JIMMA UNIVERSITY

COLLEGE OF NATURAL SCIENCE

DEPARTMENT OF BIOLOGY

PROBIOTIC POTENTIAL OF LACTIC ACID BACTERIA ISOLATED FROM SELECTED TRADITIONALLY FERMENTED BEVERAGES

By

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PROBIOTIC POTENTIAL AND ANTI MICROBIAL ACTIVITY OF LACTIC ACID BACTERIA ISOLATED FROM SELECTED TRADITIONALLY FERMENTED FOOD PRODUCTS

By

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A Thesis Submitted to the Department of Biology, College of Natural Sciences, Jimma University, in Partial Fulfillment of the Requirement for the Degree of Master of Science in Biology (Applied Microbiology)

Advisor

Dr. Ketema Bacha

September, 2015

DECLARATION

I, the undersigned, declare that this thesis is my original work and has not been presented for any degree in any other university and all materials used for this research are dully acknowledged.

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Advisor's Name: Ketema Bacha (PhD)

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Date; ----------

Jimma

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List of Acronyms

ANOVA: Analysis of variance

AWDA: Agar well diffusion assay

CAM: Complimentary and alternative medicine

CFCS: Cell free culture supernatant

CFCS: Cell free supernatant

CFU: Colony forming units

GIT: Gastro intestinal tract

GRAS: generally regarded as safe

LAB: Lactic Acid Bacteria

MHA: Muller Hinton Agar

MRS: de man Rogasa and Sharpe Agar

ZOI: Zone of inhibition

Abstract

Probiotics are live microorganisms which when administered in adequate amount confer a health benefit to the host. Fermented foods are the major sources of Probiotics. Keribo, Bukuri and Korefe are among the common traditional fermented foods consumed in different parts of Ethiopia. However, the probiotic potentials of these foods were not documented although few studies were made on their microbiology. Therefore, the aim of this study was to evaluate the probiotic potential of LAB isolated from such products under laboratory condition. Samples from these three traditionally fermented food products were prepared following traditional preparation procedures as described earlier and used in the current study. The Lactic acid bacteria found in each fermented food products were isolated and characterized using standard morphological and biochemical tests. The In vitro probiotic potential was conducted simulating the GIT condition (tolerance to stomach $P^H$and Gastric enzymes, antimicrobial activities against pathogens). The antimicrobial activities of LAB isolates against *Salmonella spps*, *Pseudomonas aeruginosa*, *Staphylococcus aureus and E. coli* were determined following the standard Agar well Diffusion method. A total of 52 LAB isolates (14 from Keribo, 23 from Bukuri and 15 from Korefe) were screened. The result indicated that all the presumptive LAB isolates were gram positive, Catalase negative, none motile, none spore forming and lactic acid producers. Strains of Leuconostoc spp. were found dominating Keribo samples. Likewise, homo fermentative Lactobacillus strains were dominant in Bukuri samples. But, both the hetero fermentative and the homo fermentative LAB strains were encountered in almost equal proportion in Korefe sample. Analysis on the in vitro probiotic potential indicated that 38.5% (20/52) isolates were tolerant to stomach pH. The 20 isolates were almost equally distributed over the sample sources (7 isolates from Keribo, 6 from Bukuri and 7 from Korefe). One isolates each of the Korefe (Kr_2) and Bukuri (Bk_{10}) exhibited the highest antimicrobial activity against *Salmonella spps* with inhibition zone diameter of 19.5 ± 3.6 and 16 ± 2.8 mm, respectively. Likewise, Ke_1 from Keribo, Bk_1 from Bukuri and Kr_9 from Korefe exhibited the highest inhibitory effect against *E. coli* with inhibition zone diameter of 17.5 ± 3.8, 16.5 ± 2.2, 13 ±1.4 mm, respectively. In addition, Kr_{11} from Korefe and Bk_2 from Bukuri exhibited the highest inhibitory activity against *Staphylococcusaureus* with inhibition zone, 22 ± 2.8 and 17 ± 4.2 mm, respectively.

Moreover, Kr_1 from Korefe, Bk_1 from Bukuri and Ke_1 from Keribo exhibited the highest inhibitory activity against *Pseudomonas aeruginosa* with inhibition zone, 20.5 ± 3.7, 17.5 ± 2.8, 10.5± 2.9mm, respectively. Over all, most of the isolated LAB showed remarkable antibacterial activities both against gram positive and gram negative pathogens although the spectrum of inhibition was different for the different isolates. In conclusion, the results of the study indicated that, the 20 isolates screened from the three traditional fermented food products had promising probiotic characteristics making Keribo, Bukuri and Korefe candidate probiotic foods for further in depth evaluation of the isolates at molecular level with some more screening criteria included in the probiotic evaluation. (Bk_1, Bk_2, $Bk_{10,}$ Kr_{11} represent Lactobacillus strains, Ke_1, Ke5 are Pediococcus species while Ke_8, Kr_9 are strains of the genus Leuconostoc).

Key words: Antimicrobial activity, Bukuri, Health benefit, Keribo, Korefe, Probiotics

1. Introduction

Infectious diseases are the biggest problem in human beings and every year gastro intestinal

Infections are responsible for significant morbidity and mortality worldwide (Culligan, et al., 2009). World health organization (WHO, 2004) estimated more than four billion episodes of diarrheal diseases annually while there were 2.2 million deaths attributable to enteric infections, making it the fifth leading cause of death at all ages worldwide. The problem is very serious in rural areas of Africa particularly Ethiopia due to poor sanitary conditions and limited health facilities.

Unavailability of modern medicine with increasing out breaks of intestinal diseases is calling for an additional simple and accessible method to improve the health of these economically challenged people. It also awakened the scientific community to the prophylactic and therapeutic use of Probiotics (beneficial micro organisms) and to reconsider them as alternative to anti biotics (Ahmed, 2003). As they provide the body with beneficial bacterial strains (Probiotics), Consumption of traditional fermented food products is the best way to improve the health of the people.

Traditional fermented foods and beverages are those which are indigenous to a particular area and have been developed by people of that area themselves using traditional techniques from locally available (mostly homemade) raw materials (Reshid, 2013). Their method of preparation is not complicated and do not require expensive equipments. Therefore, in the production of these traditional fermented food products in Ethiopia, it is common to use and follow controlled natural fermentation processes.

Keribo, Bukuri and Korefe are traditionally fermented food products which are regularly consumed by some ethnic groups in Ethiopia. People who have regularly consumed these traditionally fermented food products are found in rural areas where there is no clean water and medical facilities.

Besides they are economically very poor and have no access to antibiotics and food preserving materials. Since they are uneducated, knowledge about Probiotics has not reached these areas. Thus the type and degree of health risks caused by consuming these traditional fermented beverages and improvement of these health risks have not been known.

Therefore, the people must learn about the role of these fermented food products. These will help them to boost their use and to improve their health. Moreover, it will minimize their dependence on expensive chemical drugs (antibiotics) and scarce medical facilities. The increased use of these fermented food products will also minimize the effect of pathogens caused by contaminated water and in adequate sewage disposal mechanisms.

The guide lines proposed by FAO/WHO(2002) for evaluating of Probiotics recommended that every potential probiotic strain should be correctly isolated and identified using both phenotypic and genotypic methods followed by various tests to investigate its survival ability and functional properties. The probiotic strains found in Keribo, Bukuri and Korefe have not been clinically validated and their health effects have not been documented although the microbial dynamics and the fermentation processes of most Ethiopian traditionally fermented beverages including these three fermented food products are already studied and documented.

Since the health effects and nutritional properties of Keribo, Bukuri and Korefe have not been studied and documented yet, The aim of this study was therefore; to investigate whether Keribo, Bukuri and Korefe possess probiotic potential and anti microbial activity which could alleviate and prevent gastro intestinal disorders (infections) and other health problems. This was done by first isolating the lactic acid bacteria from these spontaneously fermented food products (Keribo, Bukuri and Korefe) and identified and characterized them. Secondly the ability of the LAB isolates to survive the passage through the gastro intestinal tract was estimated by *In vitro* studies and the isolates were screened for antimicrobial activity on the growth and survival of some food borne pathogenic microorganisms.

2. Objectives of the study

2.1. General objective

- To determine the health enhancing roles of Keribo, Bukuri and Korefe samples through evaluation of the *In vitro* probiotic potential and antimicrobial activities of lactic acid bacteria isolated from these fermented food products

2.2. Specific objective

- To isolate and characterized LAB from Keribo, Bukuri and Korefe,
- To evaluate the ability of the lactic acid bacteria isolates to survive passage through the gastro intestinal tract using simulated gastro intestinal tract environment
- To evaluate the *In vitro* antagonistic effect of probiotic LAB against some food borne pathogens

3. Literature Review

3.1. Probiotics

The term probiotic was derived from the Greek word 'pro bios' which means 'for life' opposed to 'antibiotics' which means 'against life' (Yavuzdurmaz, 2007). . Probiotics, as defined in a FAO/WHO (2002) report, are live micro organisms which when administered in adequate amount confer a health benefit on the host. They are live microorganisms which help to protect the host from various intestinal disorders while increasing the number of beneficial bacteria and making the balance steady. They are also called friendly bacteria and they can be used as complementary and alternative medicine (CAM) (Yavuzdurmaz, 2007).

Now a day's food is no longer considered by consumers only in terms of taste and immediate nutritional needs but also in terms of their ability to provide specific health benefits beyond their basic nutritional value (Saarela et al., 2002). Currently, the largest segment of the functional food market is dominated by healthy food products targeted towards improving the balance and activity of the intestinal micro flora (Saarela, 2002). Consumption of food containing live bacteria is the oldest and still most widely used way to increase the number of advantageous bacteria called probiotics in the intestinal tract (Salminen *et al*., 2004).

The human body is highly colonized by micro organisms. There are about 100 trillion microbial cells in the human body (Simon and Gorbach, 1984). That is, 10x greater than the human cells in the body. These are from 400-500 different species of micro organisms (Simon and Gorbach, 1984).The micro organisms are both good and bad (both beneficial and pathogenic).For optimum gut flora balance, the friendly micro organisms such as lactic acid bacteria (although not all of them are beneficial) like lacto bacillus, streptococcus and bifido bacteria Fungi like Saccharomyces cervisae and Aspergellus oryzae must dominate. They must be, greater than 85% of the total microbial flora (Spanhaak *et al*., 1998).

The friendly (beneficial) micro organisms that are the predominant will inhibit the growth of pathogenic micro organisms and maintain our health by competing with them for essential nutrients, adhesive sites, by producing organic acids, by stimulating our immunity, by producing nutrients, adhesive sites, by producing organic acids, by stimulating our immunity, by producing inhibitory compounds, by attenuation of virulence, suppression of toxin production and by degradation of toxic receptors (Spanhaak *etal*., 1998).

In this way the beneficial micro organisms limit the multiplication of other undesirable microbes. However if the natural microbial balance of the body upsets due to diet, climate, ageing, medication,illness,stress, and lifestyle, the pathogenic microorganisms like, clostridia, salmonella, Shigella, helicobacter pylori and Rota virus will get strength and have the opportunity to thrive resulting in accelerated ageing, poor nutritional response, reduced efficacy to medication, physiological disfunction,cancer,deficient immune responses, susceptibility to infection, physiological discomfort (Sanderson and Walker,1993).

Therefore, to be healthy, the microbial balance of the body must be maintained (constant).The beneficial micro organisms play a fundamental role in human health by preventing diseases. By taking these beneficial micro organisms, we can restore the body's natural microbial balance, boost our health and maintain our well being (Holzapfel *et al*., 1998). Fermented dairy products such as milk and yoghurt and traditionally fermented beverages are the most accepted food carriers for live probiotic delivery to the human GIT.

3.2. History of Probiotics

The history of Probiotics began with the history of man consuming fermented foods and beverages. The Probiotic bacteria have been historically used to treat a variety of ailments, including infections of mucosal surfaces such as the female genital organs and gastrointestinal tract (FAO/WHO, 2002). However with the discovery and development of antibiotics in the twentieth century, these traditional therapies lost their value.

The use of live micro organisms for enhancement of consumers health as an aid to cure some type of gastro intestinal disorders has been recognized for almost a century since the first report by Russian scientist and novel laureate Elie Metchnikoff in 1910 (Ketema *et al*., 2009).

He proposed a scientific rationale for the beneficial effect of the bacteria in yoghurt. In his book, "the prolongation of life" he postulated that aging results from the activity of putrefactive proteolytic) microbes manufacturing toxic substances within the giant bowel (R.Ilayaraja, 2012)

proteolytic bacteria like clostridia that are part of the gut micro flora manufacture toxic substances (Ammonia, phenols, indole etc) from the breakdown of proteins resulting in intestinal "auto intoxication" causing physical changes related to adulthood (R.Ilayaraja, 2012).

He found out that milk fermented with lactic acid bacteria inhibits the expansion of proteolytic bacteria and he attributed the longevity of Bulgarian peasants to their intake of yoghurt containing lacto Bacillus species. Metchnikoff himself used bitter milk fermented with the bacteria that he referred to as Bulgarian bacillus benefited his health. Later in 1920 Rettger renamed them as Lacto bacillus delbruekii.Bifido bacteria were first isolated from the breast fed infant by Henry fissier. The isolated bacterium named bacillus bifidus communis was later renamed to the genus Bifido bacterium (Gomes, 1999). Tissier found that bifido bacteria which are dominant in the gut flora of breast fed babies were advantageous in preventing diarrhea by displaying the pathogenic bacteria (Gomes, 1999).

As the result of long and repeated studies, scientists have investigated several health benefits of probiotics. These include, prevention and treatment of diarrheal diseases, prevention of systemic infections, management of inflammatory bowel disease, immune modulation, prevention and treatment of allergies, anti cancer effects, treatment of cholestrolaemia, alleviation of lactose intolerance ,lowering blood pressure, reduction of helicobacter pylori infections, prevention of

osteoporosis and prevention of urogenital infection(Çakır 2003, Schrezenmeir and De Vrese 2001, Dunne *et al*., 2001, Dugas *et al*., 1999).

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3.3. Selective criteria and possible modes of action (Mechanisms) of Probiotics

In order to carry out their benefic effects, probiotic strains must survive the passage through the gastro intestinal tract (GIT) by tolerating any antagonistic activity that may encounter with in the GIT of humans. As Ilayaraja (2012) stated, to produce a given health effects, the probiotics must have the following properties, they must be, none toxic and none pathogenic, Human in origin, capable of surviving and metabolizing in the GIT, genetically stable, clinically validated and documented health effects, safe for food and clinical use, antagonistic for pathogens and carcinogenic bacteria, immuno stimulatory, viable in field conditions and they must also have, good sensory properties, antimicrobial activity against pathogens and accurate taxonomic identification.

Besides to provide health benefits, probiotics must overcome extremely low pH and the detergent effect of bile salts and arrive at the site of action in a viable physiological state (Çakır, 2003; Schrezenmeir and De Vrese 2001; Dunne *et al*., 2001; Dugas *et al*., 1999). A probiotic should preferably not possess any anti biotic resistance gene and if it does, it should be unable to transfer the antibiotic resistant gene between micro organisms (Teuber *et al*., 1999; Saarela, 2002).

There are also studies on how pro biotics work in the gastro intestinal tract of humans, these include, the production of inhibitory substances, blocking of adhesive sites, competition with pathogens for nutrients and available energy, stimulating of immunity, degradation of toxic substances, suppression of toxic production, reduction of gut p^H, enzymatic contribution to digestion and attenuation of virulence. (Çakır, 2003; Schrezenmeir and De Vrese, 2001; Dunne, *et al*., 2001 Dugas *et al*., 1999).These considerations were also valid for the LAB isolated from these fermented food products. .

It is believed that most probiotics do not permanently adhere in the intestinal mucosa but exert their effects as they metabolize and grow during their passage through the intestine. This daily

consumption of these bacteria is probably the best way to maintain their effectiveness(Fuller, 1992; Medina *et al*., 2001).

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3.4. Safety aspects of Probiotics

Safety is one of the recommended attributes in the FAO/WHO guidelines (2002) on evaluation for Probiotics. The safety aspects of Keribo, Bukuri and Korefe are dependent on the potential micro organisms that protect the undesirable microbes. As Marteau (2001) indicated, the safety aspects of Probiotics bacteria include the following requirements, lack of pathogenicity and toxicity, tolerance to harsh conditions along the digestive tract, adhesion to mucosal surfaces and validated and documented health effects.

Moreover, they should not carry transmissible antibiotic resistance genes (Saarela, 2002).
This is because antibiotic resistant genes, especially those encoded by plasmids could be transferred between micro organisms. The potential strains need to be assayed for their antibiotic resistance to prevent the undesirable transfer of resistance to other endogenous bacteria. Furthermore, the presence of an antibiotic may facilitate the growth of antibiotic resistant mutants (Marteau, 2001).

3.5. Lactic acid Bacteria

Lactic acid bacteria are a group of gram positive, rod or cocci, catalase negative, non spore forming, non motile, bacteria. They are devoid of cytochromes and are often non aerobic habit but are aero tolerant, fastidious, acid tolerant and strictly fermentative (Aslam andQazi, 2010).They produce lactic acid as a major end product from the fermentation of carbohydrates. They are found in a variety of habitats such as, mucosal membranes of humans and animals (oral cavity, intestine, female genital) in plant materials such as silage, in food stuffs and agricultural products particularly milk, cheese and fermented beverages (Johanson *et al*., 993).

LAB are generally recognized as safe (GRAS) and play an important role in fermentation and preservation either as a natural Microflora or starter culture added under controlled condition. They also inhibit the growth of pathogenic microorganisms in the gastrointestinal tract of

humans. Together with Bifidobacterium, they are the majority of micro organisms used as probiotics (Suskovic and Gorete, 2001).

Within the group of LAB, *Lacto bacillus* species are the most commonly utilized groups of micro organisms for their potential beneficiary properties as probiotics. They make up a major part of the healthy human intestinal Microflora and are thought to be involved in the control of the flora and maintenance of its normal state (Ilayaraja, 2012).

Many reports suggest that LAB and their fermented products have potential effects against mucosal injury in the stomach. Bacteriocins produced by LAB are the subject of intense research because of their antipathogenic activity and bio preservative capacity against food borne pathogens and these features of LAB also served as a suitable index for screening them as a probiotic from the environment (Salminen *et al*., 2004)

The lactic acid bacteria are grouped as either homo fermenters or hetero fermenters based on the end product of their fermentation. The homo fermenters produce lactic acid as the major and only product of fermentation of glucose. The hetero fermenters produce a number of products besides lactic acid, including CO_2, acetic acid and ethanol from the fermentation of glucose.

An important feature of probiotic culture is its ability to kill pathogens which infect the gastro intestinal tract. In the FAO/WHO draft guidelines of 2002, an evaluation of probiotics in food, antimicrobial activity against potentially pathogenic micro organisms was one of the recommended attributes for potential probiotic strains.LAB have been reported to produce anti microbial products and exert a strong antagonistic activity against food contaminating micro organisms (Quwehand and vester Lund 2004; Cakir, 2003).Antimicrobial activity targets the enteric undesirables and pathogens (Klaenhammer Kulle,1999).

Antimicrobial effects of Lactic acid bacteria are formed by producing some substances such as organic acids (lactic, acetic, propionic, acids), carbon dioxide, hydrogen peroxide, diacetyl, low

molecular weight antimicrobial substances such as, reuterin, cytolysin, nisin, plantaricin, acidophilucin A and bacteriocins (Quwehand and Lund, 2004; Cakir ,2003).

3.6. Traditional fermented beverages

Traditional fermented beverages are those that are indigenous to a particular area and have been developed by the local people using age old techniques and locally available row materials. Early man probably used short time fermented beverages as a safe substitute for water. Since the alcohol content is too low in early stages of fermentation to produce intoxication (Rose, 1977).Indigenous fermented beverages from different parts of Africa are described. Among these are, Egyptian bouza,Tanzanian wanzuki, gongo, and tembo-mnazi and gara, Nigerian palm-wine, Kenyan muratna and uragela, South African Kaffir beer (Bahru etal., 2001).Indigenous Ethiopian fermented beverages include,Tej (Vogel and Gobezie:1983;Fite etal.,1991,Bahru;2000;Bahru etal.,2001),Tella(Sahle and Gashe,1991;Nigusie;2008),Borde and Shamita (Ashenafi and Mehari,1995;Bacha etal.,1998),Areki,traditional liquor (Desta,1977;Fite etal.,1991).These drinks are relatively cheap to prepare and are therefore important alternatives for low income consumers who cannot afford imported or industrially processed beverages. However, more effective and rational production could give increased income for those who are involved in the production. The microbiology, fermentation process and the health effects of some Ethiopian traditional fermented beverages are already studied and documented. These include, fermentation process and microbial dynamics during fermentation of Tella (Samuel Sahle and Berhanu Abegaze, Gashe, 1995), Borde (Ketema Bacha etal., 1998) and Shamita (Ketema Bacha etal, 1999).But, limited microbial and physico chemical information is available on Keribo, Bukuri and Korefe. Therefore, further studies are needed on health and nutrition perspectives.Therefore, the probiotic potential and anti microbial activity of Lactic acid bacteria strains found in these three beverages were studied in this paper.

3.6.1. Traditional fermentation of Keribo

Keribo is barley based traditional fermented beverage and is widely consumed in the southern and western part of Ethiopia including Jimma zone. It is produced mainly from barley and sugar. Fermented Keribo constitute a major part of beverages being served on holidays, wedding ceremony and also as a source of income of many households in Jimma zone (Reshid Abafita, 2013).

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The popularity of this traditional fermented beverage is more reflected among the groups and those who do not like drinks containing alcohol. Since it is none or low alcoholic beverage, it is consumed by both adults and children. It has poor keeping quality with shelf life of not more than three or four days having pronounced characteristics of the deteriorating beverage at the end of 72 hrs of fermentation.

3.6.2. Traditional fermentation of Bukuri

Bukuri is a cereal based traditional fermented beverage prepared from locally available cereals (maize, barley, sorghum, millet, wheat, teff and malt flours).It is mainly prepared by people who don't like drinks containing high alcohol. The drink is very common and more popular in Wollega zone and the surrounding areas (Biratu Chali, 2014). Bukuri is also used by Muslims who are living in wollo. It is commonly consumed during religious ceremonies. Although it is served on holydays and wedding ceremonies, it is mainly prepared for social gatherings (Debo work). It is also prepared by many households in Wollega and the surrounding areas as a source of income (for cell).It is considered as alcohol free drink although the preparation follows a lot of fermentation process.

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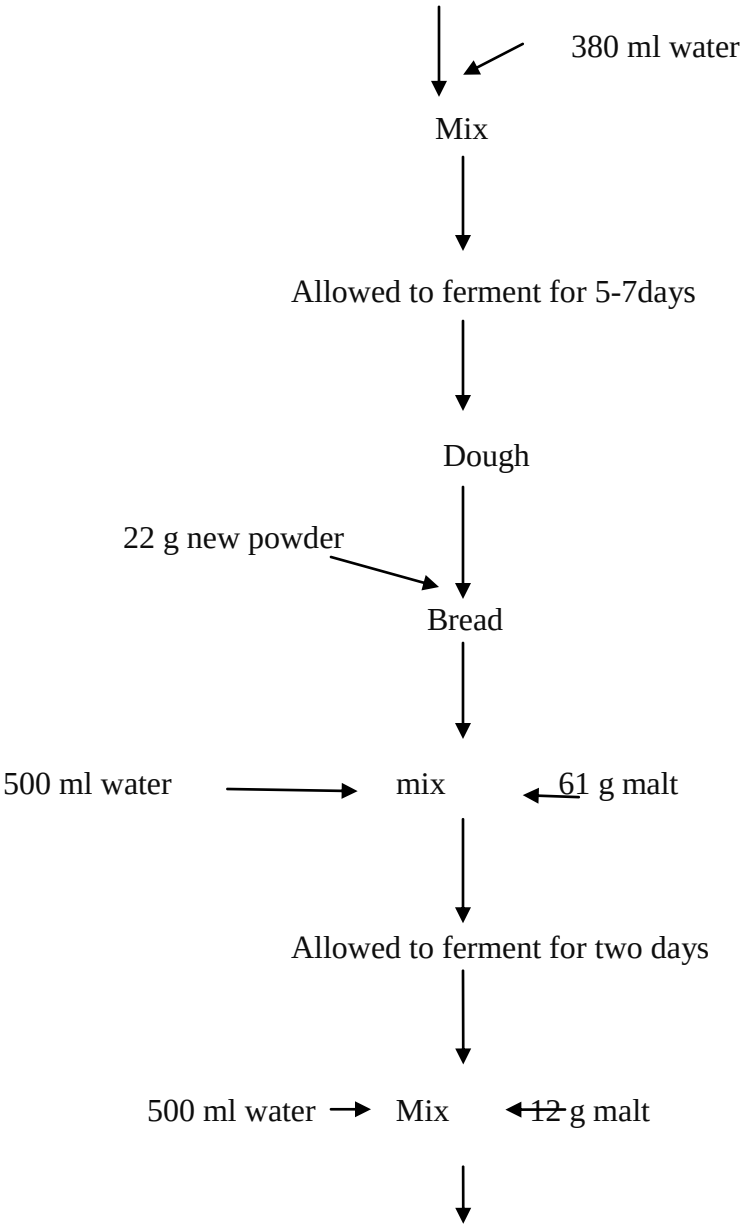
380 g cereal powder (Maize, Millet, Sorghum)
380 ml water
Mix
Allowed to ferment for 5-7days
Dough
22 g new powder
Bread
500 ml water
mix
61 g malt
Allowed to ferment for two days
500 ml water
Mix
12 g malt

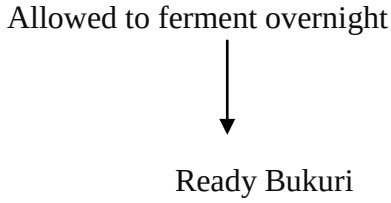

Figure.1.Flow chart of Bukuri preparation (Biratu Chali, 2014)

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3.6.3. Traditional fermentation of Korefe

As, Hailemichael and Peter (2011), stated, farmers in the northern part of Ethiopia call barley, king of crops emphasizing its suitability for diverse use. It plays an important role in the preparation of various fermented foods and beverages. One common fermented beverage which can be made from barley is Korefe. Koumant ethnic groups who are living in Gonder zone call their Darekot Tella, Korefe (Hailemichael and Peter 2011). The main focus of this paper was to study the Korefe made in northern part of Wello and southern part of Tigray which is similar to Borde than Tella. Korefe is not known in Jimma zone and Oromia region.

The name Korefe is derived from the production of a large quantity of white bubbles (Hailemichael and Peter, 2011). Unlike other beverages; it seems to be specific to the above parts of Ethiopia. It has a lower alcoholic content than Tella. Korefe is not mainly used in religious and wedding ceremonies. But served in social gatherings and as a source of income by many households. In north wello and wag Hemra zone and in the southern part of Tigray called Maichew and Korem, it is produced by many households as a source of income (for cell).Korefe is called by the local people as mother’s breast milk since it serves as a replacement of meal in most cases.

4. Materials and methods

4.1. Sample preparation

Bukuri is not known in Jimma zone by many households. Korefe is not known in Oromia region. Keribo is rarely accessed from market. It is prepared by many households only during religious festivals and for family consumption. Therefore, Keribo and Korefe were prepared locally following the traditional preparation techniques as described earlier and brought to the Microbiology laboratory, Department of Biology, Jimma University for the isolation of lactic acid bacteria. Bukuri was prepared in Microbiology laboratory following the procedures given by Biratu Chali (2014). Samples from each three fermented beverages were kept separately in a refrigerator at 4^{O}C until processed. All the microbiological analyses were conducted at Microbiology laboratory of Department of Biology, Jimma University from December, 2014 to June, 2015.

4.1.1. Bukuri preparation

Bukuri was prepared in the laboratory following the preparation procedures given by Biratu Chali (2014). It was prepared using the traditional equipments and recipe at Jimma University Microbiology Laboratory. The appropriate equipments and ingredients were purchased from open market of Jimma town. Malt was prepared from Dehusked barely and maize and soaked in water over night and then taken out of water and kept in a bowl for 3-5 days for germination

(Biratu Chali, 2014). The malt was exposed to the sun to be dried and then milled. Roasted barley and maize was milled and mixed with water to make dough. After 5-7 days of fermentation, new flour was added to the fermented dough and mixed for two hours and baked to become bread. The bread was broken in to pieces and kept in a pitcher containing malt and water and sealed. Finally, on the second day of fermentation, the remaining malt and water were added in to the previous fermented Bukuri and sealed until drinking on the third day.

4.1.2. Korefe preparation

In Korefe preparation, barley was left in water over night to be Dehusked and then dried in the sun and milled. Keeping some barley flour aside, the remaining flour was mixed with water and baked on the *mitad* to prepare lightly roasted kita. Germinated grains of barley bought in

the local market or prepared at home were dried and milled. The lighter colored kita which is different from dark brown kita and enkuro of Tella was broken in to small pieces and added in to a clay container containing water. To this, the milled malt (bikil) was mixed. Finally, when the mixture rises, it was thoroughly mixed with the remaining barley flour and sealed. After about two days, it formed bubbles and became readily fermented Korefe (duration of fermentation depends on the temperature of the surrounding environment). It could also be filtered in a pot using clean cotton cloth by adding water similar to filtered Tella. If it is filtered in the afternoon, it would be ready to serve in the morning of the next day. Tella is brown in color but Korefe is lighter in color, since the kita is not roasted very dark and the remaining flour is not roasted to become asharo. Normally Korefe is not purified but clear when served.

4.1.3. Keribo preparation

A kg of barley was roasted on a griddle of iron (mitad) and added in a large plastic bucket containing 3000ml of boiled water. To this, 250g sugar was added and sealed for three days. After three days of fermentation, the sealed plastic container was opened and filterd.Finally, the filtered Keribo sample was taken to Jimma University Micro biology laboratory for the isolation, enumeration, characterization and further studying of the isolates.

4.2. Isolation and Enumeration of Lactic acid Bacteria

MRS (de man Rogossa and Sharpe) agar is the selective medium used for the isolation and enumeration of lactic acid bacteria. Twenty five ml of sample from each three beverages were separately mixed with 225ml sterile 0.1% buffered peptone water and homogenized in sterilized

flasks for 30 seconds using a shaker at normal speed (Mugula *et al*., 2001).The homogenate was then serially diluted and one ml from each homogenized sample was pipetted aseptically into 9ml of sterile saline solution. A dilution series of 10^{-1} to 10^{-5} was made in sterile saline solution and aliquots of 0.1ml from appropriate dilutions were spread plated in duplicate on pre dried and appropriately marked MRS agar plates , and finally incubated at 32^0C for 72 hrs under anaerobic condition using anaerobic jar (anaerobic Gas pack System using candle).

All snow white colonies of LAB were counted on each MRS agar plates using a colony counter. The viable bacteria colonies in each serial dilution were counted and expressed in colony forming units per milliliter ($CFU.ml^{-1}$) (Mugula *et al*., 2001).

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Only countable plates (30-300 colonies) were considered for enumeration of the cell density. Individual colonies with distinct morphological characteristics such as, color, shape, edge, texture and forms were randomly picked from each countable MRS agar plates as LAB isolates, and further purified by transferring to MRS broth. Purified strain was verified by three subcultures from a single colony. That is, each LAB isolated (with its own codes) was purified by repeated streak plating on MRS agar for three times. Thereafter a loopful of colony was transferred in to 5ml of MRS broth and allowed to grow by incubating at 32^0C for 72hrs. Pure colonies from each MRS agar plates were maintained on MRS agar slants at 4^0C for further characterization and identification.

4.3. Identification and Characterization of Lactic Acid Bacteria

All the LAB isolates were characterized using phase-contrast microscopy and conventional biochemical and physiological tests. All isolates of LAB were separately activated in MRS broth Overnight and active cells were used for all tests and incubated anaerobically in anaerobic jar at 32^oC.

4.3.1 Morphological Characterization

Each isolate was initially examined for cell morphology, cell grouping using Gram stain and presence or absence of spores, using light micro scope under oil emersion objectives.

Spore formation test

Smear from 4-5 days old bacterial culture was placed on clean glass slide and heat fixed. Then, the smear was covered with a piece of absorbent paper and placed on the wire gauze on a ring stand. The paper was saturated with malachite green and placed near the Bunsen burner until steam was seen rising from the surface. The slide was re-heated to keep it steaming for about three minutes. The paper was removed with forceps and the slide was rinsed thoroughly with tap water and then drained and counterstained for 45 seconds with 0.5% safranin. Finally, it was washed, dried and examined under a microscope. The vegetative cells would appear red and the spores would appear green.

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The non-spore forming (vegetative) cells which appeared red were screened for the next *In vitro* probiotic potential and anti bacterial test (Tambekar and Bhutada, 2010).

4.3.2. Biochemical and Physiological test

The isolates were further characterized using conventional biochemical and physiological tests including tests for Gram-reaction; catalase production; carbohydrate fermentation, salt and temperature tolerance, etc. Only the Gram-positive, catalase negative was considered as candidate LAB. Accordingly, all the isolates were separately activated in MRS broth and an overnight culture (inoculums) was used for each test.

Gram reaction (KOH) test

The Gram reaction test was done according to rapid method proposed by Gregerson (1978). Briefly, two drops of 3% KOH solution was placed on a clean microscope slide. An overnight culture was scrapped from the plate aseptically with a wire loop and stirred in to KOH solution. After 10 seconds, the inoculating loop was raised slowly from the mass. Formation of threads of slime when the inoculating loop was raised for 0.5 to 2cm is an indication of Gram negativity but gram positive otherwise.

Catalase test

A drop of 3% hydrogen peroxide was placed on a clean glass slide. With a wire loop, overnight culture of the isolates from MRS agar plate were picked and transferred into the drop of hydrogen peroxide. Both were mixed and observed for gas bubbles production (Hugh and Leifson, 1953). Gas production was considered as positive test for Catalase enzyme production.

Carbohydrate fermentation test

This was used to determine the ability of an organism to ferment a specific carbohydrate with or without the production of gas. Phenol red was used as an indicator in the media. An inverted Durham tube in the broth captures some of the gas the organism produces allowing production to be seen. The test was done using two sugars, lactose and glucose.

Lactose fermentation test

Ten gram phenol red broth was mixed with 6g lactose broth and the mixture was added into flask containing 600 ml distilled water.

The mixture was transferred to 52 test tubes (14 for Keribo, 23 for Bukuri and 15 test tubes for Korefe) and sterilized by autoclaving.

These were then inoculated with fresh culture of isolates and incubated at 32^{o}_{C} for 72hrs.The incubated colonies were interpreted as negative and positive by observing color change in each medium (Tambekar and Bhutada, 2010).

Gas production from glucose

To determine the homo-fermentative and hetero-fermentative characterization of isolates, CO_2 production from glucose was evaluated using inverted Durham tube. Accordingly, Durham tube was placed in a test tube containing phenol red broth base medium into which glucose (1%) was added and mixed with distilled water. The tube was placed in an inverted position. After sterilization in autoclave, each test tube was inoculated with a single colony of the bacterial culture under study and incubated at $32^{o}C$ for 48-72hrs.The isolates were designated as homo

fermentative or hetero fermentative by observing the presence or absence of gas in Durham tube (Tambekar and Bhutada, 2010).

Salt tolerance test

Isolates from each sample were tested for their tolerance against different Nacl concentration. For this purpose, 2%, 4% and 6.5% Nacl concentrations were selected (Tambekar and Bhutada, 2010). Each of these different salt concentrations was added in a test tube containing MRS broth and inoculated with fresh overnight culture of the test organism and incubated at 32^0C for 72hrs. After 72hrs of incubation, 0.1ml inoculums was transferred to MRS agar plates by pour plate method and incubated at 32^0C for 48hrs. The growth of LAB on MRS agar plates was used to designate isolates as salt tolerant (Tambekar and Bhutada, 2010).

Temperature tolerance

The selected bacterial cultures were grown at varying temperatures, i.e.25^{o}C, 37^{o}C, 40^{o}C for 48 to 72hrs in MRS broth. Then 0.1ml inoculums was transferred to MRS agar plates by pour plate method and incubated at 32^0C for 48hrs.

The growth of LAB on MRS agar plates was used to designate isolates as temperature tolerant (Tambekar and Bhutada, 2010).The reason for choosing this temperature range was to detect whether the isolates are able to grow within the range of normal body temperature or not.

5. *In-vitro* probiotic potential and antibacterial activities of LAB

5.1. Probiotic potential

5.1.1. Resistance to low P^H

The isolated bacterial cultures were inoculated in to sterile MRS broth tubes of varying p^H (pH 2 and 3) using hydrochloric acid (3.0 M) to adjust the pH, and incubated at 32^{o}Cfor 48 to 72hrs. Then, 0.1 ml aliquot from each tube was transferred into MRS agar medium by pour plate method and incubated at 32^{o}C for 48 to72hrs. Viable cell count was done after 48 to 72 hours of incubation under anaerobic condition using anaerobic jar. The growth of LAB on MRS agar was used to designate isolates as pH tolerant (Tambekar and Bhutada, 2010).

5.1.2. Tolerance to Gastric enzyme

MRS broth was adjusted to pH 2 and 3 and sterilized by autoclaving. To this experimental medium, pepsin (3mg/ml) was added aseptically to simulate the activity of gastric enzymes. An overnight culture of test LAB strain was inoculated into it and incubated at 37^{0}Cfor 3hrs. As the time it takes for digestion to be completed in the stomach is 3hrs, detection of resistance to each

p^H and pepsin was done during three hrs of incubation. The growth of LAB on MRS agar was used to designate isolates as resistant to gastric enzymes which work at a very low pH.

5.2. Determination of antibacterial activity

5.2.1. Preparation of sample filtrate (CFS).

The purified LAB isolates were activated from slants to fresh MRS broth and incubated overnight at 37^{o}C.The culture broths of each isolate were then centrifuged separately at 3000 rpm for 15min.The cell free supernatant (CFS) forming the upper layer was filtered and separated from the denser bacterial cell which settled at the bottom. The cell free supernatant broth was collected for the antibacterial study against selected food borne pathogens (Cakir, 2003; Quwehand and Lund, 2004).

5.2.2. Bacterial Test strains

Salmonella sp., Pseudomonas.aerogunesa, Staphylococcus aureus and E. coli O157:H7 were used as test strains to evaluate the antimicrobial effects of LAB isolated from Keribo, Bukuri and Korefe. *Salmonella sp, Pseudomonas aeruginosa, Staphylococcus aureus and E. coli* O157:H7 were clinical isolates which were obtained from Jimma University Microbiological laboratory. The pure cultures of these pathogenic strains were activated from stock culture by inoculating into brain heart infusion broth (BHIB). An overnight culture was used for evaluation of antimicrobial activities of filtrates of LAB isolates as indicated below.

5.2.3. Antimicrobial sensitivity test by Agar well diffusion method

After overnight incubation of the test strains, the culture broth was suspended in saline solution and a lawn of each strain was made by spreading appropriate cell suspensions over the surface of Mueller Hinton agar (MHA) plates with sterile cotton swab. The agar well diffusion method was used to determine the antimicrobial property of the filtrates of LAB isolates. The Muller Hinton agar containing the test pathogens were allowed to dry and a sterile cork borer of 5mm diameter was used to cut uniform wells in the agar. Each well was filled with 60µl culture free filtrate obtained from the LAB isolates. After drying, the plates were kept for 2hrs in a refrigerator to

facilitate diffusion of sample filtrate in the agar followed by incubation at 37^{o}C for 48hrs. Finally, the plates were observed for diameter of zone of inhibition (ZOI) around the well. Results were considered positive when the diameter (mm) of the zone of inhibition was greater than 1mm.The antimicrobial evaluation was done in duplicate and the mean value (Mean ± SD) was used for data analysis. The anti microbial activity of the bacteria was compared with standard, broad spectrum 15ug of Gentamycin.

5.3. Statistical analysis

Data was entered to Microsoft excel and analyzed using SPSS software package (Version 16.0). Descriptive statistics was used to describe data on microbial counts, biochemical analysis, probiotic properties, and antimicrobial activities. Results were presented using tables and graphs. Relationship between parameters were analyzed using one-way ANOVA and differences were determined by Duncans multiple range test ($p < 0.05$). All statistical results with $p<0.05$were considered to be statistically significant.

6. Results

6.1. LAB counts of fermented food sample

After 48-72hrs of incubation under anaerobic condition, growth was observed on MRS agar plates. Snow white and pale yellow colored colonies with clear zones were counted using digital colony counter. Mean counts (CFU/ml) of lactic acid bacteria (LAB) in *Keribo, Bukuri* and *Korefe* samples were $2.35x10^3$, $2.46x10^3$, and $2.31x10^3$, respectively. The three samples had related microbial load or cell densities with regards to LAB population although the count was a bite higher in *Bukuri* samples. A total of 52 LAB were isolated from Keribo, Bukuri and Korefe samples. Accordingly, 14 were isolated from Keribo, 23 from Bukuri and 15 from Korefe. All the isolates were found capable of growing under limited oxygen supply as the isolates successfully grown under anaerobic (candle jar condition).

6.2. Characterization and Identification of LAB

Morphologically, isolates from Keribo samples were dominated by coccoid bacteria while those from Bukuri were mainly rod shaped although Korefe had both rods and coccoid cells (Tables,2, 3, and 4). None of the 52 LAB isolates were found motile; all were catalase negative, non-spore formers, and Gram positive. Biochemical/physiological characterization of the isolates revealed

that 12 isolates from Keribo, 11 isolates each from Bukuri and Korefe were found to ferment lactose (lactose positive). The isolates were successfully grown in the broth medium producing both gas and acid.

But, the remaining isolates, twelve isolates were lactose negative as they did not produce acid and the media remained red (Tables,2, 3, and 4). Concerning gas production from glucose, nine of the fourteen (64%) LAB isolates from Keribo were found to be hetero-fermentative and five were Homofermentative. Thus, Keribo fermentation was found dominated by hetero-fermentative bacteria. Furthermore, all the twenty three bacterial isolates from Bukuri were homo fermentative. The homo fermentative bacteria were the dominant bacteria isolated from the sample. Likewise, seven of fifteen isolates (47%) were hetero fermentative while eight were homo fermentative in Korefe sample (Tables,2, 3,and4).

Table.1. Morphological and biochemical characterization of isolates from Keribo sample, 2015

Characteristics	Ke_1	Ke_2	Ke_3	Ke_4	Ke_5	Ke_6	Ke_7	Ke_8	Ke_9	Ke_{10}	Ke_{11}	Ke_{12}	Ke_{13}	Ke_{14}
Cell morphology	cocci	cocci	cocci	cocci	cocci	cocci	cocci	cocci	cocci	cocci	cocci	cocci	cocci	cocci
KOH test	_	_	_	_	_	_	_	_	_	_	_	_	_	_
Spore formation	_	_	_	_	_	_	_	_	_	_	_	_	_	_
Catalase activity	_	_	_	_	_	_	_	_	_	_	_	_	_	_
Motility test	_	_	_	_	_	_	_	_	_	_	_	_	_	_
Fermentation type(Acid and gas from glucose)	Homo (+/-)	Homo (+/-)	Hetero (+/+)	Homo (+/-)	Homo (+/-)	Hetero (+/+)	Hetero (+/+)	Hetero (+/+)	Hetero (+/+)	Homo (+/-)	Hetero (+/+)	Hetero (+/+)	Hetero (+/+)	Hetero (+/+)
Lactose Fermentation	+	+	+	+	+	+	+	+	_	+	+	+	_	+
Isolates tentative identity	*Pediococcus*	*Pediococcus*	*Leuconostoc.*	*Pediococcus*	*Pediococcus*	*Leuconostoc.*	*Leuconostoc.*	*Leuconostoc.*	*Leuconostoc.*	*Pediococcus*	*Leuconostoc.*	*Leuconostoc.*	*Leuconostoc.*	*Leuconostoc.*

Where, Homo= Homofermentative; Hetero= Heterofermentative; + (Growth) - (No growth)

Table 2. Morphological and biochemical characterization of isolates from Bukuri sample, 2015

Characteristics	Bk_1	Bk_2	Bk_3	Bk_4	Bk_5	Bk_6	Bk_7	Bk_8	Bk_9	Bk_{10}	Bk_{11}	Bk_{12}	Bk_{13}	Bk_{14}	Bk_{15}	Bk_{16}
Cell morphology	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod
KOH test	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Spore formation	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Catalase activity	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Motility test	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Fermentation type(Acid and gas from glucose)	Homo (+/-)	Homo. (+/-)	Homo (+/-)	Homo (+/-)	Homo (+/-)	Homo (+/-)	Homo (+/-)	Homo (+/-)	Homo (+/-)	Homo (+/-)	Homo (+/-)	Homo (+/-)	Homo (+/-)	Homo (+/-)	Homo (+/-)	Hom (+/-)
Lactose Fermentation	+	+	–	+	–	+	–	+	–	+	–	+	+	+	+	–
Isolates tentative identity	*Lactobacillus*	*Lactobacillus*	*Lactobacillus*	*Lactobacillus*	*Lactobacillus*	*Lactobacillus*	*Lactobacillus*	*Lactobacillus*	*Lactobacillus*	*Lactobacillus*	*Lactobacillus*	*Lactobacillus*	*Lactobacillus*	*Lactobacillus*	*Lactobacillus*	*Lactobacillus*

Table 3 Cont---

Characteristics	Bk_{17}	Bk_{18}	Bk_{19}	Bk_{20}	Bk_{21}	Bk_{22}	Bk_{23}
Cell morphology	Rod	Rod	Rod	Rod	Rod	Rod	Rod
KOH test	–	–	–	–	–	–	–
Spore formation	–	–	–	–	–	–	–
Catalase activity	–	–	–	–	–	–	–
Motility test	–	–	–	–	–	–	–
Fermentation type (Acid and gas from glucose)	Homo (+/-)	Homo (+/-)	Homo (+/-)	Homo (+/-)	Homo (+/-)	Homo (+/-)	Homo (+/-)
Lactose Fermentation	–	+	–	–	–	–	–
Isolates tentative .identity	*Lactobacillus*	*Lactobacillus*	*Lactobacillus*	*Lactobacillus*	*Lactobacillus*	*Lactobacillus*	*Lactobacillus*

Where; Homo= Homofermentative; + (Growth) −(Nogrowth)

Table 3.Morphological and Biochemical Characterization of isolates from Korefe sample

Characteristics	Kr_1	Kr_2	Kr_3	Kr_4	Kr_5	Kr_6	Kr_7	Kr_8	Kr_9	Kr_{10}	Kr_{11}	Kr_{12}	Kr_{13}	Kr_{14}	Kr_{15}
Cell morphology	Rod	Rod	Cocci	Rod	Rod	Rod	Rod	Rod	cocci	cocci	Rod	cocci	cocci	cocci	cocci
KOH test	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_
Spore formation	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_
Catalase activity	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_
Motility test	_	_	_	_	_	_	_	_	_	_	_	_	_	_	_
Fermentation type(Acid and gas from glucose)	Hom (+/-)	Hom (+/-)	Heter. (+/+)	Hom (+/-)	Hom (+/-)	Hom (+/-)	Hom (+/-)	Hom (+/-)	Heter (+/+)	Heter (+/+)	Hom (+/-)	Heter (+/+)	Heter (+/+)	Heter (+/+)	Heter (+/+)
Lactose Fermentation	+	+	_	+	_	_	+	+	+	+	+	_	+	+	+
Isolates tentative identity	Lacto-bacillus	Lacto-bacillus	Leucon-ostoc	Lacto-bacillus	Lacto-bacillus	Lacto-bacillus	Lacto-bacillus	Lacto-bacillus	Leuco-nostoc	Leuco-nostoc	Lacto-bacillus	Leuco-nostoc	Leuco-nostoc	Leuco-nostoc	Leuco-nostoc

Where, Homo= Homofermentative; Hetero= Heterofermentative; + (Growth) - (No growth)

6.3. In vitro Probiotic potential and antimicrobial activity

Resistance to low p^H

As clearly stated in the methodology section, the dominant lactic acid bacteria isolated from the three fermented beverages were subjected to primary screening for acid tolerance in MRS broth adjusted to $p^{H\cdot}2$ and p^H 3 with 1N HCl.The determination of survival was performed by single streaking on MRS agar plates and the growth was observed after 48-72hrs incubation under anaerobic incubation at 32^0C. Isolates which were grown on the agar plates were considered to be acid tolerant strains. Accordingly, only seven isolates out of fourteen from Keribo (50%), six out of twenty-three isolates (26%) from Bukuri and seven out of fifteen isolates (46.7%) from Korefe were found to grow both at P^H2 and P^H3(Table.4). These isolates which were resistant to the acidic conditions of the stomach were selected for further screening against other criteria being used for evaluation of probiotic potentials of isolates.

Survival in simulated Gastric transit

All the selected acid resistant isolates, namely the seven isolates from Keribo (labeled as Ke_1, Ke_2, Ke_5, Ke_8, Ke_{10}, Ke_{12} and Ke_{14}), six isolates from Bukuri (labeled as BK_1, BK_2, BK_4, BK_8, BK_{10}, BK_{12}) and the seven isolates from Korefe (labeled as Kr_1, Kr_2, Kr_4, Kr_8, Kr_9, Kr_{11}, Kr_{13}) were found growing in an acid and enzyme mix menu of stomach simulated medium (Table 4).

Table 4.Acid tolerance in LAB isolated from Keribo, Bukuri and Korefe samples, 2015

pH			PH			pH		
Keribo isolates	2	3	Bukuri Isolates	2	3	Korefe Isolates	2	3
Ke_1	+	+	Bk_1	+	+	K_{r1}	+	+
Ke_2	+	+	Bk_2	+	+	Kr_2	+	+
Ke_5	+	+	Bk_4	+	+	Kr_4	+	+
Ke_8	+	+	Bk_8	+	+	Kr_8	+	+
Ke_{10}	+	+	Bk_{10}	+	+	Kr_9	+	+
Ke_{12}	+	+	Bk_{12}	+	+	Kr_{11}	+	+
Ke_{14}	+	+				Kr_{13}	+	+

Where, + (Growth) - (No growth);

$Ke_{1,}$,$Ke_{2,}$,$Ke_{5,}$,Ke_{10} are *Pediococcus* species but Ke_8,Ke_{12},Ke_{14} are *Leuconostoc* species ;

$Kr_{1,}$ $Kr_{,2}Kr_{4,}$ $Kr_{8,}$ $Kr_{11,}$ are *Lactobacillus* speciesbut,Kr_9,Kr_{13} are *Leuconostoc* species

Temperature and Nacl tolerance

One of the criteria used for screening of good Probiotics is the ability to grow at different temperatures. In this research, almost all the LAB isolates were able to grow and survive at the test temperatures (25^{o}C, 37^{o}C and 40^{o}C) (Tables, 6,7and 8). The reason for choosing this temperature range was to detect whether the isolates are able to grow within the range of normal body temperature or not. They were all growing at this temperature range and this was an indication that they could survive in the human gut which is an essential feature of Probiotics to show their effectiveness.

Nacl is an inhibitory substance which could inhibit the growth of certain type of bacteria. One important criterion for good probiotics is the ability to resist high salt concentration occurring in the duodenum of small intestine due to bile salt from the liver and $NaBHCO_3$ from the pancreas. In the present study, all of the selected LAB isolates from the three fermented food products were able to survive 2% and 4% Nacl but not in 6.5% sodium chloride (Tables, 6, 7and 8).

Table5.Temperature and Nacl tolerance of selected LAB isolated from Keribo, 2015

	Temperature tolerance			Nacl tolerance		
Isolates	25°C	37°C	40°C	2%	4%	6.5%
Ke_1	+	+	+	+	+	_
Ke_2	+	+	+	+	+	_
Ke_5	+	+	+	+	+	_
Ke_8	+	+	+	+	+	_
Ke_{10}	+	+	+	+	+	_
Ke_{12}	+	+	+	+	+	_
Ke_{14}	+	+	+	+	+	_

Where, + (Growth) - (No Growth)

Ke_1 Ke_2 Ke_5 Ke_{10} are *Pediococcus* strains but,Ke_8 Ke_{12} Ke_{14} are *Leuconostoc* strains

Table.6. Temperature and Nacl tolerance of selected LAB isolated from Bukuri, 2015

	Temperature tolerance			Nacl tolerance		
Isolates	25°C	37°C	40°C	2%	4%	6.5%
Bk_1	+	+	+	+	+	_
Bk_2	+	+	+	+	+	_
Bk_4	+	+	+	+	+	_
Bk_8	+	+	+	+	+	_
Bk_{10}	+	+	+	+	+	_
Bk_{12}	+	+	+	+	+	_

Where, + (Growth) - (No Growth) ; All isolates belong to *Lactobacillus* species

Table.7. Temperature and Nacl tolerance of selected LAB isolated from Korefe, 2015

	Temperature			Nacl tolerance		
Isolates	25 °C	37 °C	40 °C	2%	4%	6.5%
Kr_1	+	+	+	+	+	_
Kr_2	+	+	+	+	+	_
Kr_4	+	+	+	+	+	_
Kr_8	+	+	+	+	+	_
Kr_9	+	+	+	+	+	_
Kr_{11}	+	+	+	+	+	_
Kr_{13}	+	+	+	+	+	_

+ (Growth) - (No Growth)

*Kr_1, Kr_2, Kr_4, Kr_8, Kr_{11}, are *Lactobacillus* species but, Kr_9, Kr_{13} are *Leuconostoc* species

6.4. Determination of antimicrobial activity of selected LAB by agar well diffusion method

The agar well diffusion method was used to assess the antimicrobial activity of the selected LAB isolates screened from three fermented food products, Keribo, Bukuri and Korefe. All the 20 bacterial cultures which were resistant to stomach p^H, pepsin, human body temperature range and different salt concentrations from three fermented food products, Keribo, Bukuri and Korefe were used. The spectrum of inhibition was different for the different isolates as indicated below (Tables, 9,10and11). Diameter of zone of inhibition (ZOI) >1mm around the well was considered as positive result.

The larger the diameter of ZOI, the greater the antimicrobial activity of the isolate. Accordingly, all the selected isolates were found to exhibit antimicrobial activity against indicator strains as shown in Tables 9, 10 and 11. Thus, all the tested isolates from Keribo, Bukuri and two isolates from Korefe (namely, Kr_9 and Kr_{11}) showed inhibitory activity against all the four tested pathogenic strains. According to result of this study, *E. coli* was not sensitive to the inhibitory substances produced by Kr_1, Kr_2, Kr_4 and Kr_8. *Furthermore, Staphylococcus aureus* was not sensitive to the inhibitory substances produced by Kr_{13}. Among the isolates screened from the three fermented food products, isolate Kr_{11} from Korefe showed the highest antimicrobial activity against *Staphylococcus aureus* (ZOI=22±2.8mm) and least for Kr_1 and Kr_{13}. In addition, isolate Kr_2 from Korefe showed the highest antimicrobial activity against *Salmonella sp* (ZOI=19.5±3.6mm) and least for Kr_8 with no inhibitory activity. Isolate Bk_1 from Bukuri showed the highest inhibitory activity against *E. coli* (ZOI=17.5±3.8mm) and the least by Kr_1,Kr_2,Kr_4 and Kr_8 having no inhibitory activity. Likewise, Kr_1 from Korefe showed the highest anti microbial activity against *Pseudomonas aeruginosa* (ZOI=20.5±3.7mm) and least for Ke_2 from Keribo with ZOI=6±1.4mm (Table 9).

Table 8. Antimicrobial activity of LAB isolated from Bukuri as assessed by agar well diffusion method, 2015 (ZOI ± SD)

Bukuri isolates*	*S. aureus*	*Salmonella* spp.	*E. coli*	*P. aeruginosa*
Bk_1	13.5±4.9[a]	15.5±2.1b	17.5±3.8c	17.5±2.8 d
Bk_2	17±4.2 [a]	13.5±3.6 b	14.5±3.5 c	16.5±2.1 d
Bk_4	12.5±3.6[a]	15±1.4 b	13.5±2.1c	14.5±3.5 d
Bk_8	12±2.8 [a]	16±1.4 b	12.5±3.6 c	13±1.4 d
Bk_8	14±4.2 [a]	16±2.8 b	15±2.8 c	13±2.8 d
Gentamycin**	31±4.2	21±1.4	36.5±1.4	29.5±5

N.B: Values represent mean of duplicates with standard deviation (ZOI±SD), *All isolates belong to *Lactobacillus* species, ** Gentamycin was used as positive control across related tests; [a,b,c,d] Superscripts of the same letter under the same column indicate absence of significant difference

As the one way ANOVA analysis result showed, there was no significant difference (P=0.770) in antibacterial activity among the six LAB isolated from Bukuri (Bk) on *Staphylococcus aureus* (Table.9). The fact that all the isolates belong to Lactobacillus species could account for such close activity pattern, though differ in specific value. The result obtained was the same (no significant difference) for the anti bacterial activity of isolates against the other tested pathogens with P-value of P=0.742, P=0.662, P=0.475 for *Salmonella, E. coli and Pseudomonas aeruginosa,* respectively (Table 9). However, there was a significant variation between the Bukuri isolates and the control drug (Gentamycin) on their antibacterial activity against *Staphylococcus aureus* (P=0.017). *E. coli* (P=0.001) and *Pseudomonas aeruginosa* (Table 9). On the other hand, the ANOVA analysis result showed that there was no significant difference between Bukuri isolates and Gentamycin on their antibacterial activity against *Salmonella*

Tabl.9.Antimicrobial activity of LAB isolated from Keribo as assessed by agar well diffusion method, 2015 (ZOI ± SD)

Keribo isolates	*S. aureus*	*Salmonella* spp.	*E. coli*	*Pseud. aeruginosa*
Ke_1	5.0±1.4 [a]	3.5±2.3 [c]	13±1.4[e]	10.5±2.9[g]
Ke_2	6.0±1.4 [a]	3.0±1.4 [c]	12±1.5[e]	6.0±1.4 [g]
Ke_5	7.5±0.5 [a]	5.0±2.8 [c]	6.0±2.8 [e]	7.0±1.4 [g]
Ke_8	3.5±2.1 [a]	6.5±2.1 [c]	7.0±1.4 [e]	10±1.4 [g]
Ke_{10}	6.5±1.5 [a]	5.0±2.8 [c]	8.5±2.1[e]	8.5±0.5 [g]
Ke_{12}	5.0±1.4 [a]	3.0±1.4 [c]	6.5±1.5 [e]	8.0±1.4 [g]
Ke_{14}	5.5±2.1 [a]	4.5±2.1 [c]	10±4.2 [e]	7.0±2.8 [g]
Gentamycin	31±4.2[b]	21±1.4 [d]	36.5±1.4[f]	29.5±5[h]

Values represent mean of duplicates with standard deviation (ZOI)

* Ke_1,Ke_2, Ke_5 and Ke_{10} are *Pediococcus* strains Ke_8,Ke_{12},Ke_{14} are *Leuconostoc* strains.A,c,e,g superscript under the same column indicate absence of significant difference.

With regards to antimicrobial activity of isolates of LAB from Keribo samples, the one way ANOVA analysis result showed that there was no significant difference among the seven isolates against *Staphylococcus aureus* (P=0.368) with almost equal activities (Table.10). The same pattern was also obtained from these isolates on their inhibitory activity against *Salmonella spps* (P=0.66), *E. coli* (P=0.097), and *Pseudomonas aeruginosa* (P=0.287). But when their activities are compared with the controlled drug (Gentamycin), there was significant difference on their inhibitory activity against *Staphylococcus aureus*, *Salmonella spps*, *E. coli* and *Pseudomonas* with $P< 0.05$ (P=0.00 each).

Table.10.Antimicrobial activity of LAB isolated from Korefe as assessed by agar well diffusion method, 2015

Korefe isolates	*S. aureus*	*Salmonella spp.*	*E. coli*	*Pseud. aeruginosa*
Kr_1	_	5±1.4	_	20.5± 3.7
Kr_2	20±2.8	19.5±3.6	_	18.5±2.2
Kr_4	12.5±2.2	9.5±3.7	_	10.5±1.4
Kr_8	19±2.4	_	_	16.5±3.7
Kr_9	18.5±2.2	12±1.4	16.5±2.2	15.5±2.2
Kr_{11}	22±2.8	10.5±2.2	14.5±3.6	17.5±3.6
Kr_{13}	_	11.5±1.4	14.5±3.6	13.5±2.2
Gentamycin	31±4.2	21±1.4	36.5±1.4	29.5±5

Values represent mean inhibition zone diameter with standard deviation (ZOI±SD); Kr_1,Kr_2,Kr_4,Kr_8,Kr_{11} are *Lactobacillus* ‘Kr_9, Kr_{13} are *Leuconostoc* spp

According to the analysis result of one way ANOVA, there was a significant difference (P=0.00) in antibacterial activity among the seven Korefe isolates against *Staphylococcus aureus, Salmonella spps, E. coli and Pseudomonas aeruginosa* with $p<0.05$(P=0.001, P=0.00, and P=0.028, respectively) (Table 11). Furthermore, there was significant difference between the Korefe isolates and the control drug (Gentamycin) regarding their antibacterial activity against *Staphylococcus aureus* (P=0.00). A significant difference was also observed between Korefe isolates and Gentamycin on their anti bacterial activity against *Salmonella* spps, *E. coli* and *Pseudomonas aeruginosa* with ANOVA results of $P<0.05$(P=0.00) for *Salmonella.*

7. Discussion

Previous studies indicated that probiotics interfere with Gastro intestinal tract pathogens and this property could be a criterion in selecting new probiotic strains from different sources. Most of the LAB commonly used as probiotics are usually associated with fermentation processes. In the current study, different species of LAB were isolated from three of the traditional fermented beverages (Keribo, Bukuri and Korefe). Accordingly, most of the bacterial strains isolated from Keribo match the characteristics of the genus *Leuconostoc* as they were gram positive, mostly coccoid cells, none spore forming, catalase negative, none motile hetero- fermentative (producing acid along with gas and other by products from glucose metabolism) and able to with stand varying range of p^H, temperature and Nacl. Thus, the study indicated that at least nine of LAB isolated from Keribo samples (isolates Ke_1, Ke_6, Ke_7, Ke_8, Ke_9, Ke_{11}, Ke_{12},Ke_{13} and Ke_{14}) resemble the characteristics features of the genus *Leuconostoc* in agreement the characteristic features set for the group by *Lavanya* et al. (2012).

Likewise, most of the isolated bacterial cultures from Bukuri match the characteristics of the genus lactobacillus as they are gram positive, mostly rod shaped cells none endo spore forming, Catalase negative, acid with or without gas forming from glucose metabolism and able to with stand varying range of p^H, temperature and Nacl concentrations and lactic acid producers from sugares.So these features confirmed that almost all the isolates from Bukuri resembled the characteristics of the genus Lacto bacillus as described by Lavanya, etal (2012).There for, the homo fermentative Lactobacillus bacterial isolates are dominating the Bukuri sample.

Out of 15 LAB isolated from Korefe, seven isolates were found to be hetero fermentative and eight of them were found to be homo fermentative. Like the isolates from Bukuri, the dominant strain from Korefe belongs to the genus Lacto bacillus. The remaining and large number of isolates belongs to the genus Leuconostoc which produce gas ($C0_2$) producing white bubbles during fermentation (the name Korefe is derived from the production of these bubbles).After identifying the isolates to their genus level, and grouping in to homo fermentative and hetro fermentative, the ability of the LAB isolates to survive the passage through the gastro intestinal

tract was estimated by Invitro studies and the isolates were screened for antimicrobial activity on the growth and survival of some food borne pathogenic microorganisms.

One of the most important criterions for pro biotics is its resistance to stress full condition of the stomach. A probiotic should survive transit through the stomach where the p^H is low around one to three. Hence tolerance to extremely acidic conditions is another important feature of probiotics (Dunne etal. 2001; Gue etal., 2009). In this study, it was observed that seven LAB isolates from Keribo (Ke_1, Ke_2, Ke_5, Ke_8, Ke_{10}, Ke_{12} and Ke_{14}), six isolates from Bukuri (Bk_1, Bk_2, Bk_4, Bk_8, Bk_{10}, Bk_{12}) and seven isolates from Korefe (Kr_1, Kr_2, Kr_4, Kr_8, Kr_9, Kr_{11}, Kr_{13}) showed better survival in both P^H2and P^H3. The rest isolates were non resistant.

The resistant isolates were selected and screened for further study. However, it was noted that the number of colonies survived decreased with decreased p^H. Therefore P^H tolerance is very important for bearing initial stress in the stomach (Klayraung etal., 2008).At the application level, when LAB enter the human body, first constraint is the gastric acidity with very low p^H.

Therefore, the 52 isolates from the three fermented food products were screened in to 20 low p^H resistant isolates for Invitro probiotic study and all the acid resistant isolates from the three fermented food products were more over tested for gastric transit, temperature and Nacl tolerance and anti bacterial activity.

With respect to simulated gastric juice condition, all the 20 isolates from the three fermented food products were able to resist and grow in p^H adjusted MRS broth containing pepsin where the isolates were transferred to MRS agar and incubated at 37^0C for three hours. After three hours of incubation, the isolates from MRS broth were transferred to MRS agar and incubated at 32-37^{o}C for 48hours.Although the results showed a significant decrease in the number of population during three hours of monitoring, all were seen growing on MRS agar plates. The results were similar with the work of Madureira. etal (2005).

WHO reported that the resistance of probiotic strains to passage through the gastro intestinal tract is strain dependant and several Para meters may determine the extent to which Probiotics strains survive passage through the upper GIT, the degree of stomach acidity and the period of exposure.

In the present study, all the selected LAB isolates from the three fermented food products were able to survive at 2% and 4% sodium chloride but not in 6.5%sodium chloride as shown in the table.8,9 and10. Sodium chloride is an inhibitory substance which may inhibit the growth of certain type of bacteria. There for one important criterion for good Probiotics is the ability to resist high salt concentration occurring in the duodenum of small intestine due to bile salt from the liver and $NaBHCO_3$ from the pancreas. Although they were not grown at 6% sodium chloride, they showed good resistant to 2% and 4%sodium chloride. Their resistance to varying range of salt concentration is an indication of tolerating bile salt concentration in the small intestine.

Thus, even though bile salt tolerance of isolates were not studied due to the absence of bile salt, the acid and salt tolerating isolates from the three fermented food products(20 isolates) would certainly tolerate bile salt in the duodenum. Another important feature of Probiotics culture is its ability to kill pathogens which infect the gastro intestinal system. The twenty LAB isolates which were tested for their probiotic potential from the three fermented food products were checked for their anti microbial activity against salmonella spps, E.coli, staphylococcus aureus and pseudomonas aeruginosa which are common food borne pathogens that infect the gastro intestinal tract. The results showed that almost all the 20 isolates with the exception of very few isolates from the three fermented food products could inhibit all the four tested pathogens, however at different inhibition levels.

Several previous studies have observed that strains which can produce antimicrobial substances are active against pathogenic bacteria (Topisirovic *etal*., 2008).The difference in inhibition potential among the screened isolates could be due to different intrinsic factors induced by food origins (Klayraung etal., 2008).

Those LAB isolates from the three traditionally fermented beverages which tolerated a very low p^H of the stomach, the gastric enzymes, human body temperatures and varying salt concentrations were tested for their anti microbial activity and the results showed that all the seven screened and selected LAB isolates from Keribo, the six selected isolates from Bukuri and the seven selected isolates from Korefe could inhibit the

growth of the four tested pathogens although at different inhibition levels. From the total of 20 selected LAB isolates (7 isolates from Keribo, 6 isolates from Bukuri and 7 isolates from Korefe), all the seven isolates from Keribo, all the six isolates from Bukuri and only two isolates from Korefe showed anti bacterial activity against all tested pathogenic strains. But the controlled drug (antibiotic), namely Gentamycin, was highly effective against all tested pathogens compared to some LAB isolates.

The anti bacterial activity of the CFS of selected LAB isolates was done based on the assumption that the cell free supernatant of the isolates would be equally effective to the antimicrobial activity of Gentamycin (control drug) against tested pathogens. But the data showed that there was significant difference ($P<0.05$) between the antibacterial activity of Gentamycin and some selected LAB isolates (especially isolates from Keribo). Gentamycin was highly effective against all tested pathogenic strains compared to CFS of LAB isolates. But some isolates (those from Bukuri and Korefe) for example, Bk1, Bk2, Bk5from Bukuri and Kr_1, Kr2, Kr5, Kr11from Korefe were shown almost equal anti bacterial activity against the tested human pathogens when compared to Gentamycin. This does not totally mean that isolates from Keribo and some isolates from Bukuri and Korefe are non effective. The number (load) of the isolates which release anti bacterial metabolites must be taken in to consideration. Because as their number in our body increases, the concentration of metabolites will increase and this will change the sample size and as the sample size changes, the P-value and significant level will change.

LAB strains showing effective inhibition spectra can be used as Probiotics to replace chemical antibiotics in humans and feed industry. Considering probiotic properties, all the isolated strains can be used as potential Probiotics with further detailed studies.

The other important criterion for good Probiotics is their role in carbohydrate metabolism. The presence of lactose fermenting isolates in these fermented food products suggests that these drinks may be suitable for lactose intolerant people who couldn't metabolize lactose due to the lack of Enzyme β galactosidase. Twelve isolates including all the selected ones(Ke_1, Ke_2, Ke_3,Ke_4,Ke_5, Ke_6,Ke_7,Ke_8, Ke_{10}, $Ke_{11}Ke_{12}$ and Ke_{14})from Keribo, Eleven isolates including the selected ones(Bk_1, Bk_2, Bk_4, Bk_6,Bk_8, Bk_{10}, Bk_{12} ,Bk_{13},Bk_{14},Bk_{15},Bk_{18}) from Bukuri and eleven

isolates including the selected ones (Kr_1, Kr_2, Kr_4, Kr_8,Kr7,Kr8, Kr_9,Kr10. Kr_{11}, Kr_{13},Kr14,Kr15) from Korefe showed fermenting lactose in to lactic acid and/or other compounds. There for, if these fermented food products are consumed by lactose intolerant people, they will help them to eat lactose containing foods without the usual appearance of associated symptoms (abdominal pains, cramps, diarrhea etc.) by breaking lactose in the intestine in to lactic acid and or other by products.

The quality of the product is closely linked to the amount of beneficial micro organisms and to the individual strains in the product. Therefore, in terms of quality, Bukuri>Korefe>Keribo. Because the dominant strain in Bukuri is Lacto bacillus which is the most commonly used micro organism and desirable member of the intestinal micro flora having generally recognized as safe (GRAS) status and the Bacterial load in Bukuri is greater compared to the other two.

The capability of probiotics in these traditionally fermented food products that inhibit the growth of pathogens confirms the health benefits on the consumption of these products. In general, this study suggests that probiotics are helpful in the protection and improvement of our intestinal flora. There for, drinking Keribo, Bukuri and Korefe can help humans to improve their health by protecting against diarrhea, food poisoning and enteric infections. Besides, the selected 20 LAB isolates from the three fermented food products are highly tolerant to the stressful conditions of the stomach (acidic p^H, gastric enzymes).

These good characteristics of the LAB isolates make the three fermented food products as important alternative and natural medicines to relieve gastric ulcer by competing and inhibiting the growth of *H.pylori*.

8. Conclusion

The results obtained from this study demonstrated remarkable probiotic potential and anti bacterial activity against human pathogenic bacteria. The isolated LAB cultures had the ability to tolerate a very low P^H and varying salt concentrations. Based on these Invitero tests, there is a high probability that the isolates can resist stressful conditions of the stomach and able to reach the intestinal tract in good numbers. The number of beneficial micro organisms per milliliter in each of the three fermented food products is very large.

The study also showed that the cell free supernatant which was extracted from LAB isolates have good anti bacterial activity against food borne human pathogens. As the result of these positive and desirable results of the study, the isolation and growing of these potential LAB cultures under optimum condition can lead to the production and extraction of their useful metabolites which serve as bio preservative and therapeutic agents. Besides, Keribo, Bukuri and Korefe can be the basic sources for the discovery of new potential LAB for controlling and treatment of infectious diseases.

Since the three traditionally fermented food products are rich in beneficial LAB, they can be the possible candidates for the formulation of starter culture that can be used to produce safe and bio protective compounds. Besides, by protecting and improving the intestinal flora of poor villagers, they can minimize their dependence on expensive drugs and can be used as alternative medicines to supplement the existing anti biotic dominating medicine.

9. Recommendation

The pro biotic potential of the isolates in the intestinal tract and their antibiotic sensitivity was not tested by this study due to the absence of bile salt and antibiotic discs. No environment in the GIT other than the stomach is more stressful and harshy. Therefore, it is possible to conclude that all if not most of the isolates can resist the bile salt in the duodenum since they resist the harshy conditions of the stomach and different salt concentrations. Besides, the antibiotic sensitivity of LAB in Keribo and Bukuri were studied by prior researchers. Anyway, further work can also be required to identify which isolates are resistant to antibiotic and bile salt and which are not.

People who have regularly consumed these drinks are economically challenged and they don't have an access for antibiotics and even if they get money and buy antibiotics, the emergence of antibiotic resistant bacteria worsen their problem. Therefore, alternative strategies must be developed and studies about the health effects of these traditionally fermented food products must be continued both Invitero and in vivo as the Probiotics found in these drinks will offer the local people the choice of a natural means of overcoming illnesses and at a very least, maintaining good health and well being. Since the people are strongly linking their lives to the consumption of these drinks, Jimma University and other stake holders must work hard up to looking for an investor that can built a company that can isolate the Probiotics from these drinks and manufacture alternative medicines in supplement (Capsule or as sachet preparations) form. In addition to its health benefits, it will open job opportunity and develop the health and economic status of the local people. Finally, this study, will serve as a useful informative base line data for future studies.

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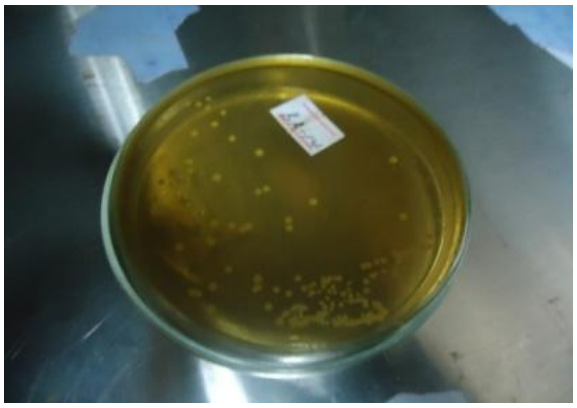
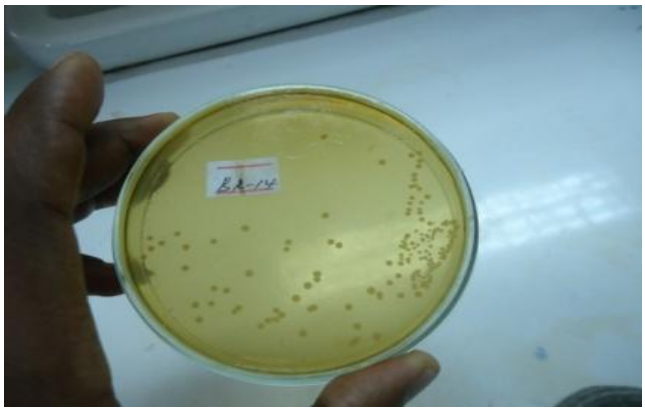
11. Annex

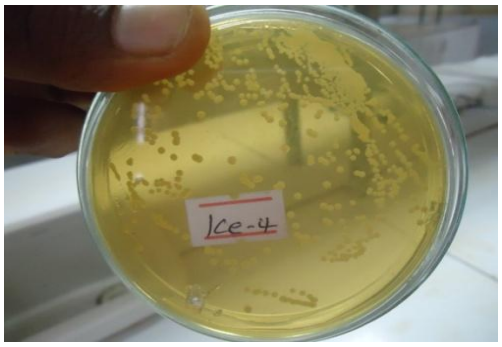
11.1. Colony of LAB isolated from Bukuri, Keribo and Korefe samples.





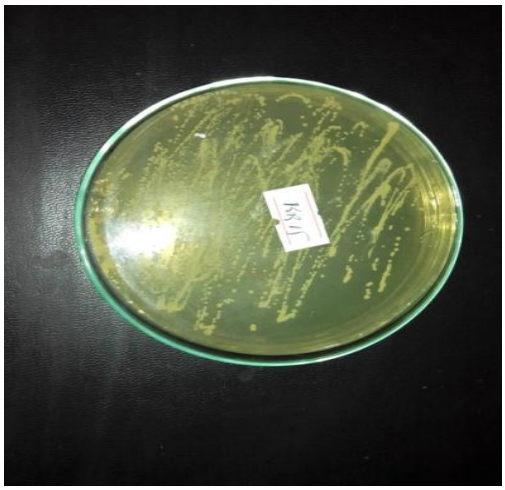
Bukuri isolates

Bukuriisolates

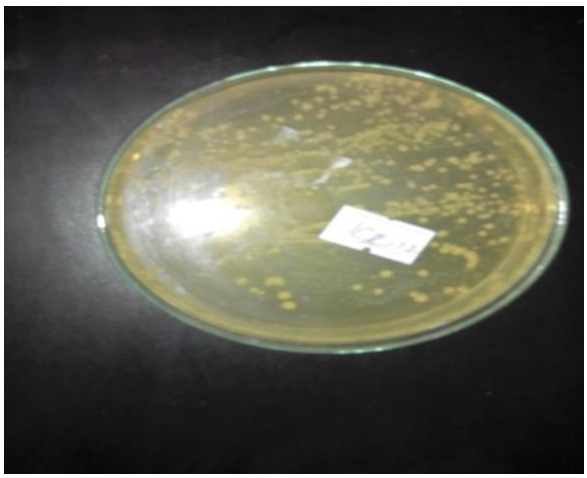


Keribo isolates

Keribo isolates

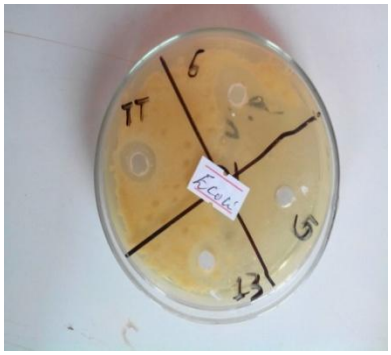


Korefe isolates

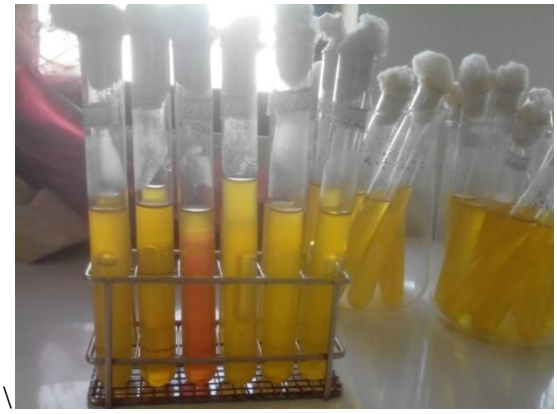


Korefe isolates

11.2 The anti bacterial activity of CFS extracted from LAB isolates



11.3 Carbohydrate fermentation test results

\ 

11.4 Row data of anti bacterial activity of lab isolates from the three fermented food products.

isolates	duplicates	S.aureus	salmonella	E.coli	pseudomonas	
bk1	1	17.0mm	17.0mm	15.0mm	15.5mm	
	2	10.0mm	14.0mm	20.0mm	19.5mm	
Bk2	1	14.0mm	16.0mm	17.0mm	18.0mm	
	2	20.0mm	11.0mm	12.0mm	15.0mm	
Bk4	1	10.0mm	14.0mm	15.0mm	17.0mm	
	2	15.0mm	16.0mm	12.0mm	12.0mm	
Bk8	1	14.0mm	15.0mm	15.0mm	14.0mm	
	2	10.0mm	17.0mm	10.0mm	12.0mm	
Bk9	1	17.0mm	18.0mm	13.0mm	15.0mm	
	2	11.0mm	14.0mm	17.0mm	11.0mm	
Bk12	1	14.0mm	15.0mm	13.0mm	17.0mm	
	2	17.0mm	11.0mm	15.0mm	14.0mm	
Ke_1	1	6mm	2mm	12mm	12.5mm	
	2	4mm	5mm	14mm	8.5mm	
Ke_2	1	7mm	2mm	13mm	7mm	
	2	5mm	4mm	11mm	5mm	
Ke_5	1	7mm	7mm	8mm	6mm	
	2	8mm	3mm	4mm	8mm	
Ke_8	1	2mm	5mm	6mm	9mm	
	2	5mm	8mm	8mm	11mm	
Ke_{10}	1	7.5mm	7mm	10mm	9mm	
	2	5.5mm	3mm	7mm	8mm	
Ke_{12}	1	4mm	2mm	7mm	9mm	
	2	6mm	4mm	6mm	7mm	
Ke_{14}	1	5mm	4mm	12mm	8mm	
	2	6mm	5mm	8mm	6mm	
Kr_1	1	-	4mm	-	22mm	
	2	-	6mm	-	19mm	
Kr_2	1	22mm	22mm	-	20mm	
	2	18mm	17mm	-	17mm	

Continue. Row data

Kr_4	1	14mm	12mm	-	11.5mm	
	2	11mm	7mm	-	9.5mm	
Kr_8	1	17mm	-	-	19mm	
	2	21mm	-	-	14mm	
Kr_9	1	20mm	13mm	18mm	17mm	
	2	17mm	11mm	15mm	14mm	
Kr_{11}	1	25mm	12mm	17mm	20mm	
	2	21mm	9mm	12mm	15mm	
Kr_{13}	1	-	12.5mm	12mm	15mm	
	2	-	10.5mm	17mm	12mm	
Gentamycin	1	27mm	20mm	40mm	35mm	
	2	35mm	22mm	33mm	24mm	