature	Incubation time (h)	pH values							
		CJ 18				CCUG 43820T			
		Inoculation rate							
		Control	5%	10%	15%	Control	5%	10%	15%
20°c	0	6.67 ± 0.017 a	6.42 ± 0.017 a	6.28 ± 0.017 a	6.18 ± 0.017 a	6.67 ± 0.017 a	6.46 ± 0.012 a	6.28 ± 0.023 a	6.14 ± 0.032 a
	3	6.60 ± 0.000 b	6.35 ± 0.006 b	6.08 ± 0.021 b	5.83 ± 0.012 b	6.60 ± 0.000 b	6.43 ± 0.000 b	6.27 ± 0.000 b	6.08 ± 0.012 b
	6	6.59 ± 0.010 b	5.85 ± 0.012 c	5.55 ± 0.006 c	5.50 ± 0.023 c	6.57 ± 0.015 b	6.19 ± 0.015 c	5.93 ± 0.000 c	5.74 ± 0.026 c
	9	6.44 ± 0.012 c	5.53 ± 0.025 d	5.41 ± 0.015 d	5.28 ± 0.025 d	6.48 ± 0.030 c	5.96 ± 0.021 c	5.56 ± 0.030 c	5.50 ± 0.021 c
30°c	0	6.46 ± 0.023 a	6.36 ± 0.017 a	6.22 ± 0.029 a	6.18 ± 0.017 a	6.46 ± 0.017 a	6.27 ± 0.025 a	6.15 ± 0.000 a	6.04 ± 0.026 a
	3	6.36 ± 0.036 b	6.23 ± 0.035 b	6.09 ± 0.012 b	5.93 ± 0.012 b	6.42 ± 0.017 b	6.23 ± 0.000 b	6.07 ± 0.030 b	5.93 ± 0.023 b
	6	6.33 ± 0.020 b	5.68 ± 0.023 c	5.47 ± 0.015 c	5.36 ± 0.030 c	6.35 ± 0.040 c	5.68 ± 0.023 c	5.46 ± 0.026 c	5.36 ± 0.031 c
	9	6.12 ± 0.021 c	5.11 ± 0.012 d	5.08 ± 0.015 d	4.98 ± 0.015 d	5.94 ± 0.006 c	5.31 ± 0.010 c	5.06 ± 0.067 c	4.94 ± 0.032 d
37°c	0	6.49 ± 0.072 a	6.32 ± 0.026 a	6.21 ± 0.021 a	6.18 ± 0.017 a	6.46 ± 0.023 a	6.32 ± 0.026 a	6.14 ± 0.017 a	6.08 ± 0.017 a
	3	6.32 ± 0.025 b	6.24 ± 0.023 b	6.15 ± 0.045 b	5.91 ± 0.023 b	6.41 ± 0.017 b	6.26 ± 0.023 b	6.12 ± 0.015 b	5.99 ± 0.006 b
	6	6.24 ± 0.012 b	5.54 ± 0.030 c	5.47 ± 0.021 c	5.36 ± 0.012 c	6.16 ± 0.031 c	5.56 ± 0.031 c	5.44 ± 0.026 c	5.33 ± 0.006 c
	9	5.92 ± 0.015 c	5.05 ± 0.012 d	4.96 ± 0.015 d	4.92 ± 0.026 d	5.82 ± 0.015 d	5.12 ± 0.006 d	5.05 ± 0.010 c	4.97 ± 0.012 d
45°c	0	6.68 ± 0.015 a	6.35 ± 0.006 a	6.08 ± 0.021 a	5.75 ± 0.157 a	6.67 ± 0.015 a	6.46 ± 0.012 a	6.28 ± 0.023 a	6.07 ± 0.012 a
	3	6.35 ± 0.035 a	5.88 ± 0.015 b	5.62 ± 0.012 b	5.55 ± 0.015 a	6.39 ± 0.000 b	6.10 ± 0.000 b	5.91 ± 0.015 b	5.62 ± 0.023 b
	6	6.20 ± 0.010 b	5.46 ± 0.023 c	5.14 ± 0.020 c	5.05 ± 0.015 b	6.31 ± 0.010 c	5.42 ± 0.026 c	5.32 ± 0.020 c	5.02 ± 0.020 c
	9	6.21 ± 0.021 c	4.76 ± 0.021 d	4.73 ± 0.000 d	4.55 ± 0.000 c	6.13 ± 0.015 d	4.72 ± 0.021 d	4.58 ± 0.012 d	4.55 ± 0.000 d

[a]Each value is a mean of three replicates of pH meter readings.
[b]Data with a different letter within a column is significantly different ($p<0.05$).
[c]Time is the incubation time in hours.

3.4 Discussion

Fermented dairy products are generally produced using traditional methods (Shiby and Mishra, 2013). They are known to play an essential role in health and nutrition (Özer and Kirmaci, 2010). They do so by acting as vehicles of probiotic strains that beneficially influences the health of the host by improving the composition of intestinal microflora (Robinson, 2005). In addition fermented products have a longer shelf life (García *et al*., 2010). There is an increased demand for natural nutrients and probiotic products resulting to increased consumption of fermented dairy products (Shori, 2015). The physical and chemical changes observed during fermentation of cow milk was due to the growth and fermentative activities of lactic acid bacteria used as starter cultures (Panesar, 2011b). The acid is responsible for development of characteristic body and texture of the fermented milk products, contributes to the overall flavour of the products and enhances preservation (Hati *et al*., 2013).

Fermentation of milk to sour milk generates lactic acid, taste, flavor compounds and textural properties using LAB. The flavor and aroma are as results proteolytic activity. Proteolytic system of the starter culture is vital for the growth of microorganisms and it is involved in casein utilization within LAB cells and results to the development of organoleptic properties of fermented milk products (Holzapfel, 2002).

Pasteurization of milk together with use of starter culture results to faster pH drop and development of acidity by giving the starter culture a competitive advantage. In raw milk, there is competition for nutrients by several bacterial flora in cow milk.

Thermophilic bacteria such as the test bacteria in the current study *Sii* CJ 18 and *Sii* CCUG 43830T have an optimum growth30^{o}C to 45^{o}C. The fast decrease in pH and development of lactic acid bacteria isolated from subtropical regions have been previously reported (Caplice and Fitzgerald, 1999).

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The traits of *Sii* being highly adapted and competitive traditional bacterial group was reported (Jans *et al*., 2013a). This explains its ability ferment raw Cow milk as indicated by development of acidity and pH decrease.

3.5 Conclusion

In conclusion, *Sii* CJ 18 and *Sii* CCUG 43830T strains can be potentially applied in dairy industry as starter cultures that results to faster pH drop and conversion of sugars to organic acids from 30 to 45^{o}C.

3.6 Recommendations

I recommend carrying out fermentation trials at the community level and testing overall acceptability of products.

CHAPTER 4

4 Inhibition effect of *Streptococcus infantarius* subsp. *infantarius* strains against common food-borne pathogens

Abstract

Fermentation serves a key role in inhibiting pathogens and spoilage microorganisms, mainly by acidification and production of antimicrobial compounds, thereby ensuring safety and preservation of food where refrigeration is not available and contributing a healthy nutritional basis. This study aimed at determining inhibition potential of *Streptococcus infantarius* subsp. *infantarius* (*Sii*) strains CJ18 and CCUG 43820T against selected food-borne pathogens; *Staphylococcus aureus*, *Listeria monocytogenes*, *Escherichia coli,* and *Salmonella.* Antimicrobial activity was performed using disc diffusion and competition assays. Disc diffusion was done as follows; a lawn of each indicator strain was made by spreading cell suspension over the surface of Muller Hinton agar plates (Oxiod) with sterile cotton swabs and allowed to dry. Sterile discs soaked with *Sii* strains cell-free supernatants for 5 minutes, excess carefully removed, were placed on the surface of Muller Hinton agar plates, and allowed to dry for 10 minutes. After incubation at 37^{o}C for 48h, the plates were observed for a zone of inhibition around the discs. Inhibition trials were also done using competition assay as follows; Raw/pasteurized cow milk were divided into equal aliquots of 100 mL, spiked with known CFU/mL of each pathogenic bacterium, inoculated with 15% v/v *Sii* CJ 18 and *Sii* CCUG 43820T starter cultures independently, and incubated at 37^{o}C. Appropriate double serial dilutions, plated in duplicate onto appropriate selective

or semi-selective growth medium after every 3 hours and enumerated. Enumeration of *E. Coli*was done by surface plating 0.1 ml of each sample on brilliance *E. coli* coliform agar (Oxiod).*Salmonella* was enumerated by pour plating onto Violet Red Bile Agar (Merck) and plates incubated at 37^{o}C for 24 h. *Staphylococcus aureus* was enumerated by surface plating onto Baird Parker Agar media (Oxoid) and plates incubated at 37 ^{o}C for 24 h. *L. monocytogenes* was surface plated in *Listeria* semi-selective agar (Oxiod).

In disc diffusion assay, the growth of *Staphylococcus aureus* and *Listeria monocytogenes* were inhibited as indicated by clear halo regions around the discs. The halo diameters in *S. aureus* ATCC 25923 were 6.3±0.6 mm for *Sii* CJ 18 and 7.8 ± 1.5mm for *Sii* CCUG 43820T while that of *L. monocytogenes* were 5.3±0.6 for *Sii* CJ 18 and 7.3±1.2mm for *Sii* CCUG 43820T. CCUG 43820T showed a better antimicrobial activity than CJ18. In competition assays, Growth of pathogens increased within 9 h of fermentation in control samples whereas growth in decreased after 3 h of fermentation in samples inoculated with *Sii* strains within 9 h. The growth was higher in raw milk than pasteurized milk. In conclusion, *Sii* strains can to be used as novel starter cultures to inhibit the growth of pathogens and spoilage microorganism in fermented milk products upon elucidation of their safety. However, further *in vitro* and *in vivo* studies are required, such as resistance to antibiotics, acid tolerance, viability, safety, and organoleptic properties.

4.1 Introduction

Milk is a balanced food of high biological value that provides the right proportion of nutrients in an easily digestible form. It is therefore important in supplying nutritious and economical food for human beings. Milk also serves as a source of income for small-scale farmers in Sub-Saharan Africa that enables them (poor communities) to buy other foodstuffs which significantly contributes to the household food security (Abate *et al*., 2015;Gemechu *et al*., 2015; Panesar, 2011).

Milk provides an excellent growth medium for many microorganisms (Swai & Schoonman, 2011). This is because of its high water content, nearly neutral pH, and being nutritious hence an excellent medium for growth and multiplication of microorganisms (Abate *et al*., 2015). It is a very perishable commodity. Sources of contamination of raw milk include ill health of animals, production under low hygiene level, using improperly cleaned and sanitized equipment (Swai & Schoonman, 2011). This often results in spoilage, low quality, and eventual loss of the product (Abate *et al*., 2015; Gemechu et al., 2015).The bacteria often detected in raw milk are obligate and opportunistic pathogens including *Streptococcus agalactiae, Staphylococcus aureus*, *Listeria* spp. and *Enterococcus* spp. associated with serious foodborne pathogens and human infections (Abate *et al*., 2015). This has a negative impact on the health and economic value of the product.

Consumption of raw milk and milk products is associated with risks (Tassew & Seifu, 2011). These risks were demonstrated by the presence of *E. coli, Salmonella* spp. *Listeria* spp. and *S. agalactiae* in raw milk from East and Central Africa (Jans *et al*., 2012; Ortolani *et al*., 2009; Swai & Schoonman, 2011b; Tassew & Seifu,

2011).Therefore, heat-treatments such as pasteurization followed by cold storage is recommended to reduce foodborne pathogens. However, cold storage is often not available in Sub-Saharan Africa rendering heat-treated milk susceptible to recontamination (Hetzel *et al*., 2004).

Small-scale household milk fermentation serves to extend the shelf-life by inhibiting pathogens and spoilage microorganisms through acidification and production antimicrobial compounds, thereby enhancing safety and preservation of food without refrigeration and contributing to healthy nutritional basis by the action of Lactic acid bacteria (Djadouni *et al*., 2012; Widyastuti *et al*., 2014; Panesar, 2011). The acids lower the pH of milk and can thus prevent or reduce the growth of some pathogens and their ability to produce toxins (Fraga *et al*., 2013; Griffiths & Tellez, 2013). Traditional fermentation process involves slow unpredictable lactic acid fermentation by inherent milk microorganisms (Yam *et al*., 2014). Therefore, by improving these processes and enhancing the traditional fermentation processes the milk products will be safer and gain market value (Panesar, 2011) and further reduce potential health risks to consumers (Šušković *et al*., 2010).

Indigenous sourced LAB starter cultures can be used to improve the quality and safety of traditional products without changing local community preferences. Fermented dairy products (FDP) are interestingly predominated by *Streptococcus infantarius* subsp. *infantarius,* a member of *Streptococcus bovis/Streptococcus equinus complex*, present at high titers of 10^8 viable cells per mL in FDP (Abdelgadir *et al*., 2008; Jans *et al*., 2013; Jans *et al*., 2015). African dairy *Sii* was reported to

produce a bacteriocin-like inhibitory substance (BLIS) against other fermentative bacteria and food-borne pathogens e.g. *Listeria* spp. potentially enhancing food safety (Jans *et al*., 2012). The high prevalence of African dairy *Sii* in traditionally fermented dairy products, suggests pivotal role it plays in traditional African fermentations (Jans *et al*., 2013). Functional and genomic analysis also indicates an adaptation to the dairy environment similar to that of *Streptococcus thermophilus* (Jans *et al*., 2013). The extent, range and mechanism of antimicrobial activity including activity against species other than *Listeria* need to be elucidated. The current study provides the basis of the application potential of *Sii* as an Africa-specific starter culture to reduce foodborne pathogens and produce traditional FDP of high quality and safety.

4.2 Materials and methods

4.2.1 Bacterial strains resuscitation and preparation of *Sii* cell-free supernatants

4.2.1.1 Bacterial strains and growth conditions

Two strains of *Sii* – CJ18 and CCUG 43820T were used in inhibition assays against four pathogens; *S. aureus* ATCC 25923, *E. coli* ATCC 25922, *S. typhimurium* ATCC 722569 and *L. monocytogenes* ATCC 7644 independently. All isolates were stored frozen at -80 ^{o}C in 30% (v/v) glycerol and were activated for growth by culturing them in Brain Heart Infusion (BHI) agar (Oxoid) and aerobically incubated at 37^{o}C for 24 h before use.

4.2.2 Preparation of filtrate for antimicrobial activity

Sii CJ18 and *Sii* CCUG 43820T were sub-cultured in MRS broth at 37^{o}c for 48h. The culture broth was centrifuged at 10,000 rpm for 30mins. To confirm the antimicrobial activity, the cell free supernatants were collected for antimicrobial activity against selected pathogens

4.3 Antimicrobial activity of *Sii* against selected human pathogens using disc diffusion assay

Disc diffusion assay was used to conduct antimicrobial activity of *Sii* strains against the following pathogens; *Staphylococcus aureus*, *Escherichia coli, Salmonella typhimurium* and *Listeria monocytogenes* (KIMURA *et al*., 1998). *S. aureus* ATCC 25923, *E. coli* ATCC 25922, *S. typhimurium*ATCC 722569 and *L. monocytogenes* ATCC 7644were grown aerobically on TSA (Oxiod) at 37^{o}C for 24 h and suspended in 0.85% normal sterile water. A lawn of each indicator strain was made by spreading cell suspension over the surface Muller Hinton agar plates (Oxiod) with

sterile cotton swabs and allowed to dry. Sterile discs soaked with overnight *Sii* cultures, CJ18 and CCUG 43820T) for 5 minutes, excess removed carefully were placed on the surface of the plates and allowed to dry for 10 minutes. After incubation at 37^{o}C for 48h, the plates were observed for zone of inhibition around the discs (Fraga *et al*., 2008).

4.4 Antimicrobial activity of *Sii* against selected human pathogens using competition assay

4.4.1 Preparation of *Sii* starter cultures

A first overnight culture of *Sii* (CJ 18 and CCUG 43820T strains) was prepared in M17 broth. This M17 broth was used to inoculate UHT milk at 1% v/v and incubated overnight to provide inoculum for fermentation.

4.4.2 Inhibition pattern of *Sii* against foodborne pathogens by enumeration

Inhibition patterns of African *Sii* strains isolates (*Sii* CJ18 and *Sii* CCUG 43820T) against *S. aureus* ATCC 25923, *E. coli* ATCC 25922, *S. typhimurium* ATCC 722569 and *L. monocytogenes* ATCC 7644 were conducted by competition assay using local raw and pasteurized milk. Raw/pasteurized cow milk was divided into equal aliquots of 100 mL, spiked with known CFU/mL of each pathogenic bacterium then inoculated with 15% v/v separate starter cultures of *Sii* CJ 18 and *Sii* CCUG 43820T and incubated at 37^{o}C. Serial dilution of milk + culture *Sii* + pathogen (Know CFU) were prepared using sterile dilution solution of 0.85% saline water. Appropriate double series dilutions were prepared and plated in duplicate onto appropriate selective or semi-selective growth medium after every 3 h for enumeration of the survival of specific microorganisms with time during the fermentation process. Enumeration of *E. coli* and *S. typhimurium* was done by pour plating onto Violet Red

Bile Agar (Merck) and plates incubated at 37^{o}C for 24 hours. *S. aureus*was enumerated by surface plating 0.1 mL onto Baird Parker Agar media (Oxoid) supplemented with 20% egg yolk and Potassium Tellurite (Oxoid) and plates incubated at 37 ^{o}C for 24 h. *L. monocytogenes* was surface plated in *Listeria* semi-selective agar (Oxiod) with X 010 and 072 supplements.

4.5 RESULTS

4.6 Disc diffusion assay inhibition technique of the 4 selected pathogens by *Sii* CJ18 and *Sii* CCUG 43820T

Inhibition assay of two strains, *Sii* CJ18 and *Sii* CCUG 43820T reference strain was performed against *S. aureus* ATCC 25923, *E. coli* ATCC 25922, *S. typhimurium* ATCC 722569 and *L. monocytogenes* ATCC 7644. *S. aureus* ATCC 25923 and *L. monocytogenes* ATCC 7644 were inhibited. *E. coli* ATCC 25922 and *S. typhimurium* ATCC 722569 were not inhibited (Table 1).

Table 5: Inhibitory effect of *Sii* strains against selected indicator organisms

	Diameter of Halo region in millimeters	
Indicator strains	CJ 18	CCUG 43820T
Staphylococcus aureus ATCC 25923	6.33 ± 0.577 mm	7.67 ± 1.527 mm
Listeria monocytogenes ATCC 7644	5.33 ± 0.577 mm	7.33 ± 1.155 mm
Salmonella typhimurium ATCC 7222569	-ve	-ve
Escherichia coli ATCC 25922	-ve	-ve

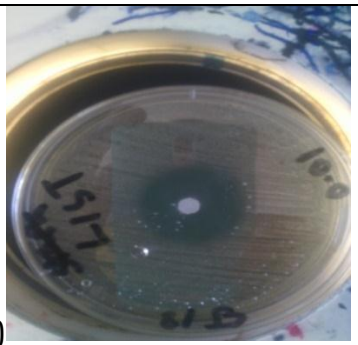
i)  ii) 

Figure 2: Inhibition halos of the growth of i)*S. aureus* by *Sii* CCUG 43820T and ii) *L. monocytogenes* by *Sii* CJ 18 in Muller Hinton agar during the quantification of by the disc diffusion method.

4.6.1 Competitive Assays against *S. typhimurium* in raw and pasteurized cow milk

4.6.1.1 Growth of S. typhimurium in raw milk inoculated with Sii CCUG 43820T and Sii CJ 18 starter culture

In competition assay, two biological replications were used to determine the survival rate of spiked pathogens during fermentation. Growth of *S. typhimurium* increased from 4$\log_{10}$ CFU/mL to 7$\log_{10}$ CFU/mL within 9 h in control samples in both *Sii* strains. The growth in raw milk inoculated with *Sii* CCUG 43820Tincreased within 3 h as follows; 5.0 to 5.5 $\log_{10}$ CFU/mL, 4.0 to 5.8 $\log_{10}$ CFU/mL, 3.0 to 4.3 $\log_{10}$ CFU/mL, and 2.0 to 2.6 $\log_{10}$ CFU/mL in fermenters A to D respectively. The growth decreased after 3h as follows;-5.5 to 5.0 $\log_{10}$ CFU/mL, 5.8 to 3.8 $\log_{10}$ CFU/mL, 4.3 to 3.8 $\log_{10}$ CFU/mL, and 2.6 to 2.0 $\log_{10}$ CFU/mL in fermenters A to D respectively within 9 h (Figure 3:i).

The growth rate of *S. typhimurium*in raw milk inoculated with *Sii* CJ 18 increased from 5.0 to 5.3 $\log_{10}$ CFU/mL, 4.0 to 5.2 $\log_{10}$ CFU/mL, 3.0 to 4.5 $\log_{10}$ CFU/mL, and 2.0 to 3.4 $\log_{10}$ CFU/mL in fermenters A to D respectively within 3 h. The growth decreased after 3 h as follows;-5.3 to 4.6 $\log_{10}$ CFU/mL, 5.2 to 4.6 $\log_{10}$ CFU/mL, 4.5 to 4.3 $\log_{10}$ CFU/mL, and 3.4 to 2.5 $\log_{10}$ CFU/mL in fermenters A to D respectively within 9 h (Figure 3:ii).

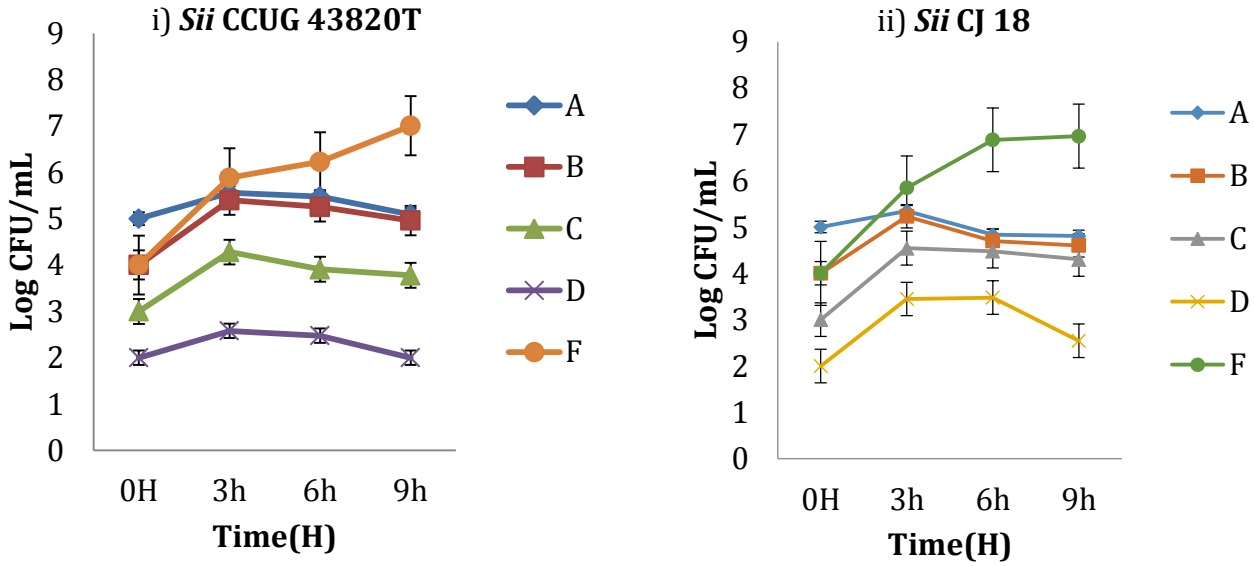


Figure 3: i) The growth of *Salmonella* introduced at a predetermined concentration of log 5-2 $\log_{10}$ CFU/mL (A-D) in raw milk inoculated with *Sii* CCUG 43820T, ii) with *Sii* CJ18 starter cultures. F, are fermenters without *Sii* starters

Legend: A-*Sii* co-culture +Raw milk + *Salmonella* (5 $\log_{10}$ CFU/mL); B- *Sii* co-culture + *Salmonella* (4 $\log_{10}$ CFU/mL), C- *Sii* co-culture + *Salmonella* (3 $\log_{10}$ CFU/mL), D- *Sii* co-culture + *Salmonella* (2 $\log_{10}$ CFU/mL), and F- *Salmonella* (4 $\log_{10}$ CFU/mL) (Control)

4.6.1.2 Growth of S. typhimurium in pasteurized milk inoculated with Sii CCUG 43820T and Sii CJ18 starter culture

Growth of *S. typhimurium* increased from 4log $_{10}$ CFU/mL to 5.8 and 5.9 $\log_{10}$ CFU/mL in *Sii* CCUG 43820T and *Sii* CJ 18 control samples respectively in pasteurized milk within 9 h. The growth of *S. typhimurium* in pasteurized milk inoculated with *Sii* CCUG 43820T increased within 3 h of fermentation as follows;5.0 to 5.4 $\log_{10}$ CFU/mL, 4.0 to 4.1 $\log_{10}$ CFU/mL, 3.0 to 3.8 $\log_{10}$ CFU/mL, and 2.0 to 3.7 $\log_{10}$ CFU/mL in fermenters A to D respectively. The growth decreased after 3 h as follows;-5.4 to 4.0 $\log_{10}$ CFU/mL, 4.1 to 3.9 $\log_{10}$ CFU/mL, 3.8 to 3.5 $\log_{10}$ CFU/mL, and 3.7 to 3.0 $\log_{10}$ CFU/mL in fermenters A to D respectively within 9 h of fermentation (Figure 4:i).

Growth in pasteurized milk inoculated with *Sii* CJ 18 increased as follows;- 5.0 to 5.3 $\log_{10}$ CFU/mL, 4.0 to 5.1 $\log_{10}$ CFU/mL, 3.0 to 3.6 $\log_{10}$ CFU/mL, and 2.0 to 3.0 $\log_{10}$ CFU/mL in fermenters A to D respectively within 3 h. The growth decreased after 3 h as follows;-5.3 to 4.5 $\log_{10}$ CFU/mL, 5.1 to 4.3 $\log_{10}$ CFU/mL, 3.6 to 3.4 $\log_{10}$ CFU/mL, and 3.0 to 2.9 $\log_{10}$ CFU/mL in fermenters A to D respectively within 9 h of storage (Figure 4:ii).

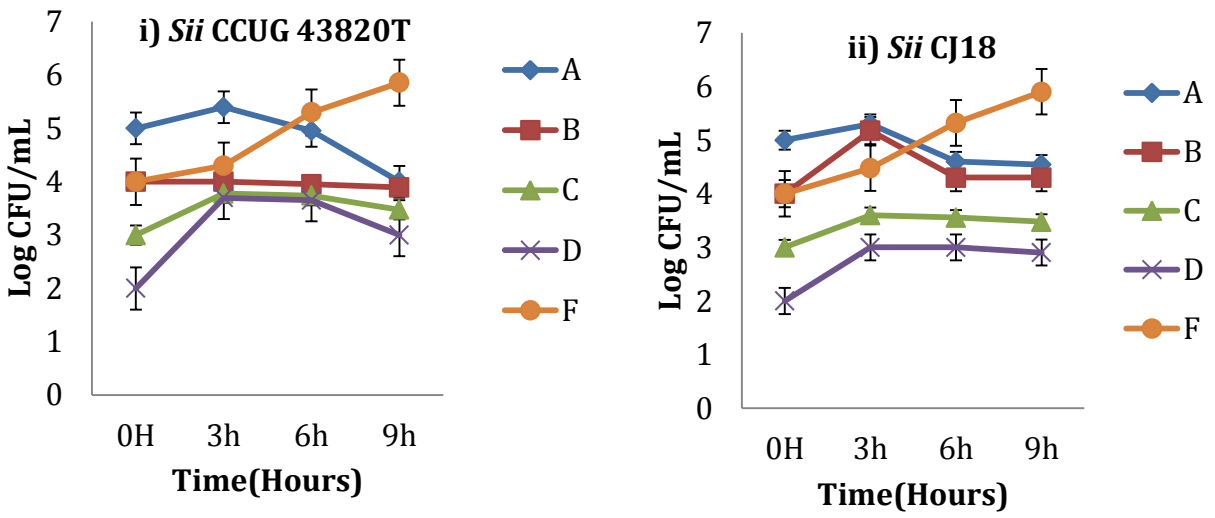


Figure 4:i) The growth of *Salmonella* introduced at a pre-determined concentration of 5-2 $\log_{10}$ CFU/mL (A-D) in pasteurized milk inoculated with *Sii* CCUG 43820T, ii) with CJ18 starter cultures.

Legend: A-*Sii* co-culture +Pasteurized milk + *Salmonella* (5 $\log_{10}$CFU/mL), B- *Sii* co-culture +Pasteurized milk + *Salmonella* (4 $\log_{10}$ CFU/mL) +, C- *Sii* co-culture +Pasteurized milk + *Salmonella* (3 $\log_{10}$CFU/mL), D- *Sii* co-culture +Pasteurized milk + *Salmonella* (2 $\log_{10}$ CFU/mL), and F- *Salmonella* (4 $\log_{10}$ CFU/mL) (Control)

4.6.2 Competitive assays against *E. coli* in raw and pasteurized cow milk

4.6.2.1 Growth of E. coli in raw milk inoculated with Sii CCUG 43820T and CJ 18 starter cultures

The growth of *E. coli* in raw milk increased from 4log $_{10}$ CFU/mL to 6and 6.2 $\log_{10}$ CFU/mL within 9 h in control samples inoculated with *Sii* CCUG 43820T and CJ 18 respectively within 9 h. The growth of *E. coli* in raw milk inoculated with *Sii* CCUG 43820Tincreased within 3 h as follows;-5.0 to 5.4 $\log_{10}$ CFU/mL, 4.0 to 4.8 $\log_{10}$ CFU/mL, 3.0 to 3.5 $\log_{10}$ CFU/mL, and 2.0 to 3.2 $\log_{10}$ CFU/mL in fermenters A to D respectively. The growth decreased after 3 h as follows;-5.4 to 4.6 $\log_{10}$ CFU/mL, 4.8 to 4.3 $\log_{10}$ CFU/mL, 3.5 to 2.9 $\log_{10}$ CFU/mL, and 3.2 to 2.9 $\log_{10}$ CFU/mL in fermenters A to D respectively within 9 h (Figure 5:i).

In fermenters inoculated with CJ 18, growth increased from 5.0 to 5.0 $\log_{10}$ CFU/mL, 4.0 to 4.8 $\log_{10}$ CFU/mL, 3.0 to 4.3 $\log_{10}$ CFU/mL, and 2.0 to 3.0 $\log_{10}$ CFU/mL in fermenters A to D respectively within 3 h. The growth decreased after 3 h as follows;-5.0 to 4.0 $\log_{10}$ CFU/mL, 4.8 to 3.9 $\log_{10}$ CFU/mL, 4.3 to 3.8 $\log_{10}$ CFU/mL, and 3.0 to 3.5 $\log_{10}$ CFU/mL in fermenters A to D respectively within 9 h (Figure 5:ii).

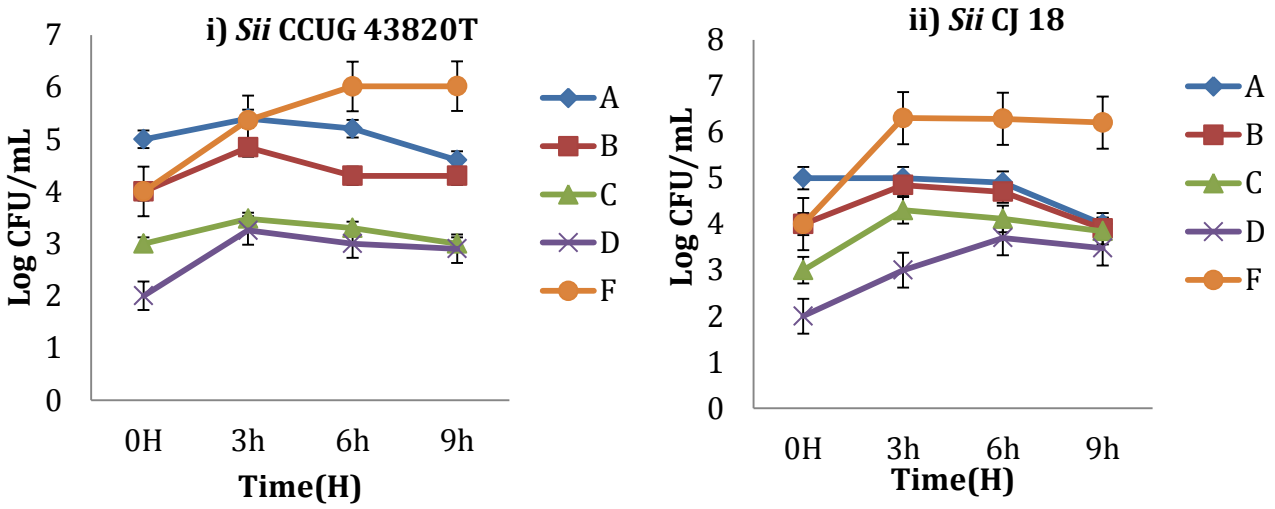


Figure 5: i) The growth of *E. coli* introduced at pre-determined concentration of 5-2 $\log_{10}$ CFU/mL (A-D) in raw milk inoculated with *Sii* CCUG 43820T: ii) *Sii*CJ18 starter culture. F, fermenters without *Sii* starter cultures

Legend: A-*Sii* co-culture +Raw milk + *E. coli* (5 $\log_{10}$ CFU/mL), B- *Sii* co-culture +Raw milk + *E. coli* (4 $\log_{10}$ CFU/mL), C- *Sii* co-culture +Raw milk + *E. coli* (3 $\log_{10}$ CFU/mL), D- *Sii* co-culture +Raw milk + *E. coli* (2 $\log_{10}$ CFU/mL), and F- *E. coli* (4 $\log_{10}$CFU/mL) (Control)

4.6.2.2 The growth of E. coli in pasteurized inoculated with Sii CCUG 43820T and Sii CJ18starter cultures

The growth of *E. coli* in pasteurized increased within 3 h of fermentation followed by either leveling off or decreasing after 3 h of fermentation inoculated with *Sii* CCUG 43820T and *Sii* CJ 18. The growth within 3 h of fermentation was as follows; 5.0 to 5.0 $\log_{10}$ CFU/mL, 4.0 to 5.0 $\log_{10}$ CFU/mL, 3.0 to 4.4 $\log_{10}$ CFU/mL, and 2.0 to 2.3 $\log_{10}$ CFU/m in fermenters A to D respectively. The growth decreased after 3 h as follows;-5.0 to 4.7 $\log_{10}$ CFU/mL, 5.0 to 4.6 $\log_{10}$ CFU/mL, 4.4 to 4.3 $\log_{10}$ CFU/mL, and 2.3 to 2.0 $\log_{10}$ CFU/mL in fermenters A to D respectively within 9 h (Figure 6:i). In fermenters inoculated with *Sii* CJ 18, growth within 3 h was as follows; 5.0 to 5.0 $\log_{10}$ CFU/mL, 4.0 to 5.0 $\log_{10}$ CFU/mL, 3.0 to 3.7 $\log_{10}$ CFU/mL, and 2.0 to 3.3 $\log_{10}$ CFU/mL in fermenters A to D respectively within 3 h. The growth decreased after 3 h as follows;-5.0 to 4.8 $\log_{10}$ CFU/mL, 5.0 to 4.7 $\log_{10}$ CFU/mL, 3.7 to 3.0 $\log_{10}$ CFU/mL, and 3.3 to 2.8 $\log_{10}$ CFU/mL in fermenters A to D respectively within 9 h (Figure 6:ii).

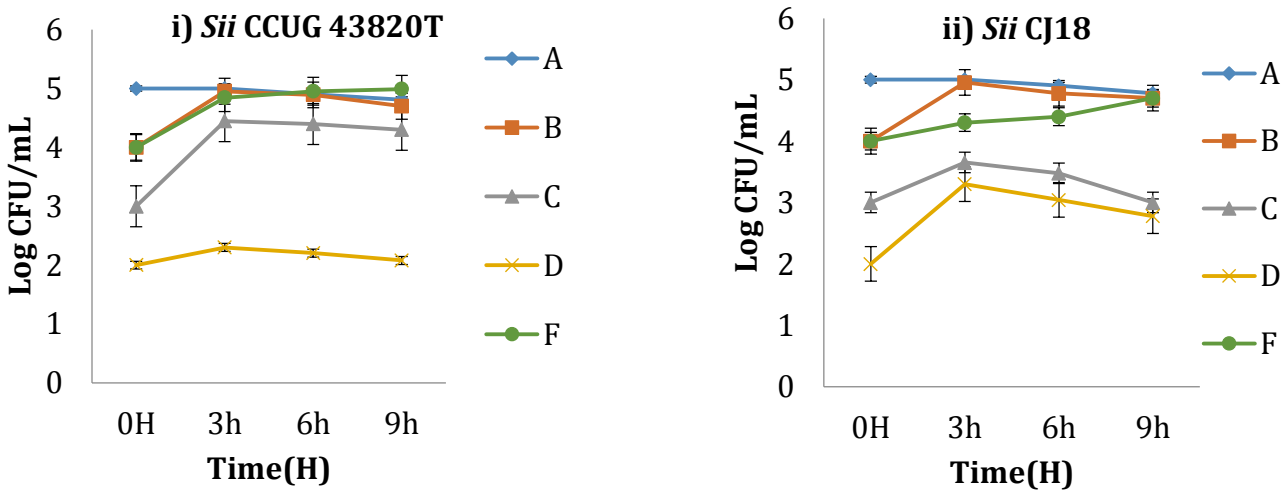


Figure 6: i)The growth of *E. coli* in pasteurized introduced at pre-determined concentration 5-2 $\log_{10}$ (A-D) CFU/mL inoculated with *Sii* CCUG 43820T: ii) with *Sii* CJ18 starter cultures. F, fermenter without *Sii* starter culture

Legend: A-*Sii* co-culture +Pasteurized milk + *E. coli* (5 $\log_{10}$ CFU/mL), B- *Sii* co-culture +Pasteurized milk + *E. coli* (4 $\log_{10}$ CFU/mL), C- *Sii* co-culture +Pasteurized milk + *E. coli* (3 $\log_{10}$ CFU/mL), D- *Sii* co-culture +Pasteurized milk + *E. coli* (2 $\log_{10}$ CFU/mL), and F- *E. coli* (4 $\log_{10}$ CFU/mL) (Control)

4.6.3 Competitive assays of *Sii* against *S. aureus* in raw and pasteurized milk

4.6.3.1Growth of S. aureus in raw milk inoculated with Sii CCUG 43820T and Sii CJ18starter culture.

The growth within 3 h of fermentation increased as follows;5.0 to 5.9 $\log_{10}$ CFU/mL, 4.0 to 4.9 $\log_{10}$ CFU/mL, 3.0 to 4.8 $\log_{10}$ CFU/mL, and 2.0 to 4.6 $\log_{10}$ CFU/mL in fermenters A to D respectively. The growth decreased after 3 h as follows;-5.9 to 3.8 $\log_{10}$ CFU/mL, 4.9 to 2.9 $\log_{10}$ CFU/mL, 4.8 to 2.0 $\log_{10}$ CFU/mL, and 4.6 to 4.0 $\log_{10}$ CFU/mL in fermenters A to D respectively within 9 h (Figure 7:i).

In fermenters inoculated with CJ 18, growth within 3 h increased as follows; 5.0 to 5.9 $\log_{10}$ CFU/mL, 4.0 to 5.8 $\log_{10}$ CFU/mL, 3.0 to 4.9 $\log_{10}$ CFU/mL, and 2.0 to 4.6 $\log_{10}$ CFU/mL in fermenters A to D respectively within 3 h. The growth decreased after 3 h as follows;-5.9 to 4.8 $\log_{10}$ CFU/mL, 5.8 to 4.1 $\log_{10}$ CFU/mL, 4.9 to 3.5 $\log_{10}$ CFU/mL, and 4.6 to 3.0 $\log_{10}$ CFU/mL in fermenters A to D respectively within 9 h (Figure 7:ii).

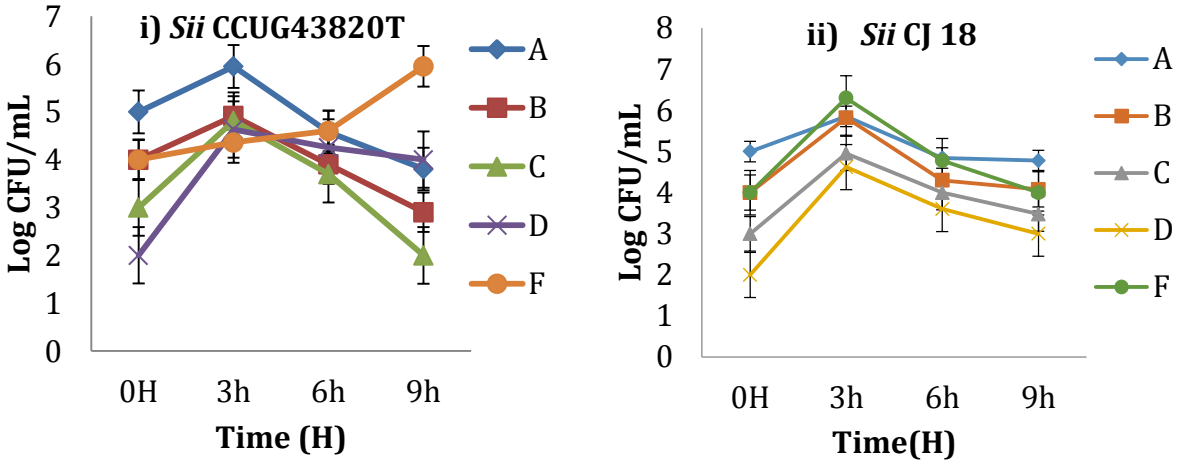


Figure 7: i)The growth of *S. aureus* in raw milk introduced at a pre-determined concentration of 5-2 $\log_{10}$ CFU/mL (A-D) inoculated with *Sii* CCUG 43820T: ii)*Sii*CJ18 starter cultures. F, fermenters without *Sii* starter cultures

Legend: A-*Sii* co-culture +Raw milk + *S. aureus* (5 $\log_{10}$ CFU/mL), B- *Sii* co-culture +Raw milk + *S. aureus* (4 $\log_{10}$ CFU/mL), C- *Sii* co-culture +Raw milk + *S. aureus* (3 $\log_{10}$ CFU/mL), D- *Sii* co-culture +Raw milk + *S. aureus* (2 $\log_{10}$CFU/mL), and F- *S. aureus* (4 $\log_{10}$ CFU/mL)(Control)

4.6.4 Competitive assays against *L. monocytogenes* in pasteurized cow milk

4.6.4.1Growth of L. monocytogenes in pasteurized milk inoculated with Sii CCUG 43820T and Sii CJ18starter cultures

The growth of *L. monocytogenes* in pasteurized milk increased from 4 $\log_{10}$ CFU/mL to 4.1 $\log_{10}$ CFU/mL within 9 h in control samples in *Sii* CCUG 43820T within 9 h. The growth of *L. monocytogenes* in pasteurized decreased as follows;-5.0 to 3.6 $\log_{10}$ CFU/mL, 4.0 to 3.3 $\log_{10}$ CFU/mL, 3.0 to 2.0 $\log_{10}$ CFU/mL, and 2.0 to 1.0 $\log_{10}$ CFU/mL in fermenters A to D respectively within 9 h (Figure 8:i).

In fermenters inoculated with CJ 18,the growth decreased follows;-5.0 to 3.5 $\log_{10}$ CFU/mL, 4.0 to 3.2 $\log_{10}$ CFU/mL, 3.0 to 2.3 $\log_{10}$ CFU/mL, and 2.0 to 1.9 $\log_{10}$ CFU/mL in fermenters A to D respectively within 9 h (Figure 8:ii).

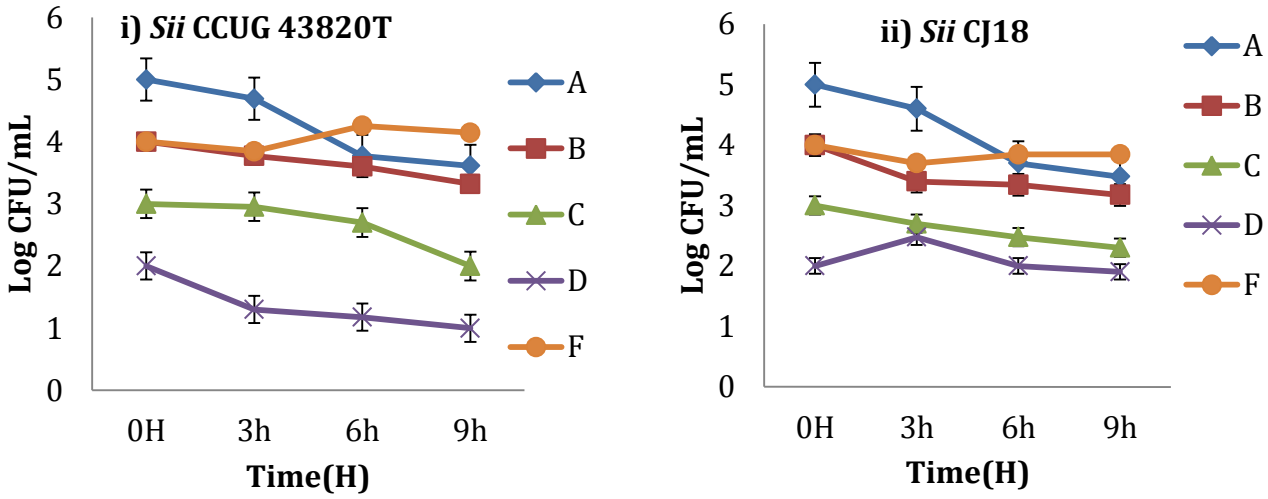


Figure 8: i) growth of *L. monocytogenes* in pasteurized milk introduced at a pre-determined concentration of 5-2 $\log_{10}$ (A-D) CFU/mL inoculated with *Sii* CCUG 43820T, ii) *Sii*CJ18 starter cultures. F, are the fermenters without *Sii* starter cultures

Legend: A-*Sii* co-culture + Pasteurized milk + *L. monocytogenes* (5 $\log_{10}$ CFU/mL), B-*Sii* co-culture +Pasteurized milk + *L. monocytogenes* (4 $\log_{10}$ CFU/mL) + *Sii*, C- *Sii* co-culture +Pasteurized milk + *L. monocytogenes* (3 $\log_{10}$ CFU/mL), D- *Sii* co-culture +Pasteurized milk + *L. monocytogenes* (2 $\log_{10}$ CFU/mL), and F- + *L. monocytogenes* (4 $\log_{10}$ CFU/mL)(Control)

4.6.4.2 Growth of L. monocytogenes in raw milk Sii CCUG 43820T and Sii CJ 18 starter culture

The growth of *L. monocytogenes* increased in raw milk inoculated with *Sii* CCUG 43820T and *Sii* CJ 18 within 3 h of storage followed by decrease within 9 h of storage. The growth increased from 4log $_{10}$ CFU/mL to 5.5 and 7.5 $\log_{10}$ CFU/mL within 9 h in control samples in both *Sii* CCUG 43820T and *Sii* CJ 18 respectively. The growth in raw milk inoculated with *Sii* CCUG 43820Tincreased within 3 h as follows;5.0 to 5.9 $\log_{10}$ CFU/mL, 4.0 to 4.6 $\log_{10}$ CFU/mL, 3.0 to 3.7 $\log_{10}$ CFU/mL, and 2.0 to 2.6 $\log_{10}$ CFU/mL in fermenters A to D respectively. The growth decreased after 3 h as follows;-5.9 to 4.1 $\log_{10}$ CFU/mL, 4.6 to 4.0 $\log_{10}$ CFU/mL, 3.7 to 2.5 $\log_{10}$ CFU/mL, and 2.6 to 1.7 $\log_{10}$ CFU/mL in fermenters A to D respectively within 9 h (Figure 9:i).

In fermenters inoculated with CJ 18, growth increased from 5.0 to 5.6 $\log_{10}$ CFU/mL, 4.0 to 5.6 $\log_{10}$ CFU/mL, 3.0 to 5.3 $\log_{10}$ CFU/mL, and 2.0 to 2.5 $\log_{10}$ CFU/mL in fermenters A to D respectively within the first 3 h. The growth decreased after 3 h as follows;-5.6 to 4.3 $\log_{10}$ CFU/mL, 5.6 to 4.0 $\log_{10}$ CFU/mL, 5.3 to 3.6 $\log_{10}$ CFU/mL, and 2.5 to 2.3 $\log_{10}$ CFU/mL in fermenters A to D respectively within 9 h of storage (Figure 9:ii).

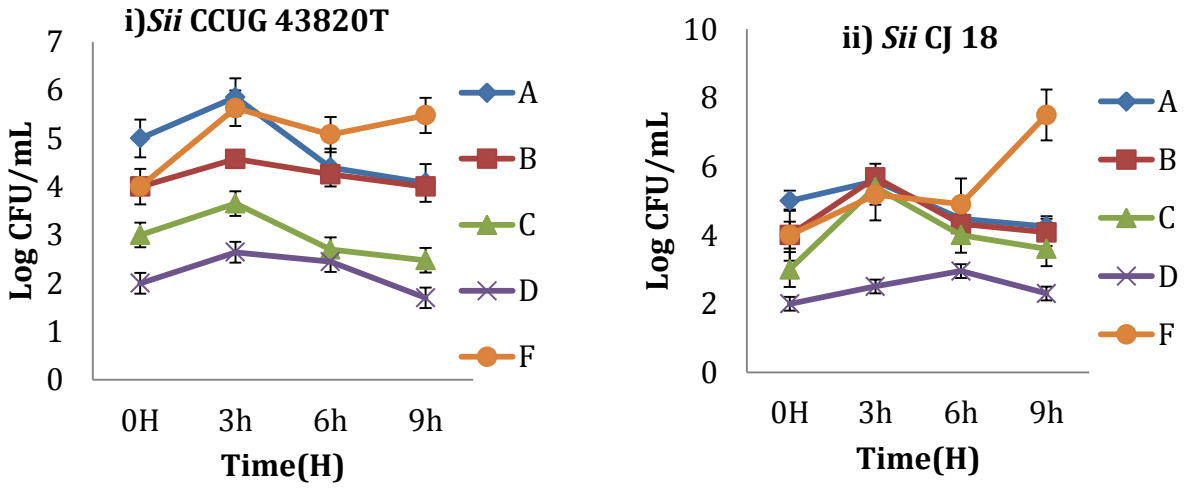


Figure 9: i) growth of *L. monocytogenes* in raw milk introduced at a pre-determined concentration of 5-2 $\log_{10}$ (A-D) CFU/mL inoculated with *Sii* CCUG 43820T, ii) *Sii* CJ18 starter culture. F-Fermenters without *Sii* starter culture.

Legend: A-*Sii* co-culture +Raw milk + *L. monocytogenes* (5 $\log_{10}$ CFU/mL) + *Sii*, B- *Sii* co-culture +Raw milk + *L.* monocytogenes (4 $\log_{10}$ CFU/mL) + *Sii*, C- *Sii* co-culture + Raw milk + *L. monocytogenes* (3 $\log_{10}$ CFU/mL), D- *Sii* co-culture + Rawmilk + *L. monocytogenes* (2 $\log_{10}$ CFU/mL), and F- *L. monocytogenes* (4 $\log_{10}$ CFU/mL) (Control)

4.6.5 Discussion

Fermented dairy products have a long tradition in African as a source of income to small-scale households, general nutrition, and preservation where refrigeration is not available (Djadouni & Kihal, 2012; Motarjemi *et al*., 1993; Rhee *et al*., 2011). In contrast to heat-treated products, fermentation results to products that are less susceptible to recontamination because of the general low pH and other active antimicrobial compounds (Šušković *et al*., 2010; Widyastuti, *et al*., 2014). In the current study, the application potential of *Streptococcus infantarius* subsp. *infantarius* as Africa-specific starter culture to reduce foodborne pathogens and produce traditional FDP of high quality and safety was demonstrated by their high antimicrobial activity against *Staphylococcus aureus* and *Listeria monocytogenes* in disc diffusion as indicated by clear halos around discs. The same pathogens were inhibited by other groups of LAB isolated from Brazilian raw milk and soft cheeses (Ortolani *et al*., 2009). The potential to inhibit food pathogens of *Sii* had been demonstrated by high antibacterial activity through a bacteriocin-like substance against *Listeria ivanovii* (Fraga *et al*., 2013; Jans *et al*., 2012).

The rate of growth of all pathogens, in competition assay reduced with fermentation time in fermenters seeded with *Sii* strains while in control there was a general increase in number throughout the fermentation period. This is attributed to little or no control over spontaneously initiated fermentation, leading to low acidification, reduced inhibition capabilities in control (Wullschleger *et al*., 2013). Inactivation kinetics of the *Sii* bioactive metabolic products varied from one pathogen to another. *S. aureus* and *L. monocytogenes* inhibited more than *Salmonella* and *E. coli.* The

reduction of total viable counts of *L. monocytogenes* and *S. aureus* with time during fermentation process has been previously reported (Ross *et al*., 2002; Savadogo *et al*., 2006). Pathogenic *E. coli* survive under low pH for a longer period compared other common food-borne pathogens (Gadaga *et al*., 2004). During early stages of fermentation, the growth of *Escherichia coli* O157:H7 is high compared to other pathogens when the levels of lactic acid are still low and adapts to acidic conditions hence survives under low acid levels (Tamime, 2008). The survival of *E. coli* in Zimbabwean naturally fermented milk has been reported. The antagonistic effect of *Sii* strains against selected pathogens was due to its acidification process the substrate, being highly competitive adapted hence had a competitive advantage, and production antimicrobial other antimicrobial compounds during the fermentation process (Heller, 2001; Tamime, 2008; Hernández *et al*, 2005; Holzapfel, 2002). The acid exerts antimicrobial activity by interfering with the maintenance of cell membrane, reducing intracellular pH, inhibiting active transport, and inhibiting metabolic functions (Griffiths & Tellez, 2013; Ross *et al*., 2005). The acids produced lowered the pH of milk and thus reduced the growth pathogens and their ability to produce toxins (Gemechu *et al*., 2015; Griffiths & Tellez, 2013). The rate of growth of the pathogens was higher in raw milk than pasteurized milk. This can be attributed to the fact that in pasteurized milk the acid development and pH drop is faster than raw milk. There is a controlled fermentation in pasteurized milk than raw milk since inherent flora is destroyed during pasteurization hence *Sii* strains dominate the fermentation process resulting in faster pH drop.

4.7 Conclusion

In conclusion, *Sii* strains can to be used as novel starter cultures to inhibit the growth of pathogens and spoilage microorganism in fermented milk products upon elucidation of their safety.

4.8 Recommendations

Further *in vitro* and *in vivo* analyses are required, such as resistance to antibiotics, acid tolerance, viability, safety, and organoleptic properties

CHAPTER 5

5 General Discussion, Conclusion and Recommendation

5.1 General Discussion

Milk fermentation serves to extend the shelf-life by inhibiting pathogens and spoilage microorganisms through acidification and production antimicrobial compounds, thereby enhancing safety and preservation of food without refrigeration and contributing to healthy nutritional basis by the action of Lactic acid bacteria (Djadouni *et al*., 2012; Widyastuti *et al*., 2014; Panesar, 2011). The acids lower the pH of milk and can thus prevent or reduce the growth of some pathogens and their ability to produce toxins (Fraga *et al*., 2013; Griffiths & Tellez, 2013).

Optimum fermentation conditions of African dairy *Sii* strains CJ 18 and CCUG 43820T for fast decrease in pH and low survival rate of pathogens were determined in the current study. The application of *Sii* for traditional fermentations as highly competitive adapted traditional starter cultures had been suggested by Jans *et al*., (2013). The strains resulted rapid acidification of the cow milk through the production of organic acids, mainly lactic acid, lowering the pH and development of characteristic flavors. The rate of fermentation was slow in control samples. There are reports on slow unpredictable lactic acid fermentation by inherent milk microorganisms (Yam *et al*., 2014).

The two strains of *Sii* exhibited antagonistic effect against tested indicator organisms. This is due to drop in pH as result of the production of acids from the fermentation of lactose. The acids lower the pH of milk and can thus prevent or

reduce growth pathogens and their ability to produce toxins (Gemechu, 2015; Griffiths and Tellez, 2013). Its potential to contribute to safety and reduce human risks was demonstrated by inhibition of growth of pathogenic bacteria. This was attributed to its ability to produce bacteriocin-like inhibitory substance (BLIS) against other fermentative bacteria and food-borne pathogens e.g. *Listeria* spp. (Jans *et al*., 2012).

The level of inhibition varied from one pathogen to another, type of strain and sample treatment. The same factors were reported by Griffiths and Tellez, (2013). The growth of the pathogenic indicator bacteria increased within three hours of fermentation. These has been previous reported (Tamime, 2008). He attributed it to high pH of milk in the initial fermentation. The decline in growth with time is due to accumulation of organic acids and drop in pH and competition for nutrients and production of other antimicrobial metabolites (Šušković *et al*., 2010). The least inhibited pathogens were *S. typhimurium* and *E. coli* whereas *L. monocytogenes* and *S. aureus* were the most inhibited. This trend of inhibition *L. monocytogenes* and *S. aureus* with time during fermentation process has been previously reported (Ross *et al*., 2002; Savadogo *et al*., 2006).

5.2 General Conclusion

The aim of the study was to determine the optimum fermentation conditions that will results to fast decrease in pH and low survival rate of the pathogens. The rate of acidification and drop in pH was high at thermophilic range of temperature. The growth of test pathogenic bacteria decreased with time in samples seeded with *Sii* strains. Therefore, upon safety assessment *Sii* CJ18 and *Sii* CCUG 43820T can be

applied as Africa-specific starter cultures that will result to faster decrease in pH and inhibition of well-known pathogens. This will ensure safety and preservation of fermented dairy products through acidification and production antimicrobial compounds without refrigeration and will contribute to a healthy nutritional basis.

5.3 Recommendations

Characterization of the antimicrobial activity of *Sii* strains is recommended to enable them to be used for the improvement of safety of food.

Further *in vitro* and *in vivo* studies are required, such as resistance to antibiotics, acid tolerance, viability, safety, and organoleptic properties.

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