Isolation, Molecular Detection and Antibiotic Activity of *Streptococcus thermophilus* isolates from Locally Fermented Milk Collected from Liben Chukala District, Oromia, Ethiopia

By: Girma Reta Taffa

A Thesis Submitted to the Program of Applied Biology Presented in Partial Fulfillment of Requirements of the Degree of Master's of Science in Applied Biology (Biotechnology)

School of Applied Natural Science

Office of Graduate Studies

Adama Science and Technology University

Adama, Ethiopia

January 2019

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Candidate's Declaration

I hereby declare that this MSc Thesis entitled **"Isolation, Molecular Detection and Antibiotic Activity of *Streptococcus thermophilus* isolates from Locally Fermented Milk Collected from Liben Chukala District, Oromia, Ethiopia"** is my original work and has not been presented for a degree in any other university, and all sources of material used for this thesis have been acknowledged.

_____	_____	_____
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Advisor's Approval Sheet

To: Applied Biology department

Subject: Thesis Submission

This is to certify that the thesis entitled **"Isolation, Molecular Detection and Antibiotic Activity of *Streptococcus thermophilus* isolates from Locally Fermented Milk Collected from Liben Chukala District, Oromia, Ethiopia"** submitted in partial fulfillment of the requirements for the degree of Master's in Applied Biology (Biotechnology), Graduate program of the department of Applied Biology and has been carried out by **Girma Reta Tafa** Id. No: **GSR/0188/09**, under my supervision. Therefore, I recommend that the student has fulfilled the requirements and hence hereby he can submit the thesis to the department.

_____	_____	_____
Name of Advisor	Signature	Date

Approval of Board of Examiners

We, the undersigned members of the Board of Examiners of the final open defense by **Girma Reta Tafa** have read and evaluated his thesis entitled **"Isolation, Molecular Detection and Antibiotic Activity of *Streptococcus thermophilus* isolates from Locally Fermented Milk Collected from Liben Chukala District, Oromia, Ethiopia"** and examined the candidate. Therefore, this is to certify that the thesis has been accepted in partial fulfillment of the requirement of the Degree of Master's in Applied Biology (Biotechnology).

Advisor	Signature	Date
Internal Examiner	Signature	Date
External Examiner	Signature	Date
Chairperson	Signature	Date
Department Chairperson	Signature	Date

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AA	Amino Acid
AMV	Annual Market Value
CFU	Colony Forming Unit
CPS	Capsular Polysaccharides
CSA	Central Statistical Agency
CTAB	Cetyl Trimethyl Ammonium Bromide
CU	Cornell University
EPS	Exopolysaccharides
GABA	Gamma Aminobutyric Acid
GRAS	Generally Recognized As Safe
PCR	Polymerase Chain Reaction
LAB	Lactic Acid Bacteria
NVI	National Veterinary Institute
TSA	Triptose Soya Agar
TSI	Triple Sugar Ion
USD	United States Dollar
VP	Vogues Proskauer

ABSTRACT

Streptococcus thermophilus is one of the most economically important Lactic Acid Bacteria (LAB) with annual market value of 40 billion USD, and milk acidification makes it the of major technologically important in industrial fermentation processes. The purpose of this study was to isolate, detect by molecular tool and test it's antibiotic activity of Streptococcus thermophilus from fermented milk samples originated from Liben Chukala District Oromia Regional State, Ethiopia. Isolation of S. thermophilus was carried out on M948 agar and characterized by biochemical tests. In the same way molecular detection of representative isolates of S. thermophilus was carried out by using two sets of primers (PA/pH and ThI/ThII) with their expected PCR product size of 1600 bp and 250 bp, respectively. This study showed that, molecular detection was more efficient than other biochemical tests for separation of S. thermophilus from other LAB. Moreover, an antibiotic activity test results revealed that S. thermophilus had the highest antibacterial activities against pathogenic Staphylococcus aurues than Clostridium difficile and E. coli with its highest zone of inhibition of 9.7±0.20 mm for S. aurues, 9.0±0.12 for Clostridium spp as well as 5.7±0.42mm for E. coli. This result implies that, gram positive S. thermophilus can inhibit other gram positive pathogens than gram negative bacterial species. On the other hand this study revealed that, the antibiotic activity test of S. thermophilus was more efficient in cellular suspension than in chemical extracts. This antibiotic activity against food borne pathogens enabled S. thermophilus to be used as biopreservatives in dairy industry. Accordingly this probiotic microorganism can be used in other industrial area to produce biosurfactants, bacteriocine, biotherapeutic, antibiotic and recombinant vaccine production due to its antibacterial activity.

Keywords: Antibiotic, Biochemical, Ethiopia, Milk, Molecular, *Streptococcus, thermophilus*

1. INTRODUCTION

1.1. Back Ground of the Study

With the annual market value of 40 billion USD*, Streptococcus thermophilus* is one of the most economically important Lactic Acid Bacteria (LAB) (Yahya and Naeima, 2014) and its rates of milk acidification are of major technological importance in industrial fermentation processes (Erkus *et al.,* 2014). An acidification rate of *Streptococcus thermophilus* strains depends on their sugar utilization ability (Morandi and Brasca, 2012). As a dairy starter culture, it is the only *Streptococcus* species used in dairy and other food industry. They are the only generally recognized as safe (GRAS) bacterium from *genus Streptococcus* (Ophelie *et al.,* 2017).

Streptococcus thermophilus is a non-pathogenic and homo-fermentative facultative anaerobic LAB with a long history of use in the home-made and modern industrial manufacture of fermented dairy products, especially yogurt, cheese, pickles and others (Wu *et al.,* 2014). In dairy industry, it can rapidly convert lactose into lactic acid, which causes a rapid reduction in pH resulting in coagulation of milk proteins or casein (Sun *et al.,* 2011).

Beside dairy industry, heteropolymeric exopolysaccharides (EPS) which contained glucose, galactose and rhamnose can be produced from *Streptococcus thermophilus Y102*. Due to the technological roles of exopolysaccharides produced by *S. thermophilus* strains, yogurt production technology has led to an increased use of this ropy starter cultures (Ispirli and Dertli, 2018). The main role of this EPS is to act as *in situ*-produced natural biothickeners that can improve the viscosity, texture and mouth feel of the dairy products, and prevent syneresis in yogurt (Purwandari *et al.,* 2007). Moreover, EPSs from *S. thermophilus* benefit the health of host animals by enhancing the immune responses due to its immunoregulatory effects related to chemical composition (Rodriguez *et al.,* 2010).

Current studies revealed that, *S. thermophilus A* has a potential use for biosurfactant production which has several advantages over chemical surfactants including lower toxicity, higher biodegradability, and effectiveness at extreme temperatures and pH values (Rodrigues *et al.,* 2006a). Biosurfactants are surface-active compounds released by microorganisms that exhibit surface activity and have been described as antimicrobial and anti-adhesive agents. The interest toward these biosurfactants has increased considerably due to their potential candidates for many commercial applications in the petroleum, pharmaceuticals, biomedical and food processing industries (Rodrigues *et al.,* 2006b).

Probiotic LAB mainly, *S. thermophilus* and the *Lactobacillus* spp. strains derived surfactants can antagonize the growth of certain pathogens like *Staphylococcus aureus, Streptococcus spp., Enterococcus faecalis, Candida albicans* (Sharma *et al.,* 2016). A great variety of alternative raw materials is currently available as nutrients for industrial biosurfactant fermentations i.e. various agricultural and industrial by-products as waste materials. A good raw material substrate for biosurfactant production is whey, as it is composed of high levels of lactose, protein, organic acids and vitamins. Whey is a by products from cheese production (Rodrigues *et al.,* 2006a).

On the other hand, *S. thermophilus* drived bacteriocins named as *Thermophilin 110* which are ribosomally synthesised antibacterial peptides that can have a narrow or broad target spectrum against food borne pathogens (Leroy and De Vuyst, 2010). *Thermophilin 110,* an apparently novel bacteriocin, was isolated from *S. thermophilus ST110* with a spectrum of activity which include related strains of *Streptococcus lactis, Pediococcus acidilactici,* and *lactococci.* The kinetics of *Thermophilin 110* production was monitored during the growth of *S. thermophilus ST110* in TYL broth at 37 °C (Gilbreth and Somkuti, 2005). Moreover, the established highly efficient bacteriocin production by *L. bulgaricus BB18* during co-cultivation in milk with the bacteriocin-insensitive technological strain of *S. thermophilus 11A* has 30% higher level of bacteriocin production; high level of colony forming units (CFU) i.e. 10^{12} CFU ml^{-1} for *S. thermophilus* 11A and $8x10^{11}$ CFU ml^{-1} for *L. bulgaricus BB18*) and the reduction of bacteriocinogenesis time by 3h are evidence of the mutual stimulation of the cell growth (Simova *et al.,* 2008).

The biopreservation systems in foods are of increasing interest for industry and consumers because of the GRAS bacteriocinogenic properties of LAB and their extracted bacteriocins are useful to control the frequent development of pathogens and spoiling microorganisms in foods and feed (Parada *et al.,* 2007). In view of the inhibition spectrum of the bacteriocin of the *S. thermophilus CHCC* 3534 strain, and its technological properties (good pH and heat tolerance) of bacteriocin production have a potential application as a biopreservative, and represent a promising candidate as starter culture for probiotic fermented dairy foods (Khali, 2009).

1.2. Statement of Problem

One of the problems of milk processing can be resolved by using industrially important adjunct culture of *S. thermophilus* species of LAB along with its major technological importance in industrial milk fermentation process (Cui *et al.,* 2016; Admassie, 2018). Beside the dairy production, this probiotic microorganism is used for production of Recombinant Probiotics for Allergen Immunotherapy (Petrarca *et al.,* 2015), biosurfactant (Rodrigues *et al.,* 2006a) and bacteriocin production (Fontaine *et al.,* 2007).

In context of our country, the use of probiotics and industrially important *S. thermophlus* in dairy production is limited. Accordingly its application in production of biotherapeutics, biosurfactants, bacteriocins and antibiotics is very few except for its application to yoghurt, and cheese production by traditional means. Even though few researches conducted for the traditional use of LAB (Admassie,2018), there was no investigation performed for isolation, biochemical characterization, molecular detection, and further use of this industrially relevant *S. thermophilus* in Ethiopia yet. Moreover, antibiotic property of *S. thermophilus* to inhibit pathogenic microbes was not well detected.

1.3. Objectives

1.3.1 General Objective

To characterize industrially relevant S. *thermophillus* isolated from fermented milk and study its antibiotic activity against pathogens.

1.3.2. Specific Objective

1. To isolate *S. thermophilus* from fermented milk sample.
2. To study the morphological and biochemical characteristics of *S*, *thermophilus* isolates
3. To detect *S*. *thermophilus* by PCR molecular tool
4. To study the antibiotic activity of *S. thermophilus* against selected food borne pathogenic microorganisms.

1.4. Scope of the Study

This study covered isolation of industrially important *S. thermophilus* and its molecular detection and biochemical characterization from traditionally fermented milk in the study area. The molecular techniques include DNA extraction for some isolates of *S. thermophillus*, and polymerase chain reaction detection. In addition, the antibiotic activity of *S. thermophilus* against food borne pathogenic microorganisms was also studied.

2. LITRATURE REVIEW

2.1. Overview of *S. thermophillus* In Dairy production and Other Industrial Application

With the involvement of living microorganism and their enzymes, industrial biotechnology is being applied for sustainable production and processing of biochemicals, fuels, dietary foods and feeds and bioenergy (Tang and Zhao, 2009). In dairy industry probiotic microbial ecosystem is very complex and responsible for the broad diversity of tastes, aromas, and textures of dairy products. Yoghurt and Cheese processing is largely based on fermentation by LAB which are added as starter cultures or are adventitiously present in the biotope and selected during the fermentation process (Pogacic *et al.,* 2010). *S. thermophilus* is a *thermophilic* LAB of major importance for the dairy industry where as the strains of this species are commonly used in the manufacture of yoghurt and many varieties of cheeses (Rizotti *et al.,* 2009).

The name of *Streptococcus* is derived from the Greek word "*Strepto*" in which means "twisted berry" it is seen under the microscope as a chain that resemble a string of beads (Figure 1). *Thermophilus* is a Greek term which means heat, referring to an organism that is able to survive extreme cases of heat (Rohit *et al.,* 2014). *S. thermophilus* is classified as a nonpathogenic, single *Streptococcus* species which characterized by having a GRAS status (Burton *et al.,* 2006). It is also considered as one the most important industrial dairy starter along with *Lactococcus lactis* (Mayo *et al.,* 2008). *S. thermophilus* which comes to our diet through variety of fermented products is a Gram-positive bacterium belonging to the *phylum Firmicutes*, order *Lactobacillales*, family *Streptococcaceae* (Table 1). It belongs to the clade of LAB which includes the species of *genera Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Leunostoc, Oenococcus, Pediococcus, Tetragenococcus, Vagococcus and Weissella* (Rohit *et al.,* 2014).

S. thermophilus is highly adapted to grow on lactose, the main carbon source in milk and rapidly converts it into lactate during growth. Lactose is transported into the cell by a lactose permease (LacS), which operates as a galactoside proton transport system or as a lactose-galactose anti-porter. Lactose is efficiently transported into the cell and subsequently hydrolyzed by an intracellular b-galactosidase (Hols *et al.,* 2005).

Table 1 Classification of Domain of *S. thermophilus*

Scientific classification of *S. thermophilus* in	schematic structure
Domain	Bacteria
Phylum	*Firmicutes*
Class	*Bacilli*
Order	*Lactobacillales*
Family	*Streptococcaceae*
Genus	*Streptococcus*
Species	*S. thermophilus*
Binomial name	*S. thermophillus*

Source: Info. Metasearch and Social Results, 2017

2.2. Classification of LAB based on by-products of sugar

Lactic Acid Bacteria may be classed depending on their by-products of carbohydrates fermentation as homofermentative or heterofermentative.

Table 2. Clasification of Lactic Acid Bacteria

Homofermentative LAB	Heterofermentative LAB
Lactic acids the main by product of this LAB	Lactic acids, CO2, Ethanol, Acetic acid are the main byproducts of this LAB.
Example L. *Bulgaricus*, *S. thermophilus, L. helviticus, Pedeococcus, Enterococcus…*	*Leuconostoc spp, L. brevis, L. fermentum, L. reutari, L. plantarum…*
Majorly as dairy starter culture	Seldom seen used as starter culture in dairy production.
No production of Carbon dioxide	There is production of CO_2, which indicates heterofermentative LAB.
Separated from by Carbohydrates fermentation profile.	Separated depending up on carbohydrates fermentation profiles
It include gram positive rod and gram positive cocci	It incorporates both gram positive rod and Gram negative cocci
It maintain the quality of dairy production	If significant number allowed to multiplicities it cause faults to produce acidity and CO_2

Source (Alquqa, 2018)

2.3. Importance of *S. thermophillus* in the Dairy Industry

LAB is a group of gram-positive, non-sporulating, anaerobic or facultative aerobic cocci or rods, fastidious on artificial media, grow readily in most food substrates. It lowers the pH rapidly to a point where competing organism are no longer able to grow as well as it is one of the main fermentation products of the metabolism of carbohydrates (Hayek and Ibrahim, 2013).

S. thermophilus has a major importance for the food industry as regard the manufacture of yoghourt and cheese (Purwandari *et al.,* 2007). It is one of the most valuable homo-fermentative lactic acid bacteria, which widely used for a long period of time as a starter for the production of fermented dairy products. The key production characteristics of *S. thermophilus,* for example the production of extracellular polysaccharide, proteolytic enzymes and flavor substances as well as acidifying capacity etc., have an important effect on the quality of dairy products (Yanhua *et al.,* 2016).

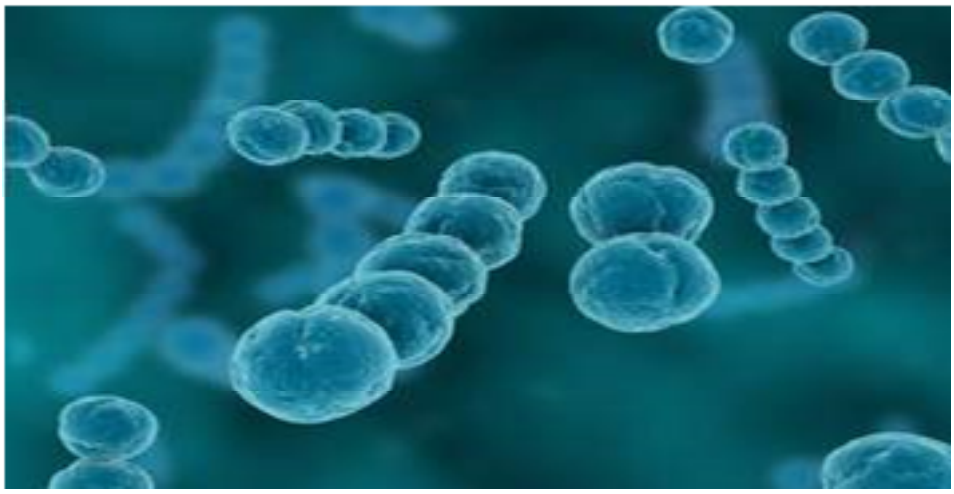


Figure 1. Microscopic representation of *S. thermophillus(*Source: Info. Metasearch & Social Results, 2017).

In various artisanal and industrial dairy productions*, S. themophilus* can improve the texture and flavor properties of these dairy products, and accelerate the acidifying rate of dairy products. Additionally, *S. thermophilus* is found to have various probiotic effects, including antioxidant activities, modulation of intestinal microbiota, inhibition of specific pathogens etc. This species has attracted broad interest in past decades because of its industrial application and probiotic effect (Iyer *et al.,* 2010).

2.4. The Propbiotic Effect of *S. thermophillus* and Other LAB

When examined world widely, various dairy products which are different in name but similar in content can be found and those products are an important part of human diet (Hugenholtz, 2013). In industry, large quantity of yogurt is being produced that is affected by number of factors such as choice of milk, milk standardization, milk additives, de-aeration, homogenization and heat-treatment, choice of culture and plant design. The milk used for yogurt production must be of the highest probiotic bacteriological quality (Soukoulis *et al.,* 2007).

Probiotics are defined as living microorganisms, which when ingested in sufficient amounts, beneficially influence the health of the host by improving the composition of intestinal microflora. In addition to improving gut health, probiotics can play a beneficial role in several medical conditions, including lactose intolerance, cancer, allergies, hepatic disease, Helicobacter pylori infections, urinary tract infections, hyperlipidemia and assimilation of cholesterol (Ejtahed *et al.,* 2011).

Probiotic microorganisms that are known to be beneficial to human health can be ingested through fermented dairy products, enrichment of various foods with these bacteria and consumption of pharmaceutical products that are obtained by using viable cells (lyophiled preparations and tablets). Probiotic dairy products have favourable effects on human health such as, reducing lactose intolerance, prevention of diarrhea and constipation, increase in the effectiveness against Helicobacter pylori infection, preservation of oral health, partial prevention of cancer, cholesterol lowering, enhancement of mineral absorption (Gobbetti *et al.,* 2010). Along with their extensive effects on human health, they have the ability to form low molecular weight components such as conjugated linoleic acid, gamma aminobutyric acid (GABA) and bacteriocin (Kanmani *et al.,* 2013).

Exposure to oxygen may induce a lack of functionality of probiotic dairy products because the anaerobic metabolism of probiotic bacteria which results loses of maintenance of their viability to provide benefits to consumer health. The exposure to oxygen along the refrigerated stored constitutes a technological challenge with respect to the maintenance of the probiotic dairy products, once a great part of the probiotic microorganisms grow in anaerobic systems. Glucose oxidase which is an enzyme produced by fungi such as

Aspergillus and *Penicillium* can constitute a potential alternative to increase the survival of probiotic bacteria in fermented milks (Cruz *et al.,* 2012). In Japan this probiotics are consumed as frozen culture tablets whilst in Europe, due to the bias against medication, probiotics are only consumed through the inclusion of probiotics to foods (Soccoll *et al.,* 2010).

2.5. Technological and Functional Properties of *S. thermophilus*

2.5. 1. Sugar Metabolism

A number of works indicate that utilization of glucose, lactose and fructose by *S. thermophilus* is a common characteristic in all existing studies, while galactose, mannose, sucrose, maltose, melibiose, and raffinose utilizations have variable profiles (Erkus *et al.,* 2014).

At the same time, it has been found that carbohydrate metabolism is essential for the colonization of *S. thermophilus* in the digestive tract of gnotobiotic rats (Thomas *et al*., 2011). The utilization of galactose is a desirable property for strains used in industrial dairy fermentations. Galactose accumulation leads to the growth of undesirable LAB in milk products, and cheese browning during. Gal-positive *S. thermophilus* strains can inhibit the growth of undesirable LAB, and prevent the browning defects (Oktay, 2014). Furthermore, galactose utilization enhances EPS production. Galactose utilization can produce nucleotide sugars, which are EPS precursors and whose low level might be a potential bottleneck in EPS production (Morandi and Braska, 2012). At the same time, high level of galactose consumption may lead to accumulation of toxic galactitol in human tissue cells (Erkus *et al.,* 2014).

2.5.2. Polysaccharide Biosynthesis

Extracellular polysaccharides are produced by a variety of bacteria, and are present as capsular polysaccharides (CPSs) or EPSs. The former are tightly linked to the surface of the microbial cell, the latter are released into the growth medium during bacteria growth and are not attached permanently to the cell surface. Most *S. thermophilus* strains can synthesize EPSs (Broabent *et al.,* 2003). It was speculated that immunoregulatory effects of EPSs are related to their chemical composition. EPSs are long-chain polysaccharides

consisting of branched, repeating units of sugars or sugar derivatives. They are classed into homopolysaccharides that consist of a single type of sugar, and heteropolysaccharides with repeating units consisting of different sugars. Most *S. thermophilus* strains synthesize heteropolysaccharide (Broadbent *et al.,* 2003).

S. thermophilus EPSs are predominantly composed of galactose, glucose, and rhamnose in different ratios. Additionally, acetyl-galactosamine, fructose, and acetylated galactose moieties have also been found in *S. thermophilus* EPSs (Zhang *et al.,* 2016). Generally, the structures of EPS have a close relationship with their functions. Therefore, the highly polymorphic structures of EPSs may be confers a broad application potential (Mozzi *et al.,* 2006). The structural diversity of EPSs molecules is correlated with the genetic diversity of the eps locus. The diversity of eps gene cluster may have a direct effect on the EPS production capacity of the strain. To date, distribution of regulatory and structural genes is conserved in known eps cluster of *S. thermophilus* strains (Yahua *et al.,* 2016).

2.5.3. Practical applications of EPS

Recent trends in yogurt production technology have led to an increased use of ropy starter cultures in yogurt production due to the technological roles of EPS produced by LAB cultures. From these isolates, *Lactobacillus delbrueckii subsp. bulgaricus Y39* and *Streptococcus thermophilus Y102* produced heteropolymeric EPS containing glucose and galactose, whereas *Leuconostoc mesenteroides Y35* and *Lactobacillus plantarum Y36* produced homopolymeric glucan (Ispirli and Dertli, 2018).

In addition, recent studies revealed the importance of the use of adjuncts cultures for yogurt and other dairy products production in order to develop the technological properties of these products as well as enhance the functional properties of these products to be used as functional foods (Ekinci and Gurel, 2008). The main technological problem in yogurt is its tendency to exhibit rheological instability during storage (Yilmaz *et al.,* 2015) and the use of ropy starter cultures that are able to produce exopolysaccharides (EPSs) is one of the main precautions to avoid the rheological and viscosity problems in yogurt especially in Europe where the use of stabilizers is prohibited for yogurt production (Ispirli and Dertli, 2018). Structurally, EPS are divided into two groups as homopolysaccharides and

heteropolysaccharides, which are composed of only one type of sugar monomer and two or more types of sugar monomers, respectively (Dertli *et al.,* 2013).

2.5.4. Proteolytic System and Amino Acid Metabolism

The growth of LAB is limited due to the low free amino acids in milk. Milk contains an abundance of proteins. Therefore, the strains must utilize proteins, produce peptides and amino acids to satisfy its nitrogen source requirement during rapid growth in milk. The protein hydrolysis system provides the basic amino acids for the growth of the cells, and also affects the sensory characteristics and flavor of fermented dairy products (Savijoki *et al.,* 2006).

The proteolytic system of LAB mainly consists of (i) an extracellular cell anchored protease capable of casein hydrolysis; (ii) a set of amino acid and peptide transport systems required for import of casein hydrolysis; (ii port of amino acids and (iii) a set of intracellular peptidases involved in the hydrolysis of casein-derived peptides essential for various housekeeping processes peptides transporters, and intracellular peptidases (Iyer *et al.,* 2010; Hols *et al.,* 2005). At present, it is found that five types of extracellular proteinases in LAB, including PrtP (*L. lactis, Lactobacillus paracasei), PrtH (Lactobacillus helveticus), PrtR (Lactobacillus rhamnosus), PrtS (S. thermophilus), PrtB (L. delbrueckii ssp. Bulgaricus* (Savijoki *et al.,* 2006).

2.5.5. Flavor

Flavor is one of the most important properties of food products, and is a key factor in determining acceptability and preference (Cheng, 2010). More than 100 different flavor compounds are found in yogurt, including alcohols, aldehydes, ketones, acids, esters, lactones, sulfur-containing compounds, and heterocyclic compounds etc. These flavor compounds include the volatiles already present in the milk and specific flavor compounds produced from milk fermentation (Liu *et al.,* 2008).

Flavor-forming capacity of LAB is an important evaluating indicator of starter culture in fermented dairy. The flavor of dairy products originated from protein, fat and carbohydrate in the milk. Casein is the main precursor of flavor compounds when LAB is growing in milk. Casein is degraded into its constituent amino acids by means of proteolytic system of

LAB. Then free amino acids are converted to various flavor compounds such as aldehydes, alcohols, and esters (Liu et al., 2008; Cheng, 2010). Research indicates that *S. thermophilus* exhibits glutamate dehydrogenase activity, which produces ketoglutarate from glutamate, and consequently is capable of catabolizing amino acids in the reaction medium without-ketoglutarate addition (Helinck *et al.,* 2004). The level of flavor compounds could be improved using molecular biotechnology. The ability to generate important metabolites of *S. thermophilus* is improved by expression of acetolactate synthase gene (als) and alcohol dehydrogenase gene (adhB). High levels of 2, 3-butanediol and ethanol are obtained by over expressing the als gene or adhB gene in *S. thermophilus* (Akyol *et al.,* 2015).

2.6. Antibiotic Properties of *S. thermophilus* and its Role in Chemical Substance Production

LAB isolated from dairy products have gained a high attention for its potential food preservative due to their antagonistic activity against many food born pathogen such as *Listeria monocytogenes* (Jamuna and Jeevaratnam, 2004). LAB are widely distributed in the nature, they are typically involved in a large number of the spontaneous food fermentation in which some its members produce bacteriocins and bacteriocins like substances that inhibit growth of spoilage and pathogenic microorganisms (Mezaini *et al*., 2009).

Bacteriocins from LAB are bioactive peptides or proteins with antimicrobial activity toward Gram positive bacteria, including closely related strains and/or spoilage and pathogenic bacteria. Bacteriocins are ribosomaly synthesized and extracellulary released bioactive peptides or peptide complexes which have bactericidal or bacteriostatic effect. Bacteriocins are innocuous due to proteolytic degradation in the gastrointestinal tract (Mezaini *et al*., 2007).

Some of *S. thermophilus* strains produce a bacteriocin named *thermophilin* which is active against several LAB and food spoilage bacteria such as *Clostridium sporogenes*. In view of its technological and biochemical properties the above bacteriocin can be considered as a potential bioprerservative (Aktypis *et al*., 2007). Some of other LAB like *Enterococcus, Lactococcus, and Pediococcus* are also widely used as natural preservatives, due to the

potential production of metabolites with antimicrobial activity such as organic acids, hydrogen peroxide, antimicrobial enzymes and bacteriocins (Mataragas *et al.,* 2003).

2.6.1. Biosurfactant Production

Biosurfactants (BS) are amphipathic molecules due to their possession of both hydrophilic and hydrophobic chemical groups. They possess surface activity; this means that they reduce the surface tension of liquids. They are also extracellular compounds synthesized by yeasts, fungus and bacteria. The most significant characteristics displayed by them are surface activity, biodegradability, antimicrobial effects, antitumor activity, anti-adhesive, low toxicity and effectiveness at extremes of temperature, pH and salinity (Saharan *et al*, 2011, Rodrigues *et al.,* 2006; Mukrnherjee *et al.*, 2006).

Due to these features, biosurfactants have great importance in bioremediation and oil recovery processes. They are important in the pharmaceutical, agriculture, cosmetics and food industries. Yeast, including Candida bombicola ATCC 22,214, is able to synthesize BS as sophorolipids at concentrations of up to of 5 g/L in a synthetic medium that emulates wastewater from the dairy industry (Daverey *et al.,* 2009). The wild type strain achieves a production of biosufectants up to 4000 mg/L at 30°C and 7500 mg/L at 37°C. The recombinant strain generates 7000 mg/L at 30°C and 10,500 mg/L at 37°C. Finally, it can be inferred that genetic engineering could improve the yields of functional compounds from industrial wastes (Colak and Kahraman, 2013).

Interest in biosurfactants has increased considerably in recent years, as they are potential candidates for many commercial applications in the petroleum, pharmaceuticals, biomedical and food processing industries. *S. thermophilus* strains can produce biosurfactants that cause their own desorption. A *S. thermophilus* strain isolated from a heat exchanger plate of a pasteurizer produced a biosurfactant that caused its own desorption from glass, leaving a completely non-adhesive coating. Similarly, yoghurt containing active *S. thermophilus* has been suggested to have beneficial effects in prolonging the lifetime of indwelling voice prostheses. It has been hypothesized that the presence of *S. thermophilus* in active yoghurt may interfere with microbial adhesion to silicone rubber, which was confirmed by artificial throat model experiments (Usman *et al.,* 2016).

2.6.2. The Use of *S. thermophillus* to Produce Bacteriocin

Bacteriocins of *S. thermophilus* strains, known as *thermophilins,* are thermostable and are active over a wide range of pH values, unlike nisin, which is not used in acidic foods. Apart from such stability, they are safe because of the GRAS status of *S. thermophilus.* Although several bacteriocin producing strains of *S. thermophilus* have been reported, only five bacteriocins have been characterized: thermophilin 347, produced by a yoghurt strain thermophilin A, thermophilin T, produced by a feta cheese strain thermophilin 13, which has been sequenced a bacteriocin from *S. thermophilus* 81 with a broad inhibitory spectrum and a new bacteriocin from *S. thermophilus Adria 91L 580* that inhibits Clostridium tyrobutyricum isolated from a hard cheese (Ramya *et al.,* 2010).

In this respect, food preservation through *in situ* production of bacteriocins by LAB specially using *S. thermophilus* introduced into the food system would be the most logical approach. However, there is a need to understand the relationship between bacterial growth and bacteriocin production in various types of food system (Sahar *et al.,* 2017). Bacteriocin production by LAB is dependent on a number of factors such as the types of carbon and nitrogen sources and their concentrations in the media formulation. Other factors which need to be considered are the culture conditions which include pH, temperature and aeration which greatly influence the cultivation performance of bacteriocins producing *S. thermophillus* (Figure 3). Thus, the fermentation factors which influence the production of bacteriocins by LAB and the approaches to improve the production not only in term of yield and productivity but also in term of economic and regulation are reviewed in this paper (Ramya *et al.,* 2010).

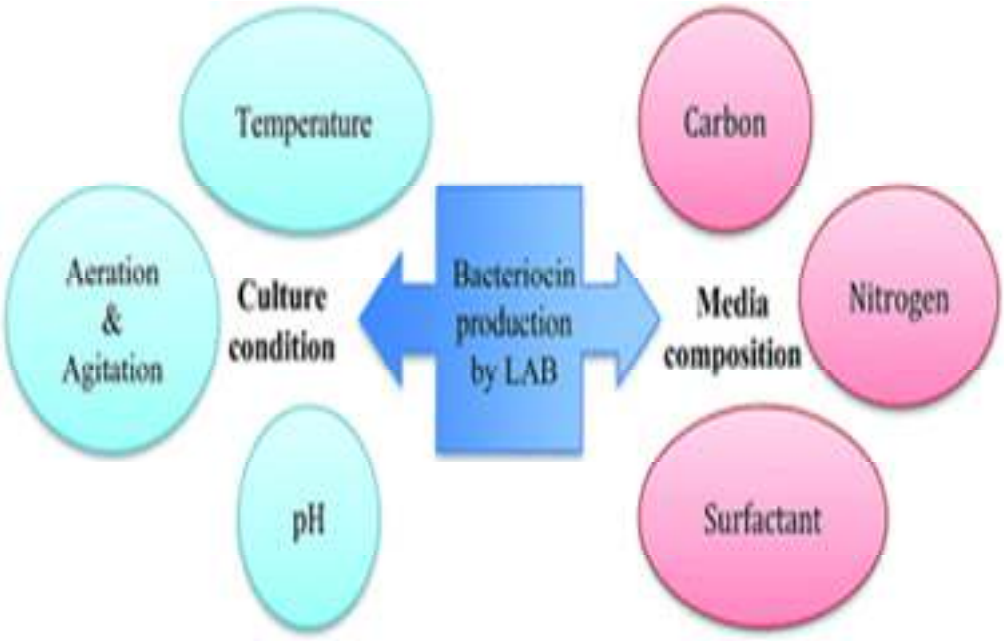


Figure 2. Production of Bactriocin by Species of Lactic acid bacteria (Source*:* Sahar *et al.,* 2017*).*

The incorporation of bacteriocins as a biopreservative ingredient into model food systems has been studied extensively and has been shown to be effective in the control of pathogenic and spoilage microorganisms. Several strains of *S. thermophilus* have been found to produce bacteriocins which are being discussed in the following table (Rohit *et al.,* 2014).

Table 3 The Bacteriocin Produced from Strains *S. thermophillus*

Strain	***Bacteriocin***	**Bacteria to be inhibited**
S. thermophilus 347	*Thermophilin347*	*Listeria monocytogenes*
S.thermophilus ST134	*Thermophilin A*	*Sensetive to cells in culture*
S. thermophilus Sfi 13	*Thermophilin 13*	*L. monocytogenes*
S. thermophilus 81	*Thermophilin 81*	*Lactococcus lactis, S. typhimurium, E. coli*
S.thermophilusACADC0040	*Thermophilin T*	*Clostridium sporogenes,C. tyrobutyricum*
S. thermophilus 580	*Thermophilin 580*	*C. tyrobutyricum*
S. thermophilus ST110	*Thermophilin 110*	*Pediococcus acidilactis*

Source: Rohit *et al*., 2014

2.6.3. Gastric Liquid product of *S. thermophillus* and Other LAB

S. thermophilus is found to be potentially therapeutic against the associated chronic gastritis. Gastritis is a common disorder in which discontinuity of the gastric mucosa is observed (Rodriguez *et al*., 2010). It is caused by various factors like excess alcohol, infection, intensive consumption of anti-inflammatory drugs with *Helicobacter pylori* or may be stress. It also, affects various mucosal defense lines such as bicarbonate secretion, mucus synthesis, and decrease of mucosal blood flow. It was able to generate immune response in mice and increased the thickness of the gastric mucus gel layer. Studies suggest that recombinant lactic acid bacteria are the excellent candidates for the production of various biotherapeutic proteins and also their delivery to specific places of requirement within the gastrointestinal tract (Daniel *et al.,* 2011). Food supplements containing *S. thermophilus* have been found to maintain a stable growth rate in children. Children consuming *S. thermophilus* containing supplements had shown better growth during a 6 month period than children who did not receive the supplement (Nopchinda *et al.,* 2002).

Image A Image B

Figure.3 Probiotic Liquid Product of *S. thermophillus* along with *L. plantarum*

2.7. Probiotic Potential of *S. thermophilus* in improving human health

2.7.1. *S. thermophilus* As Dairy Starter

A starter culture can be defined as a microbial preparation of large numbers of cells of at least one microorganism to be added to a raw material to produce a fermented food by accelerating and steering its fermentation process. *S thermophilus* is extensively used in starter cultures for dairy products like Swiss and Italian-type cheeses, Gouda cheese and yoghurt because of its metabolical traits such as production of lactic acid, flavouring compounds, exopolysaccharide production, fermentation of galactose, urease and proteolytic activity (Leroy and Vuyst, 2004). A recent study conducted at National Dairy Research Institute, India shows that *S. thermophilus* isolated from plant sources possess similar physiological and biochemical properties to those from dairy sources and can be considered for developing new starters (Mamaheswari *et al.,* 2014).

2.7.2. Antibacterial Activity against Intestinal Microbes and Diarrhoea

S. thermophilus along with other probiotic bacteria possess inhibitory effects on some of intestinal pathogenic organisms and hence it is evident that it can be administered in order to prevent or ameliorate some diseases (Hassan *et al.,* 2010). In a study conducted to determine the efficiency of probiotic drink (containing *S. thermophilus* along with *Lactobacillus casei* and *L. bulgaricus)* for the prevention of diarrhoea associated with use of antibiotics and that caused by *C. difficile. I*t was found to reduce the incidence of any disease also having the potential to decrease morbidity, healthcare costs and mortality if used routinely in patients of age greater than 50 (Hickson *et al.,* 2007).

2.7.3. Efficacy of *S. thermophilus* Against Rotavirus Induced Diarrhoea in Infants

Rotavirus-induced diarrhea poses a worldwide medical problem In a double-blind, placebo-controlled trial, infants aged 5-24 months who were admitted to a chronic medical care hospital were randomized to receive a standard infant formula or the same formula supplemented with *B. bifidum* and *S. thermophilus*. It was found that the supplementation

of infant formula with B. bifidum and *S. thermophilus* can reduce the incidence of acute diarrhoea and rotavirus shedding in infants admitted to hospital (Rohit *et al.,* 2014).

2.7.4. Role in Enteric Dialysis for Renal Failure

In a test conducted for the formulation of commensal and food grade bacteria that when ingested may become gut flora that catabolize nitrogenous toxins that accumulate in uremia flow into the gut by passive diffusion, an isolate *S. thermophilus KB19* reduced urea concentration from 300mg/dL to 20mg/dL within 249 hours at pH 6.3 when inoculated in Artificial Intestinal Fluid at initial density of 10 cfu/ml. KB19 survived 3 hours in acidic pH 3.0 with only two logs loss in cfu and was able to pass through bile. In addition, this strain evinced no resistance to 8 commonly used antibiotics. These data indicate that *S. thermophilus* bacterial isolate can be used as a urea-targeted component in an enteric dialysis formulation (Zelenaia *et al.,* 2004).

2.7.5. *S. thermophilus* for Skin Ailments

One of the most significant health benefits associated with the use of *S. thermophilus* bacteria in humans is the bacterium's ability to exert a positive effect upon the body's ceramide (a skin protective agent) levels (Marzio *et al.,* 2003). In vitro, it has shown a considerable positive impact on the ceramide levels measured in cultured human keratinocytes which have function in the formation of a barrier against environmental damage such as pathogens, heat, UV, and water loss. Secondly in vivo, it has shown an equally beneficial effect on the level of ceramides in stratum corneum, which forms a barrier to protect underlying tissue from infection, dehydration, exposure to chemicals and mechanical stress. Additionally, *S. thermophilus* has been found to positively influence the levels of sphingolipids in human skin. A study which looked at about 11 patients who were treated with topical creams containing *S. thermophilus*, in all cases there were "significant improvements in levels of bacterial sphingomyelinase" (Marzio *et al.,* 2003).

2.7.6. *S. thermophilus* as Antioxidant

The damage caused to our cells and tissues by the free radicals has a critical role in progression of disease and process of ageing. Antioxidants act as first lines of defense against the damage caused due to free radicals and thus are vital for optimal health

maintenance. It has been recently demonstrated that the probiotic microorganisms can effectively trap reactive forms of oxygen as such in the experiment conducted using rats which were deficient in vitamin E, has revealed that the intracellular extract from Lactobacillus sp. recovers this deficiency. The classical yoghurt bacteria *L. bulgaricus* and *S. thermophilus* inhibit peroxidation of lipids through scavenging the reactive oxygen radicals, such as hydroxyl radical, or hydrogen peroxide (Rohit *et al.,* 2014).

Table 4 Few Examples of Probiotic activities of *S. thermophillus*

Probiotics Activity	**Strain**
Suppression of Ulcerative Colitis	*S.thermophilus ST28,S.thermophilus ATCC 1928*
Lactose digestion	*S.thermophilus MUH 341*
Decrease in pressure	*S.thermophilusTMC 1542*
Reduction in blood cholesterol	*S.thermophilus*
Antigastric activity	*S.thermophilus CRL 1190*
Against Chronic gastritis	*S.thermophilu CRL 1190*
Anti-tumors activity	*S.thermophilus*
Anti-listerial activity	*S.thermophilus*
Food preservatives	*S.thermophilus CCHC 3534*

Source Rohit *et al.,* 2014

2.7.7. Reduction in Colonization of Nasal Pathogenic Bacteria

As obtained a study which looked upon the specific measurement of pathogenic bacteria in human nasal canals, the results showed that those patients who were given the supplemented yogurt experienced a markedly reduced level of nasal colonization of pathogenic bacteria Staphylococci aureus, *Streptococcus pneumonia* and *β haemolytic streptococci* (Gluck and Gebbers, 2003).

2.7.8. Probiotic Bacteria, the Immune System and Allergy

Probiotics are generally commensal or symbiotic gram-positive bacteria, resistant to the the microbiota, a complex system contributing to the intestine functional activity and favoring its normal physiology also considering its intimate association with the immune system. For this reason, this system may represent a possible target for the treatment of allergic diseases, being able to develop regulatory immune responses through IL-10 induction providing as well adequate stimuli for the acquisition of antigen tolerance (Veenbrgen and Samson, 2012).

Probiotic bacteria could be suited to this purpose. It has been demonstrated that certain strains of probiotics regulate the immunological homeostasis of the intestinal mucosa and partially affect the development of allergic diseases. Furthermore, epidemiological studies revealed the correlation between the composition of the intestinal microflora and the prevalence of atopy (Petrarca. 2015). Lactobacilli (LAB) are the main probiotic bacteria employed for the preparation of dairy products and of probiotics supplements for human use; actually, they are not pathogenic, do not damage the mucous membranes, do not own genes for antibiotic resistance and are not degraded by bile acids (Borchers *et al*., 2009).

2.7.9. Study with *S. thermophilus* Expressing the Allergen Rbet V1

It was revealed by some studies that, a strain of *S. thermophilus* expressing *Bet v 1*, the major allergen of *Betula verrucosa*, in order to verify its possible adjuvant or therapeutic effect in BALB/c mice made IgE-responsive to this same allergen (Petrarca, 2014). The choice of this strain stems from different considerations. First, it has been taken into account the large presence of peptidoglycan on the cellular wall and of (lipo) teichoic acids, owning immunomodulatory activities. Secondly, it has been considered its autolytic character provided by the presence of lysogenic bacteriophage expressing an enzyme able to degrade the bacterial cell wall; finally, it represents a prokaryotic expression system suitable for integrating and expressing permanently foreign genes (Petrarca et al., 2015).

The immunomodulation level and therapeutic efficacy of the ST recombinant probiotic was assessed and compared to the effects of only ST and ST+rBet v 1 association by measuring various immunological and histopathological parameters (cytokines in serum and produced by immune cells in vitro, Treg cells, inflammatory cells and cytokines in the lung tissue).

This study showed that the therapeutic treatment of pre sensitized mice with the recombinant probiotic expressing the allergen produces, upon recall airway challenge, specific local and systemic anti-inflammatory and inhibitory response along with clearance of lung eosinophilia (Petrarca *et al., 2015*) which characterizes the inflammatory response of allergic asthma in mice (Jacobsen *et al.,* 2014).

2.8. Molecular Properties of *S. thermophilus*

2.8.1 Genomic DNA Extraction Methods

In the last 10 years the application of culture-independent molecular methods based on isolation of total microbial DNA from a sample with Out prior cultivation, and amplification of the 16S rRNA gene by the polymerase chain reaction (PCR) method, have been introduced into food microbiology (Hovda, 2007). Genomic DNA/RNA isolation is the first and the most important requirement in carrying out molecular biology techniques such as PCR, restriction enzyme analysis, Southern hybridization, genomic DNA library construction (Demerdash, 2012), mutation detection or linkage analysis (Aldous *et al.,* 2007), as well as DNA microarray gene expression profiling (Omidi *et al*., 2005). It should be mentioned that, this molecular methods can be applied as culture-independent or culture-dependent, which exclusively depends on whether bacterial DNA was extracted directly from a sample, or from bacterial colonies grown on culture media (Pogacic*,* 2010). Most of the routinely used protocols for preparation of bacterial chromosomal DNA are primarily based on two strategies including either cetyl trimethyl ammonium bromide (CTAB) or lysozyme/detergent based method followed by improvement of the procedure by removal of protein and RNA contamination (Amarao *et al.,* 2008).

However, many gram positive bacteria, such as *S. thermophilus* are resistant or weakly susceptible to lysozyme due to their cell wall structure. Some problems such as poor yields and interference with Restriction enzymes digestion have been reported to be associated with some standard DNA extraction protocols (Hassen, 2012). The basis for genetic manipulation of these industrially significant bacteria begins finds a technique to facilitate the rapid purification of genomic DNA that can be used directly for molecular analysis. A modified protocol is described to isolate chromosomal DNA from various Gram positive

bacteria without the need more for phenol: chloroform extractions and caesium chloride gradients (Rossetti and Giraffa, 2005).

2.8.2. Available PCR of *S. thermophilus*

PCR is a fundamental aspect of molecular biology, and many molecular methods based on PCR have been developed to study microbial communities. In PCR, DNA serves as a template for PCR amplification of genetic targets with universal, genus- or species-specific primers to amplify target sequences of a given population. Basically, PCR consists of three major steps: denaturation temprature of DNA at 94–95^{o}C, annealing of the nucleotide primers at 37–70^{o}C and polymerization (elongation) for 10sec and DNA strand from nucleotides at 60–72^{o}C. It must also be underlined that optimization of PCR conditions for each step is inevitable in every experiment. Very often, many experimental trials must be performed to set up optimal PCR conditions (Pogacic, 2010).

3. MATERIALS AND METHODS

3.1. Description of Study Area

The study was conducted in East Showa Zone, Oromia Regional State, Ethiopia locating between 38° 03′ to 40° 05′ E longitude and from 7° 04′ to 9° 10′ N latitude covering a total area of about 13766.5 km^2 (Figure 4). According to central statistical agency of Ethiopia (CSA), the altitude of this study area ranges from 538 to 3101 m above sea level. East Shewa zone is characterized by semi-arid and sub-humid climate based on the moisture index classification of climate. Considering the long term average seasonal (June-September) rainfall, the area receives 458 – 518 mm rain. Based on mean annual rainfall and temperature of the area, the major climatic classes of the zone are dry climate and tropical rainy climate (CSA, 2012).

Dry climate includes the arid and semi-arid subdivision, while tropical rainy climate is characterized by tropical humid and sub humid climate which is good for dairy cattle survival. The agricultural activities in the dry land semi arid areas of Ethiopia in general and study area, East Showa Zone, in particular, are influenced and controlled by seasonal rain. Soil around this area is an important medium for plant growth and development owing to its power of storing water and providing anchorage for root growth and acting as a reservoir for mineral nutrients (CSA, 2012).

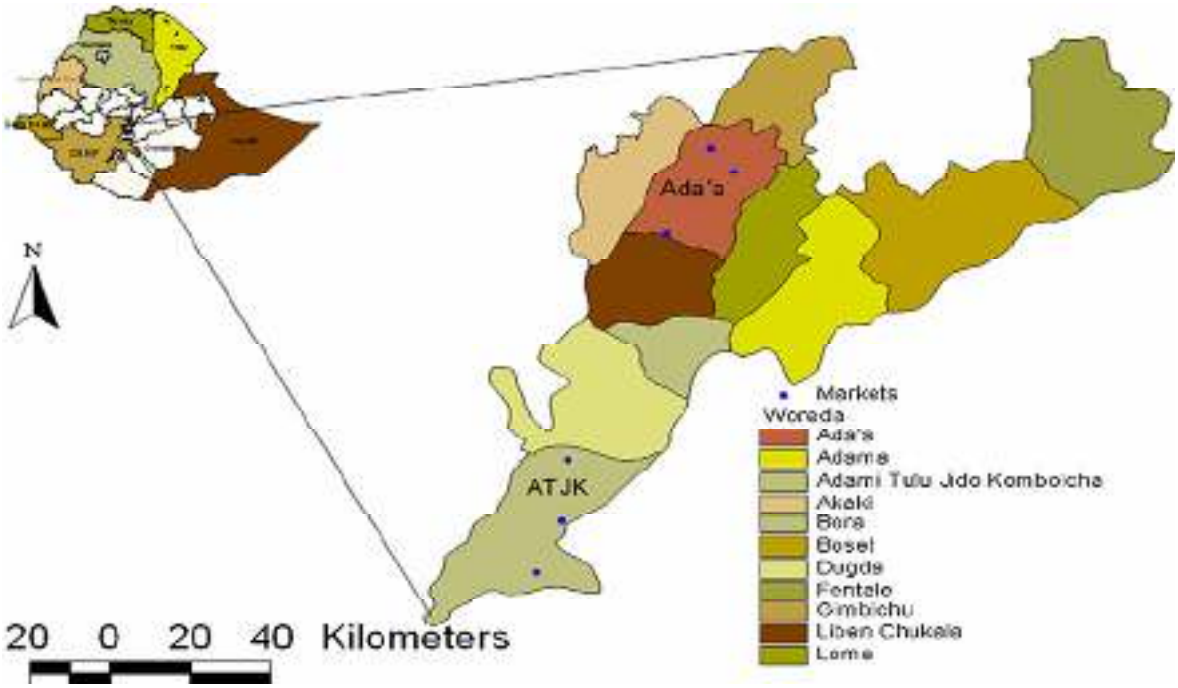


Fig. 4 The Study Area Map of East showa Oromia Regional State

3.2. Sampling Techniques and Sample size Determination

A purposive type of study with systematic random sampling techniques was applied. Thus, for convenience and availability of laboratory materials 20 samples of fermented milk were randomly collected for this research.

3.3. Fermented milk sample collection and transportation

Twenty universal glass bottles were prepared and sterilized. Then approximately 150 ml of fermented milk samples were carefully collected from fermented milk sample of producer's container and held into sterile glass bottles. Consequently, the samples were labeled and transported to bacteriology laboratory of NVI under cold chain using ice box (+4°C). Then samples were stored until biochemical characterization, molecular and antibiotic activity analysis were made

3.4. Isolation of *S. thermophillus* Species from fermented Cow milk samples

For isolation sterile M948 agar plates were used (HiMedia, 2016). An agar plates were streaked with loopful of yoghurt sample directly from the container. All the plates were incubated at 37°C for 48 hours. After the incubation, all the plates were observed for the formation of very small and white colonies (Princely *et al.,* 2013). Then bacteria was isolated from the incubated plates and transferred into another triptose soya agar (TSA) medium for further enrichment. The enriched bacterium was purified by streak again on the plate (Choi and Rogers, 2015). After the incubation, a creamy white colored of suspected *S. thermophilus* was observed. Finally purification was terminated when homogeneous colonies appeared on the plate. Before the experiment, isolated colonies were activated by successive transfer in broth and stored at +4°C (Erkus, 2007).

3.5. Biochemical and Morphological characterization of Isolates

Morphological identification of isolated colonies were carried out by Gram's staining catalase test, oxydase test, TSI test, motility test, MR-VP test and indole test (Im *et al.,* 2014).

Gram staining

First of all a loop full of overnight sub cultured fresh isolates were transferred on to slide. Gram positive bacteria became blue or purple after Gram staining; however Gram negative bacteria became pink or red (CU, 2017).

Catalase Test: - At the beginning over night activated culture was prepared. Then one drop of 3% Hydrogen peroxide was added on a clean glass slide. Aseptically a loopful of the isolates was taken and emulsified in the H_2O_2 drop. Visible bubble production indicates a positive result otherwise negative (Sridhar, 2017).

Oxidase Test: - A piece of filter paper was placed in a Petri plate and soaked with the oxidase reagent. Using a sterile swab, the bacteria were transferred to the filter paper. Then the color should be changed in to blue black for positive but negative result was observed in this study (Sheilds and Cathcart, 2010).

Motility testing: - By using aseptic techniques, an M948 broth tube was inoculated by stabbing with the needle to the approximately three-quarters of its depth. Then it was incubated at room temperature for 48 hours. Finally the growth of bacteria in tube brushing condition was examined either for motile or for non motile (Shields and Cathcart, 2016).

Methyl Red (MR) and Voges Proskauer (VP) Test: - First two tubes containing MR-VP Broth was inoculated with a pure culture of the microorganisms under investigation. Then it was incubated at 35 °C for up to 4 days. After five days about 5 drops of the methyl red indicator solution was added to the first tube. A positive reaction was indicated when the colour of the medium changes to red within a few minutes (Acharya, 2014). Then the second test tube was incubated for 24-48 hrs at 35 °C in ambient air or until turbid growth is observed. After that about 0.5ml was transferred to a glass test tube. Then twelve drops alpha naphthol and 4 drops of 40% KOH was added. Carefully the tubes was shaken and observed for 30 minutes. The tube was vigorously shaken several times during the 30 minute period. Finally the colorless test tube indicates negative result for *S. thermophilus* tests (Lynn *et al.,* 2014).

TSI (Triple Sugar iron) Test: - By using a sterile straight wire the well isolated active colony was taken. Then it was inoculated in to TSI media by first stabbing through the centre of the medium to the bottom of the tube and then streaked the surface of the slant. After that the tube was incubated at 35°C in ambient air for 18 to 24 hours. Finally the reaction was observed as it had been presented on (Pradhan, 2013). Finally the isolate was stored at +4°C until molecular characterization.

3.6. Molecular Detection of *S. thermophilus*

3.6.1. Genomic DNA Extraction Method Used

Chromosomal DNA from the Gram positive biochemically suspected *S. thermophilus* bacteria was isolated according to Demerdash (2012). Cells from an overnight culture (10 ml) were collected by centrifugation at 10000 rpm/ 3min. Then the cell pellets were re-suspended with 200 µl lysis buffers (6.7% sucrose; 50mMTris/HCl, pH7.0; 1mM EDTA). Immediately 4µl of lysis buffer was added to each isolate's suspension from 10 mg ml-1 and 100 mg-1ml of stock solutions respectively, which was then incubated at 37°C for 30

minutes. Subsequently, it was buffered with 10 µl of Proteinase K (10 mg/ml). After that the suspension was shaken on vortexes to make sure no chunks (Demerdash, 2012).

Next 7 µl of 20 % SDS were added and the suspension was incubated at 60°C for 1 hr with inverting every 15 min. The suspension was then extracted once with phenol and chloroform /isoamyl alcohol (25:24:1), mixed thoroughly by inverting for 5 min, spin at 10000 rpm for 5min. The supernatant was then transferred to a new tube. Cotton like genomic DNA was seen at this stage. DNA was ethanol-precipitated from the water phase, the DNA was re-suspended in 1/10 volume of 3M sodium acetate and 2 volume of ice cold ethanol. Afterward, it was mixed gently and re-suspended in 50 µl TE buffer. DNA yields from standard 10 ml cultures ranged between 30-50 µg (Demerdash, 2012). The presence of DNA was confirmed by electrophoresis on an agarose gel containing GelRed® (Biotium) and the gel image was taken using the gel documentation system (SynGene, Gene Genius Bio Imaging System, UK).

3.6.2. Amplification 16S RNA of S. *thermophilus* by PCR

DNA identification analysis by PCR was followed with 17µl Super mix+3µl RNase free water was used us negative control. The cellular lysates were rTM obtained with the generelease (bio Ventures, inc.) from overnight cultures as described above and the following two sets of primers coded as ThI/ThII (ThI: 5'-ACGGAATGTACTTGAGTTTC-3'; ThII: 5'-TGGCCTTTCG ACCTAAC-3') and PA/pH (PA: 5'-AGAGTTTGATCCTGGCTCAG-3'; pH: 5'-AAGGA GGTGATCCAGCCGCA') were used for this study. The amplification profile included a denaturation step of 94°C for 5 min, and then subjected to 30 cycles of 94°C for 30 s, 50°C for 30 s, 72°C for 1 min and a final soak at 4°C. DNA fragments were determined by comparing the positive control bands from reference strains with amplified fragments of wild members within indigenous flora isolates. The presence of PCR products was determined by gel electrophoresis in 1% agarose gel containing ethidium bromide. Electrophoresis in Tris-borate-EDTA was performed at 100 V, and DNA Smart ladder (Eurogentec) of 1000 bp and 2kb was used as the molecular size marker (NVIM, 2018).

Table 5 Set of primers and their maximum amplification size used for *S. thermophilus* detection

Code of primers	5'-3' Sequences of primers	Ampl.length	Source
PA Forward	AGAGTTTGATCCTGGCTCAG	Varies from spp to spp. I.e 1500-1650bp	Saraphirom and Reungsang, 2010
pH Reverse	AAGGAGGTGATCCAGCCGCA		
ThI Forward	ACGGAATGTACTTGAGTTTC	250bp	Vanatkova *et al*., 2009
ThII Reverse	TGGCCTTTCGACCTAAC		

3.7. Analysis of Antibiotic properties of *S. thermophilus*

3.7.1 Preparation of *S. thermophilus* cell free extract

The identified *S. thermophilus* isolates were inoculated into 50ml of M948 broth and incubated at 37 °C for 4 and 18hrs. Then it was centrifuged separately at 10,000 rpm for 15 minutes. After that, the supernatant was collected and passed through 0.20 μm sterile syringe filter. The cell free supernatant broth was collected for the antibacterial study against selected pathogens (Astha *et al*., 2012).

3.7.2. Agar well diffusion method for antibiotic properties Test

The agar well diffusion method was used to determine the antibacterial property of an *S. thermophilus*. A 24 hr culture of the food borne pathogens (*Clostridium deficile, E. coli,* and *S. aureus*) were grown in nutrient broth at 37 °C and used to detect anti biotic properties. A lawn of the indicator strain was swabbed on nutrient agar plates. The plates were allowed to dry and a sterile cork borer of diameter (5mm) was used to cut uniform wells in the agar. Each well was filled with 60 μL culture free filtrate and cell suspended in M948 broth that obtained from the *S. thermophilus* isolates after 4 hrs and 18 hrs incubation. After incubation at 37 °C for 24 hrs, the plates were observed for a zone of inhibition (ZOI) around the well (Astha *et al.,* 2012).

3.7.3. Controls Used for *S. thermophilus* antibiotic activity test

In this study, *S. thermophilus* active culture and pathogenic microorganism separately incubated on TSA agar was used as a negative control, pure sterile media alone was used as media control.

3.8. Data Analysis

The data was analyzed using Microsoft Word Excel, tables, bar graph and figures.

4. RESULT

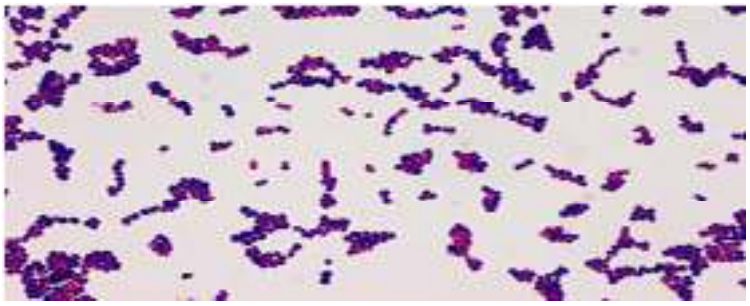
4.1. Isolation of *S. thermophilus* from Fermented Milk Sample

Out of the 20 samples all were positive for Streptococci isolates of which 14 were confirmed *S. thermophilus* isolates. *S. thermophilus* colony with white creamy color on primary culture was seen on Petri-plates (Figure 5a) streaked on triptose soya agar (TSA) media for purification to get homogeneous colonies appeared on the plate (Figure 5b).

Figure 5 The first culture of *S. thermophilus* (A) and its further purification on TSA (B).

4.2. Gram Staining and morphological Study of *S. thermophilus*

Gram's positive active colony (isolates) (Figure 6) appeared as purple blue color revealed that it was *S. thermophilus* isolates.



Figures 6. Blue colored images of *S. thermophilus* species seen under oil emersion magnification after Graham's staining.

The cultural and morphological characterization of isolates of the 20 samples was conducted in which 14 were confirmed as *S. thermophilus.* It was observed that about 70% of isolates were showed characteristic white creamy color; ovoid-regular shaped, Gram positive Cocci, and chained form (Table 5). But, 30% of isolates had varied in characters where some are yellow in color, irregular shape, large in size, cluster form and others.

Table 6: Colony structure and morphological characteristics used for *S. thermophilus* identification.

No	Sample	Colony structure on Nutrient Agar Plate	Morphological Characteristics
1	Ys1	White, regular, pinpoint	G+ve Cocci, tripled, straight chain
2	Ys2	White, regular, white, ovoidal	G+ve clusters of Cocci, and chained
3	Ys3	White pinpoint shaped & regular	G+ve cocci
4	Ys4	Ovoidal shaped, white, regular	G+ve scattered colony and Cocci
5	Ys5	White, regular, ovoidal	G+ve, Cocci, doubled & straight chain
6	St1	Ovoid, regular, smooth & white	G+ve Cocci, &chained clusters
7	St2	Circular, smooth, white and small	G+ve, Cocci, single chaine cluster
8	St3	Ovoidal, smooth, white and small	G+ve, Cocci, single and straight
9	St4	Small, smooth, ovoid and white	G+ve Cocci, cluster of chained
10	St5	Yellowish, small, circular	G-ve Bacilli, chained form
11	St6	Yellow, small, circular	G-ve Bacilli, Chained form
12	St7	White, large, ovoid	G+ve Bacilli, clustered form
13	St8	Circular, smooth, white and small	G+ve, straight chain, and clusters
14	B1	Small, ovoidal and white	G+ve scattered colony and Cocci
15	B2	Small, smooth, ovoid and white	G+ve, Cocci, single, double & chained
16	B3	Small, ovoidal, smooth white	G+ve many chained structure
17	B4	Small, ovoidal and white	G+ve Cocci, chained and scattered
18	B5	Yellow, large, irregular, circular	G+ve Bacilli, Scattered form
19	B6	Yellow, large, regular, ovoid	G-ve, Cocci, dense clustered
20	B7	White, large, irregular, circular	G-ve, Bacilli, scattered form

Ys: Yoghurt sample**; St:** *S.* *thermophilus* sample**; B:** Sample of Bottle; +ve: Positive for *S. thermophilus*; -ve: Positive for *S. thermophilus*

4.3. Biochemical Characterization of *S. thermophilus*

Result of biochemical characterization and confirmation of *S. thermophilus* cultures were shown in (Table 6). The motility test indicated that out of total sample 30% were motile and 70% were non motile as indicated on supplementary figure 1). Accordingly the biochemical and enzymatic test results was shown on (Table 6).

Table 7 The biochemical characterization result for *S. thermophilus* isolates

No	Samples	Types of Biochemical and Physical Characterization							
		Oxidase Test	TSI	Indole Test	Motility Test	Catalase Test	MR	VP	Citrate Test
1	Ys1	-ve	+ve	-ve	Non motile	-ve	+ve	-ve	-ve
2	Ys2	-ve	+ve	-ve	Non motile	-ve	+ve	-ve	-ve
3	Ys3	-ve	+ve	-ve	Non motile	-ve	+ve	-ve	-ve
4	Ys4	-ve	+ve	-ve	Non motile	-ve	+ve	-ve	-ve
5	Ys5	-ve	+ve	-ve	Non motile	-ve	+ve	-ve	-ve
6	St1	-ve	+ve	-ve	Non-motile	-ve	+ve	-ve	-ve
7	St2	-ve	+ve	-ve	Non-motile	-ve	+ve	-ve	-ve
8	St3	-ve	+ve	-ve	Non-motile	-ve	+ve	-ve	-ve
9	St4	-ve	+ve	-ve	Non motile	-ve	+ve	-ve	-ve
10	St5*	-ve	+ve	-ve	Motile	-ve	-ve	-ve	+ve
11	St6*	-ve	-ve	-ve	Motile	-ve	-ve	-ve	-ve
12	St7*	+ve	-ve	-ve	Motile	+ve	-ve	-ve	-ve
13	St8	-ve	+ve	-ve	Non motile	-ve	+ve	-ve	-ve
14	B1	-ve	+ve	-ve	Non motile	-ve	+ve	-ve	-ve
15	B2	-ve	+ve	-ve	Non motile	-ve	+ve	-ve	-ve
16	B3	-ve	+ve	-ve	Non motile	-ve	+ve	-ve	-ve
17	B4	-ve	+ve	-ve	Non motile	-ve	+ve	-ve	-ve
18	B5*	+ve	-ve	-ve	Motile	-ve	+ve	+ve	-ve
19	B6*	-ve	+ve	-ve	Motile	+ve	-ve	-ve	-ve
20	B7*	+ve	-ve	-ve	Motile	-ve	-ve	-ve	+ve

Ys: Yeast sample; B: Bottle; St; Sample of *S. thermophilus*; *: Failed sample; +ve: Positive for *S. thermophilus*; -ve; Negative for *S. thermophilus*; TSA: Triptose sugar iron test.

4.4. The Molecular Detection of *S. thermophilus*

The isolates of suspected *S. thermiphilus* were further analyzed by amplifying the 16S rRNA gene. The PCR product showed that samples coded as St1, Ys4, B2, and St8 were amplified and gave around 250bp of DNA amplifies. Lanes 1-4: were DNA indicated that amplicons of *S. thermophilus* isolates for each St1, St8, B2, and Ys4 samples (Figure 7 and 8).

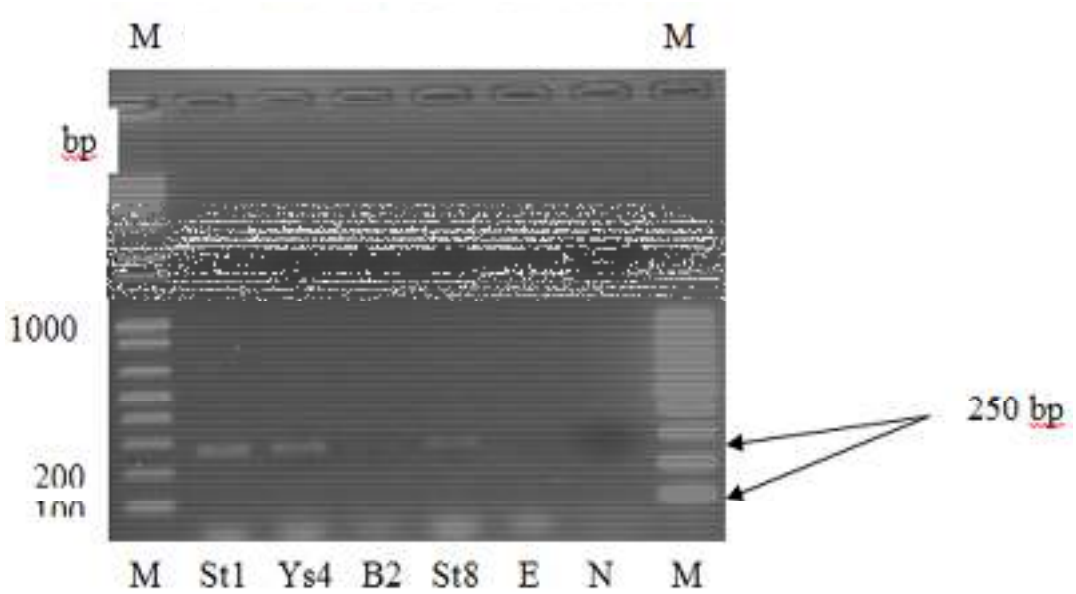


Figure 7a. The PCR products of *S. thermphilus* isolates with ThI/THII primers and it's maximum amplicon size 250bp.

Bp: Base pair; M: Molecular marker (BioLabs, Inc, New England). E: Extraction control; N: Negative control; St1, St8, Ys4, B2 (Lane 1-4) were sample code and its amplicon size.

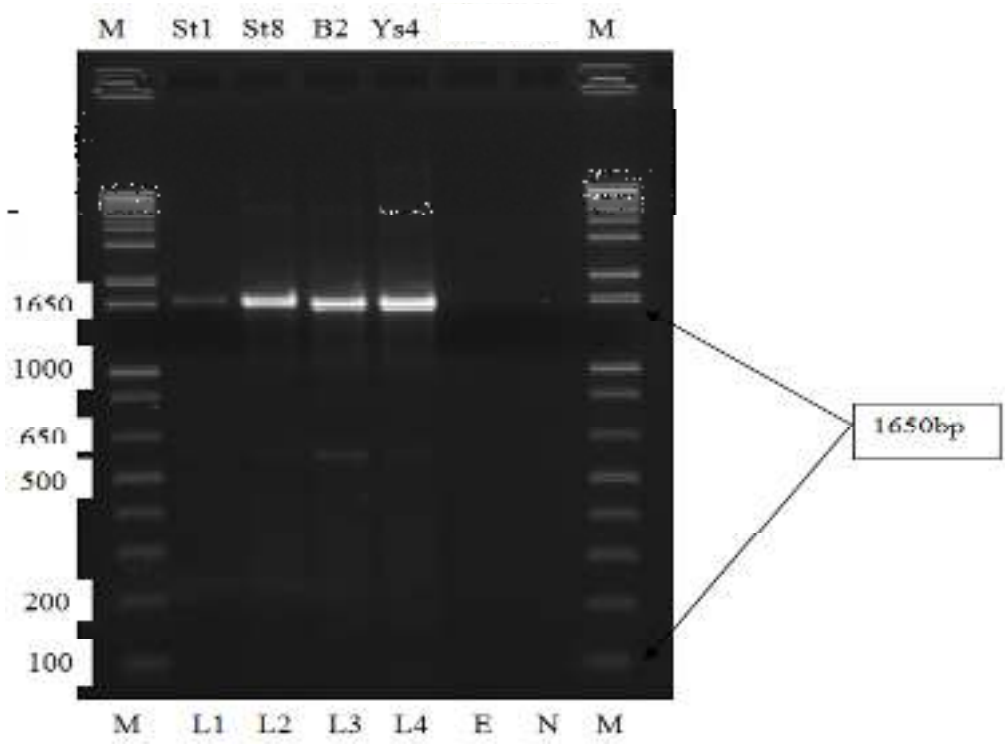


Figure 7b Agarose gel electrophoresis and the products of DNA obtained from four *S. thermophilus* isolates by using pA/pH primers. Bp: Base pair; Lane M: DNA marker used as a reference standard (BioLabs, Inc, New England). Lane E: Extraction control; Lane N: Negative control. St1, St8, Ys4, B2 (Lane 1-4) were sample code and its amplicon size.

4.5. Antibiotic Activity Test of *S. thermophilus*

Antibiotic activity of the isolated *S. thermophilus* revealed its antibiotic activity against some food born pathogenic microorganisms (Figure 8). *S. thermophilus* extracts showed the highest zone of inhibition against *S. aurues* than *C. difficile* and *E. coli* while *E. coli* was the least inhibited (Figure 9).

C. difficile *S. aurues* *E .coli*

Figures 8. Inhibition of *S. thermophilus* against food born pathogenic microbes.

The study of antibiotic activity of *S. thermophilus* was conducted three times and its mean was taken for both measures under cell suspension and cell free extracts (Table 7). The standard deviation was also measured for each *S. thermophilus* isolates.

Table 8. Antibiotic activity of *S. thermophilus* after 32 hours of incubation.

No	**Source of Samples**	**Inhibition of *S. thermophilus* in mm**					
		Suspension of cells in M948 broth			Cell free extract		
		C. difficile	*E. coli*	*S.aurues*	*C. difficile*	*E. coli*	*S. aurus*
1	Ys1	9.0±0.12	5±0.32	9.7±0.20	8.8±0.1	4.7±0.12	9.5±0.21
2	St8	8.2±0.42	5.35.3±0	9.4±0.21	8.7±0.15	5.1±0.11	9.4±0.12
3	B2	8.6±0.20	5.7±0.42	8.5±0.11	7.9±0.12	4.5±0.42	7.8±0.15
	Mean av.	8.6±0.25	.64±0.37	9.2±0.17	8.5±0.12	4.8±0.21	8.9±0.16

NB: - This pathogenic of species of *S. aurues, E.coli, C. defficile* was collected from National Veterinary Institution (NVI) bacterial store laboratory.

During this antibiotic activity detection, *S. thermophilus* isolates showed different size of zone of inhibition to ward different pathogenic microorganisms. For instance, the mean value of overtime increasing zone of inhibition of *S. thermophilus* against *S. aurues* was the highest among *E. coli* and *C. difficile* (Figure 10a). On the other hand, this study showed that the mean value of zone of inhibitions in cell suspension was more effective than of cell free extracts (Figure 10a and 10b).

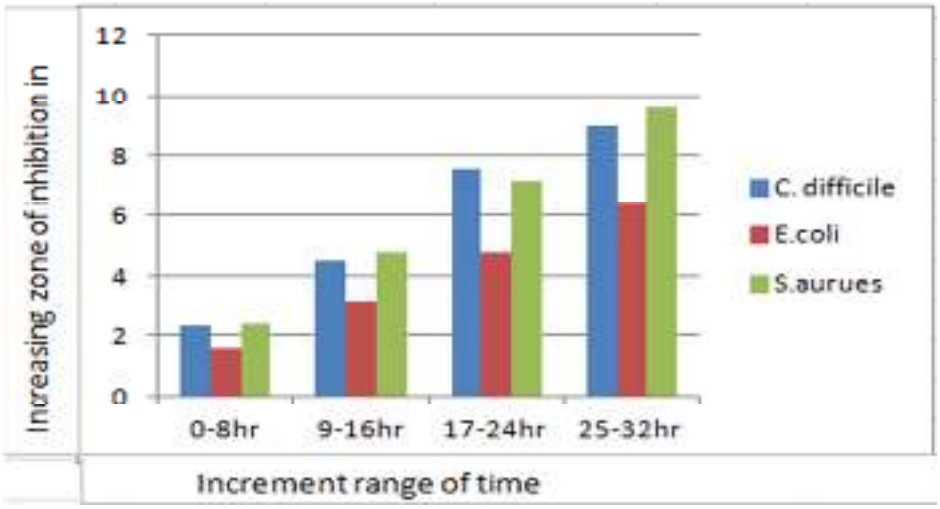


Figure 10a Zone of inhibition of *S. thermophilus* isolates against food born pathogenic microorganism using cellular suspension.

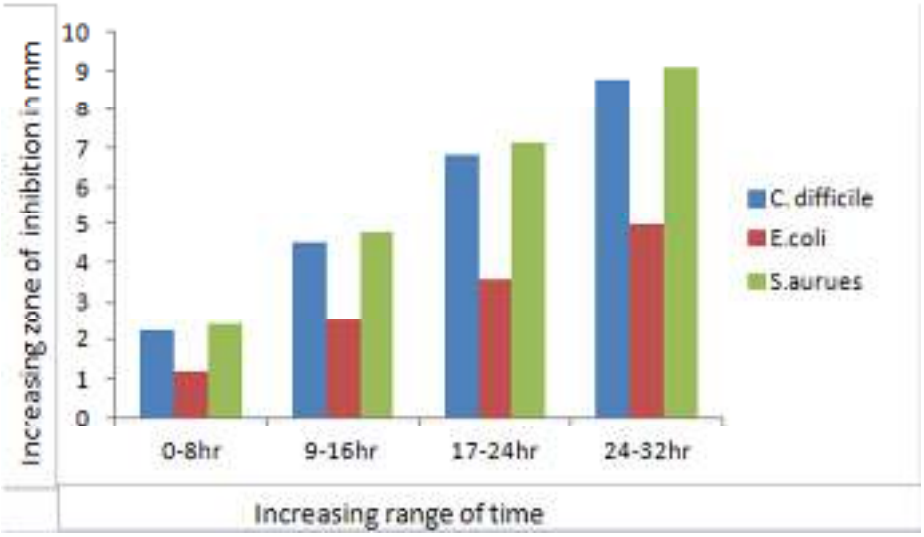


Figure 10b Zone of inhibition of *S. thermophilus* isolate against food born pathogenic microbes of using cellular free biochemical extracts.

5. DISCUSSION

5.1. Isolation and Characterization of *S. thermophilus*

Streptococcus thermophilus is one of the most economically important LAB and its rates of milk acidification are of major technological importance. Moreover, the morphological and cultural study from M948 agar showed characteristic small, ovoid shaped smooth and chain form of *S. thermophilus*. In the same way, Alquqa, (2018) reported using the morphology of the isolates coming from M17 media as of *S. thermophilus*. According to Erkus, (2007) showed, the *S. thermophilus* isolates appeared mostly as paired or chained form while Sameen *et al*. (2010) reported as, a Gram stain of *S. thermophillus* gave blue-purple color with staining.

The present isolates of S. *thermophilus* were for their capability to produce oxidase and catalase. Similarly, the report of Akpinar et al. (2011) revealed that, the enzymatic tests such as catalase and starch hydrolysis were negative for *S. thermophilus* strains coded as S1, S2, S3, and S4. Each species of the present isolates of *S. thermophilus* had given negative result for each these enzymatic tests. This was occurred because of inability of *S. thermophilus* to produce either oxidase or catalase enzyme.

The current biochemical characterization of *S. thermophilus* isolates for their non motile, positive for methyl red tests; negative for Vogues Proskauer tests; positive for triptose sugar tests; negative for indole tests were similar with the findings of other researchers (Karki, 2018; Acharya, 2014; Lynn *et al.,* 2014; Pradhan, 2013; Sameen *et al.,* 2010).

Under this study, expected size 250 bp of polymerase chain reaction products of amplified DNA of *S. thermophilus* strains was obtained with primer set ThI/ThII (ThI: 5'-ACGGAATGTACTTGAGTTTC-3'; ThII: 5'-TGGCCTTTCGACCTAAC-3'). Vanatkova *et al.,* (2009) also reported similar size of *S. thermophilus* strain DNA amplicon by using these primers and techniques. Other similar report of equal amount of DNA amplifies have been done by (Brigidi *et al.,* 2003).

Accordingly with other set of universal primers PA/pH (pA: 5'-AGAGTTTGATCCTGGCTCAG-3' and pH' 5'AAGGAGGTGATCCAGCCGCA') around 1650bp DNA amplicon of targeted 16S rRNA gene was gained for the second round amplification and confirmation of *S. thermophilus* isolates. In this PCR prodct, no significant common bands were found on the samples of negative control band (L5) and extraction control band (L6). These results along with selective medium of *S. thermophilus* could avoid ambiguity during isolation and genomic DNA extraction. This study was the first time in which strains of *S. thermophilus* isolated from fermented milk amplified with pA/pH set of primers. Due to lose of availability of data, the comparison for this study was not made in detail. On the other hand, the report of Maheswari, (2010) showed that, the molecular characterization by species specific lacZ PCR primers has been used by many labs for differentiation of *S. thermophilus* strains from other *Streptococcus spp* such as *S. mutans* and *S. salivarius*; and other LAB such as *L. delbrueckii* subspecies *bulgaricus*, *L. acidophilus, L. brevis, L. fermentum, L. helveticus, L. plantarum and Enterococcus ssp* (Maheswari, 2010).

5.2. Antibiotic Activity Evaluation of *S. thermophilus* and its Extracts

Within this study, the antibacterial activity of *S. thermophilus* was tested against food born pathogenic bacteria namely *C. difficile, E. coli* and *S. aureus*. Accordingly, *S. thermophilus* could inhibit these disease causing microorganisms in both cell free extracted supernatant and the cell suspension. The result on this figures indicated that, this antagonism against selected food born pathogen was occurred due to capability their antimicrobial chemicals. Similarly, the report of Fadela *et al*. (2008) and Suskovi *et al*. (2010) showed that, LAB can produce antimicrobial substances such as lactic acid, acetic acid, formic acid, phenyl lactic acid, caproic acid, organic acids, ethanol, hydrogen peroxide, diacetyl, bacteriocins, reuterin, reutericyclin and bactericidal proteins. They also noted that, this biochemical have a capacity to inhibit and antagonize the growth of pathogenic microorganisms like *L. monocytogens* and *C. species.*

In this study, the of zone of inhibition against time increment was also measured at every eight hours interval for 32 hours with the parameter of cell suspension antibiotic activity detection. Results indicated under this study showed that, *S. thermophilus* had the highest

antibacterial activity against *S. aurues* than *C. difficile* and *E. coli*. According to report of Akpinar et al., (2011), *L. bulgaricus* and *S. thermophilus* isolated from fermented milk appear to be the eliminator against colonization of pathogenic bacteria and the treatment of gastrointestinal tract infections including those sustained by *C. difficile* and *H. pylori.* Mezaini and Bouras (2013) showed that, some of *S. thermophilus* strains produce a bacteriocin named as *thermophilin* which is active against several food spoilage bacteria such as *C. sporogenes.*

In this study, the highest zone of inhibition by *S. thermophilus* was recorded over each pathogenic microorganism with the mean and standard deviation value of 9.7±0.20 mm for *S. aurues*, 9.0±0.12 for *Clostridium spp* as well as 5.7±0.42mm for *E. coli* as indicated on the table above. In contrast, some other report indicated that *S. thermophilus T2* did not show any inhibitory zone activity against the gram negative bacteria like *E. coli* and *S. typhimurium* (Mezaini *et al.,* 2009). This deference was occurred because he used single strain of *S. thermophilus* not for all general strains. Different strains produce different chemical substances that have cumulative effect for inhibition activity against food borne pathogens.

According to current finding, antimicrobial activity of *S. thermophilus* was higher in the cell suspension broth of M948 than cell free extracts after overnight incubation. Thus there was a significant variation in case on zone of inhibition between *S. aureus* and *C. difficile* which were both gram positive than that of gram negative of *E. coli.* This result showed that Graham positive *S. thermophilus* can inhibit other graham positive bacteria than graham negative E. coli. Other investigator's studies have revealed that *S. thermophilus T2* strain showed the wide inhibitory spectrum against the Gram positive bacteria (Mezaini *et al.,* 2009).

As a whole, the result of this study revealed as *S. thermophilus* have antagonistic activity against selected food born pathogenic microorganism such as *S. aurues*, *C. difficile* and *E. coli* in which further study will be required to produce deferent biochemicals used for food bio-preservatives, bio-surfactants and recombinant vaccine production. Some of *S. thermophilus* strains which were isolated from home made yoghurts had shown antimicrobial activity against some food borne pathogens and spoilage microorganisms

especially *L. monocytogenes, E. coli, K. pneumonie* (Akpinar *et al.,* 2011). This antibiotic activity against food borne pathogens enabled *S. thermophilus* to be used as biopreservatives in dairy industry (Suskovi *et al.,* 2010). Accordingly this probiotic microorganism can be used in other industrial area to produce biosurfactants, bacteriocine, biotherapeutic, antibiotic and recombinant vaccine production due to its antibacterial activity (Fadela *et al.,* 2008).

6. CONCLUSION AND RECOMMENDATION

The isolation of dairy adjuncts culture *S. thermophilus* on M948 was followed by biochemical, molecular and antibiotic property detection revealed the presence of *S. thermophilus* in traditional dairy industry in Ethiopia. It was confirmed where 20 of the 14 were identified as *Streptococci* isolates 14 were identified as *S. thermophilus*. All representative isolates that were subjected for their antibiotic property test revealed that, *S. thermophilus* has the highest antimicrobial activity against *Clostridium* spp and *S. aurues.* But, for *E. coli* it was low. This implies that Graham +ve *S. thermophilus* could also inhibit other Graham +ve *microbials than gram negatives.*

Identification, characterization and antimicrobial activity test of *S. thermophilus* is not only enough steps for the use of these adjuncts microorganism in dairy industry. Therefore, it should also be screened for their technological parameters. For yoghurt starters, these technological parameters mainly includes acidifying activity, production of aroma compounds especially acetaldehyde and diacetyl, exopolysaccharide production, and bacteriophage resistance. Determination of such characteristics would be helpful for dairy industry applications and biochemical productions in the future. Moreover they need to be test against other food borne pathogens, spoilage microbes and contaminants. The standardization of the antimicrobial extracts for human health and its industrialization of this isolates should be recommended.

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Appendixe

Biochemical Characterization Test Results

Motility Test **TSI Test** **VP Test**

Figure 1 **Figure** 2 **Figure** 3

On motility test: The first red on the left was control. The 4 tubes in the middle were non motile species of *S. thermophilus*. The last one was failed to be the target species.

On TSI test: The all yellow color represents the triptose sugar ion test of *S. thermophilus*. The presence of yellow/yellow for acidic slant/acidic butt (A/A) of TSI test *S. thermophilus* revealed as it was TSI positive.

On VP test: The image above shows the result of VP Test which was–ve result for *S. thermophilus*. If it were positive, it would have had pink/red color.

MR Test **Oxidase Test**

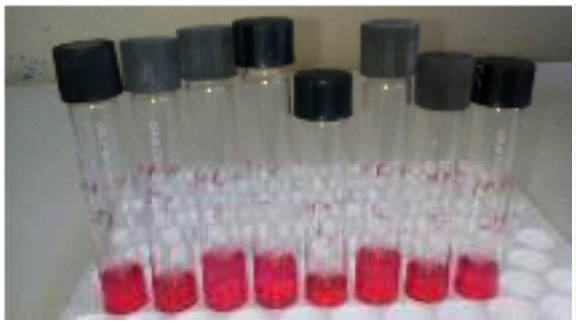

Figure 4 **Figure** 5

This figure on the left was an indicator of methyl red test result for *S. thermophilus*. All test tubes were pink-reddish colored which was one of the main characters of *S. thermophillus* to be MR +ve (Figure 4)**.** But the one right showed the result of isolates of *S. thermophilus* subjected to Oxidase reaction which was negative (Figure 5).