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Formulation and Evaluation of Bio-Protective Probiotic Starter Culture for the Production of *Ergo* and *Ayib*

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Abstract

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Probiotics are live microorganisms that confer a beneficial effect on the host when administered in enough amounts. They have the ability to modulate the intestinal biome of the consumer. The aim of this study was to formulate bio-protective probiotic starter culture, evaluating their antagonistic effect against some food borne pathogens such as *E. coli*, *S. aureus*, *S.* Typhimurium and *L. monocytogens*. The study also aimed at evaluating the impact of the formulated starter cultures on the sensory acceptance and shelf life of *ergo* and *ayib*. Accordingly, seven lactic acid bacteria and one yeast isolates were screened among previously isolated 243 lactic acid bacteria and 26 yeast isolates. The selected isolates were combined in different proportions to formulate starter cultures for the production of *ergo* and *ayib.* Nine formulations were made in different proportions based on the compatibility of the isolates. *Ergo* prepared in this study was shown to have pH of 3.99 - 4.38, titratable acidity of 0.60 - 0.97 at 48 hrs of fermentation, total solid of 15.97 - 17.87%, protein 3.19 - 4.28%, fat 4.16 - 5.50%, ash of 0.60 - 0.98%, solid nonfat of 11.63 - 13.43%, lactose 2.49 - 5.07% and syneresis (whey) content of 9.46- 10.58%. The formulate F2 and F6 showed inhibition to all the test pathogens completely at 48 hrs of fermentation except *S. aureus* in F6. The *ergo* products F2, F4, F6, and *ayib* product F2(AY2) were found to have better overall sensory acceptability with mean score of 3.75, 3.56, 3.73 and 4.03/5.00, respectively. *Ergo* products F2 and F6 and *ayib* products F2(AY2) were found to have shelf life of more than 15 days and 18 days, respectively, at ambient temperature of 18-24^{o}C. The use of this formulated starter culture for homemade production of *ergo* and *ayib* could improve the quality and organoleptic consistency of the final product as well as safety by preserving typical sensory quality of the traditional fermented product and inhibiting the growth of undesirable microorganisms.

Keywords: *Ayib*; Bio-protective; *Ergo*; Shelf life; Starter Culture

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List of Abbreviations and Acronyms

A_o	Absorption at 0 hours
ANOVA	Analysis of Variance
AOAC	Association of Official Analytical Chemist
ATCC	American Type Culture Collection
BPS	Buffered Phosphate Saline
BS	Bile Salt
BSH	Bile salt Hydrolase
CFU	Colony Forming Units
EPS	Exopolysaccharide
FAO	Food and Agricultural Organization
GRAS	Generally Regarded as Safe
H%	Percent of Hydrophobicity
H_2O_2	Hydrogen Peroxide
LAB	Lactic Acid Bacteria
Ml	Milliliter
MRS	de-Man Rogosa and Sharpe agar
MYPG	Malt Extract-Yeast Extract-Peptone-Glucose Agar
OD	Optical Density
TA	Titratable acidity
TTA	Total Titratable Acidity
WHO	World Health Organization
XLDA	Xylose Lysine Desoxycholate Agar

1. Introduction

Milk is one of the major livestock products; it is produced under urban, peri urban and pastoral dairy system in Ethiopia. Due to its short shelf life, it is consumed immediately or processed in to shelf stable products. Milk fermentation and processing is the oldest traditional practices in Ethiopia (O'Conner, 1995; Redda, 2001). Milk is fermented into different milk products in different part of the world in order to improve the organoleptic characteristics, shelf life and safety of the products (Akaband, 2010a).

Traditional fermentation is also called spontaneous fermentation which is usually natural, with no defined starter cultures used to initiate it. Raw milk is either left at ambient temperatures or kept in a warmer place to ferment; no attempt is made to control the fermentation. In some cases, the fresh milk is inoculated with part of previous fermented batch. This traditional method is still used in some part of the societies around the world. Currently, most dairy industries use modern techniques of milk fermentation process by inoculating pasteurized milk with defined starter cultures of known characteristics for the production of consistent fermented milk products. The fresh milk was pasteurized and then inoculated with defined starter culture for the production of physco-chemically consistent product (Mogessie Ashenafi, 2006).

The current as well as the ancient societies has been using traditional fermentation process for many years for processing of yoghurt and cheese. Lactic acid bacteria play a key role in food fermentations, where they contribute to the development of the desired sensory properties in the final product as well as to ensure the microbiological safety of the final product (Grazina *et al*., 2012). Fermented milk products also serve as the delivery vehicles for probiotic bacteria which have a long history of association with dairy products because the same bacteria that are

associated with fermented dairy products are normal microflora of human gastrointestinal tract (Parmjit, 2011).

In Ethiopia 98% of milk is produced in rural areas by subsistence farmers (Reda, 1998), and dairy processing is dominantly at household level. The unhygienic milk production, processing and absence of starter cultures resulted in *ergo* of variable characteristics, short shelf-life and higher spoilage risks due to the wide variety of microorganisms in the product (Mahari Tesfeye and Abegaz Gashe, 1990; Almaz Gonfa *et al*., 1991). To scale up the production of *ergo* at large scale, it has to be started with isolating and evaluating as many lactic acid bacteria as possible from traditionally produced products from the various ecological zones of the country. These cultures have to be identified and various combinations should be tested under controlled fermentation. The organoleptic quality, physicochemical, microbial safety and keeping quality of the product in relation to various starter combinations should be evaluated. Those combinations having favorable organoleptic property may then be tested for further characteristics before they are used for large scale production. It may then be possible to define an *ergo* brand in terms of its microbiology and biochemistry.

Yoghurt is one of the common product of lactic acid fermentation of milk made by addition of a defined starter culture (Steinkraus, 1997; Tamine and Robinson, 2007). The origin of yoghurt was in Eastern Europe, but it is now widely consumed throughout the world, mainly in northern European countries, Middle East and Indian sub-continents and Africa. The popularity of fermented milk is attributed to their attractive test as well as their extended shelf life and inhibition of pathogenic microorganisms (Tamine, 2002).

Ergo is the most common homemade traditionally fermented milk in Ethiopia. It is analogues to the commercial yoghurt, can be consumed as it is, or can be further processed into other dairy

products, notably *ayib* (cottage cheese), whey and butter (Coppock *et al.*, 1995). The fermentation process of *ergo* takes place as a result of the activities of natural microbial flora present in the milk or those introduced from the surrounding. During fermentation of *ergo*, the raw milk is not pasteurized, and the fresh milk is simply kept at ambient or warmer temperature for 24 hrs. (Mogessie Ashenafi, 2002). This makes the microbial safety of the product to be in doubt (Mogessie Ashenafi, 2006).

Unhygienic milk production and absence of starter cultures result in *ergo* with variable organoleptic characteristics, non-reproducible, short shelf life and higher spoilage risks due to the wide variety of microorganisms in the product (Mahari Tadese and Belay Gashe, 1990; Almaz Gonfa *et al.,* 1991). Since *ergo* is fermented spontaneously at home without a defined starter culture, their consistency and flavors vary within or among households (Mogessie Ashenafi, 1996).

Ayib is a traditional Ethiopian cottage cheese made from sour milk after the fat is removed by churning and produced by slowly heating naturally soured milk until a discrete curd mass develops and floats over the whey. *Ayib* is a well-known popular milk products widely consumed by various ethnic group in different part of the country (Anteneh *et al*., 2011). It may be consumed as fresh as side dish or spiced with hot spices. *Ergo* and *ayib* are the major homemade fermented milk products produced in Ethiopia by smallholder farmers using traditional methods (Almaz Gonfa *et al.*, 2001). According to Yigrem Seleshi and Welearegay Habteyesus (2015), among the main problems related to processing of *ergo* in most of the *ergo* shops of Hawassa City, South Ethiopia, were slow fermentation, excessive whey production and low quality of the raw milk supplied.

It is often necessary to carry out controlled fermentation with specific organisms and this has led to the selection of microbial cultures, usually referred to as starter cultures. The principal organisms used as starter for milk fermentation include species of lactic acid bacteria either in pure or mixed cultures. The proper selection and balance of starter culture are critical for the production of fermented milk products of desirable texture and flavor. The quality and reproducibility of fermented milks and processes are ensured by using defined starter cultures (Wouters *et al*., 2002).

Mixed cultures are able to bring about multistep transformations that would be impossible for a single microorganism. In a mixed culture one microorganism may produce needed growth factors or essential growth compounds such as carbon or nitrogen sources beneficial to a second microorganism. It may alter the pH of the medium, thereby improving the activity of one or more enzymes. Even the temperature may be elevated and promote growth of a second microbe (Clifford *et al*., 2014). Driessen (1981) demonstrated that when these species were grown separately, 24 mmol and 20 mmol, respectively, of acid were produced; together, with the same amount of inoculum, a yield of 74 mmol was obtained. The number of *S. thermophilus* cells increased from 5×10^8 cfu/ml to 8.8×10^8 cfu /ml with *L. bulgaricus*. Therefore, this study was intended to formulate bio-protective probiotic starter cultures of LAB and yeast for the production of *ergo* and *ayib*; evaluating their antagonistic effect on some food borne pathogens; their sensory attributes for consumer acceptability and their effect in extending the shelf life of the product stored at ambient temperature.

1.1. Statement of the problem

During the manufacturing process of industrial/commercial fermented dairy products, such as yogurt, pasteurization of the milk is a prerequisite step before inoculation of the milk with fermentative starter cultures. This process destroys contaminant microorganisms including pathogenic and spoilage organisms in the raw milk (Tamim, 2006), and makes the fermented product safer for consumers. In addition to the absence of defined starter culture in Ethiopian traditional production process of fermented milk products, there is no practice of pasteurization of milk prior to spontaneous fermentation of milk, as this delays the spontaneous fermentation process (Sintayehu Yigrem and Haile Welearegay, 2015). Therefore, microbial safety of traditionally produced *ergo* is in doubt due to the use of poor quality of raw milk for processing, absence of formal and regular quality control system in monitoring and controlling the quality of milk produced in the country (Gurmessa Terfa, 2015).

Zelalem Yilma *et al*. (2007) reported the existence of *Entrobacteriaceae* in Ethiopian traditionally fermented milk. Kidist Fikere *et al*. (2015 also reported the existence of fecal coliform, *S. aureus, Salmonella spp*. and *Shigella* in traditionally fermented *ergo.* According to Mogessie Ashenafi (1992; 1993) food borne pathogens of *Salmonella spp*., *B. cereus*, *S. aureus*, and *L. monoctyogenes* are found in 12 and 24 hrs fermented milk. A study by Sintayehu Yigrem and Haile Welearegay (2015) on *ergo*–samples collected from Hawassa City, South Ethiopia, showed that the mean counts of *S. aureus* and Coliform were 5.56 and 5.55 log cfu/ml, respectively. Therefore, in order to reduce the risk of food borne pathogens, due attention should be given to the health and safety of consumer through production of safe *ergo* and other fermented dairy products.

Kidist Fikere *et al.* (2015) reported the count of Salmonella 1.5 log cfu/ml, *S. aureus* 4.0 log cfu/ml, coliform of 6.4 log cfu/ml and fecal coliform of 4 log cfu/ml in homemade *ergo* and salmonella, coliform and fecal coliform were not detected in commercial yoghurt. However commercial yoghurt has *S. aureus* count of 0.7 - 3.7 log cfu/ml. This implies that homemade *ergo* has low microbial quality than commercial yoghurt and no safe in human health perspective.

Different chemical preservatives of salt, benzoic acid and different spices have been used to extend the shelf life and safety of cheese by inhibiting the growth of spoilage and pathogenic microbes, but it is not organic and not the interest of consumers (Neeraj, 2007). Hence, an alternative and safe method of bio-preservation is a potential preservation substitute (Ross *et al*., 2002; Carocho *et al.,* 2015). Generally, bio-preservation of fermented products can be materialized by formulating and developing starter culture with bio-preservative qualities. During the process of developing starter cultures, cultures are screened not only for their fermentation capability but also for their bio-protective as well as probiotic qualities. The development of starter culture with a bio-preservative and probiotic qualities in addition to fermentation capability has enabled one to produce bio-preserved and safe products. This will lay down the basic foundation for the preparation of consistent, reproducible, standard products and more importantly will pave the way for industrialization of the products.

1.2. Objectives

1.2.1. General objective

The general objective of this study was to formulate and evaluate bio-protective probiotic starter culture isolated from traditionally fermented *ergo* and *kocho* for the production of safe and shelf stable standard *ergo* and *ayib.*

1.2.2. Specific objectives

Specific objectives of this study were:

- Evaluate LAB and yeast isolates based on their probiotic attributes and antagonistic effect on some food borne pathogens.
- Formulate bio-protective probiotic starter cultures of lactic acid bacteria and yeasts for production of *ergo* and *ayib.*
- Optimization of raw material (raw milk, handling and processing), the fermentation process (temperature) and length of fermentation.
- Evaluate the antagonistic effect of the formulates on some food borne pathogens.
- Evaluate the physco-chemical, sensorial quality and shelf life of *ergo* and *ayib* made with the formulated starter culture.

1.3. Significance of the Study

This study is significant for providing defined starter cultures for the production of local fermented milk products, *ergo* and *ayib*. It is also significant for producing *ergo* and *ayib* with extended shelf life, organoleptically stable and reproducible. *Ergo* and *ayib* produced by bio-protective probiotic starter culture contain live probiotic microorganisms which has health benefiting. The development of bio-protective probiotic starter culture provide a potential data and initial steps for the scale up of the production of safe and standard *ergo* and *ayib* to the future industrial scale production.

2. Literature review

Fermentation

Fermentation is the breakdown of carbohydrate under either anaerobic or aerobic conditions. In dairy product fermentation is the conversion of lactose to lactic acid by lactic acid bacteria or lactose fermenting microorganisms. Most common fermenting microorganisms in dairy products are *L. delbrueckii* subsp. *bulgaricus, L. acidophilus, L. fermentum, L. helveticus, L. lactis, L. paracasei* and *S. thermphillus.* Fermentation with these microorganisms leads to improvements in texture, flavor, nutritional value, shelf life and acceptability of food products (Marcelino, 2013). The process promotes the development of essential and safe microflora, which play crucial role in preventing the outgrowth of spoilage microorganisms and food borne pathogens (Gibbs, 1987). Historically, fermentation process involved unpredictable and slow souring of milk caused by the activities of natural flora present in the food or added from the surroundings (Panesar, 2010). Fermented foods are defined as products obtained through desirable biochemical changes caused by the action of microorganisms or their enzymes. They are produced using microorganisms from various raw materials and though different manufacturing techniques. Most fermented foods are dependent on lactic acid bacteria (LAB) and yeasts to mediate the fermentation process.

Lactic acid bacteria are divided into two physiological groups based on their fermentation pathway. The homolactics produce mainly lactic acid as an end product of fermentation of glucose and heterolactics these produce other end-product besides lactic acid (Jay *et al.*, 2005). The lactobacilli are the largest group of LAB that contain more than 145 species. They are facultative hetero fermenters, obligate hetero fermenters, or obligate homo fermenters, and have complex nutritional requirements (Thiago *et al*., 2015).

2.1. Ethiopian traditional fermented dairy products

In Ethiopia, a large proportion of fermented milk is consumed. The major fermented milk products produced in Ethiopia by smallholder farmers through traditional methods are *ergo* (naturally fermented milk*), ayib* (Ethiopian cottage cheese), *Kibe* (traditional butter) and *Arera* (defatted sour milk). Among the fermented products, *ergo* and *ayib* are the most common dairy product consumed in the country (Mogessie Ashenafi, 2006).

2.2. *Ergo* (naturally fermented milk)

In Ethiopia, milk processing is based on fermenting milk mainly at ambient temperature, due to consumer's preference and increased keeping quality of sour milk (O'Mahony, 1988). *Ergo* is a naturally processed, indigenous Ethiopian fermented dairy product, which is commonly prepared at household level and the fermentation of this product is dominated by LAB (Almaz Gonfa *et al.*, 1999; Mogessie Ashenafi, 2002). According to Mogessie Ashenafi (1996), *ergo* is a product obtained by spontaneous fermentation and cannot be defined in terms of its microbiological or biochemical properties. Its processing does not have defined temperature and duration of incubation. Fermentation is carried out at ambient temperatures and precipitation of casein is usually the sign of completion of fermentation. Consistency and flavor of *ergo* varies within or among households. In most cases, household preparation of *ergo* requires one-day incubation at ambient temperature. The milk coagulates within 24 hrs and usually consumed preferably at this time due to its good flavor. Longer keeping of the fermented milk is not desirable because further drop in pH and souring will results in increased wheying off, which, in turn results in loss of protein as whey (Mogessie Ashenafi, 2006).

Researchers have tried to isolate and study starter cultures from naturally fermented dairy products. Among the bacteria, the most dominant group responsible for fermentation are the lactic acid bacteria of obligate homo fermentative *L. acidophilus, L delbruki, L. helvaticus,* the obligate hetro fermentative *L brevis, L. buchneri, L. fermentum*, *L. reuteri* and the facultative hetro fermentative *L. casi, L. plantarum, L. lactis* are these naturally accepted as safe for human consumption (Parmjit, 2011). Different strain of starter cultures significantly varies in their production of volatile flavor compounds and metabolites including organic acids, and as a result the sensory properties of the cultured milk also differ (Fekadu Beyene *et al*., 1998). *Ergo* is a naturally fermented milk at ambient temperature, which can be served directly as a refreshment or used as a raw material for the production of shelf stable milk products such as butter and *ayib* (Ethiopian cottage cheese). *Ergo* is churned to produce butter using traditional churns such as clay pot and calabash. The defatted fermented milk, which is obtained as a byproduct of the butter-making process is heated on a low fire of 40-70°C for 60 minutes for the manufacturing of *ayib* (Almaz Gonfa *et al*., 1999).

2.3. *Ayib* (Ethiopian cottage cheese)

Cheese is produced in almost every country of the world and there are more than 2000 varieties, made from milk of several mammals, industrially processed or by traditional methods. However, the basic steps required in any cheese processing are essentially the same, and slight variations in any of these steps may result in products of different quality and types (Marcelino, 2013).

Ayib is a traditional Ethiopian cottage cheese which is produced through slow heating of naturally soured milk until distinct curd mass forms and floats over the whey (Anteneh Tesfeye *et al*., 2011a). It is an important source of nutrients and serves as a staple diet and may be consumed fresh as side dish, or it may be spiced with hot spices, salt and other herb (Almaz

Gonfa *et al*., 2001). For the production of *ayib,* raw milk is collected in a clay pot and kept in a warm place (about 30^{o}C) for 24 to 48 hrs to sour spontaneously. Churning of sour milk is carried out by slowly shaking the contents of the pot until the fat is separated. The fat is then removed and the defatted milk is heated to about 50^{o}C until a distinct curd mass forms and floats over the whey. The temperature can be varied between 40^{o}C and 70^{o}C without markedly affecting product composition and yield, after gradual cooling, the curd is recovered from the whey (O'Mahony, 1988; Mogessie Ashenafi, 2006).

2.4. Microbial quality of *ergo* and *ayib*

Microbial food safety is associated with public health concern and various epidemiological reports indicate that, unpasteurized milk and milk products are the main cause of food-borne illness (Jermen Mamo *et al.,* 2016). In developing countries, the safety of dairy products is of great concern as the production takes place under unsanitary conditions (Mogessie Ashenafi, 1992; Anteneh Tesfaye, 2011a). Study done on *ergo* samples collected from five North Shoa districts by Jermen Mamo *et al*. (2016), showed that the total aerobic plate count, aerobic spore forming, Coliforms*, Staphylococci*, yeasts and molds are in the range of 6 to 9.5 log cfu/ml, 3.4 to 9.4 log cfu/ml, 2.1 to 4.7 log cfu/ml, 2.4 to 5.4 log cfu/ml, and 3.2 to 6.1 log cfu/ml respectively. On the other hand, a study by Sintayehu Yigrem and Haile Welearegay (2015) showed that, *ergo* samples collected from dairy shop in Hawassa city have aerobic mesophilic bacterial count, Coliform count, *Staphylococcus* count and lactic acid bacterial count of 6.85 cfu/ml, 6.14 cfu/ml, 6.13 cfu/ml and 7.19 log cfu/ml respectively. Zelalem Yilma (2012) also reported a mean coliform count of 4.51 log cfu/ml in traditional fermented milk, *ergo.* This indicates that the traditionally processed milk in the market was not safe in human health perspective.

In traditional a*yib* making, the milk itself may have a high initial count of microorganisms and further processing may results high in microbial load in the final product (Berhanu Andualem and Tsehayneh Geremew, 2014). However, cooking of the curd and its low pH is expected to decrease the count of microorganisms into a lower microbial load after heating (Mogessie Ashenafi, 2016). The high microbial load in a*yib* could come from handlers and plant parts used for packaging and for imparting flavor. Study by Mogessie Ashenafi (1995), showed that *ayib* samples collected from an open market around Hwassa region had counts of mesophilic aerobic bacteria, yeasts and enterococci at 10^8, 10^7 and 10^7 cfu/g, respectively. *B. cereus* and *S. aureus* were in the range of 10^2 - 10^3 cfu/g. Tekletsadik Seoyum and Kasahun Tsige (2011) reported that aerobic mesophilic bacteria, enterobacteriaceae, yeasts and mold count of 6.13, 5.96, 5.84 and 5.5 log cfu/g respectively, in *ayib* samples collected from Jimma zone. The aerobic mesophilic bacteria, aerobic spore forming, coliforms, staphylococci and yeasts and molds counts in homemade *ayib* samples collected from five North Showa districts were in the range of 5 to 8 log cfu/g, 0 to 3 log cfu/g, 2.4 to 4.8 log cfu/g, 2.7 to 4.3 log cfu/g and 3.1 to 4 log cfu/g respectively (Jermen Mamo *et al*., 2016). In a study on microbiological safety of *ayib* sold in an open market around Hwassa district, *S. aureus* were found in the samples in the range of 10^2 -10^3 cfu/g (Mogessie Ashenafi, 1990a). This indicates that the traditionally processed *ayib* sold in open market as well as milk product shop of Ethiopia was sub standards of Common market of south and east Africa.

2.5. Starter cultures

Traditional fermentations are taking place as a result of the activities of natural flora present in the food or added from the surroundings. Former scientists have tried to isolate and study the

characters of such desirable organisms. Among these organisms, the most important dominant groups responsible for fermentation are the lactic acid bacteria and yeasts (Panesar, 2010).

Currently, most dairy industries use starter cultures for rapid fermentation, because relatively small quantities of the starter cultures in raw materials can ferment the products (Wouters *et al*., 2002). Modern production of most fermented dairy foods depend entirely on the use of defined-strain starters, which replaced the natural or traditional fermentation methods that have been used for the manufacture of the products (Ross *et al.*, 2002). To achieve the required quality attributes such as texture, aroma and flavor, food industries widely use lactic acid bacteria as starter culture (Leory and Vuyst, 2004).

Dairy starter cultures are carefully selected microorganisms, which are deliberately added to milk to initiate and carry out fermentation under controlled conditions for the production of fermented milk products. Most of them belong to lactic acid bacteria and in some cases, few non-lactic starter bacteria and yeasts are also used along with lactic acid bacteria during manufacturing of specific fermented milk products (Gandhi, 2006). The starter culture, in addition to reducing the pH of milk, they produce different antimicrobial compounds such as hydrogen peroxide, carbon dioxide, acetaldehyde, diacetyl and bacteriocins.

2.6. Probiotics

Probiotics are live microorganisms that confer a beneficial effect on the host when administered in enough amounts (Kalliomaki *et al*., 2001). FAO/WHO (2001) describes the beneficial effects of food with added live microbes on human health, and in particular, of milk as health professionals are increasingly promoting these products on children and other high-risk populations. Nobel laureate Elie Metchnikoff, (father of probiotics) proposed that consuming

beneficial microorganisms could improve consumer's health and he introduced the concepts of probiotics in the early 20th century. He investigated this idea, and the term probiotics meaning "for life" finally came into use (Sanders *et al*., 2013). Currently, it is used to name microorganisms associated with beneficial effects for humans and animals. Probiotics may contain a variety of microorganisms; the most common are bacteria that belong to groups of *Lactobacillus* and *Bifidobacterium*. In addition to these two groups, many other types of bacteria such as *E. faecium, L. lactis, P. freuenreichii, P. jensenii,* and yeasts such as *S. boulardii, S. cerevisiae* are incorporated (Chaminda *et al*., 2017).

Microorganisms considered to be probiotic have to survive passage through the stomach and maintain their viability and metabolic activities in the intestine (Emiliane *et al.,* 2012). In addition, it should not only be capable of surviving passage through the digestive tract but also have the capability to proliferate in the gut. This means they must be resistant to gastric juices and be able to grow in the presence of bile salt and alkaline conditions in the intestines, or be consumed in a food vehicle that allows them to survive passage through the stomach and exposure to bile (FAO/WHO, 2001).

Probiotic microorganisms are important for (i) improving intestinal health by the regulation of micro biota, (ii) stimulation and development of the immune system, (iii) synthesizing and enhancing the bioavailability of nutrients, reducing symptoms of lactose intolerance, (iv) reducing the risk of certain other diseases and (v) provision of special therapeutic or prophylactic properties as reducing cancer and control of serum cholesterol levels (Kumar *et al*., 2009a). The primary clinical interest in the application of probiotics has been in the prevention and treatment of GI infections and diseases (Parvez *et al.*, 2006).

The use of orally applied microorganisms is a major aim of the concept of functional food (Kumar *et al.,* 2008). Several studies indicated that, the consumption of certain probiotic products could lower total plasma cholesterol and low-density lipoprotein (Lebeer *et al.,* 2010; Wang *et al.,* 2012a; Wang *et al.,* 2012b; Kumar *et al.,* 2013). The beneficial properties of strains of some *Saccharomyces* spp. were well studied (Rodrigues *et al*., 2000). According to this study, in addition to their nutritive value (e.g. provision of vitamins B group), probiotic yeasts are generally resistant to gastrointestinal passage.

2.7. Probiotic attributes of isolates

In vitro and *in vivo* tests are critical to assess the effectiveness and safety of probiotic microbes. In addition, it is useful to gain knowledge of strains and the mechanism of their probiotic effect. However, it was noted that the currently available tests are not enough to predict the functionality of probiotic microorganisms for human body (FAO/WHO, 2002). So that, correlation of target-specific *in vitro* tests with *in vivo* results are recommended. The main *in vitro* tests recommended for studying probiotic properties include, tolerance to bile salt, resistance to gastric acidity, adherence to epithelial cells and antimicrobial activity against pathogenic microorganisms (Conway *et al*., 1987).

Probiotic microorganisms have different mechanisms of tolerating low pH. Cells try to maintain intracellular pH (pH_i) above some critical pH, against pH changes in the environment and cytoplasm. They try to prevent irreversible changes of cellular components and metabolic activities. There are three mechanisms to maintain pH_i constant: the homeostatic response, the acid tolerance response and the synthesis of acid shock proteins. The homeostatic response maintains pH_i by pumping protons from cytoplasm. The acid tolerance response maintains pH_i

by production of inducing proteins. The synthesis of acid shock proteins is the third way that cells regulate pH_i (Osman and Faruk, 2016).

There are two possible factors in which probiotic microorganisms can reduce serum cholesterol (Dilmi-Bouras, 2006). One is the ability to metabolize dietary cholesterol, thereby reducing the amounts absorbed in blood. The other possibility is that they deconjugate bile salts and prevent their reabsorption in the liver. The liver, in turn, uses more serum cholesterol to synthesize bile salts and indirectly helps reducing cholesterol level in serum. *S. boulardii* probiotic yeast isolates have already been extensively studied in terms of their ability to limit inflammation and infection in the gastrointestinal tract (Pothoulakis, 2009).

Lactose-intolerant individuals are unable to produce lactase (β-galactosidase) in the small intestine. When they consume milk, lactose molecules are not hydrolyzed in or absorbed from the small intestine but passed to the colon. They are then hydrolyzed in the colon by lactase of different bacteria to glucose and galactose and then further metabolized to produce acids and gas, resulting in fluid accumulation, diarrhea, and flatulence. Consumption of probiotic organisms within yogurt and other dairy products reduces the symptoms in lactose-intolerant individuals. This benefit is attributed to the ability of probiotic organisms to produce lactase (β-galactosidase) which hydrolyzes lactose into glucose and galactose in fresh milk during fermentation (Yanyong Deng *et al.*, 2015). Probiotic microorganisms these survive stomach acidity well and colonize the small intestine can subsequently supply lactase which hydrolyzes lactose in stomach. Probiotics with bile salt hydrolase (BSH) activity are capable of hydrolyzing bile salt and tolerate the bile salt inhibitory effect in the stomach and small intestine (Máire *et al*., 2006).

2.8. Starter culture use as bio-protective agents

The antimicrobial activity of starter cultures and probiotic bacteria has been attributed to the production of metabolites such as organic acids (lactic and acetic acid), hydrogen peroxide, ethanol, diacetyl, acetaldehyde and other low molecular mass compounds with antimicrobial activity such as bacteriocins ((DeVuyst and Vandamme, 1994).

Probiotic bacteria with bio-protective qualities in yoghurt and cheese, in addition to prolonging the shelf-life of the products, they will simultaneously provide the consumer with a health advantage at a low cost (Gomes *et al.,* 1998). Lactic acid bacteria isolates recovered from traditional yoghurt, raw-milk and cheeses, were shown to have several antibacterial activity against pathogens such as *L. monoctyogenes*, *S. aureus*, *Salmonella* and *E. coli (*Marcelino, 2013). The synergistic action of some LAB and yeasts prevented the growth of a broad range of food borne pathogens, improving microbiological safety of the food products without changing their sensory characteristics (Florou-Paneri *et al*., 2013). Lactic acid bacterial strains inoculated into cottage cheese prevented the growth of cheese spoilage *P. commune* by 25 days longer than cottage cheese that did not contain LAB (Cheong *et al*., 2014). Mixed strains of LAB starter is the best way to treat the acute or lethal infections due to *C. difficile* and methicillin resistant *S. aureus* (Reddy and Reddy, 2016). Dineen *et al.* (1998) reported that mixed thermophilic LAB cultures showed a stronger inhibition of *E. coli* O157:H7 than pure cultures during fermentation of dairy products.

These antagonistic activities of starter cultures rely on the competition for nutrients, production and tolerance of high concentrations of ethanol, as well as the synthesis of a large class of antimicrobial compounds, known as killer toxins, which has a large spectrum of activity against

food spoilage microorganisms and other pathogenic microorganisms (Serena and Cristina, 2015). Syal and Vohra (2013), isolated seven yeast species having 100% tolerance to pH 2 and 1% bile salt, assimilate cholesterol in the range of 57-88.5% from traditional Indian fermented foods. They also produce antimicrobial substances against the enteropathogens; *E. coli*, Salmonella spp., *S. aureus*, *V. cholerae* and Pseudomonas spp.. *S. cerevisiae* and *S. boulardii* are differing from other yeast species in that have the ability to survive gastric acidity and bile salt. They do not appear to alter or adversely affect the normal flora of the gastrointestinal tract and can be consumed with normal probiotic bacteria (Boddy *et al*., 1991; Elmer and McFarland, 2001). Inclusion of *S. boulardii* and *S. cerevisiae* in the standard medical treatment for *C. difficile* infection has been reported to reduce the risk of recurrence in patients experiencing renewed infection (Aloysins *et al.*, 2005). *Saccharomyces* spp. has been shown to protect against various enteric pathogens and members of the family *Enterobacteriaceae* in animal studies (Czerucka and Rampal, 2002). *S. boulardii* reduced the growth of *E. coli, S. typhi, S. dysenteriae, S. enteritidis* (Czerucka and Rampal, 2002), and *C. difficile* (Izadnia *et al.*, 1998). Lactobacilli appear to enhance the beneficial effects of *S. boulardii* on intestinal mucosa (Buts, 1999). Aloysins *et al.* (2005), suggested that *S. boulardii* and *Lactobacilli* can be used to prevent antibiotic associated diarrhea. *S. cerevisiae* may also have value in the treatment of *C. difficile* associated diarrhea (Martins *et al.*, 2005).

In Ethiopia, pure and mixed LAB starter cultures inhibitory study on food borne pathogens against , *E. coli* and *S. aureus* was done by Anteneh Tesfeye *et al.* (2011a). The authors showed that pure LAB starter culture reduced the pathogens by 3 log units and mixed lactic acid bacteria cultures by 5 log units during fermentation and storage of *ergo* at 72 hrs.

2.9. Sensory shelf life estimation

Shelf-life is the period of time during which a product could be stored until it becomes unacceptable from safety, nutritional, or sensory perspectives. Sensory shelf-life is determined as the time required for overall liking scores of the product to fall below a predetermined value. In order to estimate sensory shelf-life, a dispersion diagram of average overall liking scores against storage time is obtained and a linear regression is performed (Gambaro *et al*., 2006; Gimenez *et al*., 2007). Using a consumer-based approach, sensory shelf-life of a food product could be regarded as the length of time during which the product is still accepted by the ultimate consumer as having the expected level of quality. The 5- point scale standard quality limit was used to estimate the shelf-life based on consumer overall liking data, collected using a hedonic scale (Gimenez *et al*., 2012).

2.10. Physicochemical properties of *ergo* and *ayib*

Yoghurt is more nutritious than many other fermented milk products because it contains a high level of milk solids in addition to nutrients developed during the fermentation process, and its sensory attributes have a large effect on consumer acceptability (Saint- Eve *et al*, 2008: Igbabul *et al*., 2014). The chemical composition of yoghurt has been described by several workers (Saint-Eve *et al*, 2008;Yaygin and Kihc 1980). The Physicochemical composition and organoleptic properties of yoghurt products may differ from similar products processed in different processing methods (ILCA, 1992). According to O'Connor (1994), the chemical composition of milk may be determined by measuring its content of fat, protein and total solids, which is affected by genetic and environmental factors. Breed, feeding, individuality within the breed, stage of lactation, age, health and interval between milking are among the factors responsible for variation in milk composition of cows (Zelalem *et al.,* 2004; O'Connor, 1994). Harun *et al.*

(2015) reported that cow milk yoghurt has a total solid content of 12.1%, fat 3.30%, protein of 3.38%, pH of 3.93 - 4.15 and titratable acidity of 1.27 to 1.98%.

3. MATERIALS AND METHODS

3.1. Description of the study area

The study was conducted in Holeta Agricultural Research Center and Holeta National Agricultural Biotechnology Research Center from November 2017 to April 2018. both centers are located in 29 Km 9”00 N and 38^{o}30 E to the western of Addis Ababa, capital city of Ethiopia. The altitude of the town is 2400 m above sea level with mean annual rainfall of 1144 mm, humidity 60.6%, minimum and maximum temperature of 6.2 – 24.1 ^{o}C respectively.

3.2. Lactic acid and yeast isolates

Candidate lactic acid bacteria (LAB) and yeast isolates used in the current study were obtained from Ethiopian National Agricultural Biotechnology Research Center. These isolates of LAB and yeasts were previously isolated based on their probiotic attributes from traditional fermented *ergo* and *kocho* samples collected from different agro-ecologies of Ethiopia by the staff members of the Microbial Biotechnology Department of the Ethiopian National Agricultural Biotechnology Research Center. A total of two hundred forty-three LAB and twenty six yeast isolates were obtained from the center, Among these, seven lactic acid bacteria (GB-15, SB-7, G-23, BB-60, NZ-44, BB-33, NZ-26) and one yeast isolate (Y-72) were further screened based on their probiotic potential, starter culture attributes and antagonistic effect against some food borne pathogens and used for formulation of the starter culture.

3.3. Identification of the isolates

The isolates were identified by cultural, morphological, biochemical and physiological characteristics. Morphological characteristics of each LAB isolates observed after 48 hrs of growth on De Man, Rogosa and Sharpe agar (composition per liter: Glucose 8.5g, Agar 13.5g, Pancreatic digest of gelatin10.0g, Beef extract 8.0g, Yeast extract 4.0g, Sodium acetate 3.0g, K_2HPO_4, 2.0g, Ammonium citrate 2.0g, Polysorbate 80 1.0g, $MgSO_4$:$7H_2O$ 0.2g, $MnSO_4 \cdot 4H_2O$ 0.05g, pH 6.2 ± 0.2 at 25°C) for LAB isolates and yeast extract agar (Composition per liter: Agar 15.0g, Proteose peptone10.0g, NaCl 5.0g, Yeast extract 3.0g, pH: 7.3 ± 0.2 at 25°C) for yeast isolates included colony appearance; shape, elevation, edge, cell shape, and colony surface. Cultural characteristics were supported by the microscopic identification of cells.

3.3.1. Spore production test

This test was done to detect the presence of bacterial endospores. Each isolate of LAB was grown in MRS agar slant and the yeast isolates were grown on yeast extract agar slant, respectively, for 24 - 36 hrs. Heat-fixed smears of the pure isolate were prepared on separate slides and flooded with 5% malachite green solution and steamed for one minute. The stain was washed off with water and counter stained with 2 drops of safranin solutions for 20 sec. The slides were allowed to air dry and examined under oil immersion objective (100x) lens. Endospores stained green while vegetative cells stained pink (Cheesbrough, 2006).

3.3.2. Biochemical and Physiological test

3.3.2.1. Motility test

A sterile needle was used to pick a loop full of a 24 hrs old culture and inoculated into semi solid nutrient agar in a test tubes. The tubes were incubated at 37 °C for 24-48 hrs. Non-motile

bacteria had growth confined to the stab line with definite margins without spreading to surroundings area while motile bacteria gave diffused growth extending from the surface and line of inoculation (Olutiola *et al*., 2000).

3.3.2.2. Gram staining

A thin smear of each of the pure 24 hrs old culture was prepared on clean grease-free slides, fixed by gently passing over flame. Each heat-fixed smear was stained by addition of 2 drops of crystal violet solution for 60 sec and rinsed with water. The smeared glass slides were again flooded with lugol's iodine for 30 sec and rinsed with water, decolorized with 70% alcohol for 15 sec and rinsed again with distilled water. They were then counter stained with 2 drops of safranin for 60 sec and finally rinsed with water, then allowed to air dry. The smears were mounted on a microscope and observed under oil immersion objective lens. Gram negative bacteria appeared pink or red, while gram positive bacteria appeared purple (Fawole *et al*., 2004).

3.3.2.3. Catalase test

A loopfull of 24 hrs old culture was transferred into a drop of 3% hydrogen peroxide solution on a clean glass slide with the aid of sterile inoculating loop. Gas bubbles seen as white froth indicates the presence of catalase enzyme (Cheesbrough, 2006). Isolates with gram positive and catalase negative reactions were finally used for further identification.

3.3.3. Temperature and salt tolerance test

Gram positive and catalase negative isolates were tested for their growth at different temperatures values (15, 37, and 45 ^{o}C) and tolerance to different concentrations of NaCl (3, 4 and 6.5%). A loopfull of young culture (18-24 hrs) of each isolates was inoculated in to 10 ml modified MRS broth (modified by increasing yeast extract to 2%). From the inoculate 1 ml was

pour plated on to MRS agar for LAB isolates and yeast extract agar for yeast isolates and incubated at 37^{o}C. The left broth was incubated at different temperature of 15, 37and 45^{o}C separately for 48 hrs After 48 hrs 1 ml of aliquots were pour plated on to MRS agar for LAB isolates and yeast extract agar for yeast isolates. The plates were incubated at 37 ^{o}C for 48 hrs. The colony was counted and viability of greater than >50% was considered to be tolerant (Tambekar and Bhutada, 2010). The growth of the isolates were determined as follows:

$$\text{Growth at n temp in \%} = 1 - \frac{\text{Initial cell count at 0 hrs}}{\text{Final cell count at 48 hrs}} \text{x100}$$

Salt tolerance of each isolates was assessed by inoculating young cultures of (18-24 hrs) of LAB from MRS agar transferred to 10ml tube containing MRS broth with 3%, 4% and 6.5% NaCl. The inoculated broth was incubated at 37 ^{o}C for 48 hrs. One milliliters of the broth pour plated on MRS agar and incubated at 37^{o}C for 48 hrs. For yeast isolates, yeast extract agar and yeast extract broth was used. The colony was counted and viability greater than >50% was considered to be tolerant (Hoque *et al*., 2010). The growth of the isolates were determined as follows:

$$\text{NaCl tolerance in \%} = 1 - \frac{\text{Initial cell count at 0 hrs}}{\text{Final cell count at 48 hrs}} \text{x100}$$

3.3.5. Citrate test

This test detects the ability of an organism to use citrate as a sole source of carbon and energy. About 2.4 g of Simmons citrate agar was dissolve in 100 ml of distilled water. About ten milliliter of citrate medium was dispensed into each tube and the tubes were capped, sterilized by autoclaving. The tubes were inoculated by streaking the organisms once across the surface and incubated at 37 ± 0.2^{o}C for 24 hrs. A change from green to blue indicates utilization of the citrate (Melanie *et al*., 2009)

3.3.6. Starch hydrolysis test

This is used to assay the ability of microorganisms that can produce enzymes for degradation of substrate with carbon compounds. Nutrient agar was prepared with 1% soluble starch and was sterilized by autoclaving. The medium was poured into sterile plates and inoculated by streaking the organisms once across the plates after solidifying. The plates were incubated at 37 °C for 24 hrs, after which they were flooded with Gram's iodine. Un-hydrolyzed starch forms a blue color with the iodine. Hydrolyzed starch appears as a clear zone due to alpha amylase activity while reddish brown zones around the colony indicate partial hydrolysis of starch (Evans *et al*., 2004).

3.3.7. Sugar fermentation

Sugar fermentation test of glucose, sucrose, lactose, mannitol, starch, sorbital, mannose, fructose, esculin, xylose and melibiose was carried out to determine the ability of organisms to with production of acid and gas. Sugar indicator broth was prepared using peptone water medium containing 1% fermentable sugar and 0.01% phenol red. About ten milliliters of sugar broth was dispensed into each of the test tubes and Durham tube which would trap the gas if produced was inverted and placed in a tube carefully. The test tubes were autoclaved and inoculated with a loopfull of 24 hrs old culture of the isolate, and then they were incubated for 24-48 hrs at 37 ± 2°C and observed for acid and gas production. Yellow coloration indicates acid production, while gas production was indicated by formation of gas bubble in the Durham tube (Fawole *et al.*, 2004).

3.4. Probiotic properties of the isolates

3.4.1. Acid tolerance test for LAB isolates

Acid tolerance of the isolates was tested by the method of (Liong and Shah, 2005) with slight modifications. In brief, approximately 10^6cfu/ml of each isolates was adjusted using mass spectrometer at 600 nm and inoculated into MRS broth having a pH adjusted to 2.0, 2.5 and 3.0 using 1M HCl and incubated at 37^{o}C for 24 hrs. Culture without pH adjustment is used as control sample Tubes incubated at 37 ^{o}C were checked for survival or acid tolerance at 0, 3 and 6 hrs by spreading 0.1 ml of the sample onto MRS agar with respective pH values. All plates were incubated at 37 ^{0}C for 48 hrs in anaerobic jar (Jose *et al.,* 2015). Survival percentage of each LAB was determined by comparison with the initial concentration on MRS agar as indicated below.

$$\text{Acid tolerance} = \frac{\text{Initial concentration of isolates (CFU/ml)}}{\text{Final conc. of isolate (CFU/ml) after T hrs}} \text{x } 100$$

3.4.2. Acid tolerance test for yeast isolates

The effect of exposure to low pH was determined by inoculating 10^6cfu/ml yeast cultures which adjusted using mass spectrometer at 600 nm into Potato Dextrose Broth of pH adjusted to 2.0, 2.5, and 3.0 using 1M HCl and incubated at 37 ^{o}C for 24 hrs. Samples of 0.1 ml were taken and spread on yeast extract agar at 0 hrs and at, 3, and 6 hrs incubation at 37 ºC for 48 hrs (Syal and Vohra , 2013). The survival rate of each isolate was calculated as follows:

$$\text{Acid tolerance} = \frac{\text{Initial concentration of isolate (cfu/ml)}}{\text{Final conc. of isolate (cfu/ml) after T hrs}} x100$$

3.4.2. Bile salt tolerance test

3. 4.2.1. Bile salt tolerance test for LAB isolates

The bile salt tolerance of these acid-tolerant LAB isolates was examined by adding 10^6 cfu/ml of each acid-tolerant LAB into 10 ml of MRS broth containing 0.3, 0.5 and 1.0% Ox gall bile salt, respectively. All tubes were incubated at 37 ^oC and growth was checked at 0, 3 and 6 hrs by spreading 0.1 ml aliquots on MRS agar and incubated at 37 ^oC in anaerobic jar. Bile salt tolerance of each isolate was determined by comparing the plate counts after 6 hrs of exposure to bile salts with the initial count at 0 hrs (Serrano *et al*., 2016). Survival rate was calculated according to the following equation:

$$\text{Survival rate (\%)} = \frac{\log \text{cfu N1}}{\log \text{cfu N0}} x100$$

Where: N_1= Viable count of isolates after incubation in bile salt; N_0= Initial viable count.

3.4.2.2. Bile salt tolerance test for yeast isolates

The effect of bile salt on the viability of the yeast isolates resistant to low pH was determined by inoculating 1% of activated yeast cultures into phosphate buffered salt containing 0.3, 0.5, and 1% bile salt. Inoculated tubes were incubated at 37ºC for 24 hrs. Growth was checked at 3 and 6 hrs by spreading 0.1 ml on yeast extract agar and incubated at 37 ^0C (Barnett *et al.,* 2000; Nayak, 2011). The survival rate of each isolate was expressed as the percentage of viable cells in the presence of bile salt compared to initial viable count as the above equation.

3.4.3. Cholesterol reduction assay

Cholesterol measurement was done by the method described by Searcy and Bergusst (1996). Each LAB and yeast isolates were inoculated into modified MRS broth (modified by increasing yeast extract to 2%) supplemented with 0.3% bile salts and 10 mg of cholesterol dissolved in 500

µL of ethanol. The cultures were grown for 24 hrs at 37^{o}C. The cells were harvested by centrifugation at 8,000 rpm for 10 minute at 4°C. The spent broth was collected to assay for cholesterol. The broth which is uninoculated with the isolates were used as controls. To the 1 ml of spent broth 3 ml of 95% ethyl alcohol was added followed by addition of 2 ml of potassium hydroxide and the contents were mixed after addition of each component. Then the tubes were heated for 10 minute at 60^{o}C in water bath, after cooling 5 ml of hexane was dispensed to all tubes and mixed with vortex for 5 minute. Then 3 ml of water was added and mixed thoroughly. Tubes were allowed to stand for 20 minute at 30 ^{o}C to permit the phase separation. hexane layer of 2.5 ml was transferred to a fresh tube and it was allowed to dry completely. Ferric chloride reagent of 1.5 ml was added to it and allowed to stand for 10 minute. One ml of concentrated sulphuric acid was added from the sides of the test tubes and the mixtures were mixed and allowed to stand for 45 minute at 30 ^{o}C. One control sample having cholesterol without culture was used. The optical density of the supernatant was measured at 540 nm in UV spectrophotometer and the concentration of cholesterol was calculated as follows:

$$A = 100 - \left(\frac{B}{C}\right) x100$$

Where,

A = % of cholesterol removed,

B = absorbance of the culture supernatant

C = absorbance of the control.

3.5. Compatibility test of isolates

In order to formulate bio-protective and probiotic consortia, compatibility of these isolates among each other were determined. In this method, the inter-compatibility of the candidate

isolates were determined by cross streaking each isolate against each other on modified MRS agar plate (modified by decreasing glucose content to 1% and yeast extract to 2%) and incubated at 37 ^{o}C for 48 hrs (Anupama, 2015). Their growth patterns were noticed after 48 hrs of incubation. The isolate showing best compatibility was chosen for starter formulations.

3.6. Formulation and propagation of starter culture

These isolates were formulated using Design Expert version 7.0.0, 2005, Statistical software. The number of combinations of formulated starter culture depends on the number of compatibility of candidate isolates (Komi *et al*., 2017)

3.6.1. Propagation of isolates

The lactic acid bacterial isolates were streaked on MRS agar medium and incubated anaerobically at 37^{o}C for 48 hrs and yeast isolate was streaked on yeast extract, peptone, dextrose (YPD) medium (1% yeast extract, 2% peptone, 2% dextrose) supplemented with chloramphenicol (100 μg/ml) and incubated aerobically at 37 ^{o}C for 72 hrs. Preparation of the inoculum was done according to the method of (Komi *et al.,* 2014). Colonies from fresh cultured plates were transferred to sterile skim milk and incubated at 37 ^{o}C for 18 hrs.

3.6.2. Formulation of starter culture

For formulating the starter culture, viable cells of the pre-cultured, each pure isolate having 10^{6} cfu/ml was mixed in different proportions in a flask containing 800 ml of pasteurized whole milk to determine suitable combination for the production of *ergo*.

3.7. Preparation of *ergo* using the formulated starter culture

3.7.1. Sample preparations

Fresh cow milk of 10 liters were collected with carefully cleaned and sterilized vessels from bulk samples of Holeta Agricultural Research farm and filtered with sterilized cheese cloth The milk was pasteurized at 85 °C for 30 minute (Patroklos *et al*., 2015). For *ergo* making, heat treatment is more severe than that used for pasteurization of milk.

3.7.2. Homogenization

The milk was homogenized using homogenizer (model FG30043, Germany) after pasteurization to prevent the formation of a cream layer on the surface of the product during incubation. Then it was cooled to 40 - 45 °C and 5% of the formulated starter culture was added (Aswal *et al.*, 2012). It was incubated at a temperature of 37 ± 2 ^{0}C for 4 - 6 hrs to ferment, after which the *ergo* was ready for consumption.

3.8. Antagonistic test against some spoilage and food borne pathogens

Test organisms

The test organisms of *Escherichia coli* ATCC 43895, *Staphylococcus aureus* ATCC 25923, *Salmonella typhimurium* ATCC 14802 and *Listeria monoctyogenes* ATCC 15313, were procured from Ethiopian Public Health Research Institute (EPHRI), Addis Ababa, Ethiopia and Holeta Dairy Research Laboratory. These test organisms are some of enteric pathogen and serious food borne pathogens or food spoilage bacteria.

3.8.1. Co-culturing of the formulates and test pathogens

Co-culturing method was used to test the bio-protective or antagonistic ability of each formulates on these test pathogens. The experimental culture consisted of mixed formulated starter culture

and the test pathogen together in 300 ml of pasteurized milk in triplicate to give a final inoculums level of 6 log cfu/ml and 3 log cfu/ml for each mixed starter and the test pathogen, respectively. All flasks were incubated at 37^{o}C for 48 hrs in anaerobic jar. Each test pathogen was separately inoculated in 300 ml of unpasteurized milk in triplicate to give final inoculums of 3 log cfu/ml as a control.

Samples of 1 ml from each co-cultured (formulated starter culture - test pathogen) and control sample were drawn at 0 hrs before incubation and at 24 and 48 hrs of incubation. A dilution of 10^{-1} was made and 0.1 ml of diluents was pour plated on selective media; Manitol salt agar (oxoid) (g/l of 75.0 g NaCl, 15.0 g Agar, 10.0 g D-Mannitol, 5.0 g Pancreatic digest of casein, 5.0 g Peptic digest of animal tissue, 1.0 g Beef extract, 0.025 g Phenol RedpH adjusted to 7.4 ± 0.2.) for *S. aureus*, Xylose Lysine Desoxycholate Agar (oxoid) (g/l of 15.0 g Agar, 7.5 g Lactose, 7.5 g Sucrose, 6.8 g Na_2 S_2O_3, 5.0 g L-Lysine, 5.0 g NaCl, 3.75 g Xylose, 3.0 g Yeast extract, 2.5 g Sodium deoxycholate, 0.8 g Ferric ammonium citrate, 0.08 g Phenol Red pH adjusted to 7.4 ± 0.2) for , Eosin methylene blue agar (oxoid; g/l of 13.5 g Agar, 10.0 g Pancreatic digest of casein, 5.0 g Lactose, 5.0 g Sucrose, 2.0 g K_2HPO_4, 0.4 g Eosin, 0.065 g Methylene blue pH adjusted to 7.2 ± 0.2 at 25 ^{o}C) for *E. coli,* and PALCAM (Difco) (23.0 g Peptone, 15.0 g LiCl, 10.0 g Agar, 10.0 g Mannitol, 5.0 g NaCl, 3.0 g Yeast extract, 1.0 g Starch, 0.8 g Esculin, 0.5 g Ferric ammonium citrate, 0.5 g Glucose, 0.08 g Phenol Red, 10.0 mL, PALCAM selective supplement and pH adjusted to 7.2 ± 0.2) for *L. monoctyogenes.* Characteristic colony formed was enumerated after incubation at 37 ± 2^{o}C for 24 - 48 hrs. When growth of test pathogens was not detected (< 1 log cfu/ml), 1 g of each sample was enriched in 9 ml of nutrient broth at 37 ± 2 ^{o}C for 24 hrs. The enriched samples were streaked on respective media as before and the plates were checked for the absence of perceptive colonies of target test pathogen complete inhibition.

3.9. Preparation of *ayib*

Ayib (Ethiopian cottage cheese) was prepared following the method of Anteneh Tesfeye *et al.* (2011); pasteurized milk of 800 ml was fermented by inoculating formulated starter culture of 5% and incubated at 37± 2 ^{o}C for 4 - 6 hrs. Soured milk was defatted by churning with a shaker at 100 rpm for 1 hrs. The floating fat was removed with sterile spoon. Defatted soured milk samples were separately cooked at 50 °C in a water bath for 55 minute. The curd (*ayib*) was separated from the whey after cooling the cooked solid mass to room temperature and filtered through sterile cheese cloth. *Ayib* of 200 g were wrapped in sterile cheese cloth and dipped into pasteurized whey inoculated with 8 log cfu/ml of formulated starter cultures for 30 minute and re-drained for 1 hrs.

3.10. Physicochemical and proximate analysis of *ergo* produced with different formulated starter culture

3.10.1. Determination of the pH

The products were analyzed for pH using standard methods of Association of Official Analytical Chemist procedure (AOAC, 2000; Mamoona *et al.,* 2013). The pH of the samples was measured by potentiometric method i.e. by potential difference between the sample and electrolyte solution present inside the electrode of pH meter using digital pH meter (Hanna instruments, HI 2483, Italy). First the pH meter was calibrated with fresh pH 4.0 and 7.0 standard buffer solution (Behrad *et al.,* 2009). The electrode of the pH meter was directly dipped into the samples of *ergo* ready for consumption and the pH reading was recorded.

3.10.2. Determination of the titratable acidity

Titratable acidity of each product was assayed by titration method (AOAC, 2000). Titratable acidity was measured as free and bound hydrogen ions by titrating with 0.1 N NaOH and expressed in percent (m/v). *Ergo* sample and distilled water 1:9 (m/v) mixture was titrated with standardized 0.1 N NaOH using 3 to 5 drops of 0.1% phenolphthalein as color indicator until faint color persists, the end-point has been reached at pH of 8.2 (Shah *et al.*, 2000).

Titratable acidity produced during fermentation was calculated as:

$$TA\% = \frac{\text{V NaOH in liter x 0.1N x 90.08 g/mol}}{\text{10 ml}} \text{x 100\%}$$

Where: V is volume of NaOH consumed.

0.1 N is the concentration of NaOH in normality

90.08 g/mol is the molecular weight of lactic acid

3.10.3. Determination of syneresis

Syneresis is defined as the liquid phase of curd which most consumers consider to be a defect (Megenis *et al*., 2006). Susceptibility of *ergo* to syneresis was determined according to the methodology proposed by Farnsworth *et al*., (2006). *Ergo* samples of 20 g were centrifuged at 500 rpm for 5 minute and the clear supernatant was collected and weighed (Ayman, 2009). Syneresis was calculated according to the following equation;

$$\% \text{ Syneresis} = \left(\frac{\text{Weight of supernatant (g)}}{\text{Weight of } ergo \text{ sample (g)}}\right) x100$$

3.10.4. Protein content determination

The macro Kjeldahl method as described by AOAC (2005) was used to determine the crude protein content. From each sample, 2 g was introduced into the digestion flask. 10 g of copper

sulphate and sodium sulphate in the ratio of 5:1 and 25 ml of concentrated sulphuric acid was added to the digestion flask. The flask was placed in a digestion block in fume cupboard and heated until frothing ceased given a clear and light blue coloration. The mixture was allowed to cool and diluted with distilled water until it reaches 25 ml of volumetric flask. Then after, 10 ml of the mixture was poured into the distillation apparatus and 10 ml of 40% sodium hydroxide was added. The released ammonia by boric acid was allowed to continue until 10 ml of boric acid is treated with 0.02 M of hydrochloric acid and the green color change to purple. Finally, the percentage of nitrogen in the sample was determined and calculated as the following:

$$\%\ \text{Crude protein} = \frac{10 \times 1000 \times \text{sample weight (2 g)}}{\%\ \text{Nitrogen} \times 6.25}$$

3.10.5. Fat content determination

The soxhlet solvent extraction method as described by AOAC (2005) was used to determine the fat content. In this method 2 g of the sample was weighed in a flat bottom flask of known weight with the extractor mounted on it. The thimble was held half way into the extractor and the weighed sample was carefully transferred into the thimble, and the thimble was plugged with cotton wool. The extraction was carried out at the temperature of 40 – 60 ^{o}C for 8 hrs. The solvent was removed by evaporation and then, the remaining part in the flask was dried in the oven at 80 ^{o}C for 30 minute and then cooled in desiccators. The flask was reweighed and the percentage fat was calculated as follows;

$$\%\ \text{Fat} = \left(\frac{\text{Weight of extracted fat}}{\text{Weight of sample used}}\right) x100$$

3.10.6. Determination of total solid contents

The total solids were determined as described by AOAC (2005). Sample of 3 g was weighed into a dry crucible of known weight. The sample was pre-dried for 30 minute on water bath and then dried in air drying oven at 100 ± 2 °C till constant weight was achieved between consecutive measurements at 30 minute interval.

The total solid of the sample was calculated as follows:

$$\% \text{ Total Solid} = \frac{w2-w}{w1-w} \text{x } 100$$

Where:

W = Weight of the crucible

W_1 = Weight of crucible plus sample

W_2 = Weight of oven dried crucible plus sample

3.10.7. Determination of ash contents

The ash content was determined by the direct heating method as indicated in AOAC (2005). In this method, 3 g each of the sample was measured into a crucible of known weight, the sample was burned to ash in a muffle furnace for 5 $\pm$ 1 hrs at 550^0C. It was then cooled in a desiccators and the percent of ash was finally determined as follows;

$$\% \text{ Ash} = \frac{W2-W1}{W} \text{x } 100$$

Where:

W1 = Weight of crucible

W = Weight of sample test portion

W_2 = Weight of dish and dry sample

3.10.8. Determination of lactose content

The content of lactose was determined by difference as described by Ihekoronye and Ngoddy (2002).

$$\% \text{ Lactose} = \%(\text{Ash} + \text{Protien} + \text{Fat} + \text{Moisture})$$

3.10.9. Moisture content determination

The moisture content was determined by oven method as described by AOAC (2005). In this process 3g of the sample was dried in a hot air oven for 24 hrs at 102 ± 2^{o}C. The lost in weight was determined and recorded as the moisture content and expressed as;

$$\text{Moisture} = \frac{W2 - W1}{W1} x\ 100$$

Where;

W1 = Initial weight of the sample

W2 = Weight of the dried sample

3.10.10. Determination of total solids-non-fat

The total solids-not-fat was determined as described by AOAC (2005). It was obtained by taking the difference between % total Solids and % fat content.

$$\% \text{ SNF} = \%\text{total solid} - \%\text{fat}$$

3.11. Sensory evaluation of the products

Eleven *ergo* products (nine produced by different formulated starter culture and two control) and nine *ayib* product treated with the formulate starter culture and one control were provided for sensory test. The *ergo* and *ayib* samples were randomized and coded with three digit numbers and served at 7 to 10°C in plastic cups separately. A test form comprising five sensory attribute was given to each assessor. The sensory assessments were conducted by involving ten semi-trained panelists of adult males and females. The panelists were provided with glasses of water and, instructed to rinse with water between, while working with the samples (Yang *et al*., 2010). The panelists given written instructions and evaluated the products for color, flavor, odor, texture

and overall acceptability using five-point hedonic scale: 1 = Dislike Extremely, 2= Dislike Moderately, 3= Neither like nor dislike, 4= Like Moderately, and 5= Like Extremely. In the evaluation of the sensory characteristics of the product, one was equal to the worst and five was equal to the best.

3.12. Estimation of sensory shelf life of the products

Ergo product made with different formulated starter culture and *ayib* sample treated with the formulated starter were stored at ambient temperature. Sensory test was done at the 1st day and every three day for *ergo* and every four days for *ayib* products until the products were unfit for sensory test. The shelf life cut-off point was 3.5, as it was indicated by Yuthana and Panuwat, (2016). Sensory shelf life of the products was estimated using overall acceptability versus storage time linear regression and first order kinetic reaction. The sensory overall acceptability of each product was changed to natural log, and the linear regression equation of natural log of sensory overall acceptability versus storage time was drown to get rate constant (K). Using rate constant K, the initial overall sensory acceptability (A_0) and quality limit (A_t) of overall mean score at five point hedonic scale, the shelf life of the product was calculated following Arrhenius first order reaction equation (Mahendradata *et al*., 2007; Tsironi *et al*., 2009).

$$\text{Shelflife(in days)} = \frac{\text{InA0/At}}{\text{K}}$$

Where:

A_o = Natural log of initial (1st day overall sensory mean score)

At = quality limit (Cut off point = 3.5)

K= rate constant

3.13. Experimental design

The experiments of antagonistic test and proximate composition were done in Complete Randomized Design (CRD) with three replications and Sensory analysis was done using five point hedonic scales.

3.14. Statistical analysis

Before statistical analysis of data, normality of data was tested using Kolmogorov- Smirnov test. Accordingly, if P value was greater than 0.05 the data did not significantly deviates from normality. The results obtained from proximate and physicochemical composition of the products were analyzed by one way ANOVA using SAS ver. 9.3, 2014. Significant difference between treatment mean was tested at $\alpha = 0.0\ 5$ level of significance using Duncan multiple range test to separate the mean difference between treatment. Data obtained from the antagonistic effect of starter culture on test organisms were analyzed using two ways ANOVA. Data obtained from sensory analysis was analyzed using repeated measure ANOVA (Treatment-by -panelist design), which blocks the variability due to panelist from variability due to treatment

Sensory shelf life of the product was analyzed using overall acceptability versus storage time linear regression and first order kinetic equation.

4. Results

4.1. Morphological, biochemical and physiological identification of the isolates

Among previously isolated lactic acid bacteria and yeasts, seven lactic acid bacteria and one yeast isolate were screened based on their probiotic attributes of acid tolerance, bile salt tolerance, cholesterol reduction capability and their antagonistic effect on some food borne pathogens. All the lactic acid bacteria isolates except G-23 which was grey white colony the rest were white colony appearance and rod shape, non-motile non-spore forming. The yeast isolate was white cream colony and oval shape. All the isolates were gram positive, catalase and citrate negative and can grow in 2 and 3% of NaCl. All isolated were grow in 15 and 37^{o}C and only isolates SB-7, NZ-44, BB-3 can grow in 10^{o}C. Three isolates namely SB-7, NZ-26, and Y-72 (37.5%) were hetro-fermentative; they produced CO_2 from carbohydrates fermentations and five isolates (*GB-15, G-23, BB-60, NZ-44, BB-3)* were homo fermentative and they found out that they did not produce CO_2 while fermenting carbohydrates (Table 2). All the LAB and yeast isolates were able to ferment glucose and mannitol, whereas three isolates; GB-15, SB-7 and BB-60 ferment sucrose and lactose. All the LAB and yeast isolates were able to grow in 2% and 3% NaCl, whereas five isolates (SB-7, BB-60, BB-3, NZ-26 and Y-72) and one isolates (BB-60) were able to grow in 4% and 6.55% NaCl respectively (Table 3).

Table 1: Morphological characteristics of isolates

Characteristics	Isolates							
	GB-15	**SB-7**	**G-23**	**BB-60**	**NZ-44**	**BB-3**	**NZ-26**	**Y-72**
Glucose ferment	Homo	Hetro	Homo	Homo	Homo	Homo	Hetro	Hetro
Colony color	white	white	grey-white	white	white	white	white	White creamy
	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth
Colony shape	circular	circular	circular	circular	circular	circular	circular	circular
Colony								
Margin	Entire	Entire	Entire	Entire	Entire	Entire	Entire	Entire
Cell shape	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Oval
Spore	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent
Motility	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve

GB-15, SB-7, G-23, BB-60, NZ-44, BB-3, NZ-26 = LAB isolates; Y-72= Yeast isolates; + = reaction, - = no reaction.

Table 2: Biochemical and physiological identification of isolates

Characteristics	**GB-15**	**SB-7**	**G-23**	**BB-60**	**NZ-44**	**BB-3**	**NZ-26**	**Y-72**
Gram Stain	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ ve
Catalase	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Citrate	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Glucose	+	+	+	+	+	+	+	+
Sucrose	+	+	-	+	-	-	-	-
Lactose	+	+	-	+	-	-	-	-
Mannitol	+	+	+	+	+	+	+	+
Starch	+	+	-	+	-	+	+	+
H_2S	-	-	-	-	-	-	-	-
Gas	-	+	-	-	-	-	+	+
Sorbital	-	+	+	+	-	-	-	+
Mannose	-	+	+	+	-	-	-	+
Fructose	-	+	+	+	+	-	-	+
Esculin	-	+	-	+	-	-	-	+
Xylose	-	+	+	+	-	-	-	+
Melibiose	-	+	+	+	-	-	-	+

GB-15, SB-7, G-23, BB-60, NZ-44, BB-3, NZ-26 = LAB isolates; Y-72= Yeast isolates; + = reaction, - = no reaction.

Table 3: Growth at different salt concentrations and temperature

Characteristics	Isolates							
	GB-15	**SB-7**	**G-23**	**BB-60**	**NZ-44**	**BB-3**	**NZ-26**	**Y-72**
2% NaCl	+	+	+	+	+	+	+	+
3% NaCl	+	+	+	+	+	+	+	+
4% NaCl	-	+	-	+	-	+	+	+
6.5% NaCl	-	-	-	+	-	-	-	-
Growth at temperature								
10°C	-	+	-	-	+	+	-	-
15°C	+	+	+	+	+	+	+	+
37°C	+	+	+	+	+	+	+	+
45°C	+	+	+	+	+	+	+	-

GB-15, SB-7, G-23, BB-60, NZ-44, BB-3, NZ-26 = LAB isolates; Y-72= Yeast isolates; + = reaction, - = no reaction, NaCl= sodium chloride.

4.2. Formulation of starter culture

The isolates were mixed in different combinations and proportions using design of expert software version 7.0.0/ 2005. Nine combinations/ formulates of starter cultures were made in different proportions. Two control samples of cont A = commercially available yoghurt and cont B traditionally (spontaneously) fermented *ergo* was used in the study.

Table 4: Proportion of isolates in formulates (%).

Formulates	Isolates								
	GB-15	SB-7	G-23	BB-60	NZ-44	BB-33	NZ-26	**Y 72**	Total starter
F1	-	0. 5	0. 5	0. 5	-	0. 5	0. 5	2. 5	5%
F2	1.50	-	0.75	-	0.75	0.50	1.0	0.50	5%
F3	0.75	0.75	-	2.25	-	1.5	-	-	5%
F4	0.75	0.75	0.75	0.75	0.5	-	1.25	0.25	5%
F5	1.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	5%
F6	1.5	-	-	0.5	2		0.5	0.5	5%
F7	-	2.75	0.75	0.75	-	0.75	-	-	5%
F8	0.5	0.5	0.5	-	0.5	2.5	-	0.5	5%
F9	0.75	0.75	0.5	-	2.5	-	0.5	-	5%
Control A					-				5%

Control A= commercially available starter culture; F1, F2…F9= formulated starter culture; LAB= Lactic acid bacteria isolate; GB-15, SB-7 , G-23, BB-60, NZ-44, BB-33 and NZ-26 = Lactic acid bacteria isolates; Y 72= yeast isolates.

4.3. Probiotic property of isolates

4.3.1. pH tolerance

All the isolates have shown tolerance to pH of 2.0, 2.5 and 3.0 with more than 60% survival or tolerance value. (*Fig.* 1). The three pH values (2.0, 2.5 and 3.0) tolerance of the isolates were found in (*Fig.* 1). Among the isolates NZ-44 and Y-72 showed relatively higher tolerance to pH of 2.0, 2.5 and 3.0. Relatively lower tolerance of 63.6% and 61.9% to pH 2.0 was exhibited by G-23 and NZ-26 in that order.

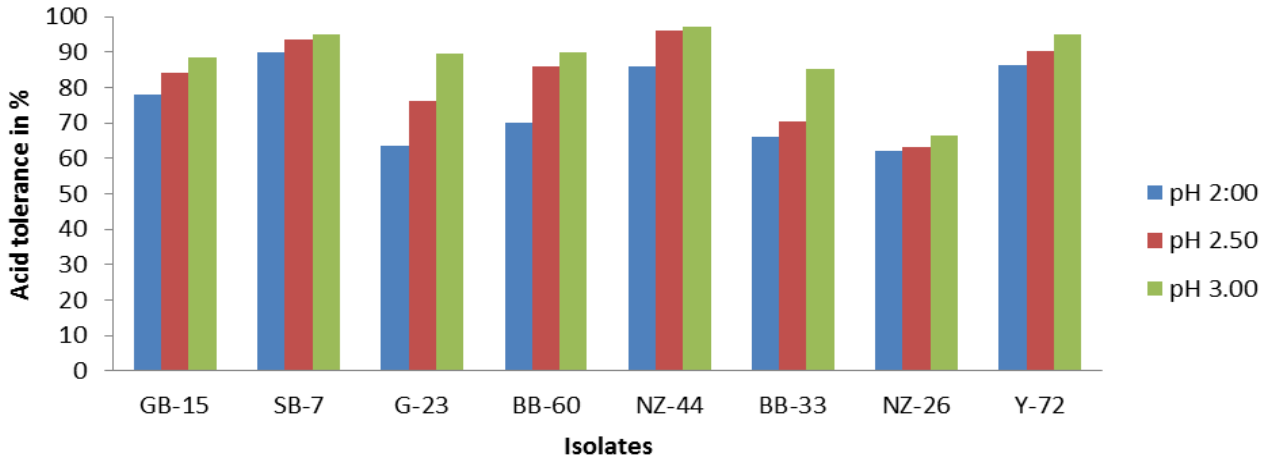


Figure 1 : : pH tolerance of each isolates for 6 hrs of incubation

GB-15, SB-7; G-23; BB-60; NZ-44; BB-33; and NZ-26 were lactic acid bacterial isolates; Y-72= yeast isolate.

4.3.2. Bile salt tolerance

Figure 2 showed the bile salt tolerance of each isolate. All the seven lactic acid bacterial and yeast isolates were found to be tolerant to bile salt of 0.3%, 0.5% and 1% at varying ranges. Relatively, highest tolerance of 98.37% and 92.45% to bile salt concentration of 0.3% and 0.5% were observed by isolate GB-15 and relatively lowest tolerance of 88.66% and 77.60% to bile salt concentration of 0.3% and 0.5% were observed by isolate BB-33. The average bile salt tolerance of whole isolates was greater

than 72%. Tolerance or viability of the isolates decreased as the concentration of bile salt was increasing from 0.3% to 1% (*Fig*. 2).

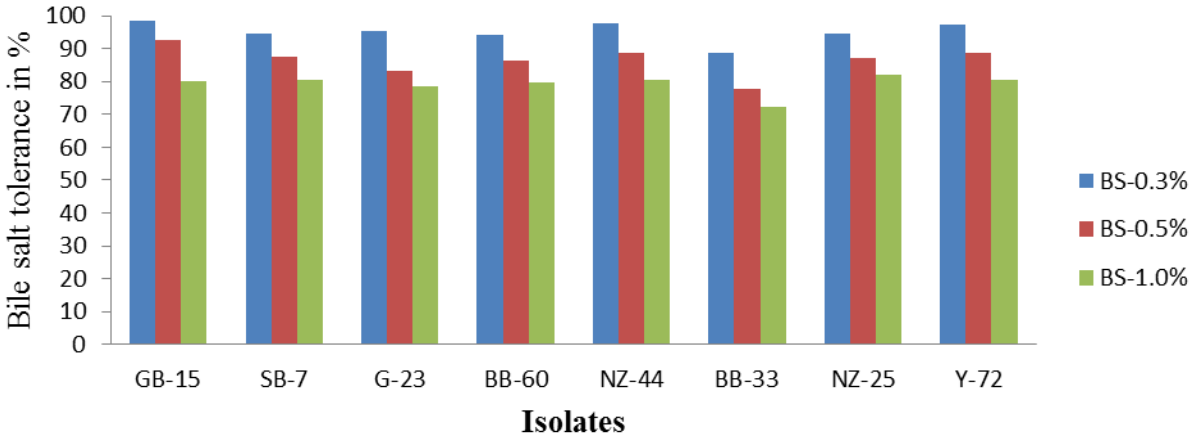


Figure 2: Bile salt tolerance of isolates in percent during 6 hrs of incubation**.**

. *GB-15, SB-7; G-23; BB-60; NZ-44; BB-33; and NZ-26 were lactic acid bacterial isolates; Y-72= yeast*

4.3.3. Cholesterol reduction

The *in vitro* cholesterol reduction of each probiotic starter culture candidates of lactic acid and yeast isolates was shown in *Fig*. 3. The LAB isolates were capable of reducing cholesterol in the range of 14.57 to 45.81%. The highest (45.81%) cholesterol reduction capability was observed by lactic acid bacteria isolate (GB-15) and the lowest (14.57 %) by LAB (NZ-26) isolate. The yeast isolate used in this study (Y-72) had relatively the least (9.83%) cholesterol reduction. The control sample (cholesterol without starter culture) has not shown any cholesterol reduction i.e. 0% of cholesterol reduction (*Fig*. 3).

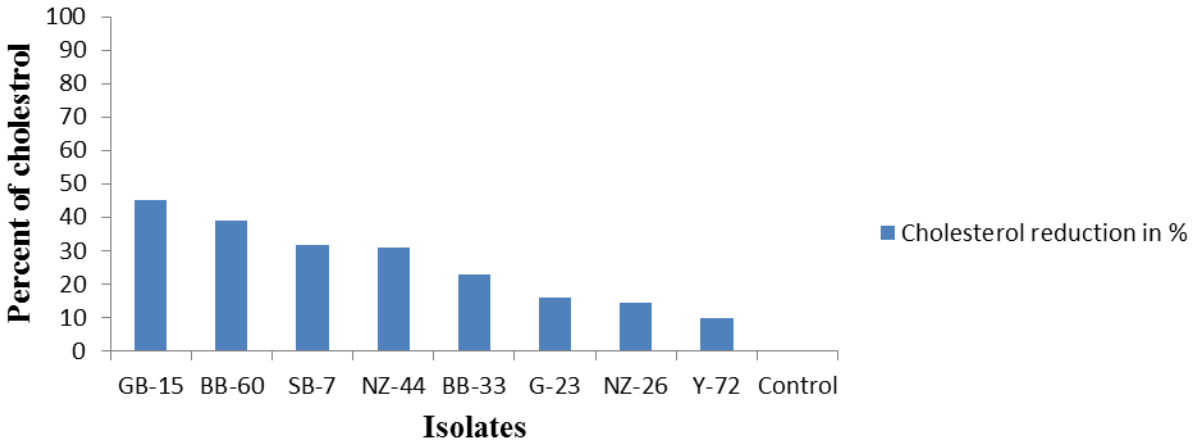
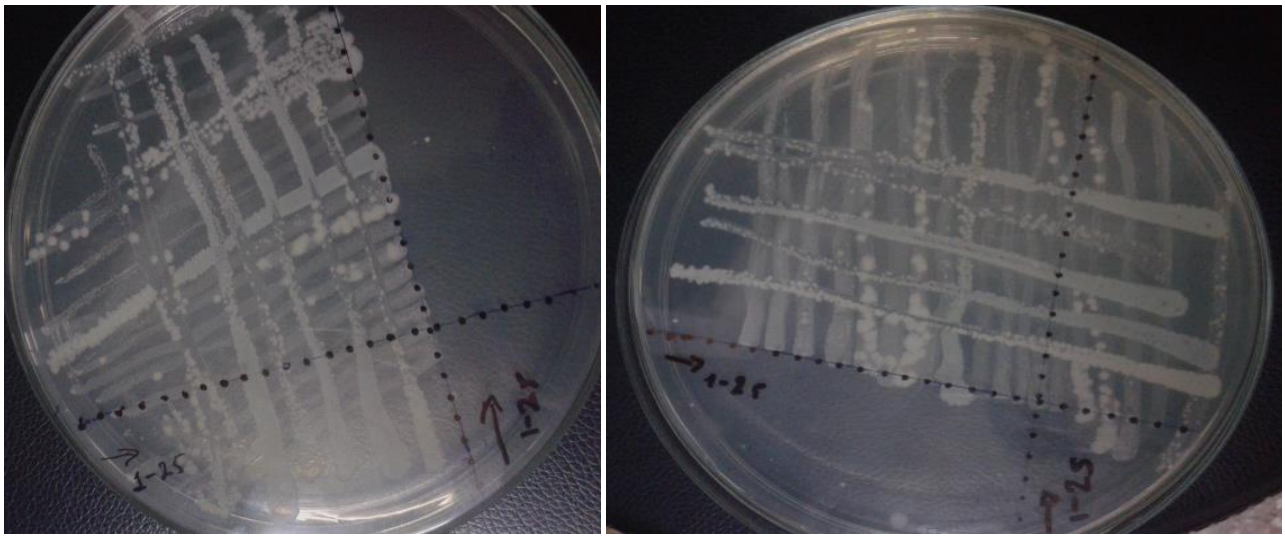


Figure 3: Isolates *in vitro* cholesterol reduction capability with 0.3% bile salt and 10mg of bile salt.

GB-15, SB-7; G-23; BB-60; NZ-44; BB-33; and NZ-26 were lactic acid bacterial isolates; Y-72= yeast isolate.

4.4. Compatibility test

All the seven lactic acid bacteria (NZ-44, SB-7, BB-60, BB-33, GB-15, G-23 and NZ-26) and the yeast (Y-72) isolate were found to be compatible. None of the isolates was found inhibiting the other. They have no antagonistic effect on one another (*Fig*.4).



a). Compatibility between LAB isolates b). Compatibility between LAB and yeast isolate

Figure 4: Compatibility test of each lactic acid and yeast isolates

4.5. Antagonistic effect of starter culture formulates on some food borne pathogens

The pH of the fresh milk at the time of inoculation of formulate (0 hrs) was 6.80, and was shown to progressively decrease as fermentation continue. Analysis of variance result showed pH of the products was highly significant ($P < 0.001$) difference at 24 and 48 hrs during the course of fermentation of *ergo* (Table 5). The pH values of *ergo* fermented by nine different formulated starter culture ranged from 4.74 by F5 to 4.01 by F2 at 24 hrs and from 3.99 by F2 to 4.38 by F7 at 48 hrs. Relatively lower pH value product was observed during 48 hrs of fermentation. Among the *ergo* product produced with the formulated starter culture, lower pH of 3.99 and 4.00 was observed in product F2 and F6 at 48 hrs of fermentation respectively. The pH of each product was significantly ($P < 0.05$) lower than the control fermented *ergo* (cont B) at 24 hrs of fermentation (Table 5). The initial (0 hrs) titratable acidity of fresh milk was 0.21% and increased to 0.97% during fermentation of *ergo* for 48 hrs. Titratable acidity of the product ranged from 0.87% by F6 to 0.49% by F8 at 24 hrs. Relatively maximum titratable acidity of 0.97% by F2 and F6 and minimum titratable acidity of 0.71% was observed by F8 at 48 hrs during the fermentation of *ergo*. Titratable acidity of each product produced by different starter culture formulates was significantly ($P < 0.05$) higher than the titratable acidity of control fermenting *ergo* (cont B) at 48 hrs. Rather than F8 and F9 the titratable of all products was significantly ($P < 0.05$) higher than the titratable acidity of control fermented *ergo* (cont B) at 24 hrs.(Table 5).

Table 5 shows the antagonistic effect of each formulated starter cultures on food borne pathogens when co-cultured in milk during the course fermentation of *ergo.* The mean initial (0 hrs) counts of the separate test organisms (*E. coli, S. aureus, S.* Typhimurium, and *L. monocytogenes*) of the challenge study was between 3.14 and 3.20 log cfu/ml. The reduction in the number of *E. coli* ranges between the highest 3.14 log cfu/ml by F2 and F6 and the lowest 1.16 log cfu/ml by F5 during 48 hrs of the challenge study, whereas the maximum reduction in number of *E. coli* was 0.86 log cfu/ml in F2 and

minimum of 0.15 log cfu/ml in F1 and F9 at 24 hrs. The number of *E. coli* was reduced by 2.14 log cfu/ml, 2.04 log cfu/ml, 1.99 log cfu/ml, 1.49 log cfu/ml, 1.45 log cfu/ml, and 1.31 log cfu/ml by F4, F1, F7, F3, F9 and F8 in that order at 48 hrs. The antagonistic effect of each formulated starter culture on the growth *E. coli* was significantly ($P < 0.05$) higher than the effect of control fermenting *ergo* (cont B) at 48 hrs during the course of fermentation of *ergo.*

S. aureus co-cultured with F2, F6, F4, F7, F1, F3, F9, F8 and F5 starter culture formulates in fermenting *ergo* was reduced by 3.14 cfu/ml, 2.14 cfu/ml, 2.04 cfu/ml, 1.69 cfu/ml, 1.71 cfu/ml, 1.61 cfu/ml, 1.45 cfu/ml, 1.40 cfu/ml and 1.27 log cfu/ml in that order during the course of fermentation of *ergo* at 48 hrs (Table 4). Maximum reduction in number of *S. aureus* co-cultured with the formulated starter culture was 1.1 log cfu/ml by F4 and minimum reduction of 0.25 log cfu/ml by F7 was observed during 24 hrs. Antagonistic effect of each formulates was significantly ($P < 0.05$) higher than the effect of control fermented *ergo* on the growth of *S. aureus* at 48 hrs during the course of fermentation of *ergo*.

Inhibition of *S.* Typhimurium co-cultured with each starter culture formulate during the course of fermentation of *ergo* was 3.08 log cfu/ml by (F2 and F6), 2.08 log cfu/ml (F4), 2.01 log cfu/ml (F3), 1.93 log cfu/ml (F8), 1.84 log cfu/ml (F9), 1.78 log cfu/ml (F7), 1.72 log cfu/ml (F1), and 1.43 log cfu/ml (F5) at 48 hrs. Maximum reduction of 1.05 log cfu/ml by F2 and minimum reduction of 0.4 log cfu/ml by F8 was observed on the number of *S.* Typhimurium co-cultured with each formulated starter culture at 24 hrs. Reduction of growth of *S.* Typhimurium by each formulated starter culture was significantly ($P < 0.05$) higher than the control fermented *ergo* (cont B*)* at 24 hrs (Table 5).

Analysis of variance result showed that *L. monoctyogenes* co-cultured with each formulated starter culture was noted being significantly ($P < 0.05$) reduced during the course of fermentation of *ergo* at both 24 and 48 hrs. However, antagonistic effect of each formulated starter culture on the growth of *L. monoctyogenes* was significantly ($P < 0.05$) higher than the control fermented *ergo* (cont B) at both 24

and 48 hrs. Maximum reduction of 3.20 log cfu/ml by F2, F4 and F6 and minimum reduction of 1.2 log cfu/ml of *L. monoctyogenes* was observed by F3 at 48 hrs. Other formulates reduced *L. monoctyogenes* count by 2.2 log cfu/ml (F1 and F7), 2.05 log cfu/ml (F5) and 2.03 log cfu/ml (F8 and F9) at 48 hrs, during fermentation of *ergo.* Maximum reduction of *L. monoctyogenes* raged from 3.20 log cfu/ml by F2 and minimum of 0.76 log cfu/ml by F7 was observed at 24 hrs during the course of fermentation of *ergo*.

Generally, among each starter culture formulates co-cultured separately with each test pathogens maximum antagonistic effect on the growth of each test pathogens was observed by F2 and F6 at 48 hrs during the course of fermenting *ergo* (Table 5).

Table 5: Antagonistic effect of each formulates against food borne pathogens

Formulates	Time (in hrs)	pH	TA (%)	*E. coli* Mean ±SD (Log cfu/ml)	*S. aureus* Mean ±SD (Log cfu/ml)	Mean ±SD (Log cfu/ml)	*L. monoctyogenes* Mean ±SD (Log cfu/ml)
M1	0	6.80±0.02^{a}	0.21±0.04^{i}	3.14±0.06^{a}	3.14±0.02^{a}	3.08±0.03^{a}	3.20±0.02^{a}
F1	24	4.43±0.02^{fg}	0.48± 0.13^{k}	2.89±0.01^{b}	2.72±0.02^{ed}	2.60±0.02^{d}	1.53±0.02^{d}
F1	48	4.22±0.6^{ji}	0.60± 0.01^{hi}	1.10±0.17^{ji}	1.43±0.38^{j}	1.36±0.10^{i}	1.00±0.00^{g}
F2	24	4.01± 0.04^{l}	0.86 ±0.04^{b}	2.28±0.03^{e}	2.14±0.04^{g}	2.03±0.03^{g}	0.00±0.00^{f}
F2	48	3.99 ±0.14^{l}	0.97 ±0.02^{a}	0.00±0.00^{k}	0.00±0.00^{l}	0.00±0.00^{m}	0.00±0.00^{f}
F3	24	4.38 ±0.03^{hg}	0.65± 0.04^{hg}	2.54±0.01^{d}	2.62±0.01^{e}	2.34±0.02^{e}	2.15±0.15^{c}
F3	48	4.14 ±0.06^{jk}	0.89 ±0.01^{b}	1.65±0.05^{j}	1.53±0.06^{j}	1.07±0.12^{ml}	2.00±0.15^{e}
F4	24	4.63± 0.04^{e}	0.73± 0.03^{fe}	2.52±0.05^{d}	2.04±0.04^{g}	2.22±0.08^{f}	1.00±0.00^{g}
F4	48	4.02 ±0.02 ^{l}	0.81 ±0.02^{dc}	1.00±0.00^{l}	1.10±0.17^{k}	1.00±0.00^{m}	0.00±0.00^{f}
F5	24	4.74 0.02^{d}	0.62± 0.03^{hi}	2.61±0.01^{cd}	2.32±0.02^{f}	2.09±0.09^{g}	2.15±0.15^{c}
F5	48	4.37± 0.02^{hg}	0.81 ±0.01^{dc}	1.98±0.03^{f}	1.87±0.03^{h}	1.65±0.05^{h}	1.15±0.15^{e}
F6	24	4.21 ±0.04^{ji}	0.87 0.03^{b}	2.66±0.05^{c}	2.84±0.03^{cd}	2.60±0.02^{d}	1.40±0.1^{d}
F6	48	4.00 ±0.13^{l}	0.97± 0.02^{a}	0.00±0.00^{k}	1.00±0.00^{jk}	0.00±0.00^{n}	0.00±0.00^{f}
F7	24	4.52 ±0.02^{f}	0.69 ±0.02^{fg}	2.88±0.03^{b}	2.89±0.03^{c}	2.70±0.04^{cd}	2.44±0.06^{b}
F7	48	4.38± 0.03^{hg}	0.78 ±0.01^{de}	1.15±0.15^{i}	1.45±0.15^{j}	1.30±0.00^{i}	1.00±0.00^{e}
F8	24	4.74 ±0.01^{d}	0.49± 0.07^{jk}	2.83±0.02^{b}	2.82±0.04^{cd}	2.68±0.04^{cd}	2.01±0.53^{c}
F8	48	4.26 ±0.01^{i}	0.71± 0.01^{f}	1.83±0.13^{g}	1.74±0.04^{hi}	1.15±0.15^{kl}	1.17±0.29^{e}
F9	24	4.66± 0.10^{de}	0.53 ±0.01^{j}	2.89±0.01^{b}	2.85±0.04^{cd}	2.59±0.06^{d}	2.14±0.04^{c}
F9	48	4.28 ±0.03^{hi}	0.73 ±0.04^{fe}	1.69±0.09^{i}	1.69±0.09^{i}	1.24±0.24^{kj}	1.17±0.29^{e}
Cont B	24	5.15±0.05^{b}	0.46±0.01^{k}	3.02±0.02^{a}	3.05±0.06^{ab}	3.08±0.02^{a}	3.01±0.01^{a}
Cont B	48	4.92± 0.06^{gf}	0.68± 0.06^{f}	2.96±0.03^{ab}	2.95±0.02^{cb}	2.97±0.02^{ab}	2.98±0.02^{a}

All values are mean ± SD in triplicate; Values in the same column carrying the same superscript are not significantly different;
Control B = control fermented ergo; 0 hrs= fresh pasteurized milk, ,M1= fresh raw milk before inoculation; F1, F2… F9= ergo product; TA= titratable acidity.

4.6. Proximate composition of *ergo*

Table 6 showed, the proximate analysis results of *ergo* products made by different formulated starter cultures. The result of total solid content was significantly ($P < 0.05$) different among the products. The total solid of the products was ranging from the highest 17.87% in product F2 to the lowest 15.97% in product F4. The total solid of all products rather than F4 was significantly ($P< 0.05$) higher than the total solid content of control fermenting *ergo* (cont B). Analysis of variance results of protein contents of each *ergo* product produced by each formulated starter culture showed that there was significant ($P < 0.05$) difference among the products. The protein content of the products varying from highest (4.28%) in F6 to the lowest (3.19%) in F7. The protein content of F2 (4.22%) and F6 (4.28%) was significantly ($P < 0.05$) higher than the protein content of control fermented *ergo* (cont B) and control A (commercial yoghurt) (Table 4). The fat content of the product was a significant ($P < 0.05$) difference among *ergo* products produced by different starter culture formulates. The fat content of the products range from maximum of 5.50% in F2 to minimum of 4.16% in F7. Among the products, the fat content of, F2 (5.50%) and F6 (5.47%) was significantly ($P<0.05$) higher than control fermented *ergo* (cont B) and commercial yoghurt (cont A) (Table 6).

The ash values of the products was varied from the highest 0.98% in product F3 to the lowest 0.60% in F8. The analysis of variance result showed that there was no significant ($P > 0.05$) difference among the ash content of the products produced by different starter formulates, the control fermented *ergo* (cont B) and cont A (commercial yoghurt) (Table 6). Analysis of variance results of solid nonfat (SNF) of *ergo* products produced by different starter formulates were shown significantly ($P < 0.05$) differing (Table 6). The result showed that, SNF of F2 (13.43%). and F1 (12.97%), was significantly higher than the SNF of the control fermented *ergo*

(cont B) and cont A (commercial yoghurt). While analysis of variance results of lactose content of the product showed that, there was a significant ($P < 0.05$) difference among the products. High lactose content of 5.07% in product F9 and low lactose content of 2.49% was observed in product F2. The lactose content of F2 (2.49%), F5 (3.15%) and F6 (3.10%) were significantly ($P < 0.05$) lower than the control fermented *ergo* (cont B) and cont A (commercial yoghurt).

Except F4, the moisture content of all the products was significantly lower than the control fermented *ergo* (cont B). However, the moisture content was significant ($P < 0.05$) different among the products. The moisture content of the products varied from the highest 84.03% in F4 to the lowest 82.13% in F2. The syneresis (whey) content of the products ranged from a minimum of 9.46% in F3 to a maximum of 10.68% in F1. The syneresis content, 10.58% in F5, 10.40% in F7, 9.91% in F6, 9.78% in F2 and 9.71% in F4 were significantly ($p < 0.05$) lower than the control fermented *ergo* (cont B*)* and cont A (commercial yoghurt). The syneresis content 10.68% in F1, 10.50% in F9 and 9.46% in F3 did not show significant difference with the syneresis content of control fermented *ergo* (cont B) and cont A (commercial yoghurt) (Table 4).

Table 6: *Ergo* made with formulated starter culture proximate analysis

Product	Total solid (%) Mean ±SD	Protein(%) Mean ±SD	Fat(%) Mean ±SD	Ash(%) Mean ±SD	SNF(%) Mean ±SD	Lactose(%) Mean ±SD	Moisture(%) Mean ±SD	Syneresis(%) Mean ±SD
F1	17.04 ±0.59^{dcb}	3.72±0.12^{ab}	4.67±0.06^{bc}	0.67±0.15^{ab}	12.97±0.59^{ab}	4.58±0.54^{a}	82.96±0.59^{dbc}	10.68±0.54^{cad}
F2	17.87±0.10^{a}	4.22±0.82^{a}	5.50±0.50^{a}	0.74±0.00^{ab}	13.43±0.21^{a}	2.49±0.04^{d}	82.13±0.1^{e}	9.78±0.47^{ced}
F3	17.79±0.05^{a}	3.61±0.289^{ab}	4.43±0.12^{bc}	0.98±0.36^{a}	12.29±0.51^{cdb}	3.61±0.53^{cb}	82.21±0.05^{e}	9.46±0.15^{cab}
F4	15.97±0.13^{e}	3.56±0.159^{ab}	4.33±0.29^{bc}	0.75±0.015^{ab}	11.63±0.30^{de}	3.66±0.26^{cb}	84.03±0.13^{a}	9.71±0.59^{ced}
F5	17.17±0.10^{cb}	3.69±0.06^{ab}	4.50±0.50^{bc}	0.76±0.02^{ab}	12.67±0.51^{cab}	3.15±0.13^{cd}	82.83±0.10^{dc}	10.58±0.496^{ed}
F6	16.57±0.26^{d}	4.28±0.095^{a}	5.47±0.06^{a}	0.82±0.04^{ab}	12.47±0.38^{cdb}	3.10±0.09^{cd}	83.43±0.26^{b}	9.91±0.51^{ced}
F7	16.69±0.196^{dc}	3.19±0.24^{b}	4.16±0.29^{c}	0.75±0.02^{ab}	12.53±0.42^{cdb}	4.57±0.63^{a}	83.31±0.196^{bc}	10.40±0.34^{ed}
F8	16.82±0.067^{dc}	3.79±0.19^{ab}	4.63±0.42^{bc}	0.60±0.17^{ab}	12.15±0.23^{cdeb}	4.61±0.58^{a}	83.18±0.07^{bc}	10.18±0.12^{cedb}
F9	16.57±0.02^{d}	3.37±0.30^{b}	4.83±0.29^{b}	0.77±0.01^{ab}	11.75±0.27^{cde}	5.07±0.06^{a}	83.14±0.02^{b}	10.50±1.39^{cadb}
Cont A	17.45±0.40^{ab}	3.82±0.67^{ab}	4.10±0.36^{c}	0.68±0.24^{ab}	11.99±0.44^{cde}	4.23±0.88^{ab}	82.55±0.40^{de}	11.24±0.93^{ab}
Cont B	15.92±0.63^{e}	3.24±0.15^{b}	4.07±0.12^{c}	0.55±0.35^{ab}	11.29±0.95^{e}	4.52±0.04^{a}	84.08±0.63^{a}	11.55±0.78^{a}

All values are means ± SD of triplicate data *in percent; Values in the same column carrying the same superscript are not significantly different; F1, F2 ...F9 = ergo product produced with different starter formulates; cont A= commercial yoghurt, cont B= control fermented ergo; SNF= Solid nonfat.*

4.7. Sensory characteristics of *ergo* produced by different starter formulates

Sensory qualities of flavor (aroma + test) and texture are crucial for consumer acceptance. Products can have quite distinct flavor and texture properties depends on the starter cultures capability of producing flavor enhancing compounds and curd formation. Generally, the *ergo* product F6 was exhibited to have a better sensory mean score of color 3.85 and flavor 3.67 (Table 5). Analysis of variance result showed statistically significant ($P < 0.05$) difference for color sensory attributes among the products. *Ergo* represented by F2, F6 and F4 were shown to have better overall sensory acceptability with mean score of 3.75, 3.73 and 3.56, respectively (Table 7). Overall sensory acceptability mean score of *ergo* product F8 (2.80) and F9 (2.73) were observed significantly ($P < 0.05$) lower than the overall mean score of control fermented *ergo* (cont B) and cont A (commercial yoghurt). Among the sensory overall acceptability of *ergo* products only product F2, F4 and F6 were significantly ($P < 0.05$) higher than these of the control fermented *ergo* (cont B). The sensory overall acceptability mean scores of the control fermented *ergo* (cont B) and cont A (commercial yoghurt) was 3.43 and 3.20, respectively (Table 7).

Table 7: Sensory characteristics of *ergo* made with the formulated starter culture

	Score for sensory characteristics (Mean score ± SD)				
Product	**Color**	**Flavor**	**Odor**	**Texture**	**Overall acceptability**
F1	3.04±0.59^{cdb}	2.91±0.54^{cdb}	3.11±0.54^{cd}	3.38±0.73^{ab}	3.02±0.53^{cb}
F2	3.77±0.58^{a}	3.74±0.44^{a}	3.72±0.42^{ab}	3.79±0.45^{a}	3.75±0.47^{a}
F3	3.16±0.67^{cb}	3.81±0.87^{a}	3.23±0.37^{cdb}	3.42±0.48^{ab}	3.39±0.46^{ab}
F4	3.59±0.26^{ab}	3.5±0.37^{ab}	3.59±0.47^{cab}	3.42±0.38^{ab}	3.56±0.37^{a}
F5	3.14±0.66^{cb}	3.19±0.56^{cab}	3.82±0.47^{a}	3.78±0.41^{a}	3.38±0.56^{ab}
F6	3.85±0.47^{a}	3.67±0.48^{a}	3.65±0.42^{ab}	3.74±0.56^{a}	3.73±0.43^{a}
F7	3.13±0.18^{cb}	3.32±0.22^{cab}	3.60±0.21^{cab}	3.55±0.58^{a}	3.35±0.12^{ab}
F8	2.78±0.28^{cd}	2.78±0.26^{cd}	2.85±0.59^{d}	2.92±0.25^{b}	2.80±0.37^{c}
F9	2.55±0.42^{d}	2.55±0.50^{d}	3.09±0.26^{cd}	2.97±0.31^{b}	2.73±0.39^{c}
Cont A	3.34±0.56^{cab}	3.22±0.76^{cab}	3.57±0.64^{cab}	3.76±0.34^{a}	3.43±0.65^{ab}
Cont B	3.31±0.65^{cab}	2.92±0.61^{cdb}	3.39±0.34c^{ab}	2.99±0.54^{b}	3.20±0.47^{b}

All values are Mean score ± SD of triplicate data; F1, F2…. F9 = Ergo product; Cont A commercial yoghurt; Cont B= control fermented ergo; Values in the same column carrying the same superscript are not significantly different

4.8. Sensory characteristics of *ayib* treated with the starter culture

The sensory attributes of *ayib* such as color, flavor, odor and texture revealed significant ($P < 0.05$) difference among products. Higher overall sensory acceptability values of 4.03 and 3.79 were observed for F2(AY2) and F6(AY6), respectively, and least sensory overall acceptance mean score of 3.31 was observed for product F9(AY9) (Table 8). The overall sensory acceptance mean score for control AYB (*ayib* not treated with any formulated starter culture) was indicated as 3.35. Among *ayib* treated with different formulated starter cultures only F2(AY2) was significantly ($P < 0.05$) higher sensory overall acceptable mean score than the control (Table 8).

Table 8: Sensory characteristics of *ayib* treated with the formulated starter culture

	Score for sensory characteristics (Mean score ± SD)				
Product	**Color**	**Flavor**	**Odor**	**Texture**	**Overall acceptability**
F1(AY1)	3.58 ± 0.37^{b}	3.13 ± 0.38^{d}	3.41 ± 0.29^{cb}	3.72 ± 0.23^{cab}	3.47 ± 0.32^{cb}
F2(AY2)	3.89 ± 0.53^{ab}	3.91 ± 0.36^{ab}	4.02 ± 0.24^{a}	4.15 ± 0.39^{a}	4.03 ± 0.35^{a}
F3(AY3)	3.65 ± 0.41^{ab}	3.69 ± 0.33^{cab}	3.58 ± 0.41^{ab}	3.85 ± 0.32^{cab}	3.69 ± 0.36^{cab}
F4(AY4)	3.60 ± 0.41^{ab}	3.44 ± 0.37^{cd}	3.69 ± 0.37^{ab}	3.55 ± 0.31^{cdb}	3.57 ± 0.36^{cb}
F5(AY5)	3.80 ± 0.42^{ab}	3.79 ± 0.48^{cab}	3.82 ± 0.44^{ab}	3.55 ± 0.34^{cdb}	3.62 ± 0.38^{cab}
F6(AY6)	4.13 ± 0.42^{a}	3.97 ± 0.28^{a}	3.39 ± 0.29^{cb}	3.66 ± 0.24^{cb}	3.79 ± 0.3^{ab}
F7(AY7)	3.76 ± 0.46^{ab}	3.76 ± 0.4^{cab}	3.63 ± 0.41^{ab}	3.92 ± 0.60^{ab}	3.77 ± 0.42^{ab}
F8(AY8)	3.78 ± 0.40^{ab}	3.67 ± 0.44^{cab}	3.38 ± 0.25^{cb}	3.67 ± 0.60^{cb}	3.63 ± 0.41^{cab}
F9(AY9)	2.86 ± 0.32^{c}	3.35 ± 0.35^{cd}	3.63 ± 0.45^{ab}	3.38 ± 0.29^{cd}	3.31 ± 0.34^{c}
AYB	3.66 ± 0.78^{ab}	3.47 ± 0.36^{cdb}	3.05 ± 0.69^{c}	3.10 ± 0.56^{d}	3.35 ± 0.54^{cb}

All values are means ± SD of triplicate data; F1(AY1), F2(AY2)… F9(AY9) were ayib product treated with formulated starter culture; AYB = Ayib not treated with starter culture; Values in the same column carrying the same superscript are not significantly different.

4.9. *Ergo* sensory shelf life estimates

The shelf life of *ergo* produced with each starter culture and stored at ambient temperature (18-24^{o}C) was found varying from 15 days of F6 to 6 days of F1 (Table 9). The shelf life of *ergo* product produced by different starter culture formulates was shown as 12 days for F2, 10 days for F4, 9 days for F3, 8 days for F7 and F9, 7 days for F5 and F8, and 6 days for F1 (Table 9). The shelf life of control fermented *ergo* (cont B) and control A (commercial yoghurt) were shown as 6 and 7 days in that order.

Table 9: Sensory shelf life of *ergo* stored at ambient temperature of 18-24^{o}C

Product	Quality criteria	Storage temp.	Quality limit	R^2	Estimated shelf life (in days)
F1	Sensory Overall acceptability	18-24^{o}C	3.5	0.99	6
F2			3.5	0.97	12
F3			3.5	0.94	9
F4			3.5	0.97	10
F5			3.5	0.98	7
F6			3.5	0.95	15
F7			3.5	0.99	8
F8			3.5	0.98	7
F9			3.5	0.97	8
Cont A			3.5	0.98	7
Cont B			3.5	0.94	6

cont B= Ergo produced by natural fermentation, cont A= Ergo from commercial supermarket; R^2 = coefficient of determination; F1, F2...F9 = ergo product.

4.10. *Ayib* (cottage cheese) shelf life estimates

The shelf life of *ayib* treated with each starter culture and stored at ambient temperature (18-24^{o}C) exhibited variation from 18 days for F2(AY2) and F6(AY6) to 6 days for F8(AY8). The shelf life of each *ayib* products was shown as 18 days for F2(AY2) and F6(AY6), 12 days for F4(AY4) and F7(AY7), 11 days for F9(AY9), 9 days for F5(AY5), 8 days for F3(AY3), 7 days for F1(AY1), and 6 days for F8(AY8). The shelf life of control AYB was found as 4 days (Table 10).

Table 10: Sensory shelf life of *ayib* stored at ambient temperature of 18-24^{o}C.

Product	Quality criteria	Storage temp.	Quality limit	R^2	Estimated shelf life (in days)
F1(AY1	Overall acceptance	18-24^{o}C	3.5	0.94	7
F2(AY2			3.5	0.92	18
F3(AY3			3.5	0.92	8
F4(AY4			3.5	0.99	12
F5(AY5			3.5	0.94	9
F6(AY6			3.5	0.89	18
F7(AY7			3.5	0.95	12
F8(AY8			3.5	0.93	6
F9(AY9			3.5	0.87	11
AYB			3.5	0.88	4

AYB= Ayib not treated with the formulates, F1(Ay1), F2(Ay2)... F9(Ay9) = Ayib product treated with different starter culture; R^2= coefficient of determination.

5. Discussions

In this study, seven LAB and one yeast isolates were screened for the formulation of bioprotective probiotic starter culture based on their probiotic and starter culture attributes. These isolates were mixed in different proportions to formulate bio-protective probiotic starter cultures. The screened isolates were found to have high tolerance to low pH (2.0 – 3.0) for 24 hrs of incubation. Among the screened LAB, isolate NZ-44 showed the best tolerance to pH 2.0, 2.5 and 3.0, followed by the yeast isolate Y-72 (pH 2.0, 2.5 and 3.0) (*Fig* 1). Acid tolerance of the isolates were found in decreasing order as NZ-44 >Y-72 > SB-7 > BB-60 > GB-15 > BB-33 > G-23 > NZ-26 to pH of 2.0, The result indicated that, the isolates could survive in the acidic stomach environment and reach the small intestine after being ingested. Syal and Vohare, (2013), reported that, probiotic yeasts, isolated from Indian traditionally fermented food having 56 - 100% tolerant to a pH 2.0 for 3-6 hrs of incubation. Mary and Ammani (2004). reported that lactic acid bacteria isolated from traditionally fermented food having 88-91% tolerance to pH of 2.0 and 3.0 for 6 hrs. This acid tolerance might be due to homeostatic response or through synthesis of acid shock protein (Osman *et al*., 2016).

Tolerance to bile salts also considered as the main prerequisite for growth, colonization and metabolic activity of probiotics in the host's gut (Liong and Shaha, 2005). After passage of the organisms through the acidic stomach condition, it has to survive the bile salt in the intestine, the normal level of which is around 0.3%. In this study, all the LAB and yeast isolate were found-to be tolerant to inhibitory action of bile salt ranging from 0.3% to 1% for 24 hrs of incubation. The LAB isolates of this study were able to survive 1% bile salt concentration for 6 hrs at survival rate ranging from 72.38% to 82.24%. This result is also in agreement with Damayanti, (2014), reported that lactic acid bacteria isolated from proventriculus of broiler chicken having more

than 100% tolerance to bile salt of 0.3% for 2hrs. Syal and Vohare (2013), also found probiotic yeasts isolated from Indian traditionally fermented food having 1% bile salt tolerance of 80-100% at 3-6 hrs incubation. This result indicates that the isolates could survive bile toxicity during their passage through gastrointestinal system and brought the desired effect. The bile salt tolerance capability might be due to bile salt hydrolase (BSH) activity of the isolates (Osman *et al*., 2016).

High levels of serum cholesterol have been associated with the risk of coronary heart disease inducing colon cancer (Golnoush *et al.,* 2013). Supplementation of diet with fermented dairy products or lactic acid bacteria containing dairy products has shown the potential to reduce serum cholesterol levels (Manoj *et al*., 2012). In this study, all the probiotic isolates of LAB and yeast used for formulation of starter culture were able to reduce cholesterol at varying degrees. *In vitro* cholesterol reduction by the LAB and yeast isolates ranged from 14.57% to 45.18%. LAB isolates GB-15 showing the highest potential of cholesterol reduction (45.81%) and the yeast isolate showed cholesterol reduction capability of 9.83%. The study of Fatimah and Miremad (2014) revealed that 37 - 40.01% cholesterol assimilation of three lactobacillus strains isolated from fermented food. Kaur *et al*,. (2002) have also reported 38% reduction of cholesterol by *L. reuteri* when it was given to mice for 7 days. Cholesterol reduction of these isolates might be due to capability of the isolates hydrolyzing and utilizing cholesterol for metabolic activity or they may bind or incorporate the cholesterol directly into the cell membrane (Dilmi-Bouras, 2006)).

Each isolate of LAB and yeast isolate was found compatible and was able to grow together without any antagonistic effect against one another. This property may increase their antagonistic potential when mixed as starter culture in different proportion and may have synergistic effect against food borne pathogens. The lowest pH values of *ergo* fermented by different starter

culture combinations, was 3.99 in F2 at 48 hrs. The pH of the products were observed decreasing (become more acidic) and titratable acidity of the product was observed increasing as the time of fermentation extended from 24 hrs to 48 hrs. Titratable acidity and pH of the product were due to fermentation of lactose in the milk by starter culture formulates. These two parameters with other metabolites enhance the antagonistic effect of the formulates on food borne pathogens (Pundir *et al*., 2013).

In this study, all formulates co-cultured with the test organisms in fermented milk showed an antagonistic effect against the test pathogens. The best inhibitory effect against each of the four test pathogens (*Escherichia coli* ATCC 43895, *Staphylococcus aureus* ATCC 25923, *Salmonella* Typhimurium ATCC 14802 and *Listeria monoctyogenes* ATCC 15313) was shown by F2 and F6 formulates during the course of fermentation of *ergo* at 48 hrs. The antagonistic effects of each formulate against *E. coli* ATCC 43895 has shown variation from 3.14 log cfu/ml to 1.16 log cfu/ml by formulate F2 and F6 during 48 hrs of fermentation. During 24 hrs of incubation maximum reduction of *E. coli* count was 2.14 log cfu/ml by F4. Similarly, Anteneh *et al.* (2011a) reported mixed LAB culture totally eliminated *E. coli* at 36 hrs of co-culturing. Kasimoglu and Akgun (2004) also reported that 10^6 cfu g^{-1} of *E. coli* O157:H7 were eliminated after 48 hrs incubation in acidophilus yoghurt. Mekonen and Mogessie (2005), reported during spontaneous fermentation of *ergo* entroheamorigic *E. coli* decreased by 2 log units at 24 hrs of fermentations. Mufandaedza *et al*. (2006) reported that population of *E. coli* decreased by 2 log units during fermentation of milk with *L. biovar*. In relation to the above finding, the formulated starter cultures in this study have shown a better antagonistic effect against food borne pathogenic *E. coli* during the course of fermentation of *ergo.* This antagonistic effect might be due to

production of antimicrobial metabolites in addition to other organic acids (Tharmaraj and Shah, 2009).

Standard *S. aureus* ATCC 25923 co-cultured with formulate F2 and F6 were completely eliminated (100%) at 48 hrs. Formulate F4 showed the next high antagonism effect against *S. aureus* next to F2 and F6, reducing them by 2.04 log cfu/ml at 48 hrs. Related study done by Anteneh *et al*, (2011) reported that mixed LAB culture totally eliminated *S. aureus* at 48 hrs when co-cultured in pasteurized milk and stored at ambient temperature. Voravuthikuncha *et al.* (2006) also reported that lactic acid bacteria co-cultured with *S. aureus* ATCC 25923 reduced the population of *S. aureus* by 8 log units at 24 hrs. Charlier *et al.* (2006) also reported that, *L. lactic* co-cultured with *S. aureus* in 1:1 ratio in nutrient broth medium reduced the population of *S. aureus* by 8 log units. In relation to the above finding, the result of this study indicated that the formulated starter culture showed a better inhibitory effect and this might be due to the synergistic antagonistic effect of the formulated isolates (Komi *et al.*, 2013).

Similar to *E. coli* and *S. aureus*, *S.* Typhimurium ATCC 14802 co-cultured with F2 and F6 were eliminated at 48 hrs during the course of fermentation of *ergo*. The effect of F4 and F3 against *S.* Typhimurium was better antagonistic effect next to formulate F2 and F6 at 48 hrs. Anteneh *et al*., (2011), reported similar result, he indicated that *S.* Typhimurium DT104 co-cultured with mixed LAB culture was totally eliminated at 48 hrs when stored at ambient temperature. Related result of reduction of *S.* Typhimurium by 3.2 log units while being co-cultured with probiotic organisms in MRS medium was also reported by Tharmaraj and Shah, (2009).

L. monoctyogenes ATCC 15313 co-cultured with F2 was totally eliminated (3.20 log cfu/ml) at 24 and 48 hrs. Formulate F6 reduced the test pathogen by 1.8 log cfu/ml at 24 hrs and completely

inhibited (3.20 log cfu/ml) at 48 hrs of co-culturing in fermenting milk. Related results of 0.8 and 1.3 log cfu/ml reduction of *L. monoctyogenes* co-cultured with LAB for 12 and 24 hrs in fermented milk was reported by Mogessie Ashenafi, (1993).

The total solids of the *ergo* products produced by different starter culture formulates were varied from a maximum of 17.87% in F2 to a minimum of 15.97% in product F4. This content is relatively similar to the findings of Muhammed *et al*. (2005), who reported a yoghurt total solid of 17.11%. Joseph, *et al*. (2011), also reported yoghurt having a total solid content of 12.9% to 19.2%. Related result was also reported by Dublin-Green and Ibe, (2005) they reported total solid values of natural yoghurts ranging from 13.6% to 18.08%. However, Guler *et al*. (2011), reported 15.3% total solid yoghurt which is relatively lower than our results.

The protein content of the products was statistically significant ($P < 0.05$) difference among products. Relatively maximum protein value of 4.28% was observed in F6 and minimum of 3.19 % was observed in F7. This finding was comparable to 4.02% protein content reported by Bibiana *et al.*,(2014), 3.5% reported by Berhanu Andualem and Tsehayneh Geremew (2014), and 4.02 - 6.14% reported by Igbabul *et al.*, (2014). Guler *et al.* (2011), also reported yoghurt with protein content of 4.88%. This variation might be due to breed, lactation stage, feed and season or proteolysis of starter culture used (Davinia *et al*., 2012).

The fat content of product were significantly ($P < 0.05$) different among products. The fat content of the products varied from 4.16% in product F7 to 5.50% in product F2. Similar result of 4.0% to 5.5% was reported by Joseph, *et al.*, (2011). Our result was found to be higher than 3.25% reported by Igbabul *et al.* (2014), and 3.7% reported by Berhanu Andualem and Tsehayneh Geremew, (2014). This might be due to variation of type of breed, lactation stage, fat

content in the raw milk used, season, feed or lipolysis capability of the isolates in the formulates (Davinia *et al*., 2012).

The ash value was the total mineral content of the products. There was no significant ($P > 0.05$) difference among the ash value of the products. However, the products ash content varied from minimum 0.60% in F8 to maximum of 0.98% in F2. Guler *et al*. (2011) and Joseph *et al.* (2011), reported similar result of ash values of 0.99% and 0.71%. respectively. Igbabul *et al.* (2014) reported relatively similar yoghurt ash content of 0.41 - 1.02%. Solid nonfat of product was significantly ($P < 0.05$) different among *ergo* products fermented with different formulated starter culture. The SNF of the products were varied from 11.63% in product F4 to 13.43% in product F2 that compile with USDA (2001), yoghurt SNF specification of 9.49 to 18.77% and FDA (2009), standard of SNF not less than 8.25%.

The residual lactose content was tightly correlated to the ability of microorganisms to uptake lactose for metabolism and their power of milk acidification (Nambou *et al.,* 2013). This makes *ergo* an ideal food for lactose intolerant individuals (Ehirim and Ndimantang, 2004). The high residual lactose recorded in fermented milk may be attributed to low acidifying starter culture (Magalhaes *et al.,* 2011a). The minimal average of lactose content of the products was 2.49% in F2 and maximum of 4.61% was observed in F8. Lactose content was found to be lower in product with high titratable acidity values (F2) compared with those product with low titratable acidity values F8. This result was related to 3.25% of lactose content reported by Berhanu Andualem and Tsehayneh Geremew (2014) and smaller than 9.38-12.85% reported by Early (1998). This difference might be due to the fermentation capability of the starter culture used and difference in the raw milk used (Igbabul *et al.,* 2014).

In this study, the moisture content of the products varied from 82.13% in product F2 to 84.03% in product F4. The moisture content was significantly ($P < 0.05$) lower in product F2 and F3, respectively. Related results of 83.2% was reported by Berhanu Andualem and Tsehayneh Geremew (2014), and 78.2 - 87.1%, by Joseph, *et al.* (2011). The moisture content of yoghurt was dependent on the total solid content of the product and curd formation capability of the starter culture used for fermentations (Narayana *et al.*, 2013).

Syneresis was a measure of the quantity of whey separated from the yoghurt and one of the most important factors influencing consumers' acceptance. Higher syneresis yoghurt have low quality (Aprodu *et al*., 2012). The syneresis (whey) content of *ergo* products produced by different starter culture formulates showed variation from 9.46% in product F3 to 10.68% in F1. The result was found to be comparable to 9.65% reported by Rima *et al*. (2017).

Sensory attributes of color, flavor and texture of yogurt are important quality characteristics of the product and generally considered the most critical and important indicator of consumer acceptance (Yonca *et al*., 2017). Flavor with respect to odor and color had overwhelming influence in overall acceptability of the product (Igbabul *et al*., 2014). The entire *ergo* product prepared with different starter culture formulate were found to have significant difference ($p < 0.05$) in color mean scores. The results showed high color mean scores of 3.85 and 3.77 in product F6 and F2 respectively. These products were glossy surface appearance without excessive whey. Flavor of *ergo* product F2 (3.74), F3 (3.81) and F6 (3.67) were significantly ($P < 0.00$) higher mean score. Products flavor mean score had a range of 2.55 (F9) to 3.81 (F3) on 5 point hedonic scale. Lactic acid content of the product as well as diacetyl, ethyl acetate were the factors which influence the flavor of the yoghurt (Chaves *et al*., 2002; Beshkova *et al.*, 2003). Acidity influences the quality attributes of yoghurt such as flavor, texture, whey

syneresis and shelf life (Narayana *et al.,* 2013). The variability in titratable acidity between products might results the variability of the flavor of the *ergo* product (Beshkova *et al.,* 2003). Capability of starter culture on lactic acid and other flavor enhancing(diacetyl) aromatic compound production during incubation of the product influence flavor characteristic of final product (Beshkova *et al.,* 2003). Generally, product F9 (2.73) and F8 (2.80) were found to have low overall acceptability mean score values below (3.5) average overall acceptability. The texture of yoghurt can be influenced by milk composition, dry matter content, heating, homogenization, the type of starter culture, incubation temperature, cooling and storage time (Karsheva *et al.,* 2013; Kucukcetin *et al*., 2009).There is no homogeneity in texture of *ergo* prepared with these different starter culture formulates, texture mean score of the product ranged from 2.92 to 3.79 on 5 point scale. Other factors for the variability of the texture was the acid value of the products (Hemamali *et al*., 2016). Product F2 and F6 showed relatively higher texture mean scores of 3.78 and 3.79 this might be due to high titratable acid value of the product. Harun *et al.* (2015) reported that titratable acidity has significant effect on texture of yoghurt; as the acidity of the product increased texture (curd) of the product was increased. Igbabul (2014) also reported an increase of firmness of yoghurt as the pH decreased. Overall acceptability score reflected the scores obtained by all sensory parameters (Narayana and Gupta, 2013). *Ergo* product F2 (4.03) and F6 (3.79) had significantly ($p < 0.05$) high mean score values of overall sensory acceptability. It was a direct relationship with the mean scores of 3.7, 4.2, and 4.5 for flavor, odor, texture and color respectively reported by Macciola *et al.* (2008).

Ayib treated with formulate F2 was observed having values for color; flavor, odor, and texture mean score of 3.89, 3.91, 4.02 and 4.15 respectively in a 5 point hedonic scale. Relatively higher (3.75) sensory overall acceptability was observed in product F2(AY2).This indicated that

product F2(AY2) was relatively, better customer preference than other *ayib* products. Minimum overall acceptability score of 3.31 was observed in *ayib* product F9(AY9) Hemamali *et al*. (2016) reported cottage cheese having sensory overall acceptability of 3.2 – 4.6 in a five point hedonic scale.

Sensory shelf life of the products(*ergo* and *ayib*) were made by performing linear regression analysis on the storage time. Calculated quality indicator standard values of *ergo* and *ayib* based on 5 point scale (3.5) were used in the linear regression equation to determine the quality limit date. After storage of *ergo* and *ayib* samples at ambient temperature (18 - 24℃), the sensory evaluation was done every three and four days, respectively (Jung-Min *et al*., 2014). The expiry date was typically decided via determining a proper quality limit at which the product loses its value by sensory evaluation. In this study, the specifications of the "Animal and Plant Quarantine Agency, Process Criteria and Ingredient Standard of Livestock Products"(IDF, 2012) were used as a quality limit. The expiry date was determined by inserting the quality limit of 3.5 points into the linear regression equation. Accordingly, the shelf life of *ergo* made with the starter cultures and stored at ambient temperature (18 - 24^{o}C) was shown ranging from (15 days) of product F6 to (6 days) of product F1. Product F2 was shelf life of 12 days which is higher shelf life next to product F6. This showed that all the product have better shelf life than 2 - 4 days of naturally fermented *ergo* reported by Almaz Gonfa (2001) when stored at ambient temperature (18-24^{o}C).

Ayib treated with these different starter cultures formulates were exhibited to have varying shelf life. The shelf life of *ayib* products treated with each formulated starter culture was found ranging from 7 days in F1(AY1) to 18 days in F2(AY2) and F6(AY6). Better shelf life of 18 days was observed for *ayib* products F2(AY2) and F6(AY6) followed by (12 days) of the shelf life of F7(AY7). Nurgin *et al*. (2014) reported that cheese stored at 25^{o}C had a shelf life of 2.6

days and *ayib* shelf life of 2-3 days was also reported by Almaz Gonfa (2001). Relative to these finding our result showed that *ayib* treated with the starter culture formulates were found to have better shelf life. However, Kamleh *et al.* (2012) reported Halloumi (Greek cheese) stored at 15^{o}C has shelf life of 38.7 days. These variation in shelf life of cheese was due to that of salty of Halloumi, storage temperature, condition, post contamination and antimicrobial substance in the products from the starter cultures (Yuthana and Pennwalt, 2016).

6. Conclusions

Each isolate considered in this study was found to have better tolerance to low pH and high bile salt concentration. This study demonstrated that lactic acid bacteria and yeast formulates F2 and F6 may be better probiotics and have antagonistic effect against some common food borne pathogens. Therefore, they are capable of surviving and colonizing the host stomach. The formulates were able to reduce cholesterol up to 45.81% at in vitro conditions and capable of reducing or inhibiting food borne pathogens *E. coli*, *S. aureus*, *S.* Typhimurium and *L. monoctyogenes* during the course of fermentation of *ergo*. The use of these formulated starter cultures improved the safety and shelf life of *ergo* and *ayib.* Among e*rgo* prepared with formulated starter cultures, F2 and F6 were found having better sensory overall acceptability. Similarly, *ayib* product F2(AY2), F6(AY6) and F7(AY7) had better overall sensory acceptability. In addition, these formulated starter cultures have capability of extending the shelf life of *ergo* from 6 days to 15 days at ambient temperature of 18 – 24^{o}C storage. They were also capable of extending the shelf life of *ayib* from 7 days to 18 days at 18- 24^{o}C storage. Therefore, the results of this study indicates that the use of these formulated starter cultures were enhancing the keeping quality of both *ergo* and *ayib*, and were having enhanced consumer acceptability. Moreover, *ergo* fermented with these starter cultures and *ayib* treated with them can be a vehicles for provisions of potential health promoting probiotics. We hope that dairy products considered in this study, *ergo* and *ayib*, produced using these starter cultures may improve health problem associated with cholesterol.

7. Recommendations

- Implementation of this formulated starter culture in homemade and small scale production of *ergo* and *ayib* could improve the safety, sensory quality and physicochemical properties of the final product
- The result of the present study will contribute to the future applications and utilization of probiotic bio-protective starter cultures in the dairy industry.
- The information obtained from this study will serve as a foundation to establish small scale production of probiotic bio-protective starter cultures of *ergo* and *ayib.*
- Utilization of these starter cultures can improve physicochemical and sensorial consistency of *ergo* and *ayib* produced at home and small scale milk processors.
- There is a need of further laboratory level trial experiments on standardizing and packaging of the starter culture for a final use.
- However, more studies are needed on the efficiency of the starter cultures using *in vivo* studies, isolates toxicity test and identification of the isolates to species level is required using molecular tools.

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9. Appendices

Appendix 1*:* ANOVA table for *ergo* proximate analysis

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Total solid					
Treatment	10	12.56723258	1.25672326	12.82**	<.0001
Experimental error	22	2.15725833	0.09805720		
Total	32	14.72449091			
Protein					
Treatment	10	3.67832955	0.36783295	2.50*	0.0350
Experimental error	22	3.23385833	0.14699356		
Total	32	6.91218788			
Fat					
Treatment	10	7.25477273	0.72547727	6.56**	0.0001
Experimental error	22	2.43250000	0.11056818		
Total	32	9.68727273			
Ash					
Treatment	10	0.38497197	0.03849720	1.14ns	0.3811
Experimental error	22	0.74539167	0.03388144		
Total	32	1.13036364			
SNF					
Treatment	10	11.22508106	1.12250811	4.59**	0.0014
Experimental error	22	5.37759167	0.24443598		
Total	32	16.60267273			
Lactose					
Treatment	10	21.09361818	2.10936182	10.70**	<.0001
Experimental error	22	4.33880000	0.19721818		
Total	32	25.43241818			
Moisture					
Treatment	10	12.56723258	1.25672326	12.82**	<.0001
Experimental error	22	2.15725833	0.09805720		
Total	32	14.72449091			
Syneresis					
Treatment	10	18.12793333	1.81279333	4.19**	0.0024

Experimental error	22	9.51046667	0.43229394
Total	32	27.63840000	

Appendix 2: ANOVA table of *ergo* physicochemical

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
pH					
Product	0	2.19884091	0.21988409	232.94**	<.0001
Time	3	93.05858788	31.01952929	32861.8**	<.0001
Product*time	30	1.19159545	0.03971985	42.08**	<.0001
Experimental error	88	0.08306667	0.00094394		
Total	131	96.53209091			
Titratable acidity					
Product	10	0.11596818	0.01159682	34.8[7]**	<.0001
Time	3	0.59285455	0.19761818	594.21**	<.0001
Product*time	30	0.03472879	0.00115763	3.48**	<.0001
Experimental error	88	0.02926667	0.00033258		
Total	131	0.77281818			

Appendix 3: ANOVA table of Antagonistic effect of starter culture on some food borne pathogens

Source	DF	Sum of square	Mean Square	F Value	Pr > F
E. coli					
Product	9	4.18754000	0.46528222	10.19**	<.0001
Time	2	29.82421333	14.91210667	326.66**	<.0001
Product*time	18	5.01272000	0.27848444	6.10**	<.0001
Experimental error	30	1.36950000	0.04565000		
Total	59	40.39397333			
S. aureus					
Product	9	3.02332667	0.33592519	59.04**	<0.0001
Time	2	23.56462333	11.78231167	2070.71**	<0.0001
Product*time	18	2.6122434333	0.14512463	25.51**	<0.0001
Experimental error	30	0.17070000	0.00569000		

Total	59	29.37089333			
***S* .Typhimurium**					
Product	9	8.43084833	0.93676093	91.41**	<0.0001
Time	2	34.58083000	17.29041500	1687.14**	<0.0001
Product*time	18	6.76643667	0.37591315	36.68**	<0.0001
Experimental error	30	0.30745000	0.01024833		
Total	59	50.08556500			
List. monoctyogenes					
Product	9	6.20194348	0.68910483	6.25**	<0.0001
Time	2	38.73641170	19.36820585	175.73**	<0.0001
Product*time	18	6.69734197	0.37207455	3.38**	<0.0001
Experimental error	30	3.30652050	0.11021735		
Total	59	54.94221765			

Appendix 4*:* ANOVA of sensory attribute of *ergo* made with formulated starter culture

Source	DF	Squares	Mean Square	F Value	Pr > F
Color					
Treatment	10	7.82794545	0.78279455	3.59**	0.0006
Experimental error	77	16.78885000	0.21803701		
Total	87	24.61679545			
Flavor					
Treatment	10	5.18443182	0.51844318	3.50**	0.0008
Experimental Error	77	11.42061250	0.14831964		
Total	87	16.60504432			
Odor					
Treatment	10	5.39410227	0.53941023	3.03**	0.0028
Experimental error	77	13.70567500	0.17799578		
Total	87	19.09977727			
Texture					
Treatment	10	6.13651136	0.61365114	3.44**	0.0009
Experimental error	77	13.72137500	0.17819968		
Total	87	19.85788636			
Overall acceptability					
Treatment	10	3.51212727	0.35121273	2.30**	0.0204

Experimental error	77	11.78126250	0.15300341		
Total	87	15.29338977			

Appendix *5:* ANOVA table of sensory attribute of *ayib* treated with formulated starter culture

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Color					
Treatment	10	7.82794545	0.78279455	3.59**	0.0006
Experimental error	77	16.78885000	0.21803701		
Total	87	24.61679545			
Flavor					
Treatment	10	5.18443182	0.51844318	3.50**	0.0008
Experimental error	77	11.42061250	0.14831964		
Total	87	16.60504432			
Odor					
Treatment	10	5.39410227	0.53941023	03**	0.0028
Experimental error	77	13.70567500	0.17799578		
Total	87	19.09977727			
Texture					
Treatment	10	6.13651136	0.61365114	3.44**	0.0009
Experimental error	77	13.72137500	0.17819968		
Total	87	19.85788636			
Overall acceptability					
Treatment	10	3.51212727	0.35121273	2.30*	0.0204
Experimental error	77	11.78126250	0.15300341		
Total	87	15.29338977			

***Appendix 6**: Ergo* and *ayib* sensory test formats

Panelist code **_____X1_____**

Sample code	Sensory attribute	Score					
		1	2	3	4	5	Remark
P1	Color						
	Flavor						
	Odor						
	Texture						
	Overall acceptability						

Panelist code **_____X1_____**

Sample	Sensory attribute	Score					
		1	2	3	4	5	Remark
P2	Color						
	Flavor						
	Odor						
	Texture						
	Overall acceptability						

Panelist code_**____X1_____**

Sample	Sensory attribute	Score					
		1	2	3	4	5	Remark
P3	Color						
	Flavor						
	Odor						
	Texture						
	Overall acceptability						

NB: 1= Dislike extremely

2= Dislike

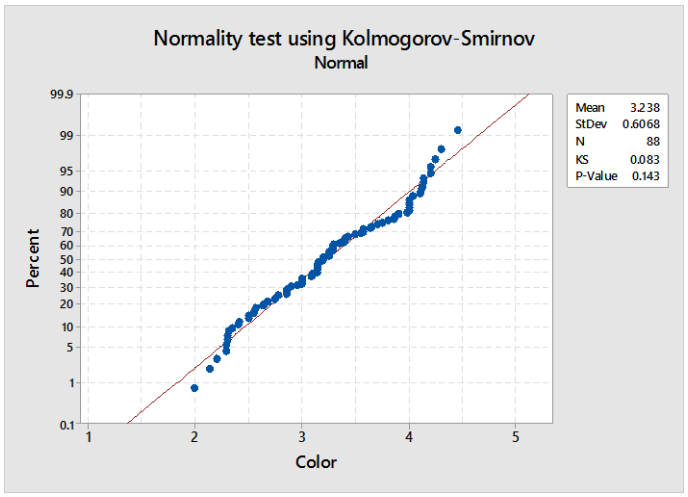
3=Neither like nor dislike

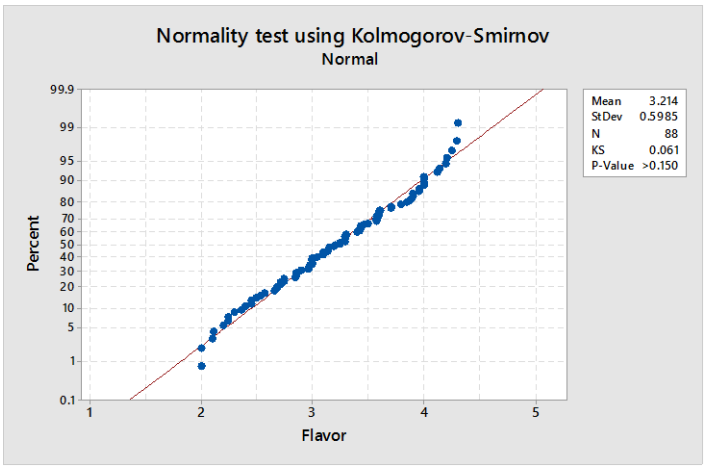
4= Like moderately

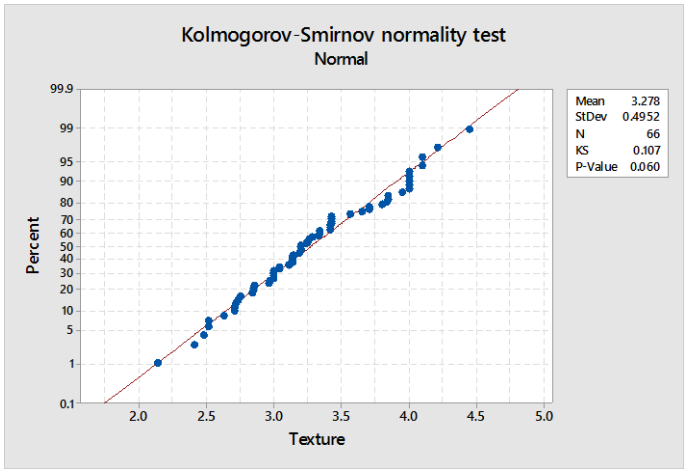
5=Like extremely

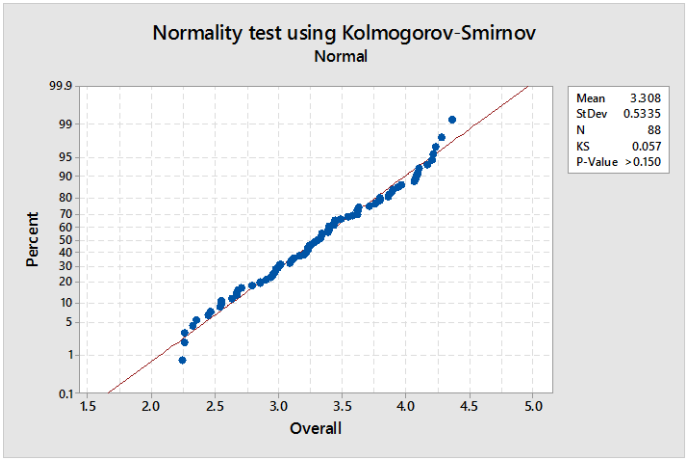
- Similar formats were used for all products.

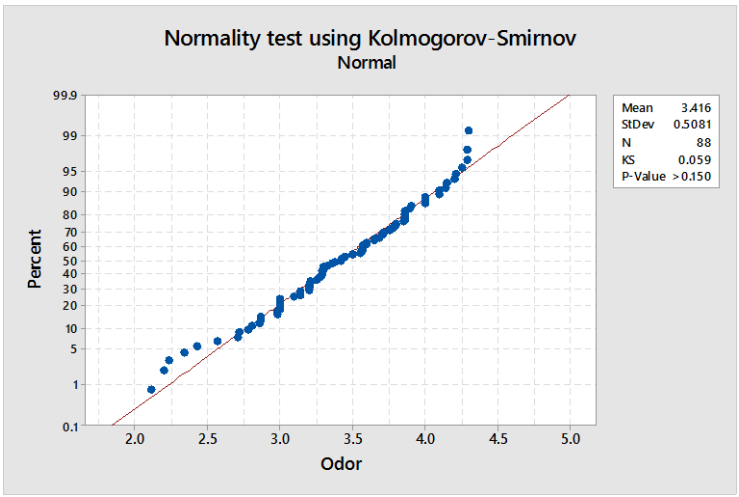
Appendix 7a *: Ergo* sensory test data normality test using Kolmogorov- Smirnov



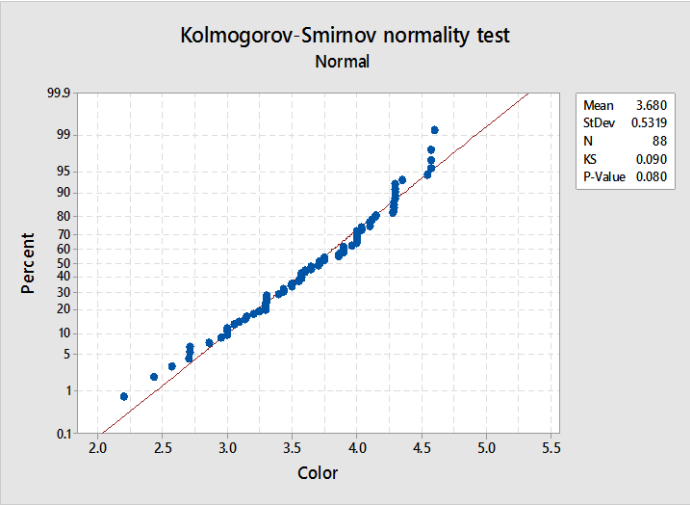


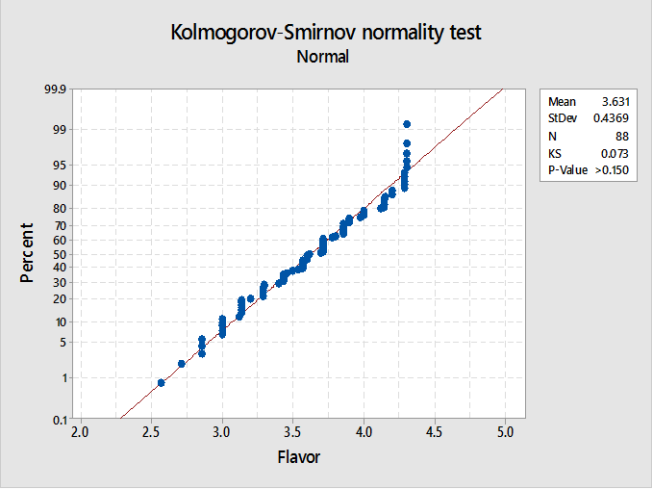




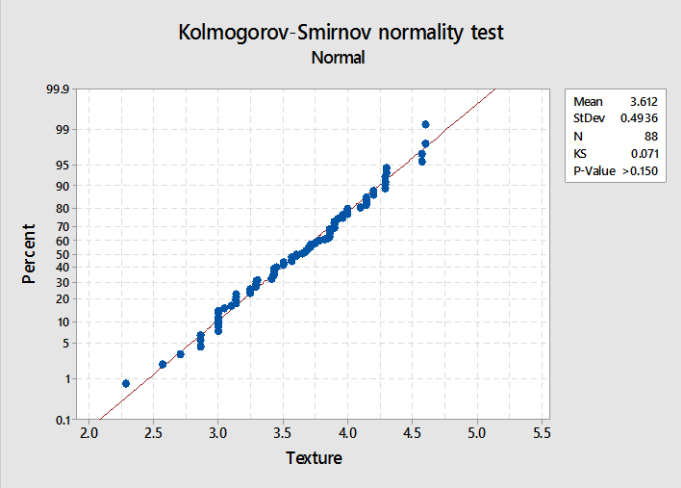


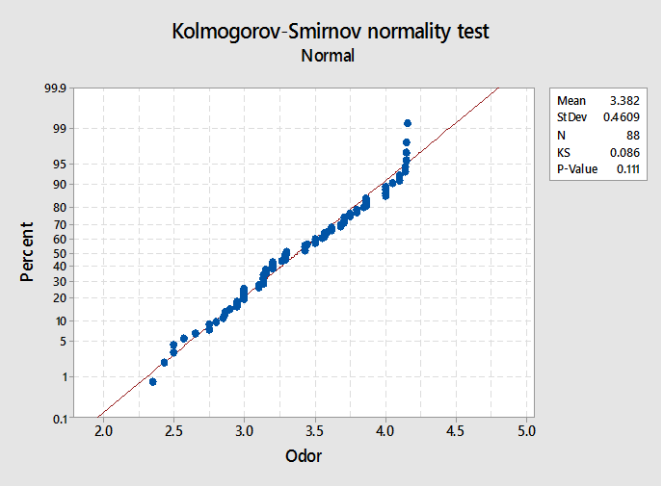
Appendix 7b: *Ayib* sensory test data

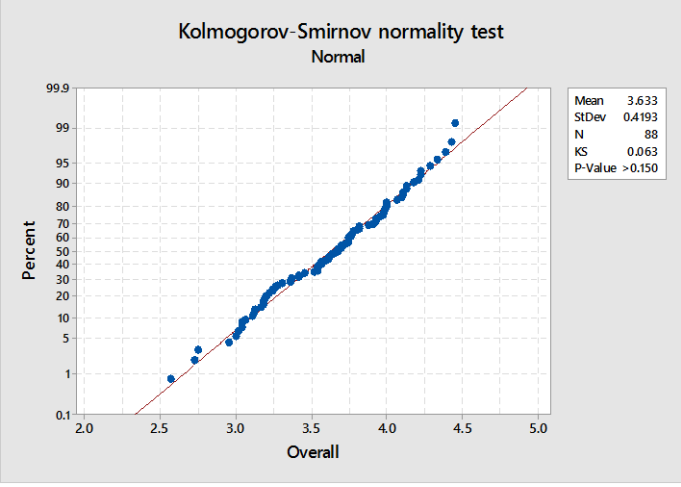




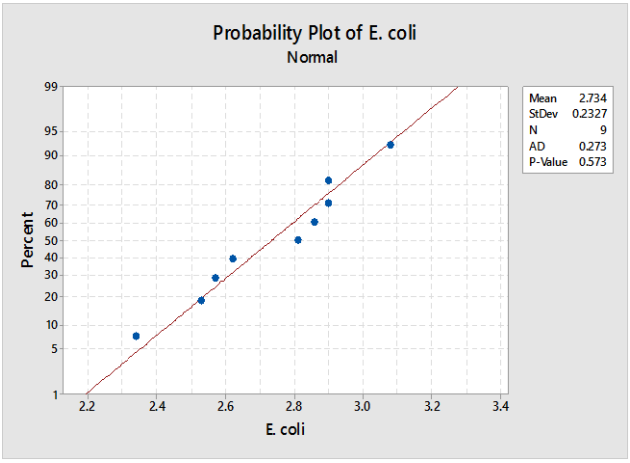
Normality test using Kolmogorov- Smirnov

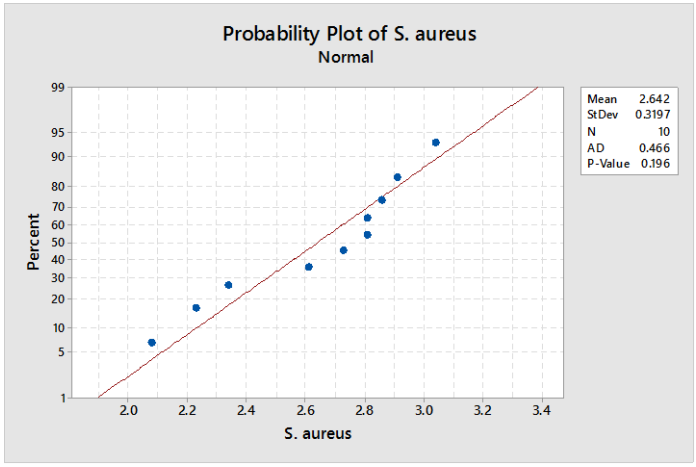


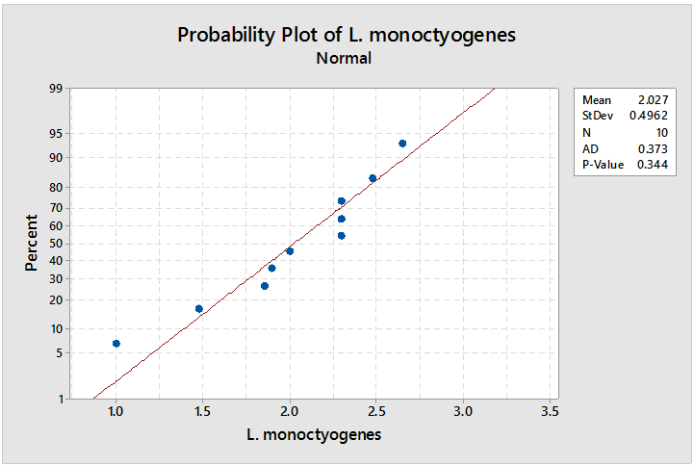




Appendix 7C: Antagonistic effect normality test

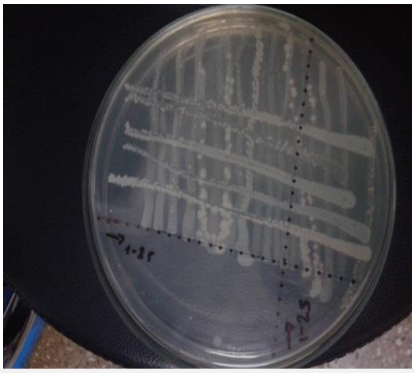






Appendix 8: *Pictures during the Laboratory analysis work*

LAB & yeast isolates



Antagonistic test

Formulation of starters

Inoculation of formulates

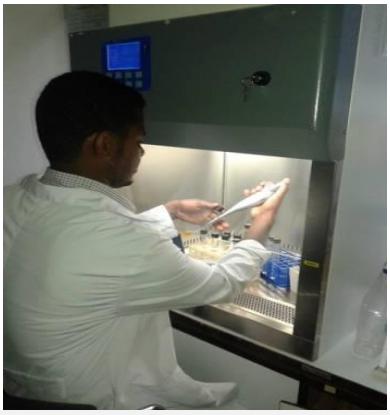
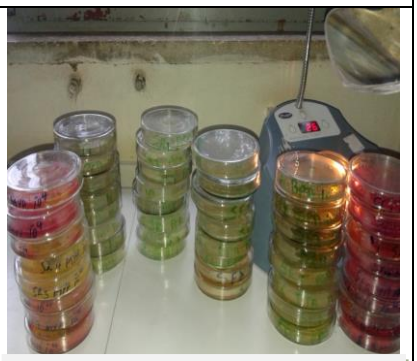
Fermentation of *Ergo*

Ergo Proximate Test

TA test

Anaerobic jar

Starch Utilization of isolates

		
pH test	CHO utilization of isolates	Antagonistic of isolates test
		
Citrate utilization test	Gram staining test	Sensory test
		
Microbial test	Syneresis test	Microbial enumeration