COLLEGE OF NATURAL SCIENCES

SCHOOL OF POST GRADUATE STUDIES

DEPARTMENT OF BIOLOGY

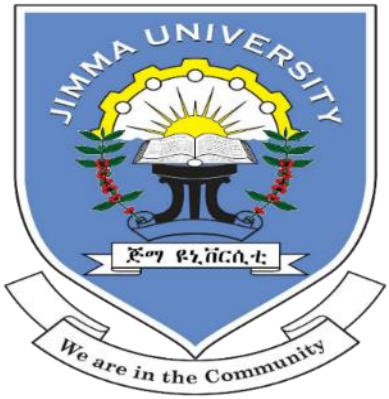
In vitro antagonistic effect of probiotic LAB isolated from traditional fermented products against some antibiotics resistant food borne pathogens

By: Alemayehu Gudisa

Presented in Partial Fulfillment of the Requirements for the Degree of Masters of Science in Biology (Applied Microbiology)

January, 2021

Jimma, Ethiopia



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Principal advisor: Meseret Guta (M.Sc. Asst. Prof.)

Co-advisor: Lata Lachisa (M.Sc.)

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DEDICATION

This thesis work is dedicated to the hero of my life time, a singer, songwriter, civil rights activist and a former political prisoner artist Hachalu Hundessa who was assassinated on June 29, 2020

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LIST OF ABBREVIATIONS/ACRONYMS

AMP	Ampicillin
CFU	Colony forming unit
CHL	Chloramphenicol
CIP	Ciprofloxacin
CRO	Ceftriaxone
DC	Dendritic cells
f	Norfoxacin
FDA	Food and Drug Administration
GM	Gentamycin
HUS	Hemolytic uremic syndrome
I	Intermediate
IARC	International Agency for Research on Cancer
IEC	Intestinal epithelial cells
Ig	Immunoglobulin
IL	Interleukin
MRS	de Man Ragosa and Sharpe
MUB	Mucus-binding protein
NA	Nalicidic acid
PAMP	Pathogen-associated molecular patterns
PPR	Pathogens is triggered by pattern recognition receptors
R	Resistant
S	Sensitive
SPI	Salmonella pathogenicity islands
SXT-TMP	Sulfamethoxazole-trimethoprim
UTI	Urinary tract infections
WHO	World health organization

ABSTRACT

Infections associated with foodborne microbial pathogens are the major causes of morbidity and mortality in the world's populations. Most of the pathogens especially bacteria have developed new resistances to the most commonly used antibiotics. In order to avoid the use of antibiotics, it is necessary to look in to an alternative way of treating the pathogens through the uses of probiotics. The aim of this study was to isolate probiotic LAB form traditionally fermented products and evaluating their antagonistic effect against antibiotics resistant foodborne pathogens such as, E. coli, Salmonella and Shigella. Accordingly sixteen (16) probiotic LAB were screened from traditionally fermented ergo and Tej. The selected isolate were screened based on their probiotic attributes of acid tolerance, bile salt tolerance, starter culture attributes and their antagonistic effect against antibiotics sresistant E.coli, Salmonela and Shigella. Inhibitions using cell-free liquid cultures of LAB on solid cultures of pathogens, showed a growth inhibition against nine antibiotic resistant pathogens diameter equal or greater than 7mm in well diffusion assay. The isolate showed a relative strong inhibition activity, moderate inhibition activity to low inhibition activity (inhibition zone < 10mm) against antibiotics resistant pathogens in well diffusion method. Each LAB co-cultured in milk separately with each nine antibiotic resistant pathogens showed antagonistic activity against the pathogens during the course of fermentation. The highest reduction in the number of pathogens was observed during 48hrs of fermentation of Ergo. The reduction in the number of growth of pathogens by each LAB isolate was significantly ($P < 0.05$) higher than the control fermented Ergo at 24hrs and 48hrs of fermentation. The use of these isolate at home made production of Ergo could help to control the proliferation of antibiotic resistant disease causing bacteria and could also serve as traditional way of treating the pathogens through the uses of fermented Ergo. Fermented products are rich in probiotic bacteria, therefore consuming fermented Ergo add a beneficial bacteria and enzymes to overall intestinal flora, increasing the health of gut microbiome and digestive system and enhancing the immune system.

Key words: Antibiotic resistant*; E .coli; Salmonella; Shigella;* Probiotics LAB

1. INTRODUCTION

Infections associated with foodborne microbial pathogens are the major causes of morbidity and mortality in the world's populations, causing the death of about 1.9 million children worldwide each year (WHO, 2001). Food-borne diseases most commonly occur in developing countries particularly in Africa, as a result of the prevailing poor food handling and sanitation practices, inadequate food safety laws, weak regulatory system, lack of financial resources to invest in safer equipment and lack of education for food-handlers (Haileselassie *et al*., 2013). Even though most of these diarrheal diseases occur in developing nations, it is not limited to these countries, it is estimated that, in the United States foodborne diseases are 48 million (cases) people sick with 128,000 hospitalizations and 3,000 deaths each year (CDC, 2011). New forms of transmission of foodborne disease and increased antibiotics resistance by pathogens, are evading the conventional control measures (WHO, 2001; Bester and Essack, 2010).

Pathogens such as *Escherichia coli, Salmonella* species, *Shigella* species, *Staphylococcus aurous, Clostridium difficile* and *Campylobacter jejuni* find their way to the gastrointestinal tract through the consumption of contaminated food, unsafe water and close proximity to animals (Afolayan *et al.*,2017). The pathogens enters the gastro-intestine are responsible for inciting intestinal infections that negatively affect the normal functions of the gastrointestinal tract, leading to diseases such as cholera, typhoid, salmonellosis, acute gastroenteritis, traveller's diarrhea and shigellosis (Walderma,1998). The disease caused by these pathogens requires antibiotic therapy. However, as a consequence of the indiscriminate use of antibiotics to treat human and animal microbial infections, most of the pathogens especially bacteria have developed new resistances to the most commonly used antibiotics (WHO, 2001; Moellering *et al*, 2007; Bester and Essack, 2010). In order to avoid the use of antibiotics and to control the proliferation of gastrointestinal disease causing bacteria efficiently, it is necessary to look into scientific basis of some traditional way of treating the pathogens through the uses of fermented products that naturally contains probiotic microorganisms.

FAO-WHO has defined probiotics as nonpathogenic live microorganisms, which when administered in adequate amounts confer a health benefit on the host (FAO/WHO, 2002). Preoperative oral administration of symbiotic can enhance immune responses, attenuate systemic

postoperative inflammatory responses and improve the intestinal microbial environment (Sugawara *et al*., 2006). Most commonly used probiotics are lactic acid producing bacteria such as *Lactobacillus, Bifidobacterium, Enterococcus,* and *Bacillus* (Sullivan and Nord, 2005). *Lactobacilli* have been successfully used in prophylaxis and in the treatment of gastrointestinal disorders and infections (Servin, 2004). They are acid resistant and can persist in the stomach for a long time than any other bacteria (Bhatia*et al.,* 1989). Lactobacilli are noninvasive and induce various epithelial cell responses by competing with pathogenic bacteria for host adhesion-binding sites, thereby improving the epithelial cell barrier function and stimulating the host immune response (Forestier *et al*., 2001). Certain lactobacilli synthesize antimicrobial compounds that are related to the bacteriocin family (Jack*et al*., 1995), while others are well known metabolic end products of lactic acid fermentation such as lactic, acetic acids and hydrogen peroxide (Vandenbergh, 1993). Studies indicated that probiotic lactic acid bacteria (LAB) are known to inhibit the growth of food-borne pathogenic microorganisms such as *Escherichia coli, Salmonella typhi, Salmonella typhimurium* and *Staphylococcus aureus* (Tadesse *et al*., 2005; Klayraung *et al*., 2008; Tesfaye *et al*., 2011), *Shigella flexneri* (Tadesse*et al*., 2005*), Pseudomonas aeruginosa, Bacillus cereus, Klebsiella pneumoniae, Proteus spp., Corynebacterium* spp., and *Streptococcus pneumonia* (Al-Allaf *et al*., 2009).

Most of the traditional fermented products of Ethiopia are consumed without further heat processing. Thus, they are a good sources and ideal vehicles to carry probiotic bacteria into the human gastrointestinal tract. *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 (Abdulkadir *et al*., 2011). 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 24hrs (Mogessie Ashenafi, 2002). *Tej* is a honey wine with alcohol content varying from 8 to 14% ABV, which is made from honey, water and leaves of gesho (*Rhamnus prinoides*). Previously, upper class were used, but now it is widespread among all social groups, consumed on holidays and at weddings as well as served in hotels and bars across the country (Fite *et al*., 1991).

Several studies demonstrated the inhibitory effect of LAB isolated from various Ethiopian traditional fermented foods, condiments and alcoholic beverages against some food-borne pathogens (Bacha *et al*., 2009; Dessalegn and Ashenafi, 2010; Tesfaye*et al*., 2011) and also evaluated the *in-vitro* (Bacha *et al*., 2009; Dessalegn and Ashenafi, 2010) and *in-vivo* (Tesfaye *et al*., 2011) antagonistic effect of probiotic lactic acid bacteria. But rare reports on the *in-vitro* anatagonistic activity of probiotic LAB against antibiotics resistant food borne pathogens. Therefore, the current study was designed to isolate potential probiotic LAB from Ethiopian traditional fermented *Ergo* and *Tej* having *in-vitro* antagonistic activity against some antibiotics resistant food borne pathogens, namely *Salmonella, Shigella* and *E.coli*.

1.1 Statement of the problem

As a result of the indiscriminate use of antibiotics to treat human and animal microbial infections, most of the pathogens especially bacteria have developed new resistances to the most commonly used antibiotics (WHO, 2001; Moellering, 2007; Bester and Essack, 2010). Antimicrobial resistance has emerged in the past few years as a major problem and many programs have been set up for its surveillance in human and veterinary medicine. These programs are aimed mainly at human pathogens, agents of zoonoses, and indicator bacteria of the normal intestinal flora (Lanz *et al*., 2003). Use of antimicrobial agents in agriculture, over prescribing by physicians and misuse by patients have been identified as the three main causes of antimicrobial resistance (Doughari, 2012). The major concern on the public health threat of food borne illness is infection by antimicrobial resistant strains that lead to more intractable and severe disease (Helms *et al*., 2002; Martin *et al*., 2004). This situation is further complicated by the potential of resistant bacteria to transfer their resistance determinants to resident constituents of the human microflora and other pathogenic bacteria (Olatoye *et al*., 2012). The food supply is an established vehicle for certain other antimicrobial resistant and/or pathogenic bacteria (Mohle-Boetani *et al*., 2001; Lanz *et al*., 2003; Oliver *et al*., 2011; Rahimi and Nayebpour, 2012).

For the mentioned reasons above, it is an essential demand to develop alternative solutions to prevent pathogen colonization and to search for additional treatment agents. Reports indicate that probiotic LAB have the potential of inhibiting the growth of pathogens. So the current study was intended to isolate the potential probiotic LAB in Ethiopian traditional fermented *Ergo* and *Tej* having antagonistic activity against antibiotics resistant food borne pathogens, namely *Salmonella, Shigella* and *E.coli*.

1.2. Objectives

1.2.1. General objective

To assess the *in vitro* antagonistic activity of probiotic LAB isolated from traditional fermented products against antibiotics resistant food borne pathogens

1.2.2. Specific objectives

- ✓ To isolate and characterize LAB from traditional fermented *Ergo* and *Tej*
- ✓ To evaluate the isolated LABs for their probiotic attributes
- ✓ Determine and screening for antibiotics resistant food borne pathogens isolated from raw milk and raw meat
- ✓ To evaluate the *in vitro* antagonistic activity of probiotic LAB against antibiotics resistant food borne pathogens

1.3. Significance of the study

This study is significant to indicate the potential probiotic LAB in traditional fermented *Ergo* and *Tej* having antagonistic activity against antibiotics resistant food borne pathogens. The probiotic LAB isolates is also significant to provide a potential defined starter culture for making locally fermented products having therapeutic function. The probiotic LAB isolate having antagonistic effect against resistant pathogens could again provide a potential data and initial steps for the large scale production of safe and standard therapeutic *ergo* for the treatment of bacterial infections.

2. LITERATURE REVIEW

2.1 Antimicrobial resistance (AMR) and its challenges

Antibiotics are vital medical materials which can be natural, synthetic or semi-synthetic and can kill or interfere the growth of bacteria, and are used both in animals and humans for control or treat infections (O'Neill, 2015;WHO, 2015). According to studies, antimicrobials can be used as growth enhancer in low subtherapuetic doses, but these doses can not destroy the bacteria and allow them to achieve more resistance to the drug. Concerns about misuse or overuse of antimicrobials as nontherapeutic and appearance of drug resistance have arisen when antimicrobial dose increased to 100 percent in aquaculture in the 1994–2004 (Cabello FC, 2006). Antimicrobial resistance pointed to the situation that a microorganism shows resistance to a drug that was effective for its killing or destroying previously (Economou and Gousia, 2015). Todays, this issue have significant effect on mortality and morbidity of humans each year and has reported that antibiotic-resistant bacteria caused death of 700,000 people globally and has predicted that this rate tend to enhance approximately to 10 million by 2050 (McCullough *et al*., 2016). Adaptation of bacteria to various environmental stresses such as antibiotics, approve that they are quite adaptive organisms.

There are two types of mechanisms for creation and spreading the resistant bacteria population: vertical gene transfer and horizontal gene transfer. The former, which is also called intrinsic resistance, occurs in evolutionary phase and genetic errors accumulate in the plasmid or chromosome of bacterial cells. However in the horizontal gene transfer or acquired resistance, the exchanges are within and between bacterial species in which the organisms gain new genes on their mobile genetic elements including plasmids, insertion sequences, phagerelated elements and integrons, transposons (Holmes *et al.*,2016). Antibacterial resistance can be spread by food chain through direct or indirect exposure. Direct exposure occurs, following the contact of human with animal or its blood, saliva, milk, semen, feces and urine which is very simple and rapid way for spreading resistant bacteria. The indirect contact occurs, following by consumption of contaminated food products such as egg, meat and dairy products which is more complex and far-reaching pathway (Chang *et al*., 2015). The other particular transport routs are related to environment which can be the source of antibiotic-resistance genes (D'Costa *et al*., 2006). As a

result, the bacteria as a reservoir of resistance genes in addition of their pathogenicity, can be a hidden hazard for public health. The appearance of antimicrobial resistance by the food chain is a cross-sectorial problem; the first, antibiotics are extensively used in veterinary, aquaculture and agriculture, the second, antibacterial-resistant bacteria and genes can simply spread at each step of the food chain, and the last can be related to infectious diseases in humans (da Costa *et al*., 2013). On the other hand, antibacterial-resistance can have globally dissemination by food chain due to extension of population, international travels and trade in food products. In preparing food animals, vegetables and fishes, in different ecosystems with numerous bacteria, large types of antibiotics are used which can cause to appearance resistant bacteria (Acar and Moulin, 2006). Todays, antibiotic-resistance, especially that which is transferred from food chain to human is a global concern, and a lot of researches have been conducted to find approaches for solving this critical problem. In the present study, it was tried to express use of probiotic LAB approache for preventing the appearance of drug-resistant bacteria.

2.2. Probiotics

Probiotics are live microorganisms such as *Lactobacillus* spp. *Bifidobacterium* spp. and *Saccharomyces boulardii*; these confer a health benefit on the host when administered in adequate amounts, (FAO/WHO, 2002). At present, the most studied probiotics are lactic acid producing bacteria, particularly *Lactobacillu* ssp. They have been proven to be useful in the treatment of several gastrointestinal diseases such as acute infectious diarrhea or pouchitis (Gill and Guarner, 2004).

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 microbiota, (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 *etal*., 2009). The primary clinical interest in the application of probiotics has been increasing in the prevention and treatment of GI infections and diseases (Parvez *et al*., 2006).

2.3. 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 (pHi) 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 pHi constant: the homeostatic response, the acid tolerance response and the synthesis of acid shock proteins. The homeostatic response maintains pHi by pumping protons from cytoplasm. The acid tolerance response maintains pH by production of inducing proteins. The synthesis of acid shock proteins is the third way that cells regulate pHi (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 those 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).

2.4. Role of Probiotics in the treatment of bacterial pathogens

Many strains of probiotic microorganisms have been shown to inhibit the growth and metabolic activity as well as the adhesion to intestinal cells of enteropathogenic bacteria to modulate (temporarily) the intestinal microflora and to have immune stimulatory or regulatory properties (Coconnier *et al*., 1991, Hudault *et al*., 1997; Gopal *et al*., 2001). The bifidobacteria, in addition to other sanogenetic actions (the improvement of vitamin and protidic metabolism) have antibacterial action especially on pathogenic species (*E. coli, Staphylococcus aureus, Shigella, Salmonella, etc.*). Most probiotics produce lactic acid, which lowers the local pH and thus prevents the growth of sensitive bacteria in acid and renders permeable the outer membrane of gram-negative bacteria (Dekeermaecker and Verhoeven, 2006). Probiotic bacteria such as *Lactobacilli, Enterococci, Bifidobacteria, Pediococcus, E.coli, Streptococcus* and *Leuconostoc* species are normally found in the human GIT, where they form normal flora (Priyodip*et al*., 2017) and are commonly included in popular fermented functional foods to make their delivery easy (Priyodip *et al*., 2017; Saarela *et al*., 2000; Prado *et al* 2015; Plessas *et al*., 2016). Probiotic products can also be in the form of lyophilized capsules or powders or aqueous solutions (Martins*et al*., 2009). Probiotic bacteria have been widely used in the treatment of infectious bacterial diseases and their efficacious applications are summarized in Table 1. These organisms

also confer other benefits, such as appropriate digestion, epithelial cell function, metabolism, enteric nerve function and angiogenesis to the host (Nami*et al*., 2015).

Table 1: Prophylactic and therapeutic properties of probiotic bacteria

Probiotic Micro-organisms	**Indicator enteric pathogens and/or animal models**	**Treatment mechanisms and outcomes**	**References**
Bifidobacterium longum subsp. *longum/ infantis*	*E.coli 0157: H7*	Prevented the production of toxin in the caecum and translocation of toxin from the GIT to the blood stream and hence reduced mortality	Yoshimura *et al.*,2010
Lactobacilli, Bifidobacterium bifidum strains Bb12 and Lactobacillus kefir	*S. Typhimurium*	Secrete molecules that prevent invasion of epithelial cells	Oelschlaeger, 2010
L. plantarum 299 v, L.rhamnosus GG, Bifidobacterium lactis Bb12 and L. rhamnosus LGG	Infection of human mucusa cells with enteropathogenic *E. coli, S.Typhimurium* ATCC 12028.	Competition for the same receptor in the GIT and stimulation of mucin production by probiotic resulted in inhibition of pathogenic bacteria adhesion to the GIT	Oelschlaeger *et al*.,2010; Collado *et al*.,2007
Lactobacillus strains, three Pediococcus strains and four Bifidobacterium strain	E.coli (EHEC) 0157: H7 in in- vitro experiment	All probiotics inhibit toxin production due to the production of organic acid, which resulted in low pH	Carey *et al*.,2008

2.5. Mechanisms of action of probiotics

2.5.1. Non immunological mechanisms

Non immunological include barriers such as the acidity of the stomach and the gastric mucosal barrier that represent the first line of defense against pathogenic bacteria. Major probiotic mechanisms of action includes enhancement of the epithelial barrier, increased adhesion to intestinal mucosa and inhibition of pathogen adhesion, competitive exclusion of pathogenic microorganisms, production of anti-microorganism substances (*Klaenhammer,* 2003).

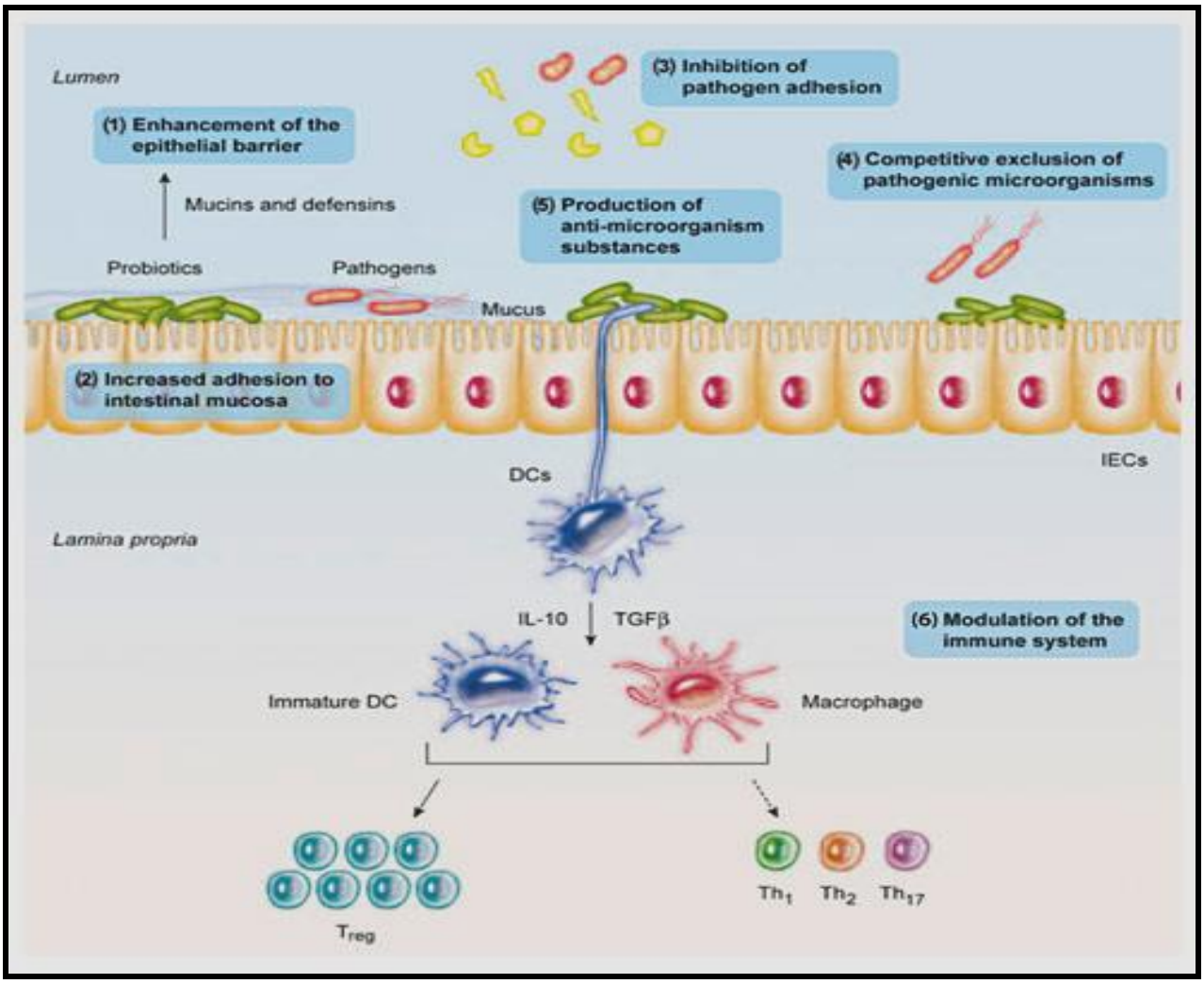

Figure 1: Mechanisms of action of probiotics (Yuying *et al*, 2018)

2.5.1.1 Enhancement of the epithelial barrier

The intestinal epithelium is in permanent contact with luminal contents and variable enteric flora. The intestinal barrier is a major defense mechanism used to maintain epithelial integrity and protect the organism from the environment. It consists of the mucous layer, antimicrobial peptides, secretary IgA and epithelial junction adhesion complex (Ohland *et al*., 2010). Once this barrier function is disrupted, pathogenic bacteria can reach the sub-mucosa and can induce inflammatory responses, which may result in intestinal disorders, such as inflammatory bowel disease (Hooper, *et al*., 2001; Hooper, *et al*., 2003; Sartor *et al*., 2006). Probiotic organisms have involvement in the intestinal barrier function and maintenance of this barrier. Study of Anderson *et al*., (2010), indicates that probiotics enhances the expression of genes involved in tight junction signaling to strengthen intestinal barrier integrity. For instance, *lactobacilli* modulate

several genes encoding adherence junction proteins, such as E-cadherin and β-catenin, in a T84 cell barrier. In addition *lactobacilli* differentially influence the phosphorylation of adherence junction proteins and the abundance of protein kinase C (PKC) isoforms, such as PKCδ, which modulating epithelial barrier function (Hummel *et al.*, 2012). Probiotics initiate repair of the intestinal barrier function after damage in addition to preventing the disruption of the mucosal barrier by enteropathogens. They restore mucosal integrity in T84 by enhancing the expression and redistribution of tight junction proteins of protein kinase C resulting in the reconstruction of tight junction complex (Zyrek *et al*., 2007; Stetinova *et al*., 2010).

2.5.1.2. Increased adhesion to intestinal mucosa and inhibition of pathogen adhesion

Adhesion has been one of the main beneficial effects of probiotics (Castagliuolo *et al*., 2005). Lactic acid bacteria (LAB) display various surface determinants that are involved in their interaction with intestinal epithelial cells (IECs) and mucus. IECs secrete mucin, which is a complex glycoprotein mixture that is the principal component of mucous, thereby preventing the adhesion of pathogenic bacteria (Collado *et al*., 2005; González *et al*., 2012). Additionally, lipids, free proteins, immunoglobulins and salts are present in mucous gel (Neutra *et al*., 2007). This specific interaction has indicated a possible association between the surface proteins of probiotic bacteria and the competitive exclusion of pathogens from the mucus (Haller *et al*., 2001; Ouwehand *et al*., 2002; Tassell *et al*., 2011). Probiotic *Lactobacillu s*sp. have proteins to promote mucous adhesion (Tassel *et al*., 2011), and display surface adhesin proteins, saccharide moieties and lipoteichoic acids that mediate attachment to the mucous layer (Buck *et al*., 2005; Velez *et al*., 2007). The usual mucus-targeting bacterial adhesin is MUB (mucus-binding protein) produced by *Lactobacillus reuteri* (Buck *et al*., 2005; Velez *et al*., 2007). This protein has mainly secreted and surface-associated proteins, either anchored to the membrane lipid moiety or embedded in the cell wall and having role in the mucous adhesion of *lactobacilli* (Goh *et al*., 2010; Ossowski *et al*., 2010; Sanchez *et al*., 2011; Ossowski *et al*., 2011). This protein has also facilitating the colonization of human gut through degradation of the extracellular matrix of cells or by facilitating close contact with the epithelium (Candela *et al*., 2007; Candela *et al*., 2009). Mucous adhesion-promoting protein (MapA) has been mediating the binding of probiotics, such as *L. reuteri* and *L. fermentum* to epithelium mucus (Ouwehand *et al*., 2002). Probiotic *L. plantarum*, have been induce MUC2 and MUC3 mucins to inhibit the adherence of enteropathogens.These enhanced mucous layers and glycocalyx overlying the intestinal

epithelium as well as the occupation of microbial binding sites by probiotics provide protection against invasion by pathogens (Hirano *et al*., 2003; Voltan *et al*., 2007; Kim *et al*., 2010). Probiotic strains can also induce the release of defensins (small peptides/proteins) from epithelial cells; these are active against bacteria, fungi and viruses. And stabilize the gut barrier function (Furrie *et al*., 2005).

2.5.1.3. Competitive exclusion of pathogenic Microorganisms

One species of bacteria dynamically competes for receptor sites in the intestinal tract than another species. The mechanisms used by one species of bacteria to exclude or reduce the growth of another species are varied, including the mechanisms of creation of unsuitable microecology, elimination of available bacterial receptor sites, production and secretion of antimicrobial substances and selective metabolites, and competitive depletion of essential nutrients (Rolfe *et al*., 2001). Specific adhesiveness properties due to the interaction between surface proteins and mucins may inhibit the colonization of pathogenic bacteria and results antagonistic activity by some strains of probiotics against adhesion of enteropathogens (Servin, 2004).

Competitive exclusion by intestinal bacteria is based on a bacterium-to-bacterium interaction mediated by competition for available nutrients and for mucosal adhesion sites. To gain a competitive advantage, bacteria can modify their environment to make it less suitable for their competitors. The production of antimicrobial substances, such as lactic and acetic acid, is one example of this type of environmental modification (Schiffrin *et al*., 2002). Some *lactobacilli* and *bifidobacteria* share carbohydrate-binding specific sites with some enteropathogens (Nesser *et al*., 2000; Fujiwara *et al*., 2001), which makes it possible for the strains to compete with specific pathogens for the receptor sites on host cells (Mukai *et al*., 2002). In general, probiotic strains are able to inhibit the attachment of pathogenic bacteria by means of steric hindrance at enterocyte pathogen receptors (Coconnier *et al*., 2003).

2.5.1.4. Production of antimicrobial substances

One of the mechanisms of the health benefits afforded by probiotics includes the formation of low molecular weight (LMW) compounds (<1,000 Da), such as organic acids, and the production of antibacterial substances termed bacteriocins (>1,000 Da). Organic acids, in particular acetic acid and lactic acid, have a strong inhibitory effect against Gram-negative bacteria, and they have been considered the main antimicrobial compounds responsible for the

inhibitory activity of probiotics against pathogens (Alakomi *et al*., 2000; Keersmaecker *et al*., 2006; Makras *et al*., 2006). The organic acid in un-dissociated form enters the bacterial cell and dissociates inside its cytoplasm and lowering the intracellular pH or the intracellular accumulation of ionized form of organic acid can lead to the death of the enteropathogens (Ouwehand, 1998; Russell *et al.,* 2008).

2.5.2. Immunological mechanisms

Probiotic bacteria can exert an immune-modulatory effects, they have the ability to interact with epithelial cells, dendritic cells (DCs) and with monocytes/macrophages and lymphocytes (Miriam *et al*., 2012). Immune system can be divided in to innate and adaptive systems. The adaptive immune response depends on β and T lymphocytes, which are specific for particular antigens. In contrast, the innate immune system responds to common structures called pathogen-associated molecular patterns (PAMPs) which shared by majority of pathogens (Gomez *et al*., 2010).

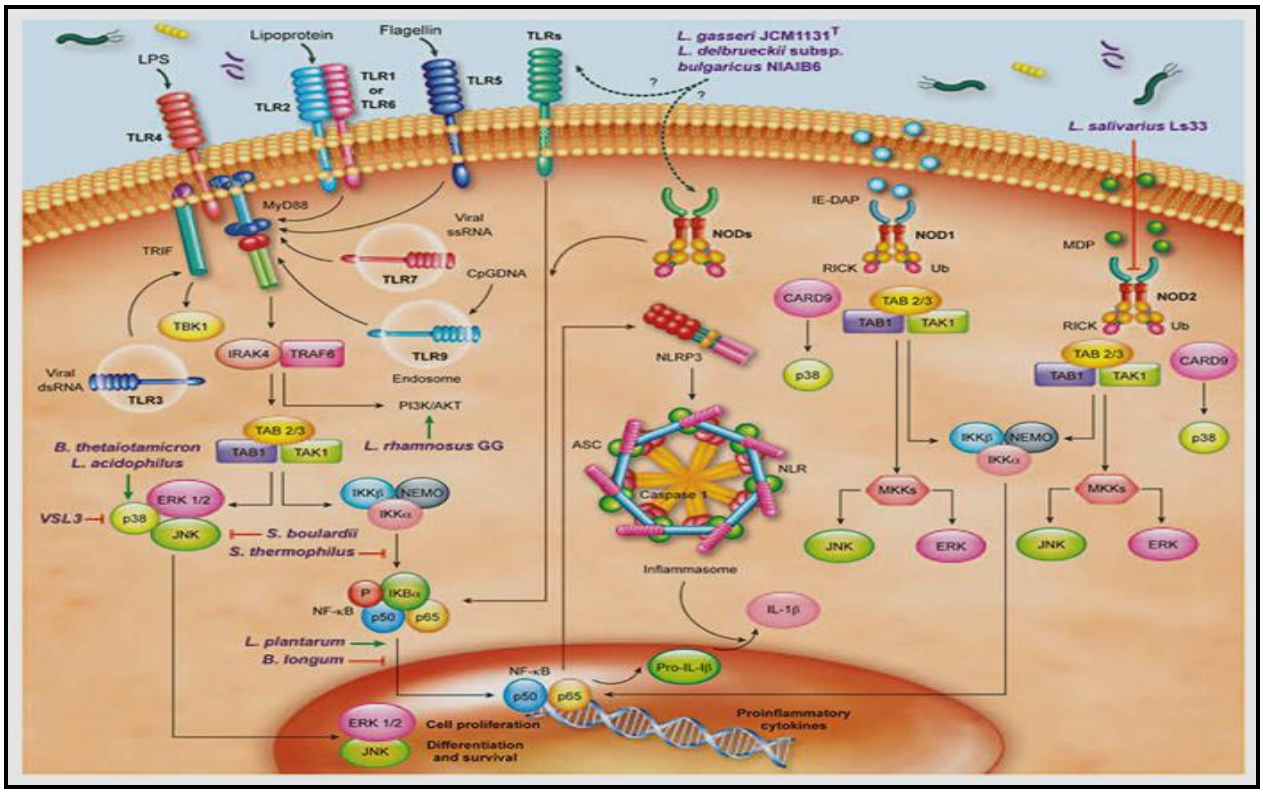

Figure 2: How probiotics may interact and modulate the immune system (He *et al.*, 2010)

The primary response to pathogens is triggered by pattern recognition receptors (PPRs), which bind PAMPs and transmit signals upon interaction with bacteria (Lebeeret *et al.,* 2010). Host cells most extensively interact with probiotics are Intestinal epithelial cells (IECs) and Probiotics can also encounter DCs, which have an important role in innate and adaptive immunity. Both IECs and DCs can interact with and respond to gut microorganisms through their PPRs (Gomez *et al*., 2010; Lebeeret *et al*., 2010).

Tall like receptors (TLRs) are trans-membrane proteins expressed on various immune cells, such as B cells, natural killer cells, DC, macrophages, fibroblasts and epithelial cells (Miriam *et al* ,2012). It is outer membrane protein associated on the surface of endosomes and primarily responds to bacterial surface associated PAMPs. Where they respond primarily to nucleic acid based PAMPs from viruses and bacteria (Gomez *et al*., 2010). Probiotics can suppress intestinal inflammation via the down regulation of TLR expression, secretion of metabolites that may inhibit TNF-1 from entering blood mononuclear cells and inhibition of NF-B signaling in enterocytes (Gomez *et al*., 2010).

Probiotics could modify the immunologic response of the host by interacting with epithelial cells and modulating the secretion of anti-inflammatory cytokines, which would result in a reduction of gastric activity and inflammation (Gill, 2003). Animal studies showed that; probiotic lactic acid bacteria regulate immune system particularly through controlling the balance of pro-inflammatory and anti-inflammatory cytokines, which results in a reduction of gastric activity and inflammation *(*Murosaki *et al.,* 2000).

2.6. Bacterial pathogens

Over 1.7 billion global cases of diarrheal disease are reported annually and are associated with an estimated 2.2 million deaths (WHO, 2012).The burden of diarrheal disease is most critical in developing countries, facilitated by unsafe water supplies, poor sanitation, and nutritional deficiencies. Diarrheal disease in children aged less than 5 years in these countries is devastating, where repeated diarrheal episodes contribute to malnutrition, which in turn puts these children at heightened risk of acquiring infectious diarrhea and is associated with stunting and impaired cognitive development (Fischer*et al*., 2012; Checkley *et al*., 2008).While less common in high-income countries, diarrheal diseases remain a significant health concern. There are an estimated

211 to 375 million episodes of diarrheal illnesses each year in the United States, with 1.8 million hospitalizations and 3, 100 deaths (Guerrant *et al.,* 2001). Many of these cases are foodborne. The Foodborne Diseases Active Surveillance Network (Food Net) at the Centers for Disease Control and Prevention (CDC) reported1,000 foodborne outbreaks that resulted in 48 million illnesses,128,000 hospitalizations, and 3,000 deaths from 10 sites in the United States (CDC, 2011). It is important to note that many cases of foodborne diarrheal illness are not part of a recognized outbreak and thus are not captured by the Food Net data (Scallan *et al*, 2011).

Diarrhea may be infectious, i.e., caused by bacteria, viruses, or parasites. In these instances, laboratory tests for infectious etiologies, including a bacterial stool culture, are useful for diagnosis by either ruling out or ruling in acommon infectious process (Guerrant*et al*., 2001). The primary mechanisms for bacterial gastroenteritis are (i) excessive secretion of fluids in the proximal small intestine induced by the action of luminal toxins expressed by enteropathogens or by minimally invasive bacteria, (ii) inflammatory or cytotoxic damage of the ileal or colonic mucosa which may produce blood and pus, or (iii) penetration of the bacterium through the mucosa to the reticuloendothelial system, as is the case with typhoid fever. Regardless of mechanism, most cases of bacterial gastroenteritis are self-limiting and with a few exceptions, neither empirical antimicrobial therapy nor bacterial stool culture is indicated (Guerrant*et al*., 2001). For most patients who present withacute diarrhea, symptoms have resolved by the time bacterial culture results are available, and these generally do not change patient management (Chan *et al*., 2003).

In this review, three enteric pathogens were discussed: *Salmonella, Shigella,* and *E.coli*. These important members of *Enterobacteriaceae* are responsible for significant morbidity and mortality, causing diarrhea and a spectrum of associated symptoms from mild to severe in most parts of the world. In the review infection and epidemiology, taxonomic classification, detection methods, susceptibility testing, treatment, prevention and control methods were covered. Discussions that pertain to individual organisms are organized into individual sections starting with *Salmonella,* followed by *Shigella*, then *E.coli*.

2.6.1. *Salmonella*

Members of the genus *Salmonella* cause a well characterized spectrum of disease in humans, ranging from asymptomatic carriage to fatal typhoidal fever. In the developed world, food-borne

acute gastroenteritis and enterocolitis are the most common forms of *Salmonella* infection, with an estimated 1.2 million annual cases of non-typhoidal Salmonellosis occurring in the U.S. (Patrick*et al*., 2004; Braden, 2006; Hanning *et al*., 2009). Though relatively uncommon in the U.S., typhoid, paratyphoid, and enteric fever constitute a very serious global public health problem, with 25 million new infections and >200,000 deaths occurring annually (Holt*et al*., 2009; Dougan and Baker, 2014).

Salmonella is a member of the family *Enterobacteriaceae*, facultatively anaerobic Gram-negative rod. Originally it is characterized by their ability to metabolize citrate as a sole carbon source and lysine as a nitrogen source, as well as their ability to produce hydrogen sulfide (Nataro *et al*., 2011).

2.6.1.1. Disease and Symptoms

Salmonella colonizes the intestinal tracts of vertebrates. Some serotypes, including *Salmonella enterica* sub sp.*enterica* serotype *Typhi (Salmonella Typhi*), are only found in human hosts. The majority of *Salmonella* cases occur as the result of ingesting contaminated food or water. *Salmonella* can also be acquired by contact with domestic animals and their food products, farm animals or animals in petting zoo, and exotic pets like turtles, hedgehogs, and iguanas (Behravesh*et al.,* 2010; Pickering *et al*., 2008; Sanyal*et al*., 1997). *Salmonella* can also be transmitted from person to person via the oral-fecal route.

The incidence of *Salmonella* infections in the United States in 2011 was 1,645 per 100,000 populations (CDC, 2012), with higher rates in late summer and early fall. Worldwide, there are an estimated 94 million cases of non typhoidal *Salmonella* gastroenteritis and about 155,000 deaths (Majowicz*et al*., 2010). In developing countries and the Indian sub continent in particular, typhoidal isolates cause the majority of disease and are associated with an estimated 21.6 million annual cases and 216,500 deaths (Crump*et al*., 2004). In sub-Saharan Africa, non-typhoidal *Salmonella*, predominantly the *Salmonella Typhimurium* ST313 strain, are a significant cause of blood stream infections in both children and adults (Feasey *et al*., 2012; Morpeth*et al*., 2009).

Non-typhoidal salmonellosis consists of diarrhea, nausea, headache, and abdominal cramps, which last for 4 to 7 days. Fever may be present and usually resolves in 24 to 48h. The disease is typically limited to the lamina propria of the small intestine, and antimicrobial therapy is not

indicated. Extra intestinal manifestations, such as bacteremia, septic arthritis, urinary tract infections, and osteomyelitis, are seen in 5% of cases (Black*et al.*, 1960; Cherubin*et al.*, 1969; Shimoni*et al*., 1999).

Typhoid fever is caused by *Salmonella Typhi*, and a similar syndrome is caused by *Salmonella Paratyphi* A, *Salmonella Paratyphi* C, and tartrate-negative variants of *Salmonella Paratyphi* B. In typhoid, the organism disseminates from the lamina propria to the reticulo-endothelial system in infected phagocytes via lymphatic and hematogenous routes. Fever, malaise, anorexia, headaches and vomiting are common symptoms of typhoid and typically start 1 to 3 weeks after infection. Patients may have diarrhea following ingestion of the organism, but many do not. Rose spots, which are blanching maculopapular lesions 2 to 4 mm in diameter, are seen in 5 to 30% of cases. A complication of untreated typhoid fever is the erosion of the blood vessels in the Peyer's patches, which can lead to intestinal hemorrhage (Crump *et al*., 2004). The organism persists in the mesenteric lymph nodes, gallbladder, and bone marrow for years. Five to 10 percent of patients will have are lapse of infection, typically 2 to 3 weeks following resolution of symptoms (Parry *et al*., 2002). Up to 10% of asymptomatic patients will become carriers, and 1 to 4% of these will shed for more than 1 year (Parry *et al*., 2002).

2.6.1.2. Classification

Salmonella, a member of the family *Enterobacteriaceae*, is a facultatively anaerobic Gram-negative rod. *Salmonella* taxonomy is a complicated matter, with two species in the genus: *Salmonella Enterica* and *Salmonella bongori*. *Salmonella enterica* has six subspecies (*S. enterica sub sp.enterica, S. enterica sub sp. salamae, S. enterica sub sp. arizonae, S. enterica* sub *sp.diarizonae, S. enterica sub sp.indica, and S. enterica sub sp.houtenae*) that can be further serotyped using the Kauffmann-White-Le Minor scheme, based on the properties of their somatic (O), flagellar (H), and capsular polysaccharide (Vi) antigens (Popoff *et al*., 2000; Popoff *et al*., 1997). There are over 2,500 serotypes of *S. enterica* (Popoff *et al*., 2000; Popoff *et al*., 1997). Because of the diversity of the genus, several isolates may be difficult to identify due to atypical biochemical reactions.

2.6.1.3. Pathogenesis

The severity of *Salmonella* disease depends on the inoculating dose (Blaser and Newman, 1982), infecting serotype (Shimoni*et al*., 1999), and predisposing host factors. Children below the age

of 5 year, elderly people and patients with immune suppression are more susceptible to *salmonella* infection than healthy individuals. Almost all strains of *salmonella* are pathogenic as they have the ability to invade, replicate and survive in human host cell, resulting in potentially fatal disease (Eng *et al*., 2015). *Salmonella* displays a remarkable characteristic during its invasion of non-phagocytic host cells (Hansen-Wester *et al*., 2002) whereby it actually induces its own phagocytosis in order to gain access to the host cell. The remarkable genetics underlying this ingenious strategy is found in salmonella pathogenicity islands (SPIs), gene clusters located at the large chromosomal DNA region and encoding for the structure involved in the invasion process (Grassl and Finlay,2008).

When bacteria enter the digestive tract via contaminated water of food, they tend to penetrate the epithelial cells lining the intestinal wall. SPIs encode for type III secretion systems, multi-channel proteins that allow salmonella to inject its effectors across the intestinal epithelial cell membrane into the cytoplasm. The bacterial effectors then activate the signal transduction pathway and trigger reconstruction of the actin cytoskeleton of the host cell, resulting in the outward extension or ruffle of the epithelial cell membrane to engulf the bacteria. The morphology of the membrane ruffle resembles the process of phagocytosis (Takaya *et al.*, 2003). The ability of salmonella strains to persist in the host cell is crucial for pathogenesis, as strains lacking this ability are non-virulent (Bakowski *et al*., 2008).

Following the engulfment of salmonella in to host cell, the bacterium is encased in a membrane compartment called a vacuole, which is composed of the host cell membrane. Under normal circumstances, the presence of the bacteria foreign body would activate the host cell immune response resulting the fusion of the lysosomes and the secretion of digesting enzymes to degrade the intracellular bacteria. However, *salmonella* uses the type III secretion system to inject other effector proteins in to the vacuole, causing the alteration of the compartment structure. The re-modelled vacuole blocks the fusion of the lysosomes and this permits the intracellular survival and replication of bacterial within the host cells. The capability of the bacteria to survive within macrophages allows them to be carried in the reticulo endothelial system (Monack *et al*., 2004).

2.6.1.4. Diagnosis

2.6.1.4.1. Culture Method

Conventional bacterial identification methods usually include a morphological evaluation of the micro-organism as well as tests for the organism's ability to grow in a various media under a variety of conditions. These methods are very sensitive, inexpensive and can give both qualitative and quantitative information on the number and the nature of microorganisms present in the food sample (Gracias and Mckillip, 2004). Although standard microbiological techniques allow the detection of single bacteria, amplification of the signal is required though the growth of a single cell into a colony. Isolation of salmonella by culture based methods requires the prolonged enrichment steps and is still the most widely used detection techniques and remains the gold standard for the detection of salmonella due to their selectivity and sensitivity (Lee *et al*.2015).

Culture methods of *Salmonella* typically involve the enrichment of a portion of the sample to recover sub-lethally injured bacterial cells due to heat, cold, acid or osmotic shock (Gracias and Mckillip, 2004) in a non-selective pre-enrichment media, such as Buffered peptone water (BPW), and to increase the number of target cells as these are generally not uniformly distributed in food, typically occur in low numbers and may be present in a mixed microbial population. Next, primary enrichment broth, such as Cysteine broth (SC), Rappaport Vasiliadis Soy broth (RVS), Tetrathionate Broth (TT), or Muller Kauffmann Tetrathionate-Novobiocin broth (MKTTn) and incubated at elevated temperature (37^0C or 42^0C for18-24 hours) before being struck on to selective agars such as Xylose Lysine Deoxycholate agar (XLD), Bismuth Iron Sulphate or sulfapyridine (BGS), modified semisolid Rappaport Vasiliadis (MSRV), Salmonella Shigella Agar, or Hektoen Enteric agar. There are several published standard methods utilizing combination of media such as the current ISO horizontal method, ISO 6579:2002 for the detection of *Salmonella*.

The conventional microbiological methods serve as the basis for analysis in many food safety and public health laboratories due to the ease of use, reliability of result, high sensitivity and specificity and lower cost compered to emerging molecular-based technologies (Gracias and McKillip, 2004; Ricke *et al*., 2006). However, these procedures need to prepare multiple subcultures required for several identification steps, taking more than 5 days for completed

isolation and confirmation. In addition, false positive results may occur due to competitive flora (e.g proteus) (Naravaneni and Jamil, 2005)

2.6.1.4.2. Immunology-based assays

Enzyme-linked immunosorbent assay (ELISA): The different ELISA systems have been developed and commercially available in kit form. In the ELISA assay, an antigen specific to *salmonella ssp*. is bound to the appropriate antibody linked to a solid matrix. After forming the antigen antibody complex, the concentration of the antigen and the presence of *Salmonella* can be measured though the change in color caused by the enzymatic cleavage of a chromogenic substrate (Tietjen and Fung, 1995; Blivet *et al*., 1998). Alternatively, the presence of antibodies in samples infected with *salmonella ssp*. can be detected using antigen coupled to solid phase of ELISA (Wiuff *et al*.; 2000). ELISA has also been used to detect antibodies for development of vaccines against *salmonella* infections (Meenakshi *et al*.; 1999).

Latex agglutination test: The agglutination technique employs latex particles coated with antibodies which react with antigens on the surface of *salmonella* cells to form visible aggregates for identification of salmonella positive samples (Tietjen and Fung.1995). The assays are specific, uncomplicated and reliable so that generally, they have been used as a confirmatory analysis technique, rather screening test for salmonella organisms (Love and Sobsey, 2007; Eijkelkamp *et al*., 2008).

2.6.1.4.3. Nucleic acid- based assays

The nucleic acid-based assays are *Salmonella* detection tests that utilize a specific nucleic acid target sequence within the organism. The assays have been most intensively explored and developed for the past decade among *salmonella* detection methods because they offer some advantages of sensitivity, specificity and inclusivity over other methods, rapidly identifying *salmonella* without obtaining pure cultures. Two major techniques of assays are direct hybridization (DNA probe) and amplification (PCR) methods. The great progress of the assays allows the detection of very low numbers of organisms in the sample and high throughput of a large number of samples for routine analysis (Mozola, 2007).

Polymerase chain reaction (PCR): Conventional methods for detection of salmonella serovars in foods are generally time consuming and labor intensive. A real time PCR method has been

developed with custom designed primers and a TaqMan probe to detect the presence of a 262-bp fragment of the Salmonella- specific invA gene (Chorng-ming et al., 2008). Several salmonella specific target gene such as oriC, fimA, HimA, himlA and stn have been identified (Chen *et al*., 2000; Sanchez *et al*., 2004; Moore *et al.*, 2007).

Although PCR is a powerful technology, the reactions can be dramatically affected by the presence of inhibitory compounds in food and selective microbiological media like bile salts and aciflavin. A problem to routine use of PCR in food testing lab is that the procedures are rather complicate and very clean environment is needed to perform the test. Further, PCR cannot distinguish between live and dead cells and hence providing more false negative results (Biswas *et al* 2008).

2.6.1.5. Treatment

Treatment of non-typhoidal salmonella infection is different from typhoidal infection. In treatment of non-typhoidal Salmonella infection antibiotics should not be used routinely, as used in typhoid. Antibiotic should be only used if required as most infection with non-typhoidal. *Salmonella* is self-limiting type and duration of diarrhea and fever are not much affected by use of antibiotics. Additionally antibiotic therapy can increase relapse of infection and also prolong the duration of gastrointestinal carrier states. The main treatment should be aimed at correcting dehydration that may arise due to prolonged diarrhea by fluid and electrolyte replacement (Jemal, 2004). In case of patient with bacteremia and other complication antimicrobial drugs with chloramphenicol, ampicillin, trimethoprim sulfamethoxazole and newer fluroquinoazoles being drug of choice sensitive salmonella. Proper management of fluid and electrolyte balance is important in all patients with salmonella gastroenteritis but is crucial in young children and elder individuals (Mohlet *et al*., 2009). Early treatment is essential for septicemic salmonellosis but there is controversy regarding the use of antimicrobial agent for intestinal salmonellosis. Oral antibiotics may alter the intestinal micro flora and interfere with competitive antagonism and prolong shading of the organism. There is also concern that antibiotic resistance strain of salmonella selected by oral antibiotic my subsequently infect human. Antibiotic such as ampicillin or cephalosporin lead to lyses of bacteria with release of endotoxin. NSAID may be used to reduce the effect of endotoxemia (Davison, 2005).

2.6.1.6. Prevention and control

Prevention is mainly depending on the proper cooking of foods of animal origin in order to limit the entrance of zoonotic salmonellosis in human food chain. Report form Malawi has highlighted the potential importance of antibody in both serum killing and intracellular oxidative killing of iNTS in African children (MacLennan *et al*., 2008; Gondwe *et al*., 2010). The virulence of NTS is dependent on its ability to grow within macrophages of the reticuloendothelial system (Fields *et al*., 1986). Extracellular replication and bacteremic dissemination also occur. Resistance to complement killing, by way of long- chain lipopolysaccharide, is an important virulence trait. Both complement and specific antibodies together are required to kill salmonella species in vitro. Although serum samples from healthy African adults were able to kill NTS, serum samples from children aged less than 16 months often did not contain specific antibody titers sufficient to kill effectively (MacLennan *et al*., 2008)

2.6.2. *Shigella*

Shigella is Gram-negative intracellular bacterial pathogens that inhabit the gastrointestinal tract of humans and are the causative agent of shigellosis. Shigellosis is a current health burden and estimated to affect 80-165 millions of individuals annually. Ninety-nine percent of infections caused by *shigella* occur in developing countries and the majority of the cases, and cases of deaths, occur among children less than 5 years of age (Kotloff *et al.*, 1999; WHO, 2005).

2.6.2.1. Disease and Symptoms

Shigella species are host adapted to humans but have been documented in rare instances from dogs and primates (Janda and Abbott, 2006). They can be acquired from ingestion of a variety of foods or water contaminated with human feces, sexually during oral-anal sex, or by laboratory workers. Transmission by person-to-person contact is common for *Shigella* spp. because of a low infectious dose of 10 to 100 organisms (Janda and Abbott, 2006). Between 2009 and 2010, *Shigella* accounted for 508/8,523 (2%) of reported illnesses associated with foodborne outbreaks (CDC, 2013). The worldwide incidence of shigellosis has been reported to be approximately 165 million cases, but the mortality has decreased substantially over the past three decades (Kotloff*et al*., 1999; Bardhan *et al.*, 2010; Van de Verg and Venkatesan, 2014).

Shigellosis and dysentery are diseases associated primarily with poor hygiene and lack of access to medical care. Approximately 150 million cases are reported annually in developing countries,

in contrast to 1.5 million cases in industrialized nations. Of importance, one multicenter study found that half of patients with culture-negative, bloody stools were positive by PCR for *Shigella*, suggesting that the actual incidence of *Shigella* is grossly underestimated (von Seidlein*et al*., 2006). Shigellosis symptoms range from watery diarrhea to mucoid and/or bloody stools, which can be accompanied by fever, malaise, and abdominal pain. In one study of 1,114 culture confirmed patients followed for 14 days or longer, 29% (241) reported diarrhea persisting for14 days (von Seidlein*et al*., 2006). Factors associated with persistence were age, fever, mucoid diarrhea, vomiting, and abdominal pain. Meningitis, pneumonia, and urinary tract infections (UTIs) are rare complications of shigellosis and are most commonly seen with *S. Flexneri* and *S. sonnei* (Bennish*et al.*, 1991; Margolin*et al*., 2003; Papasian*et al*., 1995).

HUS is the most serious complication of shigellosis. HUS occurs in 13% of cases of *S. dysenteriae* type 1 shigellosis and is attributable to the expression of Stx1 by this organism (Butler, 2012). However, in rare cases, non- *S. dysenteriae* species of *Shigella* have been isolated from children with HUS (Butler, 2012; Khan *et al*., 2013).S.dysenteriae type 1 HUS is seen mainly in children5 years old in Asia and Africa.

2.6.2.2. Classification

Shigella belongs to the family of *Enterobacteriaceae*. They share common characteristics and genetic relatedness with members of the genus *Escherichia* (in particular to enteroinvasive *E.coli* [EIEC] which is also responsible for shigellosis in humans), and are now moving towards being classified as a subtype of *E.coli* (Lan and Reeves, 2002; Parsot, 2005). Analyses on the evolution of shigella (as well as EIEC) have suggested that shigella originated from non-invasive *E.coli* but unlike most commensal and other pathogenic *E.coli* strains, have acquired the ability to invade cells through the gain of a ~220 kb virulence plasmid and other virulence genes (pathogenicity islands) as enhancers, and loss of virulence – suppressor genes (such as cadA) and genes which are no longer required for living in the intracellular niche (e.g. lactose utilization and motility). The four species/groups of Shigella are Shigella dysenteriae, Shigella flexneri, Shigella boydii, and Shigella sonnei (Lan & Reeves 2002).

2.6.2.3. Pathogenesis

Shigella pathogenesis involves translocation through ileal and colonic M cells, uptake by macrophages, basolateral invasion of epithelial cells and dissemination within the mucosa

(Phalipon and Sansonetti, 2007; Schroeder and Hilbi, 2008). Shigella has a large virulence plasmid, encoding a Type III-secretion system and a set of secreted proteins. The system results in the injection of virulence factors into host colonic epithelial cells, leading to damage to the epithelial lining, as well as proteins that antagonize the adaptive and innate immune response (Phalipon and Sansonetti, 2007; Ogawa et al.,2008). Shigella virulence is enhanced by the presence of enterotoxins, which is separate from the Shiga toxin produced in limited strains(Barry et al., 2013).The genes encoding the Shiga toxin genes are located in the genome of heterogeneous lambdoid prophages. These are highly mobile genetic elements that play a role in horizontal gene transfer and genome diversification.102Shiga toxin is made by the Shigella dysenteriae type I strains and E. coliO157:H7 among other E. colishiga toxin producing strains. Shiga-like toxin II, also seen in some E. colistrains is 56% homologous with Shiga toxin (or Shiga-like toxin I) (Donohue-Rolfe et al., 1991).

2.6.2.4. Diagnosis

Clinically *Shigella* infection was identified by the presence of bloody and mucoid stool, but the differential diagnosis should include infection caused by enteroinvasive *Escherichia coli* (EIEC), *Salmonella enteritidis*, *Campylobacter species* and *Entamoeba* histolytica (Goodman & Segreti, 1999; Sur *et al*., 2004). Usually, fresh and bright red blood is present in the stools of patients infected with *Shigella* (Niyogi, 2005). But in the laboratory diagnosis, stool culturing is the ideal method to detect the Shigella infection followed by biochemical and serological tests for further confirmation. Shigella was isolated from the fresh stool samples by culturing them in the MAC, DCA, XLD, HEK agar by incubating at 37^0C for 18-24 hrs. Suspected colonies are subjected to biochemical screening medium such as MR test medium, Motility medium, Triple sugar iron agar, Kligler iron agar, Citrate agar and Urea agar. Shigella produces an alkaline slant and an acid but due to the inability to ferment lactose aerobically in the slope and the anaerobic fermentation of glucose in the butt and fail to produce H_2S gas. Shigella is negative for motility, citrate and urea test, and positive reaction is observed in the MR test. Serological identification was done by slide agglutination test. Agglutination test is done using the polyvalent or monovalent O antigen grouping sera. Since Shigella contains distinct type of O antigen, Shigella polyvalent antiserum will agglutinate strains of the same sero group and monovalent antiserum will agglutinate the specific serotype or sub-serotype.

Other diagnosis techniques include DNA based PCR techniques and immunological assays for rapid detection of infection. A multiplex PCR assays were developed by Aranda and collegues using IpaH as probe. These PCRs were reported as specific and sensitive for rapid detection of target isolates in stools (Aranda *et al*., 2004; Na-Ubol *et al*., 2006; Gomez-Duarte *et al.*, 2010). Studies also used DNA hybridization techniques, restriction endonuclease analysis for detecting Shigella (Boileau *et al*., 1984; Venkatesan *et al*., 1988; Litwin *et al*., 1991). Molecular epidemiology of multidrug resistant *S. sonnei* outbreak in a day care center has been studied using pulse field gel electrophoresis (PFGE) and plasmid DNA analysis (Brian *et al*., 1993). Immunoassays were employed for most rapid detection of the Shigella infection. Several immunoassays include latex agglutination, immune diffusion and enzyme immunoassays (EIA) Mostly different methods of EIA were used. Monoclonal antibody-based ELISA has been developed using a 43 kDa invasion plasmid-coded protein antigen (IpaC) to identify EIEC and Shigella strains in fecal samples from children in Kuwait (Pal *et al*., 1997).

2.6.2.5. Treatment

Oral rehydration therapy with a solution containing salts is sufficient and only severe cases need intravenous therapy. Continued feeding is rather encouraged preventing hypoglycaemia and weight loss because the disease does not affect the small intestine much where most of the absorption of nutrients takes place. Shigellosis can usually be treated with antibiotics which shortens the duration of diarrhoea, fever, and period of communicability. The antibiotics commonly used for such treatment are: ampicillin, trimethoprim/sulfamethoxazole (TMP-SMX, also known as Bactrim or Septra), nalidixic acid, or ciprofloxacin. The first choice of antimicrobial agent is ciprofloxacin and azithromycin (Khan *et al*., 1997), alternatively TMP/SMX and ampicillin (resistance is common in Middle East, Latin America) (Gilbert *et al*., 2001). Existence of multidrug resistant Shigella strains made the disease more badly. Antibiotic resistance emerging among the *S. dysenteriae* 1 strains, which cause the most severe clinical features, mainly in Africa, South-Eastern Asia and South America is a major problem (Jamal *et al* 1998; Materu *et al.*,1997; Taylor *et al*., 1989; Hoge *et al*.,1998). A study reports that strains of Shigella isolated during periods of infectious diarrhea in Abidjan from 2004 to 2005 showed resistance to ampicillin and tetracyclin (90%), sulphamide (85%) and to a lesser extent to cotrimoxazole (65%)(Antoine *et al*., 2010). Also a multidrug resistant *S.dysenteriae* type I strain has been reported to cause sporadic outbreak in and around

Kolkata in India (Dutta *et al*., 2003; Pazhani *et al*., 2004; Pazhani *et al*., 2008). Studies from six Asian countries shows that *S. flexneri* isolates were resistant to amoxicillin and cotrimoxazole. Ciprofloxacin-resistant *S. flexneri* isolates were identified in China (6%), Pakistan (3%) and Vietnam (2%) (Seidlein *et al*., 2006). Emergence of ciprofloxacin resistance Shigella strains has been reported in India (Taneja, 2007). Therefore using antibiotics can actually make the bacteria more resistant

2.6.2.6 Prevention and control

The only way to prevent the spread of shigellosis is the sanitary handling of food. Special measures are required, like cooking food thoroughly rather than eating raw food, protecting food from flies, avoiding preparing food when ill with diarrhea or vomiting (Shears, 1996).

2.6.3. *Escherichia coli*

Escherichia coli is characterized as a gram-negative, rod-shaped bacterium belonging to the family *Enterobacteriaceae* with five virulence groups, including; enteroaggregative *Escherichiacoli* (EAEC), enterohemorrhagic *Escherichia coli* (EHEC) or *E. coli* (STEC), enteroinvasive *Escherichiacoli*(EIEC), enteropathogenic *Escherichia coli*(EPEC), and enterotoxigenic (ETEC). *Escherichia coli* O157: H7, one of the virulent strain under the species *Escherichia coli* is a leading cause of hemorrhagic colitis, hemolytic-uremic syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP) in man (Cobbaut *et al*., 2009; Earley and Leonard, 2006). This strain is considered as one of the severe foodborne disease-causing organism globally. The risk and consequences of the organism are severe in developing countries due to different reasons that are attributable to poor livelihood in general. The food types most commonly associated with outbreaks of food poisoning due to *Escherichia coli* O157 are mostly of bovine origin, in particular, beef and beef burgers and unpasteurized milk (Hussein *et al*., 2001; Pennington, 2010; Seoderlund,2015). However, it has been increasingly recognized that fresh vegetables and fruits other than beef or beef-product can be the sources of Shiga toxin-producing *Escherichia coli* (STEC) infection (Cheol *et al*., 2013; Gomes *et al*., 2016; Karmali *et al*., 2010).

2.6.3.1. Disease and Symptoms

The incidence of STEC infections in the United States is monitored by FoodNet. In 2012, the incidence of O: 157 STEC was 1.12 per 100,000 population, and the incidence of non-O157

STEC was 1.16 per 100,000 (CDC, 2013). Among the non-O:157 STEC strains, O26, O103,O111, O121, O45, and O145 are the most common serotypes isolated in the United States (Gould*et al*.,2013). The incidence of STEC in other developed countries varies; it is as low as 0.4 per 100,000 in Australia (Vally*et al*., 2012) and as high as 5.33 per 100,000 in Ireland. Theincidence of STEC is much higher in developing countries such as Argentina and India, but formal surveillance data are not available for these countries.

STEC disease presents as enteritis that may quickly progress to hemorrhagic colitis (Su and Brandt, 1995).The chief symptoms included bloody diarrhea, abdominal pain, nausea, and vomiting (Rohde*et al*., 2011).Importantly, not all STEC infections are associated with bloody diarrhea (Manning *et al*., 2007; van Duynhoven *et al*., 2008) and so laboratory algorithms that only test bloody specimensfor STEC are no longer considered standard of care. The most common and serious complication of STEC infection is the development of HUS, which typically presents 5 to 13 days after the onset of diarrhea (DuPont HL,2009.). HUS is life-threatening and consists of the triad of renal failure, microangiopathic hemolytic anemia, and thrombocytopenia. The mortality rate connected with HUS is 3% to 5% (Walker*et al*., 2012).It has been estimated that 61% of all HUS cases are related to STEC infection (Walker *et al*., 2012). HUS has been observed more frequently in O: 157 (11% of cases) versus non-O157 (1% of cases) STEC infections (Gould*et al*., 2013). Approximately 15% of children 10years of age develop HUS following STEC infection. However, in the recent outbreak of O104 STEC in Germany, 22% of children developed HUS (Tarr*et al*., 2005 2005; Frank *et al*., 2011; Wonget *al*., 2012). It should be noted that this outbreak was caused by an atypical STEC strain that harbored enteroaggregativ e *E.Coli* virulence factors in addition to the Shiga toxins. HUS occurs much less frequently among adults and is associated predominantly with advanced age (75 years) (Zoufaly*et al*., 2013).Increasedrates of HUS have been more frequently associated with Stx2-expressing STEC strains. Exposure to antibiotics also increases the risk of HUS in children (Wong*et al*., 2012).

2.6.3.2. Classification

The six major diarrheagenic pathotypes described to date are enteropathogenic *E. coli*, Shiga toxin-producing *E. coli (*STEC), enteroinvasive *E. coli* (EIEC), enterotoxigenic *E. coli*, enteroaggregative *E. coli*, and adherent invasive *E. coli* (Croxen *et al*.,2013). Of these, only

STEC is routinely identified by most clinical and public health laboratories, and it will be the focus of the discussion here.

2.6.3.3. Pathogenesis

Pathogenic *E.coli* strains use a multi-step scheme of pathogenesis that is similar to that used by other mucosal pathogens, which consists of colonization of a mucosal site, evasion of host defences, multiplication and host damage. Most of the pathogenic *E. coli* strains remain extracellular, but EIEC is a true intracellular pathogen that is capable of invading and replicating within epithelial cells and macrophages. Other *E. coli* strains might be internalized by epithelial cells at low levels, but do not seem to replicate intracellularly (Nataro and Kaper, 1998).

Virulence factor in *E. coli* include the ability to resist phagocytosis, utilization of highly efficient iron acquisition systems, resistance to killing by serum, production of colicins and adhere, colonize, and invade the hosts' cells. Further to these are the secretion systems, production of cell surface molecules, transport and siderophore formation. The pathogenicity of STEC O157:H7 is associated with a number of virulence factors, including Shiga toxin 1 (encoded by thestx1gene), Shiga toxin 2(encoded by the stx2gene), intimin (encoded by the eae Agene) and enterohaemolysin (encoded by the Ehlygene) (Kang *et al*., 2004).

2.6.3.4. Diagnosis

E. coli can be differentiated from other members of the *Enterobacteriaceae* on the basis of a number of sugar-fermentation and other biochemical tests. Classically an important group of tests used for this purpose are known by the acronym IMViC .These tested for the ability to produce: indole from tryptophan (I); sufficient acid to reduce the medium pH below 4.4, the break point of the indicator methyl red (M); acetoin (acetyl methyl carbinol) (V); and the ability to utilize citrate (C) (Adams and Moss, 2008). Despite *E. coli* can be identified with a variety of biochemical reactions, the indole test remains the most useful method to differentiate *E. coli* from other members of the *Enterobacteriaceae* (Xia, 2010).

Immunoassays and polymerase chain reaction technology have led to more rapid detection of *E. coli* in stools, food, and water. Techniques included in this category are PCR and DNA based techniques, immune magnetic separation, and enzyme-linked immune sorbent assays (ELISAs) (Bavaro, 2009; Clifton, 2000). Molecular-based techniques are distinctly advantageous because of their sensitivity, selectivity, and their rapid results (Parsons et al., 2016).

However, molecular-based techniques are appreciably more expensive than traditional plating techniques and are also more novel and unfamiliar. Therefore, the integration of molecular-based approaches into quality control procedures depends on the overall needs and resources of the food processing plant (Robinson and McKillip, 2010). Latex agglutination test is often used for the rapid identification of *E. coli* O157:H7. The test is best used in conjunction with Sorbitol MacConkey Agar. A positive result is indicated by agglutination with the test reagent, whilst the control reagent should appear milky and smooth (Al-Dragy and Baqer, 2014).

2.6.3.5 Treatment of pathogenic *E.coli*

The use of antibiotics in the treatment of STEC infection is controversial (Panos *et al*., 2006; Ochoa *et al*., 2007). Some authors reported that antibiotics may have beneficial effects in STEC infection and reduce the risk of STEC-associated complications (Yoshimura *et al*., 1999; Kurioka *et al*., 1999) while others reported an increase in the level of shiga toxin production and a greater risk of fatal complications following administration of antibiotics in STEC infection (Zhang *et al*., 2000; Wong *et al*., 2000). In vitro studies showing most strains are susceptible to various antibiotics, although certain antibiotics, at sub lethal concentrations may increase the release of Shiga-like toxin which has been associated with the development of HUS. No clinical studies have indicated that antibiotics are effective in reducing the duration of *E. coli* infection or duration of bloody diarrhea (Collins and Green, 2010).

2.6.3.6. Prevention and control of pathogenic *E.coli*

An effective control program to substantially reduce *E. coli* O157:H7 infections will require the implementation of intervention strategies through out the food continuum, from farm to table. Promising intervention measures at the farm include competitive exclusion bacteria, bacteriophage and targeted anima management practices addressing common points of contamination. Consumers also have a role in implementing intervention controls in food handling and preparation. Unfortunately, many consumers eat high -risk foods, improperly handle and store foods and ignore warnings regarding foods known to be unsafe (Sanchez *et al*., 2002).

3. MATERIALS AND METHODS

3.1. Description of study area

This study was conducted in Holeta Agricultural Research Center, Holeta town. Holeta is found in Oromia Regional State of Ethiopia and is situated at a distance of 29km West of Addis Ababa and located at 9°02‟ N latitude and 38°29‟ E longitude. The altitude of the town is 2400m above sea level with mean annual rainfall of 1144mm, humidity of 60.6%, minimum and maximum temperature of 6.2–24.1^{o}C respectively. The town has an area of 5550ha and administratively it is subdivided in to eight kebeles (Kidist *et al*.; 2013). Holeta is known by its potential area in production of milk and milk products like *ergo* and *ayib.* A large amount of milk and milk products transported to capital city, Addis Ababa each day. *Tej* is also a widely used traditinall fermeted beverage which is made from honey, water and leaves of gesho (*Rhamnus prinoides*).

3.2. Study design and period

Cross sectional and experimental study design was used in the study. The study was conducted from February, 2019 to Nov, 2019 in Holeta Agricultural Research Center, Holeta.

3.3. Sampling technique and Sample Collection

Purposive sampling technique was used to select representative samples of traditional fermented products in the study area. Forty (40) samples (20 traditional fermented *ergo* and 20 traditional fermented *Tej*) were collected from a total of twenty five (25) *Ergo* vendors and twenty two (22) *Tej* producing and vending houses located in Holeta town. Around five hundred (500) ml of samples were collected aseptically and immediately transported to Holeta Agricultural Research center dairy laboratory, Ethiopian Institute of Agricultural Research, for isolation and characterization of lactic acid bacteria having antagonistic activity against antibiotics resistant bacterial pathogens. In the laboratory, the samples were kept at the temperatures below 4°C and analyzed within 48hr of collection. After the samples were transported to the laboratory the pH of each samples were determined using a digital pH-meter (Hanna instruments, HI 2483, Italy), as suggested by Erkmen and Bozkurt (2004).

3.4. Isolation of Probiotic LAB

Lactic acid bacteria (LAB) were isolated from locally fermented *Ergo* and *Tej* using MRS agar (Highmedia) according to Abdulkadir *et al* (2011). Twenty five (25) ml of each sample were homogenized in 225 ml of sterile buffered peptone water. The suspension were used for making suitable serial dilutions of 10^{-1}- 10^{-7} by incorporating 1ml into 9ml of sterile buffered peptone water in sterile test tubes. From the appropriate dilutions, 0.1 ml of the samples was spread in duplicate on MRS agar medium, incubated anaerobically (Gas Pack Anaerobic System, BBL, New Delhi, India) for 48hr at 37^{o}C. Isolates with morphological characteristic of LAB were transferred to MRS broth, incubated overnight at 37^{o}C and checked for purity by streaking the culture on MRS media until only a single type of colony appeared and the purity of the cultures were also checked under microscope as well. The purified isolates were stored on MRS agar slant at 4°C under anaerobic condition for further identification. The isolates were designated with letter Y for *Ergo* LAB isolates and letter T for *Tej* LAB isolates followed with different numbers.

3.5. Characterization of LAB

The purified LAB colonies with distinct morphological characteristics, such as color, shape, and size were characterized and identified by microscopic examination. Conventional biochemical and physiological tests were also used to identify LAB isolate. The preliminary identification of LAB was made on the basis of Gram Staining, catalase reaction and production of CO_2 from glucose. Only gram positive and catalase negative isolates were considered as presumptive lactic acid bacteria (Imen *et al*., 2017).

3.5.1. Morphological Characterization

3.5.1.1. Gram Staining

Cell morphology of LAB was determined by using gram staining according to Ismail*et al*., (2018). Gram staining cell morphological identifications included color and shape of bacterial cell. Gram-positive bacteria was marked by purple or red color which indicates that the bacteria cell wall are capable of binding to crystal violet dye, where as gram-negative was characterized by the formation of pink color which indicates that the bacteria cell wall are not able to bind the crystal violet dye.

3.5.1.2. Spore production test

Spore production test was done to detect the presence of bacterial endospores. Each isolate of LAB was grown on MRS agar slant for 24 - 36hrs. 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 20sec. 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).

3.5.1.3. Gas production from Glucose/Glucose fermentation

The aim of the test was to identify homo-fermentative and hetero-fermentative LAB isolates. It was assessed based on gas production from glucose using MRS broth with inverted Durham tube according to Fguiri *et al*., (2017). A loop full of overnight cultures was transferred in to 8ml of autoclaved MRS broth media supplemented with 1% glucose with inverted Durham tube and incubated anaerobically at 32°C for 5 days. Gas accumulation in Durham tubes was taken as the evidence for CO_2 production from glucose. If CO_2 is produced from glucose, the isolate is hetro-fermentative and if not the isolate is homo-fermentative and also uninoculated broth media was used for control experiment.

3.5.1.4. Motility test

A sterile needle was used to pick a loop full of a 24hr old culture and inoculated into semisolid nutrient agar in a test tube. 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).

3.5.2. Biochemical and Physiological Characterizations

3.5.2.1. KOH string test

A loop full of bacterial colony from the culture plate was emulsified over glass slide in suspension of 3% KOH. The suspension was stirred continuously for one minute and then loop was gently pulled up from it. The test was considered positive if string were seen within first 30

seconds after mixing in KOH solution, KOH positive bacteria are Gram negative whereas KOH negative are Gram positive bacteria (Chinmaya Dash *et al*., 2016).

3.5.2.2. Catalase reaction

The test was done according to Abdulkadir *et al*., (2011). A drop of 3% hydrogen peroxide was placed on a clean microscope slide. A visible amount of bacterial growth (colony) was streaked on the glass slide with the inoculating loop and observed for gas (bubble) production. Non-catalase producer do not release any gas (oxygen) from hydrogen peroxide, and considered positive test for LAB. `

3.5.2.3. 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).

3.5.2.4. Starch hydrolysis test

The ability to degrade starch is used as a criterion for the determination of amylase production by a microbe. The ability of the isolates to hydrolyze starch was determined on starch agar (1gram starch + 1gram nutrient agar + 100ml distilled water). After activation LAB isolates in MRS broth for 24hr, 0.1ml were spread/streaked on the starch agar and incubated aerobically/anaerobically at 37°C for 2 days. The plates were then flood with gram's iodine for 15 to 30 min as an indicator. Starch in the presence of iodine produces a dark blue coloration of the medium and a yellow zone (clear zone) around a colony in a blue medium indicates amylolytic activity and was considered as positive test (Estifanos *et al*., 2016).

3.5.2.5. Carbohydrate Fermentation Test

Sugar fermentation test of sucrose, lactose, mannitol, sorbital, fructose and xylose was carried out to determine the ability of organisms to produce 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 loop full of 24hrs old culture of the isolate and then they were incubated for 24-48hrs 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).

3.5.3.1. Tolerance to Different Temperatures

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 loop full of young culture (18-24hrs) of each isolates was inoculated in to 10 ml MRS broth. From the inoculate 1 ml was pour plated on to MRS agar for LAB isolates and incubated at 37^{o}C. The left broth was incubated at different temperature of 15, 37 and 45^{o}C separately for 48hrs After 48hrs 1 ml of aliquots were pour plated on to MRS agar for LAB isolates. The plates were incubated at 37^{o}C for 48hrs. The colony was counted and viability of greater than >50% was considered to be tolerant (Tambekar and Bhutada, 2010). The growths of the isolates were determined as follows:

$$\text{Growth at n temp in \%} = \frac{\text{Final cell count at 48hrs}}{\text{Initial cell count at 0hr}} \text{X } 100$$

3.5.3.2. Tolerance to Different Salt Concentration

Salt tolerance of each isolates was assessed by inoculating young cultures of (18-24hrs) 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^{0}C for 48 hrs. One milliliters of the broth pour plated on MRS agar and incubated at 37^{o}C for 48 hrs. The colony was counted and viability greater than >50% was considered to be tolerant (Hoque *et al*., 2010). The growths of the isolates were determined as follows:

$$\text{NaCl tolerance in \%} = \frac{\text{Final cell count at 48hrs}}{\text{Initial cell count at 0hr}} \text{X } 100$$

3.5.4. Probiotic properties of the isolates

The characterization was done by using different physiological parameters (temperatures and salt tolerance).

3.5.4.1. Tolerance to Low pH

This was recorded by inoculating a loop full of an overnight LAB isolates into autoclaved MRS broth whose pH acidity was pre-adjusted to pH 2.0, 2.5 and 3.0 using 1MHCl.Culture without pH adjustment is used as control. Tubes were incubated at 37^{o}C and checked for survival or acid tolerance at 0, 3 and 6hrs by using spectrometer at 600 nm with respective pH values according to (Liong and Shah, 2005). Formation of more turbid broth was considered as the strains tolerance to the specified acidity. Alternatively, tubes incubated at 37^{o}C were checked for survival or acid tolerance at 0, 3 and 6hrs by spreading 0.1ml of the sample onto MRS agar with respective pH values. All plates were incubated at 37^{0} C for 48hrs 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.

$$\text{Survival rate in \%} = \frac{\text{Final cell count at 48hrs}}{\text{Initial cell count at 0hr}} \text{X } 100$$

3.5.4.2 Bile salt tolerance test

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^{o}C and growth was checked at 0, 3 and 6 hrs by using spectrophotometer. Alternatively by spreading 0.1ml aliquots on MRS agar and incubated at 37^{o}C in anaerobic jar. Bile salt tolerance of each isolate was determined by comparing the plate counts after 6hrs of exposure to bile salts with the initial count at 0hrs (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.

3.5.5. Assessment of beneficial activities of probiotic LAB

3.5.5.1. Acidifying Activity Test

Acidification property of LAB isolates were measured by the change in pH and TA with time. The isolates were initially grown on MRS agar and then inoculated in to skim milk. The experimental culture consisted of isolate starter culture and the test pathogen were mixed together in 300ml of pasteurized skim milk in triplicate to give a final inoculums level of 6log

cfu/ml and 3log cfu/ml for each isolate starter and the test pathogen, respectively. All flasks were incubated at 37^{o}C in anaerobic jar and pH changes were determined using pH meter (Hanna instruments, HI 2483, Italy). Change in pH was monitored at different intervals by taking samples at 0hr (initial), 24hrs and 48hrs.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 0.1N NaOH and expressed in percent (m/v) according to (Shah et al., 2000). Titratable acidity produced during fermentation was calculated as:

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

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

3.5.5.2 Compatibility test

In order to formulate starter culture 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 MRS agar plate and incubated at 37^{0}C for 48hrs (Anupama, 2015). Their growth patterns were noticed after 48hrs of incubation. The isolate showing best compatibility was chosen for starter culture.

3.6. Food borne pathogens confirmatory test

A total of Ninety (90) (each of 30 *E.coli*, 30 *Salmonella* and 30 *Shigella*) food borne pathogenic microorganisms were obtained from Holeta Agricultural Research Center, Holeta Dairy Research Laboratory,which was isolated from raw milk and raw meat in the previous study. Antimicrobial resistant pathogens were screened from the obtained pathogens. Standard reference strains of *Escherichia coli* ATCC 43895, *Salmonella typhimurium* ATCC 14802 and *Shigella* ATCC 14301 was obtained from Ethiopian Public Health Research Institute (EPHRI), Addis Ababa, Ethiopia.The standard reference strain and biochemical confirmation test was conducted for confirmation.

Biochemical confirmation: For biochemical confirmation, pathogens suspected colonies were streaked on Nutrient agar and incubated overnight at 37^{0}C. Series of biochemical tests were

conducted to characterize the isolates including Gram stain, Catalase, Citrate and fermentation of different sugars such as Glucose, Sucrose, Lactose, H_2S, Gas, Sorbitol, Fructose and Xylose.

3.7. Screening of Antimicrobial resistant food borne pathogens

Antimicrobial resistant food borne pathogens were screened from the pathogens obtained from Holeta Agricultural Research Center. Antimicrobial susceptibility testing was performed using the Kirby–Bauer disk diffusion method according to Clinical and Laboratory Standards Institute (CLSI) recommendations. In order to survey the antibiotic profile, 100µl of each overnight culture of the strains and the standard strain which have been adjusted to 0.5McFarland standard was spread on Mueller-Hinton agar plates; subsequently, antibiotic discs applied according to the Bauer-Kirby technique(CLIS, 2012). The following concentrations in antibiotics were used: Ampicillin (10µg), Ceftriaxone (30µg), Ciprofloxacin (5µg), Chloramphenicol (30µg), Gentamycin (10µg), Sulfamethoxazole-trimethoprim (25µg), Nalicidic acid (30µg) and Norfoxacin (10µg). The plates were incubated for 24h at 37°C , followed by measurement of the inhibition zone in diameters (IZDs), including the diameter of the disk (in millimeters) and classified as resistant, intermediate or susceptible according to CLSI standard break points for *Entrobactericeae.*

3.8. *In vitro* Antagonistic test against antibiotics resistant food borne pathogens

Preparing supernatant of probiotic lactic acid bacteria

Lactic acid bacteria isolates were subcultured in sterile test tube containing MRS broth at 37^0C for 24hrs and transferred in to sterile test tube containing 10ml MRS broth. And the broth culture was incubated at 37^0C for 3days in thermostat water bath. To get the culture filtrate, 3days old culture was centrifuged at 10000rpm for 20minutes (Esayas *et al*., 2008).

3.8.1. Agar Well diffusion method

The respective Lactic acid bacteria strain was incubated at 37^0C for 3days in thermostat water bath and centrifuged at 10000rpm for 20min.The supernatants were distributed in two aliquots, the non filtered (NFS) and the filtered (FS) with a sterile membrane of 0.22µm pore size (Millex

GS filter, Millipore).These procedures was performed to see the influence of the residual cells of unfiltered samples, in the antagonistic effect.

Twenty milliliters of molten Mueller-Hinton agar was poured in to 8cm sterile Petri dishes and 100µl of the overnight culture of *Salmonella, E. coli and Shigella* was cultivated on BHI agar and made 0.5McFarland turbidity using saline solution and it was evenly swabbed on MHA plates. Wells of 6mm in diameter was cut using a sterile cork borer into the Mueller Hinton agar (MHA) medium and 100µl of each type of probiotic strain supernatant(NFS and FS) and were placed into each well (Esayas *et al*., 2008) and Ampicillin was used as a negative control. The culture plates were incubated at 37°C for 24hrs and the zones of inhibition was measured in millimeters (mm).

3.8.2. Co-culturing of LAB and pathogens

Co-culturing method was used to test the antagonistic ability of each isolate on the three different pathogens. The experimental culture consisted of LAB isolate starter culture and the test pathogen together in 300ml of pasteurized skim milk in triplicate to give a final inoculums level of 6log cfu/ml and 3log cfu/ml for each LAB isolate starter and the test pathogen, respectively. All flasks were incubated at 37^{o}C for 48hrs in anaerobic jar. Each test pathogen was separately inoculated in to 300ml of unpasteurized milk in triplicate to give final inoculums of 3log cfu/ml as a negative control. Samples of 1ml from each co-cultured (isolate starter culture - test pathogen) and control sample were drawn at 0hrs before incubation, at 24 and 48hrs of incubation. A dilution of $10^{-1}$was made and 0.1 ml of diluents was pour plated on selective media; Xylose Lysine Desoxycholate Agar (oxoid) (g/l of 15.0 g Agar, 7.5 g Lactose, 7.5 g Sucrose, 6.8 g Na2 S2O3, 5.0 g L-Lysine, 5.0 g NaCl, 3.75 g Xylose, 3.0 g Yeast extract, 2.5g Sodium deoxycholate, 0.8 g Ferric ammonium citrate, 0.08 g Phenol Red pH adjusted to 7.4 ± 0.2) for *Salmonella* and *Shigella*, 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 K2HPO4, 0.4 g Eosin, 0.065 g Methylene blue pH adjusted to 7.2 ± 0.2 at 25^{0}C) for *E. coli*, and 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 (Drago *et al.*, 1997).

3.9. Data analysis

The average pH of *Ergo* and *Tej* samples from the triplicates of independent experiments were statistically analyzed using SAS version 9.3, 2014.Variation in pH between *Ergo* and *Tej* samples were compared using independent mean value at 0.05 p-values and independent sample mean value was used to determine if significant variation occurred in pH within the samples. Data obtained from the antagonistic effect of Lactic acid bacteria (co-cultured) on test organisms were analyzed using two ways ANOVA. The different inhibition zone measurements in triplicate were compared by performing One-way ANOVA ranked with Duncan's multiple range tests with descriptive analysis type. All statistical results with $p<0.05$ will be considered to be statistically significant.

4. RESULTS AND DISCUSSION

4.1. RESULTS

A total of 40 samples, 20 each of *Ergo* and *Tej* were collected and analyzed aseptically for the presence of probiotic LAB. The maximum and the minimum pH value of *Ergo* samples were 4.38 and 3.94 respectively.The maximum pH value of *Tej* samles was 3.43 with a minimum pH value of 2.90. The average pH values of *Ergo* and *Tej* sample were 4.13 and 3.27, respectively.Variations in pH values within the samples of *Ergo* and *Tej* were significantly different (Table 2). Similarly, there was a significant difference in pH values between *Ergo* and *Tej* samples $p<0.05$.

Table 2: pH values of *Ergo* and *'Tej'*samples

Sampletype	**PH**	**Sample type**	**pH**
Ergo	**Mean±S.D**	**Tej**	**Mean±S.D**
ES-1	4.36 ± 0.01^{a}	TS-1	3.31 ± 0.01^{bc}
ES-2	4.26 ± 0.06^{bc}	TS-2	3.14 ± 0.00^{d}
ES-3	4.38 ± 0.02^{a}	TS-3	3.30 ± 0.05^{bc}
ES-4	3.98 ± 0.01^{f}	TS-4	3.35 ± 0.01^{bc}
ES-5	3.94 ± 0.01^{f}	TS-5	3.36 ± 0.01^{bc}
ES-6	4.32 ± 0.08^{ab}	TS-6	3.10 ± 0.00^{d}
ES-7	4.09 ± 0.01^{d}	TS-7	3.34 ± 0.00^{bc}
ES-8	4.32 ± 0.01^{ab}	TS-8	2.90 ± 0.01^{f}
ES-9	4.33 ± 0.00^{ab}	TS-9	3.30 ± 0.00^{bc}
ES-10	4.190 ± 0.00^{c}	TS-10	3.32 ± 0.01^{bc}
ES-11	4.09 ± 0.01^{d}	TS-11	3.43 ± 0.01^{a}
ES-12	4.00 ± 0.05^{ef}	TS-12	3.32 ± 0.00^{bc}
ES-13	3.99 ± 0.01^{ef}	TS-13	3.35 ± 0.01^{bc}
ES-14	4.01 ± 0.01^{ef}	TS-14	3.33 ± 0.01^{bc}
ES-15	4.02 ± 0.01^{def}	TS-15	3.36 ± 0.00^{bc}
ES-16	3.97 ± 0.01^{f}	TS-16	3.32 ± 0.01^{bc}
ES-17	4.31 ± 0.02^{d}	TS-17	3.35 ± 0.01^{bc}
ES-18	4.06 ± 0.01^{de}	TS-18	2.96 ± 0.02^{e}
ES-19	3.98 ± 0.01^{ef}	TS-19	3.31 ± 0.01^{bc}
ES-20	4.01 ± 0.01^{def}	TS-20	3.29 ± 0.05^{c}

All values are mean ± SD in triplicate; Values in the same column carrying the same superscript are not significantly different; ES=Ergo sample, TS=Tej samples, S.D=standard deviation.

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4.1.1 Morphological, biochemical and physiological characterizations of LAB

A total of 222 different colonies were selected and purified, of which 121 colonies were confirmed as LAB. Out of these, 69 isolates (57%) and 52 isolates (43%) were from *Ergo* and *Tej* samples, respectively. The isolates were Gram positive, catalase negative, rod shaped and appeared in single, pairs, chains or tetrad cellular arrangement. Among these, Sixteen(16) lactic acid bacteria namely,Y-4, Y-5, Y-7, Y-13, Y-14, Y-16, Y-17, Y-18, Y-35, T-9, T-11, T-18, T-34, T-36, T-37 and T-38 were further screened based on their probiotic attributes of acid tolerance, bile salt tolerance, starter culture attributes and their antagonistic effect against antibiotics resistant food borne pathogens. Out of 16 lactic acid bacteria nine (9) isolates was creamy, six (6) isolates was white colony and only one isolate (T-34) was found to be grey white in colony appearance. The entire LAB isolates were circular in colony shape, rod in cell shape, non-motile and non-spore forming (Table 3). All the isolates were able to grow in 2% and 3% of NaCl and also able to grow at different remprature (15^0C, 37^0C and 45^0C) but four (4) isolates (Y- 4, T-9, T-36 and T-38) do not able to grow at 45^0C. On other hand only three isolates Y-7, T-11, T-38 isolates were able grow at 10^0C.

Four isolates (25%), namely Y-7, Y-17, T-9 and T-36 were hetro-fermentative; and they produced CO_2 from carbohydrates fermentations and twelve isolates (75%) Y- 4, Y-5, Y-13, Y-14, Y-16, Y-18, Y-35, T-11, T-18, T-34, T-37 and T-38 were homo fermentative and found out that they did not produce CO_2 while fermenting carbohydrates. The entire LAB was able to ferment glucose, lactose and mannitol, whereas four isolates; Y-13, Y-18, T-9 and T-18 do not ferment sucrose (Table 4). All the LAB isolates were able to grow in 2%, 3% and 4%NaCl but only three isolates (Y-35,T-18 and T-37) do not able to grow in 4% NaCl. Eight isolates (Y-5, Y-7, Y-13, Y-14, Y-18,Y-35, T-34 and T-37 were able to grow in 6.5% NaCl (Table 5).

Table 3: Morphological characteristics of LAB

LAB Isolates	Characteristics							LAB genera
	Colony color	Colony shape	Colony margin	Cell shape	Glucose fermentation	Spore	Motility	
Y- 4	Creamy Smooth	Circular	Entire	Rod in chains	Homo Fer.	Absent	-ve	*Lactobacillus*
Y- 5	White Smooth	Circular	Entire	Rod in chains	Homo Fer	Absent	-ve	*Lactobacillus*
Y-7	Creamy Smooth	Circular	Entire	Rod in chains	Hetro Fer	Absent	-ve	*Lactobacillus*
Y-13	White Smooth	Circular	Entire	Rod in chains	Homo Fer	Absent	-ve	*Lactobacillus*
Y-14	White Smooth	Circular	Entire	Rod in chains	Homo Fer	Absent	-ve	*Lactobacillus*
Y-16	Creamy Smooth	Circular	Entire	Rod in chains	Homo Fer	Absent	-ve	*Lactobacillus*
Y-17	White Smooth	Circular	Entire	Rod in chains	Hetro Fer	Absent	-ve	*Lactobacillus*
Y-18	Creamy Smooth	Circular	Entire	Rod in chains	Homo Fer	Absent	-ve	*Lactobacillus*
Y-35	Creamy Smooth	Circular	Entire	Rod in chains	Homo Fer	Absent	-ve	*Lactobacillus*
T-9	Creamy Smooth	Circular	Entire	Rod in chains	Hetro Fer	Absent	-ve	*Lactobacillus*
T-11	Creamy Smooth	Circular	Entire	Rod in chains	Homo Fer	Absent	-ve	*Lactobacillus*
T-18	Creamy Smooth	Circular	Entire	Rod in chains	Homo Fer	Absent	-ve	*Lactobacillus*
T-34	Grey white Smooth	Circular	Entire	Rod in chains	Homo Fer	Absent	-ve	*Lactobacillus*
T-36	White Smooth	Circular	Entire	Rod in chains	Hetro Fer	Absent	-ve	*Lactobacillus*
T-37	White Smooth	Circular	Entire	Rod in chains	Homo Fer	Absent	-ve	*Lactobacillus*
T-38	Creamy Smooth	Circular	Entire	Rod in chains	Homo Fer	Absent	-ve	*Lactobacillus*

Y-4, Y-5, Y-7, Y-13, Y-14, Y-16, Y-17, Y-18 and Y-35 were Ergo lactic acid bacterial isolates; T-9, T-11, T-18, T-34, T-36, T-37 and T-38 was Tej lactic acid bacterialisolates.

Table 4: Biochemical and physiological identification of LAB isolates

LAB Isolate	Characteristics												
	Gram stain	Catalase	Citrate	Glucose	Sucrose	Lactose	Mannitol	Starch	H_2S	Gas	Sorbitol	Fructose	Xylose
Y- 4	+ve	-ve	-ve	+ve	+ve	+ve	+ve	+ve	-ve	-ve	-ve	+ve	+ve
Y- 5	+ve	-ve	-ve	+ve	+ve	+ve	+ve	+ve	-ve	-ve	-ve	+ve	+ve
Y-7	+ve	-ve	-ve	+ve	+ve	+ve	+ve	-ve	-ve	-ve	+ve	+ve	-ve
Y-13	+ve	-ve	-ve	+ve	-ve	+ve	+ve	-ve	-ve	-ve	-ve	+ve	-ve
Y-14	+ve	-ve	-ve	+ve	+ve	+ve	+ve	-ve	-ve	-ve	+ve	+ve	+ve
Y-16	+ve	-ve	-ve	+ve	+ve	+ve	+ve	-ve	-ve	-ve	+ve	+ve	+ve
Y-17	+ve	-ve	-ve	+ve	+ve	+ve	+ve	-ve	-ve	-ve	+ve	+ve	+ve
Y-18	+ve	-ve	-ve	+ve	-ve	+ve	+ve	-ve	-ve	-ve	+ve	+ve	+ve
Y-35	+ve	-ve	-ve	+ve	+ve	+ve	+ve	+ve	-ve	-ve	-ve	+ve	-ve
T-9	+ve	-ve	-ve	+ve	-ve	+ve	+ve	+ve	-ve	-ve	+ve	+ve	-ve
T-11	+ve	-ve	-ve	+ve	+ve	+ve	+ve	+ve	-ve	-ve	+ve	+ve	+ve
T-18	+ve	-ve	-ve	+ve	-ve	+ve	+ve	+ve	-ve	-ve	-ve	+ve	-ve
T-34	+ve	-ve	-ve	+ve	+ve	+ve	+ve	-ve	-ve	-ve	-ve	+ve	+ve
T-36	+ve	-ve	-ve	+ve	+ve	+ve	+ve	-ve	-ve	-ve	-ve	+ve	-ve
T-37	+ve	-ve	-ve	+ve	+ve	+ve	+ve	-ve	-ve	-ve	+ve	+ve	-ve
T-38	+ve	-ve	-ve	+ve	+ve	+ve	+ve	-ve	-ve	-ve	+ve	+ve	-ve

Y-4, Y-5, Y-7, Y-13, Y-14, Y-16,Y-17, Y-18 and Y-35 were Ergo lactic acid bacterial isolates; T-9, T-11, T-18, T-34, T-36, T-37 and T-38 was Tej lactic acid bacterial isolates.+ = reaction, - = no reaction.

Table 5: Responses of LAB to different salt concentrations and temperature

LAB isolate	Characteristics							
	Growth at d/t salt concentration				Growth at d/t temperature			
	2% NaCl	3%NaCl	4%NaCl	6.5%NaCl	10^0C	15^0C	37^0C	45^0C
Y- 4	+	+	+	-	-	+	+	-
Y- 5	+	+	+	+	-	+	+	+
Y-7	+	+	+	+	+	+	+	+
Y-13	+	+	+	+	-	+	+	+
Y-14	+	+	+	+	-	+	+	+
Y-16	+	+	+	-	-	+	+	+
Y-17	+	+	+	-	-	+	+	+
Y-18	+	+	+	+	-	+	+	+
Y-35	+	+	-	+	-	+	+	+
T-9	+	+	+	-	-	+	+	-
T-11	+	+	+	-	+	+	+	+
T-18	+	+	-	-	-	+	+	+
T-34	+	+	+	+	-	+	+	+
T-36	+	+	+	-	-	+	+	-
T-37	+	+	-	+	-	+	+	+
T-38	+	+	+	-	+	+	+	-

Y-4, Y-5, Y-7, Y-13, Y-14, Y-16Y-17, Y-18 and Y-35 were Ergo lactic acid bacterial isolates; T-9, T-11 T-18, T-34, T-36T-37and T-38 was Tej lactic acid bacteria isolate.+ = reaction, - = no reaction.

4.1.2 Probiotic property of isolates

4.1.2.1. pH tolerance

All the isolates have shown tolerance to pH 2.0, 2.5 and 3.0 with more than 50% of survival or tolerance value. The three pH values (2.0, 2.5 and 3.0) tolerances of the isolates were indicated in (Fig.3). Among the isolates T-9, Y-14 and T-37 showed relatively higher tolerance to pH of 2.0, 2.5 and 3.0. Relatively lower tolerance of 51.52% and 51.2% to pH 2.0 was exhibited by T-38 and T-34 in the order.

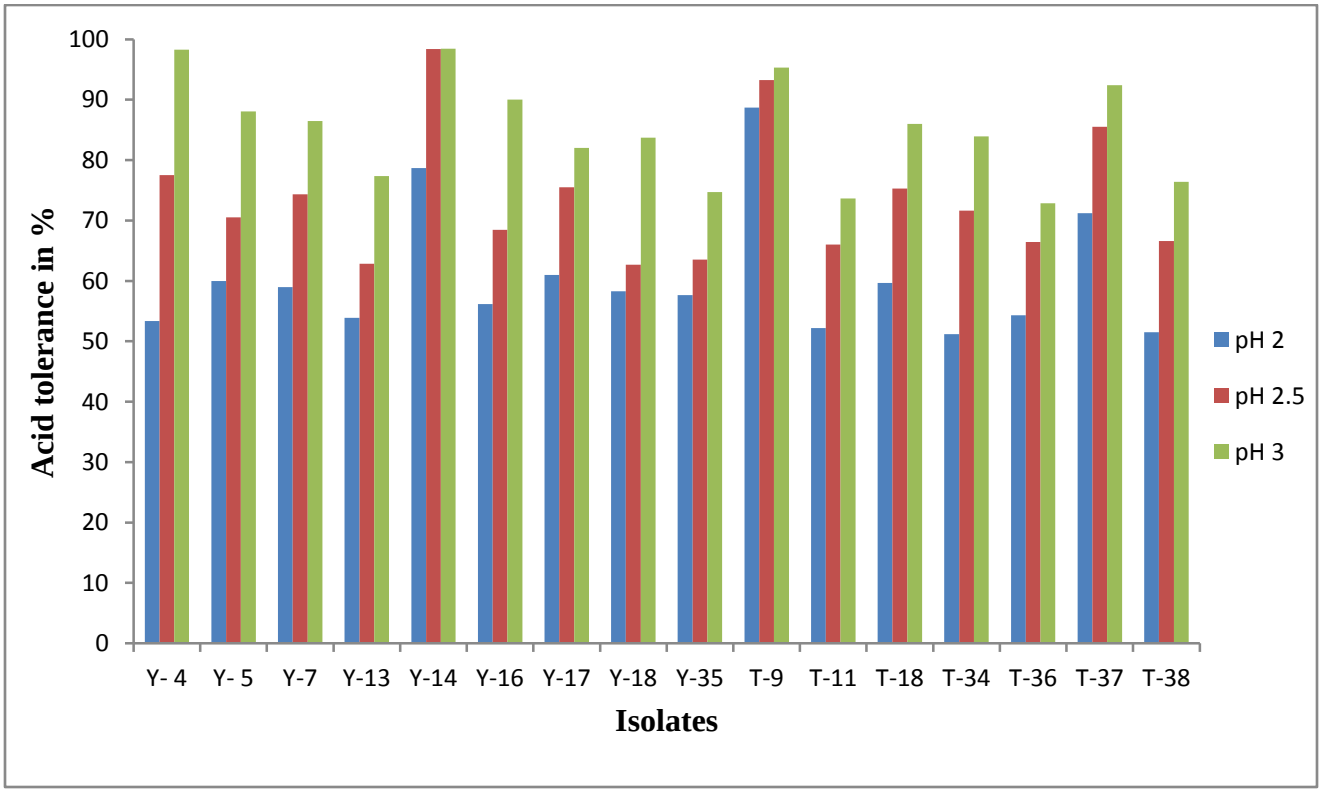


Figure 3: pH tolerance of LAB isolates for 6hrs of incubation
Y-4, Y-5, Y-7, Y-13, Y-14, Y-16,Y-17, Y-18 and Y-35 were Ergo lactic acid bacterial isolates; T-9, T-11 T- 18, T-34, T-36T-37and T-38 were Tej lactic acid bacteria isolate

4.1.2.2. Bile salt tolerance

The details of Figure 4 showed the bile salt tolerance of each LAB isolates. All the sixteen lactic acid bacterial isolates were found to be tolerant to bile salt of 0.3%, 0.5% and 1% at a vary ranges. Relatively, highest tolerance of 97.78%, 91.98% and 88.27% to bile salt concentration of 0.3%, 0.5% and 1% were observed by isolate Y-35 and relatively lowest

tolerance of 61.00% and 54.12% to bile salt concentration of 0.3% and 0.5% were observed by isolate Y-14. The average bile salt tolerance of whole isolates was greater than 67%. Tolerance or viability of the isolates decreased as the concentration of bile salt was increasing from 0.3% to 1% (Fig. 4).

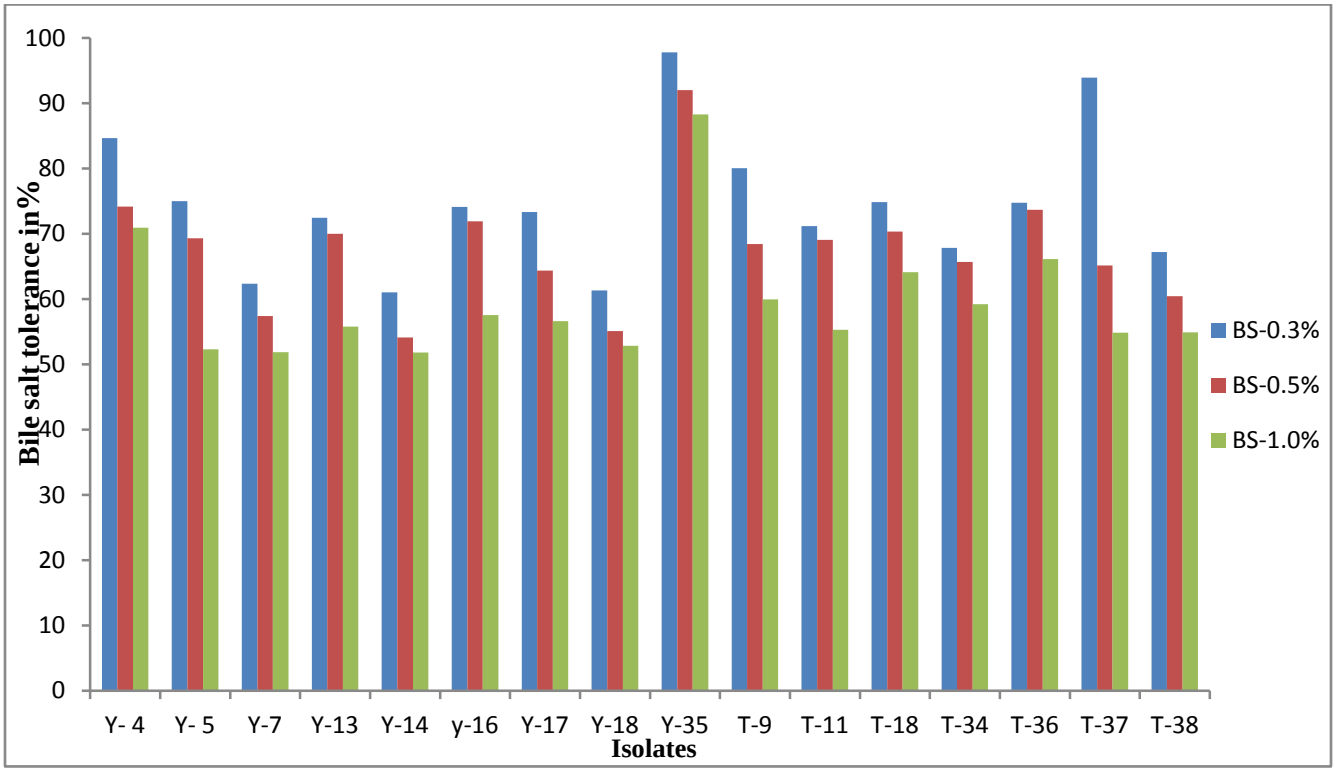


Figure 4: Bile salt tolerance of LAB isolates in percent during 6hrs of incubation

Y-4, Y-5, Y-7, Y-13, Y-14, Y-16Y-17, Y-18 and Y-35 were Ergo lactic acid bacterial isolates; T-9, T-11, T-18, T-34, T-36T-37and T-38 were Tej lactic acid bacteria isolate, BS=bile salt

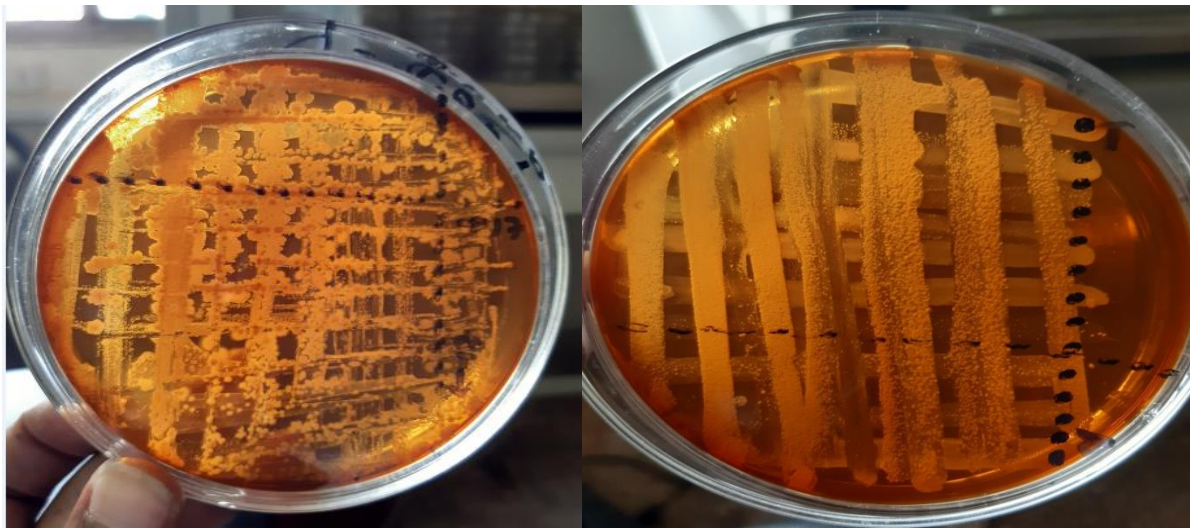
4.1.3. Acidifying capability of LAB isolates

The entire LAB isolates has the capability to acidify skim milk samples at 24hr and 48hr of fermentation. *Ergo* fermented with probiotic LAB was highly significant ($p< 0.05$) at 24 and 48hrs of fermentation. Among ergo product produced with probiotic LAB the lower pH of 3.80 and 3.76 was observed by isolate Y-16 and Y-5 respectively at 48hrs of fermentation (Table 9). The pH of each ergo feremented with LAB was significantly ($p<0.05$) lower than the control fermented ergo at 24hrs and 48hrs of fermentation. Titratable acidity of ergo ranged from 0.79% by Y-5 to 0.46% by Y-35 at 24hrs of fermentation. Relatively maximum titratable acidity of 0.85% by isolate Y-14 and the minimum titratable acidity of 0.49% were observed by Y-35

during 48hrs of fermentation of ergo. Titratable acidity of each product produced by different isolate was significantly ($P < 0.05$) higher than the titratable acidity of control fermented ergo at 24 and 48hrs of fermentation (Table 9).

4.1.4. Compatibility test of LAB isolates

All Ergo LAB isolates (Y- 4, Y-5, Y-7, Y-13, Y-14, Y-16, Y-17, and Y-18 &Y-35) and all Tej LAB isolates (T-9, T-11, T-18, T-34, T-36, T-37 and T-38) were compatible. All Ergo and Tej LAB (Y- 4, Y-5, Y-7, Y-13, Y-14, Y-16, Y-17, Y-18, Y-35, T-9, T-11, T-18, T-34, T-36, T-37 and T-38) isolates were also found to be compatible. None of the isolates were found inhibiting the growth of the other. The LAB isolates do not have any antagonistic affect on each other (Fig.5).



a) Compatibility between *Ergo* LAB isolates **b)** Compatibility between *Ergo* and *Tej* LAB isolates

Figure 5: Compatibility test of each lactic acid bacteria

4.1.5. Spoilage and food borne pathogens confirmatory test

Nine (9) food borne pathogens, three of each *Salmonella, Shigella* and *E.coli* resistant to the antimicrobials were screened. The two approaches; biochemical test and reference strains (*Salmonella typhimurium* ATCC 14802, *Shigella* ATCC 14301 and *Escherichia coli* ATCC 43895) led to identify the pathogens as *Salmonella*, *Shigella* and *E. coli.*

4.1.6. Antimicrobial Susceptibility pattern of food borne pathogens

Table 6 showed that all the food borne pathogens was found to be resistant to Ampicillin, Cefriaxone, Chloramphenicol and Sulfamethoxazole-trimethoprim except *Salmonella* ssp10 and *E.coli* ssp7 which were intermidiatly suscepitable to Sulfamethoxazole-trimethoprim. *Salmonella* ssp10 was resistant to four antibiotics and was found to be intermediately susceptible to Ciprofoxacin and Sulfamethoxazole-trimethoprim while susceptible to Gentamicin and Nalicidic acid. *Salmonella* ssp21 and *Salmonella* ssp22 were found to be resistant to six antibiotics but found to susceptible to Ciprofloxacin. *Salmonella* ssp21 was also found to be susceptible to Nalicidic acid. *Shigella* ssp12 was resistant to all of the antibiotics while *Shigella* ssp14 and *Shigella* ssp19 were found to be resistant to four antibiotics but, susceptible to ciprofloxacin, Nalicidic acid and Norfoxacin (Table 6). *E. coli* ssp7 was susceptible to four antibiotics, intermediately susceptible to Sulfamethoxazole-trimethoprim and resistant to three antibiotics (Details have shown in table 6). Both *E. coli* ssp27 and *E. coli* ssp30 was resistant to four antibiotics and intermediately susceptible to Gentamicin. In other hand *E. coli* ssp 27 was susceptible to Naldic acid while *E. coli* ssp30 was intermidiatley susceptible to Naldic acid.

Table 6: Antibiotic susceptibility patterns of pathogens

Pathogen	**Antibiotics**							
	AMP (10µg)	CRO (30µg)	CIP (5µg)	CHL (30µg)	GM (10µg)	SXT-TMP (25µg)	NA (30µg)	f (10µg)
*Salmonella*ssp10	R	R	I	R	S	I	S	R
Salmonella ssp21	R	R	S	R	R	R	S	R
*Salmonella*ssp22	R	R	S	R	R	R	R	R
Shigella ssp12	R	R	R	R	R	R	R	R
Shigella ssp14	R	R	S	R	S	R	S	S
Shigella ssp19	R	R	S	R	I	R	S	S
*E.coli*ssp7	R	R	S	R	S	I	S	S
E.coli ssp27	R	R	R	R	I	R	S	R
E.coli ssp30	R	R	S	R	I	R	I	I

AMP=Ampicillin, CRO=Cefriaxone, CIP=Ciprofoxacin, CHL=Chloramphenicol, GM=Gentamicin, SXT-TMP= Sulfamethoxazole-trimethoprim, NA= Nalicidic acid, f=Norfoxacin, R=Resistant, I=Intimidate S=Susceptible

4.1.7. Antagonistic effect of probiotic LAB against Pathogens

4.1.7.1. Well diffusion method

Inhibitions using cell-free liquid cultures of LAB from traditional fermented *Ergo* and *Tej* on solid cultures, showed a growth inhibition in a diameter equal or greater than 7mm against antibiotic resistant food borne pathogens (Table 7). Significant differences ($P<0.05$) were found for the inhibitions of LAB supernatants obtained from *Ergo* and *Tej* against the pathogens. The inhibition of both types of supernatants (F and NF) were not statically different ($P>0.05$) and they both inhibit the growth and survival of antibiotics resistant food borne pathogens (Table 8). This result confirmed that the inhibition effect was caused by the compounds secreted by probiotic LAB and not by the remaining cells in the supernatant.

Figure 6: Growth inhibition test of LAB supernatant against resistant pathogens

Table 7: Growth inhibition test on agar plates in anaerobic cultures of resistant pathogens by well diffusion of the LAB supernatants

LAB isolate	Pathogens								
	***Salmonella*ss p10**	***Salmonella* ssp21**	***Salmonella*ss p22**	***Shigella* ssp12**	***Shigella* ssp14**	***Shigella* ssp19**	***E.coli*ssp7**	***E.coli*ssp27**	***E.coli*ssp.30**
Y- 4	16.66±0.57^{ba}	18.02± 0.00^{a}	15.33±0.57^{bac}	13.66±2.88^{b}	12.03±0.00^{a}	16.33±1.52^{ba}	8.66±1.15^{g}	11.00±0.00^{ih}	14.33±2.30^{bdac}
Y- 5	14.00±2.00^{bdec}	14.04±0.00^{ed}	12.33±0.57^{fde}	16.66±2.88^{a}	14.33±0.57^{fedc}	14.00±2.00^{bdc}	16.00±1.00^{bac}	13.00±1.00^{fe}	17.33±2.30^{a}
Y-7	18.00±1.00^{a}	17.00±1.00^{ba}	13.66±0.57^{dec}	16.00±0.00^{ba}	12.66±0.57^{feg}	14.33±3.21^{bdc}	13.66±1.52^{edf}	10.00±0.00^{ij}	15.33±2.08^{bac}
Y-13	16.68±1.15^{ba}	9.33±1.15^{ij}	10.33±1.52^{fdeg}	16.66±1.15^{a}	12.66±1.15^{feg}	13.33±1.15^{dc}	11.66±1.52^{f}	12.00±0.00^{fhg}	14.00±2.00^{bdac}
Y-14	16.00±1.00^{bac}	13.33±0.57^{egdf}	12.00±1.73^{fdeg}	10.66±1.15^{c}	14.66±1.15^{bedc}	16.00±2.00^{bac}	14.66±1.52^{edc}	9.33±1.15^{j}	12.66±1.15^{bdc}
Y-16	12.66±0.57^{de}	12.33±0.57^{egf}	9.66±0.57^{hg}	16.66±1.15^{a}	12.33±1.52fg	15.66±1.15^{bac}	14.33±0.57^{edc}	12.33±1.52^{fhg}	12.33±1.52^{bdc}
Y-17	12.33±1.15^{e}	12.00±1.00^{gf}	10.00±0.00^{fhg}	16.33±1.15^{ba}	16.33±1.15^{bac}	16.33±1.52^{ba}	14.33±0.57^{edc}	15.00±0.00^{dc}	12.66±2.51^{dc}
Y-18	13.67±0.57^{dec}	13.66±0.57^{gf}	16.33±1.15^{ba}	14.33±0.57^{ba}	17.33±1.15^{a}	17.33±1.15^{a}	16.00±0.00^{bac}	11.33±1.15^{de}	11.00±0.00^{d}
Y-35	12.66±0.57^{de}	11.66±0.57^{gh}	10.00±1.00^{fhg}	14.00±2.00^{ba}	14.66±0.57^{bedc}	12.00±0.00^{d}	9.33±1.15^{g}	12.66±1.15^{feg}	11.66±1.52^{dc}
T-9	15.33±2.51^{bdac}	16.33±1.52^{bac}	15.66±2.51^{bac}	13.66±1.15^{b}	14.66±0.57^{bedc}	14.33±1.15^{bdc}	17.33±0.57^{ba}	17.00±1.00^{ba}	16.66±1.15^{a}
T-11	15.67±0.58^{bac}	16.03±0.00^{bc}	15.66±0.57^{bac}	14.00±2.00^{ba}	12.66±1.15^{feg}	15.33±0.57^{bac}	13.33±1.52^{edf}	17.66±0.57^{a}	17.66±0.57^{a}
T-18	11.67±2.89^{fe}	11.66±2.88^{gh}	11.66±2.88^{feg}	8.66±1.15^{c}	15.00±0.00^{bdc}	14.33±0.57^{bdc}	12.66±1.15^{ef}	14.00±2.00^{ihg}	14.00±3.46^{bdac}
T-34	15.33±1.15^{bdac}	15.00±0.00^{dc}	14.33±1.15^{bdac}	14.00±0.00^{ba}	16.66±1.52^{ba}	15.66±1.15^{bac}	16.00±1.00^{bac}	17.00±0.00^{ba}	15.33±1.52^{bac}
T-36	13.33±1.52^{dec}	10.00±0.00^{ih}	16.66±1.15^{a}	10.00±0.00^{c}	12.66±0.57^{feg}	12.33±0.57^{d}	15.33±1.15^{bdc}	11.00±0.00^{ih}	15.33±1.15^{bac}
T-37	11.83±1.75^{fe}	16.00±0.00^{bc}	14.00±1.00^{bdec}	11.00±0.00^{c}	8.66±1.15^{h}	16.66±1.52^{ba}	18.00±2.00^{a}	17.00±0.00^{ba}	16.33±1.52^{ba}
T-38	9.33±2.30^{f}	8.00±0.00^{j}	9.00±1.00^{h}	11.00±0.00^{c}	13.33±2.30^{fedg}	15.33±1.15^{bac}	7.33±0.57^{g}	16.00±0.00^{bc}	12.00±4.00^{dc}

Y-4, Y-5, Y-7, Y-13, Y-14, Y-16Y-17, Y-18 and Y-35 were Ergo lactic acid bacterial isolates; T-9, T-11,T-18,T-34, T-36, T-37and T-38 were Tej lacticacid bacterial isolate. Data are means ±standard deviations of the diameters of growth inhibition (mm) measured on the surface of Petri dish of pathogens culture. Means with same letter in the same column were not differ statistically (P>0.05)

Table 8: Test of growth inhibition on agar plates in anaerobic cultures of pathogens by well diffusion of LAB supernatants (NF and F)

Supernatant	***Salmonella*ss p10**	***Salmonella* ssp21**	***Salmonella*ss p22**	***Shigella* ssp12**	***Shigella* ssp14**	***Shigella* ssp19**	***E.coli*ssp7**	***E.coli*ssp27**	***E.coli*ssp.30**
NFS	14.07±2.57^{a}	13.39±2.94^{a}	12.91± 2.78^{a}	13.58 ± 2.81^{a}	13.79 ± 2.27^{a}	14.95 ±1.92^{a}	13.66 ± 3.18^{a}	13.52 ± 2.79^{a}	14.29± 2.65^{a}
FS	14.23± 2.60^{a}	13.56± 2.97^{a}	13.07± 2.80^{a}	13.75 ± 2.83^{a}	13.95 ± 2.27^{a}	14.79 ±2.86^{a}	13.83 ± 3.16^{a}	13.68 ± 2.80^{a}	14.45± 2.67^{a}

NFS=non filtered and FS =Filtered supernatant. Data are the mean ±standard deviations of the diameters of growth inhibition (mm) of none filtered and Filtered measured on the surface of Petri dish of pathogen cultures.Means with same letter in the same column are statistically similar (P>0.05).

4.1.7.2. Antagonistic effect of LAB against some antibiotic resistant food borne pathogens (Co-culturing)

The pH value of fresh milk at a time of inoculation of the isolate at 0hr was 6.84 and was progressively decreased as the fermentation proceeds. Analysis of variance result showed that the pH of *Ergo* fermented with probiotic LAB was highly significant ($p< 0.05$) at 24 and 48hrs of fermentation (Table 9). The pH values of *ergo* fermented with sixteen different probiotic LAB ranges from 4.70 by isolate T-38 to 4.02 by isolate Y-16 and T-34 at 24hrs of fermentation. During 48hrs of fermentation the pH value ranges from 4.38 by T-11 to 3.76 by Y-5 and relatively the lowest pH values was observed during 48hrs of fermentation. Among ergo product produced with LAB isolates the lower pH of 3.80 and 3.76 was observed by isolate Y-16 and Y-5 respectively at 48hrs of fermentation. The pH of each ergo feremented with the probiotic LAB was significantly ($p<0.05$) lower than the control fermented ergo at 24hrs and 48hrs of fermentation (Table 9). The initial titratable acidity of fresh milk at 0hrs was 0.21% and increased to 0.85 during fermentation of ergo at 48hrs. Titratable acidity of ergo ranged from 0.79% by Y-5 to 0.46% by Y-35 at 24hrs of fermentation. Relatively maximum titratable acidity of 0.85% by isolate Y-14 and the minimum titratable acidity of 0.49% were observed by Y-35 during 48hrs of fermentation of ergo. Titratable acidity of each product produced by different isolate was significantly ($P < 0.05$) higher than the titratable acidity of control fermented ergo at 24 and 48hrs. Other than isolate Y-35 the titratable of all products was significantly ($P<0.05$) higher than the titratable acidity of control fermented ergo at 24hrs (Table 9).

The detail of Table 8 indicates the antagonistic effect of each probiotic LAB against antibiotic resistant food borne pathogens when co-cultured in skim milk during fermentation of ergo. The mean initial (0hrs) counts of the separate test organisms (*Salmonella* ssp10, *Salmonella* ssp21, *Salmonella*ssp22, *Shiegella* ssp12, *Shigella* ssp14, *Shigella* ssp19, *E. coli* ssp7, *E. coli*ssp27 and *E .coli*ssp.30) of the challenge study was between 2.55 and 2.30 logcfu/ml. The reduction in the number of *Salmonella*spp10 co-cultured with each probiotic LAB isolate during the course of fermentation of ergo ranges between the highest 1.08logcfu/ml by isolate T-34 and the lowest 0.00logcfu/ml by isolate Y-7, Y-13, Y-16, T-37 and T-38 at 48hrs where as the maximum reduction in number of *salmonella* ssp10 was 1.11logcfu/ml by isolate Y-35 and 0.60logcfu/ml by isolate T-38 at 24hrs of fermentation. The reduction of *salmonella* ssp21 was between 0.78logcfu/ml by isolate Y-5 and 0.00logcfu/ml by isolate Y-18, Y-7, T-18 and T-34 at

48hrs.The maximum reduction in *Salmonella* ssp21 during the 24hrs of fermentation was between 2.00logcfu/ml by isolate T-9 and 1.30logcfu/ml by isolate Y-14.The reduction in the number of *Salmonella* ssp22 co-cultured with LAB isolate was between 1.43logcfu/ml by isolate Y-7 and 0.00logcfu/ml by isolate Y-5,Y-5,Y-14 and Y-18 at 48hrs of fermentation. During the 24hrs of fermentation the reduction of *Salmonella*ssp22 was between 1.96logcfu/ml and 0.60logcfu/ml. Reduction of the growth of *Salmonella* by each LAB isolate was significantly ($P< 0.05$) higher than the control fermented ergo at 24hrs and 48hrs ferementation (Table 9).

The antagonistic effects of each LAB co-cultured with *Shigella* spp12 during the course of fermentation of 48hrs was between 2.01logcfu/ml by isolate Y-5 and 0.00logcfu/ml by isolate Y-13, Y-16, T-37 and T-38. During the 24hrs of fermentation the number of *Shigella* ssp12 was reduced between 2.261logcfu/ml by isolate Y-4 and 1.00logcfu/ml by isolate T-38. Inhibition of *Shigella* spp14 co-cultured with LAB isolate during the course of fermentation of ergo was between 2.01logcfu/ml by isolate T-36 and 0.00logcfu/ml by isolate Y-7, Y-18, T-37 and T-38 at 48hrs. Maximum reduction of 2.21logcfu/ml by isolatesT-36 and the minimum reduction of 1.00logcfu/ml by isolate T-37 were observed on the number of *Shigella* spp14 co-cultured with isolate at 24hrs of fermentation. The Antagonistic effect of each isolate was significantly ($P<0.05$) higher than the controled fermented ergo on the growth of *Shigella* spp14 at 48hrs during the course of fermentation of ergo. The number of reduction in the number of *Shigella* ssp19 was between 2.01logcfu/ml by isolate Y-4 and 0.48logcfu/ml by isolate Y-5 during 48hrs of fermentation. During the 24hrs of fermentation the number of *Shigella* ssp19 was reduced between 2.41logcfu/ml by isolate Y-7 and 1.00logcfu/ml by isolate Y-16. Reduction of the growth of *Shigella* by each LAB isolate was significantly ($P< 0.05$) higher than the control fermented ergo at 24hrs and 48hrs ferementation (Table 9).

Analysis of variance result showed that *E.coli* co-cultured with each LAB isolate was noted being significantly ($P<0.05$) reduced during the course of fermentation of ergo at both 24hrs and 48hrs. Antagonistic effect of each isolates on the growth of *E.coli* was significantly ($P<0.05$) higher than the controlled fermented ergo at both 24hrs and 48hrs.The inhibition *E.coli* ssp7 co-cultured with each LAB isolate during the fermentation of ergo was between 2.00logcfu/ml by isolate Y-17 and 0.00logcfu/ml by isolate Y-35,T-9 and T-38. The reduction of *E.coli* ssp7 during the fermentation of 24hr was between 2.22logcfu/ml by isolate Y-17 and 1.46logcfu/ml

by isolate T-37.*E.coli* spp27 was reduced between 2.08logcfu/ml by isolate Y-7 and 0.48logcfu/ml by isolate T-9 during 48hrs of fermentation. LAB isolates reduced *E.coli* spp27 between 2.41logcfu/ml by isolate Y-7 and 2.36logcfu/ml by isolate Y-5 during 24hrs of fermentation. *E.coli* ssp30 was reduced between 1.25logcfu/ml by isolate T-38 and 0.48logcfu/ml by isolate Y-13, Y-35 and T-36 at 48hrs of fermentation. During 24hrs of fermentation *E.coli* ssp30 was reduced between 2.42logcfu/ml by isolate Y-14 and 1.32logcfu/ml by isolate T-38. Reduction of growth of *E.coli*ssp30 by each LAB isolatewas significantly ($P < 0.05$) higher than the control fermented ergo at 24hrs and 48hrs (Table 9).

Generally each LAB isolates co-cultured separately with each pathogens showed antagonistic activity against pathogens. The Antagonistic effect of each LAB isolates on the growth of each pathogens was significantly ($P<0.05$) higher than the controlled fermented ergo at both 24hrs and 48hrs of fermentation (Table 9).

Table 9: Antagonistic effect of each isolate against resistant food borne pathogens (co-culturing of LAB with pathogens)

LAB isolate	Time (In hrs.)	pH	TA	Pathogens								
				***Salmonella* ssp10**	***Salmonella* ssp21**	***Salmonella* ssp22**	***Shiegella* ssp12**	***Shigella* ssp14**	***Shigella* ssp19**	***E.coli*ssp7**	***E.coli* ssp27**	***E.coli* ssp.30**
		Mean ±SD	**Mean ±SD**	**Mean ±SD (Log cfu/ml)**	**Mean ±SD (Log cfu/ml)**	**Mean ±SD (Log cfu/ml)**	**Mean ±SD (Log cfu/ml)**	**Mean ±SD (Log cfu/ml)**	**Mean ±SD (Log cfu/ml)**	**Mean ±SD (Log cfu/ml)**	**Mean ±SD (Log cfu/ml)**	**Mean ±SD (Log cfu/ml)**
M1	0hr	6.84±0.00^{a}	0.21±0.00^{a}	2.40±0.00^{b}	2.39±0.00^{b}	2.39±0.00^{b}	2.30±0.00^{b}	2.33±0.00^{ba}	2.31±0.00^{d}	2.55±0.00^{a}	2.51±0.00^{a}	2.54±0.01^{a}
Y-4	24hr	4.54±0.00^{f}	0.68±0.00^{j}	1.83±0.00^{j}	1.48±0.00^{r}	1.96±0.00^{f}	2.26±0.00^{d}	2.27±0.00^{ba}	2.21±0.00^{f}	2.08±0.00^{g}	2.41±0.00^{d}	2.40±0.00^{e}
	48hr	4.16±0.00^{p}	0.80±0.00^{d}	1.43±0.00^{r}	1.30±0.00^{u}	0.00±0.00^{a}	1.69±0.00^{q}	1.68±0.00^{fgh}	2.01±0.00^{h}	1.78±0.00^{p}	1.95±0.00^{r}	2.07±0.00^{i}
Y-5	24hr	4.22±0.00^{k}	0.79±0.00^{e}	1.73±0.00^{k}	1.70±0.00^{n}	1.79±0.00^{k}	2.21±0.00^{f}	1.69±0.00^{fgh}	1.96±0.00^{i}	2.04±0.00^{j}	2.36±0.00^{f}	2.36±0.00^{f}
	48hr	3.76±0.00^{g}	0.82±0.00^{b}	1.34±0.00^{t}	0.78±0.00^{y}	0.00±0.00^{a}	2.01±0.00^{h}	1.34±0.00j^{k}	0.48±0.00^{x}	1.69±0.00^{q}	1.82±0.00^{u}	1.82±0.00^{r}
Y-7	24hr	4.12±0.00^{s}	0.50±0.00^{v}	1.96±0.00^{d}	1.79±0.00^{k}	1.83±0.00^{i}	1.96±0.00^{j}	1.71±0.00^{fhg}	2.41±0.00^{b}	1.42±0.00^{z}	2.41±0.00^{d}	2.21±0.00^{h}
	48hr	3.93±0.00^{c}	0.51±0.00^{u}	0.00±0.00^{e}	0.00±0.00^{d}	1.43±0.00^{p}	0.48±0.00^{i}	0.00±0.00^{n}	1.95±0.00^{j}	1.04±0.00^{f}	2.08±0.00^{l}	2.01±0.00^{k}
Y-13	24hr	4.19±0.00^{n}	0.50±0.00^{v}	1.78±0.00^{j}	2.24±0.00^{d}	1.73±0.00^{l}	1.79±0.00^{m}	2.25±0.00^{bac}	2.36±0.00^{c}	1.52±0.00^{w}	2.18±0.00^{k}	1.96±0.00^{m}
	48hr	4.01±0.0^{w}	0.51±0.00^{u}	0.00±0.00^{e}	1.63±0.00^{o}	1.34±0.00^{r}	0.00±0.00^{k}	1.69±0.00^{fhg}	1.82±0.00^{n}	0.00±0.00^{l}	1.90±0.00^{s}	0.48±0.00^{d}
Y-14	24hr	4.20±0.00^{m}	0.76±0.00^{g}	1.87±0.00^{f}	1.30±0.00^{u}	1.48±0.00^{o}	2.24±0.00^{e}	2.21±0.00^{bdac}	1.88±0.00^{m}	2.19±0.00^{e}	2.38±0.00^{e}	2.42±0.00^{d}
	48hr	4.18±0.00^{o}	0.85±0.00^{a}	1.43±0.00^{r}	0.30±0.00^{c}	0.00±0.00^{a}	1.63±0.00^{t}	2.01±0.00^{bdec}	1.43±0.00^{q}	1.90±0.00^{n}	2.06±0.00^{m}	1.95±0.00^{n}
Y-16	24hr	4.02±0.00^{v}	0.58±0.00^{q}	1.00±0.00^{z}	1.80±0.00^{j}	1.04±0.00^{v}	2.04±0.00^{g}	2.21±0.00^{bdac}	1.00±0.00^{u}	1.04±0.00^{f}	2.24±0.00^{h}	2.36±0.00^{f}
	48hr	3.80±0.00^{f}	0.59±0.00^{p}	0.00±0.00^{e}	0.60±0.00^{a}	0.78±0.00^{x}	0.00±0.00^{k}	2.01±0.00^{bdec}	0.00±0.00^{z}	0.69±0.00^{j}	1.63±0.00^{x}	1.81±0.00^{r}
Y-17	24hr	4.35±0.00^{i}	0.68±0.00^{i}	1.47±0.00^{p}	1.70±0.00^{n}	1.88±0.00^{h}	2.27±0.00^{c}	1.96±0.00^{fdec}	2.24±0.00^{e}	2.22±0.00^{d}	2.04±0.00^{n}	2.40 ±0.00^{e}
	48hr	3.95±0.00^{a}	0.80±0.00^{d}	1.30±0.00^{v}	1.34±0.00^{t}	1.43±0.00^{p}	1.68±0.00^{r}	0.48±0.00^{m}	1.63±0.00^{p}	2.00±0.00^{k}	0.00±0.00^{g}	2.07±0.00^{i}
Y-18	24hr	4.13±0.00^{r}	0.47±0.00^{x}	1.69±0.00^{l}	1.71±0.00^{n}	1.00±0.00^{w}	1.70±0.00^{p}	1.96±0.00^{fdec}	1.30±0.00^{s}	1.68±0.00^{r}	2.22±0.00^{i}	2.21±0.00^{g}
	48hr	3.98±0.00^{z}	0.53±0.00^{t}	0.77±0.00^{b}	0.00±0.00^{d}	0.00±0.00^{a}	1.34±0.00^{z}	0.00±0.00^{n}	0.30±0.00^{y}	1.30±0.00^{d}	2.00±0.00^{p}	2.00±0.00^{l}

Y-35	24hr	4.24±0.00^{j}	0.46±0.00^{y}	1.11±0.00^{w}	1.04±0.00^{w}	1.96±0.00^{f}	1.23±0.00^{b}	1.96±0.00^{fdec}	1.93±0.00^{k}	0.00±0.00^{l}	2.27±0.00^{g}	1.96±0.00^{m}
	48hr	4.10±0.00^{t}	0.49±0.00^{w}	0.85±0.00^{a}	0.70±0.00^{z}	0.48±0.00^{z}	1.11±0.00^{c}	0.92±0.00^{l}	1.40±0.00^{r}	0.00±0.00^{l}	1.68±0.00^{w}	0.47±0.00^{d}
T-9	24hr	4.05±0.00^{u}	0.57±0.00^{r}	1.82±0.00^{h}	1.96±0.00^{g}	1.62±0.00^{m}	1.65±0.00^{s}	1.75±0.00^{fheg}	1.93±0.00^{k}	1.25±0.00^{e}	1.96±0.00^{q}	1.82±0.00^{r}
	48hr	3.84±0.00^{e}	0.58±0.00^{q}	1.30±0.00^{v}	0.48±0.00^{b}	1.08±0.00^{u}	1.30±0.00^{a}	1.64±0.00^{hig}	1.40±0.00^{r}	0.00±0.00^{l}	0.48±0.00^{f}	1.30±0.00^{z}
T-11	24hr	4.61±0.00^{e}	0.66±0.00^{l}	1.30±0.00^{v}	2.00±0.00^{f}	1.93±0.00^{g}	1.90±0.00^{k}	1.69±0.00^{fhg}	1.88±0.00^{m}	1.85±0.00^{o}	2.41±0.00^{d}	2.04±0.00^{j}
	48hr	4.38±0.00^{h}	0.80±0.00^{d}	0.30±0.00^{d}	0.60±0.00^{a}	1.40±0.00^{q}	0.60±0.00^{h}	1.34±0.00^{jk}	1.43±0.00^{q}	0.95±0.00^{g}	2.08±0.00^{l}	1.46±0.00^{v}
T-18	24hr	4.22±0.00^{k}	0.79±0.00^{e}	1.32±0.00^{u}	1.72±0.00^{l}	1.57±0.00^{n}	1.83±0.00^{l}	1.96±0.00^{fdec}	1.93±0.00^{k}	2.04±0.00^{i}	1.96±0.00^{q}	2.21±0.00^{h}
	48hr	3.93±0.00^{c}	0.81±0.00^{c}	0.60±0.00^{c}	0.00±0.00^{d}	1.00±0.00^{w}	1.40±0.00^{y}	0.47±0.00^{m}	1.40±0.00^{r}	1.46±0.00^{y}	0.48±0.00^{f}	2.01±0.00^{k}
T-34	24hr	4.02±0.00^{v}	0.58±0.00^{q}	1.62±0.00^{m}	1.00±0.00^{x}	1.32±0.00^{s}	1.62±0.00^{u}	1.96±0.00^{fdec}	2.19±0.00^{g}	1.96±0.00^{l}	2.00±0.00^{p}	1.96±0.00^{m}
	48hr	3.80±0.00^{f}	0.59±0.00^{p}	1.08±0.00^{x}	0.00±0.00^{d}	0.60±0.00^{y}	0.70±0.00^{g}	0.00±0.00^{n}	1.90±0.00^{l}	1.38±0.00^{b}	0.60±0.00^{e}	0.47±0.00^{d}
T-36	24hr	4.05±0.00^{u}	0.57±0.00^{r}	1.57±0.00^{n}	2.00±0.00^{f}	1.00±0.00^{w}	1.08±0.00^{d}	2.21±0.00^{bdac}	1.82±0.00^{n}	2.08±0.00^{h}	2.21±0.00^{j}	1.46±0.00^{v}
	48hr	3.88±0.00^{d}	0.58±0.00^{q}	1.00±0.00^{z}	0.60±0.00^{a}	0.00±0.00^{a}	0.78±0.00^{f}	2.01±0.00^{bdec}	1.30±0.00^{s}	1.39±0.00^{a}	2.01±0.00^{o}	0.47±0.00^{d}
T-37	24hr	4.70±0.00^{d}	0.60±0.00^{o}	0.00±0.00^{e}	1.83±0.00^{i}	1.93±0.00^{g}	1.72±0.00^{n}	1.00±0.00^{l}	1.04±0.00^{t}	1.46±0.00^{y}	1.08±0.00^{c}	1.90±0.00^{p}
	48hr	4.24±0.00^{j}	0.64±0.00^{m}	0.00±0.00^{e}	1.40±0.00^{s}	1.40±0.00^{q}	0.00±0.00^{k}	0.00±0.00^{n}	0.70±0.00^{v}	0.48±0.00^{k}	0.78±0.00^{d}	0.78±0.00^{b}
T-38	24hr	4.70±0.00^{d}	0.68±0.00^{j}	0.60±0.00^{c}	1.62±0.00^{p}	1.88±0.00^{h}	1.00±0.00^{e}	1.08±0.00^{lk}	1.93±0.00^{k}	1.55±0.00^{v}	1.83±0.00^{t}	1.32±0.00^{y}
	48hr	4.24±0.00^{j}	0.78±0.00^{f}	0.00±0.00^{e}	0.70±0.00^{z}	1.43±0.00^{p}	0.00±0.00^{k}	0.00±0.00^{n}	1.40±0.00^{r}	0.00±0.00^{l}	1.40±0.00^{z}	1.25±0.00^{a}
Cont.	24hr	5.16±0.00^{b}	0.45±0.00^{z}	2.39±0.00^{a}	2.40±0.00^{a}	2.40±0.00^{a}	2.48±0.00^{a}	2.48±0.00^{a}	2.48±0.00^{a}	2.44±0.00^{c}	2.45±0.00^{c}	2.45±0.00^{c}
	48hr	4.99±0.00^{c}	0.46±0.00^{y}	2.38±0.00^{c}	2.38±0.00^{c}	2.38±0.00^{c}	2.48±0.00^{a}	2.48±0.00^{a}	2.48±0.00^{a}	2.41±0.00^{d}	2.42±0.00^{d}	2.45±0.00^{c}

All values are mean ± SD in triplicate; Values in the same column carrying the same superscript are not significantly different; Control = controled fermented ergo; 0hrs= fresh pasteurized milk, M1= fresh raw milk before inoculation; Y-4, Y-5, Y-7, Y-13, Y-14, Y-16Y-17, Y-18 and Y-35 were Ergo lactic acid bacterial isolates; T-9,T-11, T-18,T-34, T-36, T-37and T-38 were Tej lactic acid bacterial isolate. TA= Titrable acidity.

4.2. DISCUSSION

In this study, 40 samples of traditionally fermented products were collected and analyzed for the presence of probiotic LAB. The mean pH values of *Ergo* and *Tej* samples were 4.13 and 3.27, respectively.Variations in pH values within the samples of *Ergo* and *Tej* were significantly different. The difference in the pH values could be due to the duration of fermentation and type of microorganisms involved in fermentation. In the present study the pH value of *Ergo* was lower than the one reported by Almaz (2001) where the relative low pH value of ergo was ranging from 4.3 to 4.5. The difference in the pH value of the present study and other related studies could be due to the duration of fermentation or type of microorganisms involved during fermentation as the samples were collected from different local *Ergo* producers in open markets. Bekele *et al*. (2006) reported that the pH value of tej was < 4 which were similar result with the present study.

In this study a total of sixteen (16) probiotic LAB were isolated based on their probiotic attributes, stater culture attributes and their antagonistic effect against antibiotics resistant food borne pathogens. The screened isolate were found to have high tolerance to low pH (2.0, 2.5 and 3.0) for 6hrs of incubation. Among the screened LAB isolate T-9 showed best tolerance to pH 2.0, 2.5 and 3.0 followed by isolate Y-14 and T-37. The result indicated that the isolate could survive in acidic stomach environments and could reach the small intestine after being ingested. 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 6hrs of incubation. The acid tolerance might be due to homeostatic response or through synthesis of acid shock protein (Osman *et al*., 2016). The screened probiotic LAB were also found to show tolerance to 0.3%, 0.5% and 1% of bile salt concentrations for 6hrs of incubation. Among probiotic LAB, isolate Y-35 showed best tolerance to 1% bile followed by isolate Y-4 and T-36. The LAB isolates of this study were able to survive 1% bile salt concentration for 6hrs at survival rate ranging from 88.27% to 51.81%. This result is also in agreement with Damayanti, (2014) report, that lactic acid bacteria isolated from proventriculus of broiler chicken having more than 100% tolerance to bile salt of 0.3% for 2hrs. Tolerance to bile salts were 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%. 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).

This study also conducted the acidifying activity and compatible test, in order to formulate a starter culture and the study found that each isolate of probiotic LAB were found to be compatible. This property may increase their antagonistic potential when mixed as starter culture in different proportion and may have synergistic effect against resistant food borne pathogens. The lower pH of 3.80 and 3.76 was observed by isolate Y-16 and Y-5 respectively at 48hrs of fermentation. The pH of each ergo feremented with the LAB isolates was significantly ($p<0.05$) lower than the control fermented ergo at 24hrs and 48hrs of fermentation. The pH of the products was observed decreasing (become more acidic) and titratable acidity of the product was observed increasing as the time of fermentation extended from 24hrs to 48hrs. Changes in Titratable acidity and pH of the product were due to fermentation of lactose in the milk by the probiotic LAB. These two parameters with other metabolites enhance the antagonistic effect of isolates on food borne pathogens (Pundir *et al*., 2013).

In this study, the antimicrobial activity of the cell free supernatant of sixteen (16) probiotic LAB against antibiotics resistant pathogens was evaluated. The obtained results revealed that the whole probiotic LAB showed a growth inhibition against each nine antibiotics resistant pathogens a diameter equal or greater than 7mm in well diffusion assay. There was a significant difference in inhibition assay of supernatant obtained from LAB isolates against *Salmonella* ssp., *Shigella* spp and *E.coli* spp. The detail result showed isolate Y-7 and Y- 4 showed a strong growth inhibition of 18.00mm against *Salmonella* spp. There was also a significant difference in inhibition assay of supernatant obtained from LAB isolates against *Salmonella* spp. The antimicrobial activity of the cell free supernatant of sixteen (16) probiotic LAB isolates against *Shigella* spp. showed inhibitions of a diamerer equal or greater than > 8mm. There is also a significant difference in inhibition assay of supernatant obtained from LAB isolates against the *Shigella* spp. The antimicrobial activity of the cell free supernatant of sixteen (16) probiotic LAB isolates against *E.coli* showed inhibitions of a diameter equal or greater than > 7mm. Significant

difference also observed in inhibition assay of supernatant obtained from LAB isolates against *E.coli* spp. The result of this study revealed that the inhibitions of probiotic LAB against antibiotics resitant food borne pathogens were greater than that reported by Arias *et al* (2013) in which the inhibition of probiotic LAB on anaerobic solid cultures of antibiotics resistant pathogenic strains, showed a growth inhibition of a diameter equal or greater than 5 mm. Similarly Afolayan *et al.,* (2017) reported that the cell free supernatant of LAB isolated from traditionally fermented Ogi had antagonistic activity against antibiotics resistant *Salmonella, Shigella* and *E.coli*.

The inhibition of both types of supernatants (F and NF) were not statically different (P>0.05) and they both inhibit the growth and survival of the pathogens. This result confirmed that the inhibition effect was caused by a compounds secreted by probiotic LAB and not by the remaining cells in the supernatant.The inhibition could be due to metabolites like bacteriocin and other bioactive comounds such as lactic, acetic acids and hydrogen peroxide.Several studies reported that LAB produces a wide range of antibacterial compounds, including sugar catabolites suchasorganicacids, Lacticacid and aceticacid); oxygen catabolites such as hydrogen peroxide; andproteinaceous compounds such as bacteriocins (Servin 2004, Valerio *et al.;* 2004, Canani *et al.;* 2007).

In this study, each probiotic LAB co-cultured with each pathogens in fermented milk showed antagonistic effect against the pathogens. Probiotic LAB co-cultured with antibiotic resistant pathogens showed an inhibitory activity both at 24hrs and 48hrs of fermentation.The best inhibitory effect of the probiotic isolates against nine (three of each *Salmonella* spp., *Shigella* spp. and *E.coli* ssp) pathogens was shown by isolate Y-13, Y-14, Y-18, Y-35, T-9, T-18, T-34, T-36, T-37 and T-38 during the course of fermentation of *ergo* at 48hrs. The reduction in the number of growth of pathogens by each LAB was significantly (P< 0.05) higher than the control fermented *Ergo* at 24hrs and 48hrs of fermentation. Also, the inhibition of pathogens was significantly different for each probiotic LAB co-cultured with the pathogens. During 24hrs of fermentation the maximum reduction of *Salmonella* ssp. count was 0.60logcfu/ml. Similarly Kailasarapathy and Chinn (2000) demonstrated the antagonism of 15 *Bifidobacterium* strains (*B. animalis*, *B. globosum* and *B. breve*) against six *Salmonella* strains (*Salmonella enteritidis* and *Salmonella Typhimurium*). They found that all strains of *Bifidobacterium* were effectively

antagonistic against *Salmonella* strains. The antagonistic effects of each probiotic LAB against *Shigella* spp. showed variation between 2.01logcfu/ml to 0.00logcfu/ml at 48hrs of fermentation. The best inhibition of *E.coli* co-cultured with each LAB isolate during the fermentation of ergo was shown at 48hrs of fermentation. Afolayan *et al* (2017) demonstrated that *W. paramesenteroides* AFN004, *L. fermentum* AFN018, and *L. plantarum* AFN021, which have been isolated from uncooked Ogi effectively inhibited the growth of *Shigella* spp ,*Salmonella* spp. and *E.coli* either when inoculated after 8 hours and 24 hours of growth of pathogen or when cultured overnight and then incubated with the pathogens. The best reduction of *E.coli* ssp7 during the fermentation of 24hr was shown by isolate T-37. *E.coli* spp27 was reduced best by isolate T-9 during 48hrs of fermentation. Probiotic LAB isolates reduced *E.coli* spp27 by isolate Y-5 during 24hrs of fermentation. Similarly *E.coli* ssp30 was best reduced by isolate Y-13, Y-35 and T-36 at 48hrs of fermentation. During 24hrs of fermentation *E.coli* ssp30 was best reduced by isolate T-38. Arias *et al*. (2013) reported the probiotic LAB co-cultured with *E. coli* O157:H7 found effective in inhibiting the growth of *E. coli* O157:H7.

5. CONCLUSION

In this study a total of sixteen (16) probiotic LAB were isolated and characterized from traditionally fermented products and they were found to have better tolerance to low pH and high bile salt concentration.This study demonstrated that lactic acid bacterial isolatesY-13, Y-14, Y-18, Y-35, T-9, T-18, T-34, T-36, T-37 and T-38 have better probiotic and antagonistic effect against some antibiotics resistant food borne pathogens. Therefore, they are capable of surviving and colonizing the host stomach. The isolate were also capable of reducing or inhibiting the growth of antibiotic resistant food borne pathogens such as *E. coli*, *Salmonella* and *Shigella* during the course of fermentation of *ergo*. Therefore, the result of this study indicates that the use of these isolate cultures were able to reduce or inhibit the growth of antibiotic resistant food borne pathogens. Moreover, ergo fermented with these isolates can be a vehicles for provisions of potential health promoting probiotics.

6. RECOMMENDATION

From the results obtained in this study the following recommendations were forwarded:-

- Antibiotics disturb the normal functioning of micro flora and pathogenic microorganisms are developing resistance to antibiotics. Beside clinical therapy is often accompanied by unwanted side effects of using multi antibiotics therefore, probiotic LAB of this study can be an alternative way of controlling and treatment agent of antibiotics resistant pathogens.
- The result of the present study will also contribute to the future applications and utilization of probiotic starter cultures in the dairy industry.
- The information obtained from this study will serve as a foundation to establish small scale production of probiotic *ergo* for therapeutic purpose.
- Fermented products are rich in probiotic bacteria so commsuming fermented *Ergo* is preferable than consuming raw milk as fermented ergo provide many health benefits such as anti-microbial activity.
- There is a need of further studies on the formulation of probiotic Starter Culture and sensory acceptances of ergo.

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8. APPENDICES

Appendix 1: ANOVA table of *Ego* pH

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
pH					
Product	19	1.40990	0.074205	107.03	0.000
Experimental error	40	0.02773	0.000693		
Total	59	1.43763			

Appendix 2: ANOVA table of *Tej* pH

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
pH					
Product	19	1.09454	0.057607	127.08	0.000
Experimental error	40	0.01813	0.000453		
Total	59	1.11267			

Appendix 3: ANOVA table of *Ergo* and *Tej* pH comparison

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
pH					
Product	1	22.154	22.1536	1025.02	0.000
Experimental error	118	2.550	0.0216		
Total	119	24.704			

Appendix 4: Biochemical characterizations of pathogens

Pathogens	Characteristics										
	Gram stain	Catalase	Citrate	Glucose	Sucrose	Lactose	H_2S	Gas	Sorbitol	Fructose	Xylose
***Salmonella* ssp10**	-ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve	+ve	-ve
***Salmonella* ssp21**	-ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve	-ve	+ve	-ve
***Salmonella* ssp22**	-ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve	+ve	-ve
***Shigella* ssp12**	-ve	+ve	-ve	+ve	-ve	-ve	-ve	-ve	+ve	+ve	+ve
***Shigella* ssp14**	-ve	+ve	-ve	+ve	-ve	-ve	-ve	-ve	+ve	+ve	+ve
***Shigella* ssp19**	-ve	+ve	-ve	+ve	-ve	-ve	-ve	-ve	+ve	+ve	+ve
***E.coli* ssp7**	-ve	+ve	-ve	+ve	+ve	+ve	-ve	+ve	+ve	+ve	+ve
***E.coli* ssp27**	-ve	+ve	-ve	+ve	+ve	+ve	-ve	+ve	+ve	+ve	+ve
***E.coli* ssp30**	-ve	+ve	-ve	+ve	+ve	+ve	-ve	+ve	+ve	+ve	+ve

+ = reaction, - = no reaction

Appendix 5: CLSI break points for *Entrobactericeae* available for these antimicrobial

Antimicrobial	Disc code	Potency	Susceptible (mm)	Intermediate (mm)	Resistant (mm)
Ampicillin	AMP	10µg	≥17	14 –16	≤13
Cefriaxone	CRO	30µg	≥23	20–22	≤19
Ciprofoxacin	CIP	5µg	≥21	20–22	≤20
Chloramphenicol	CHL	30µg	≥18	13–17	≤12
Gentamicin	GM	10µg	≥15	13–14	≤12
Sulfamethoxazole-trimethoprim	SXT-TMP	25µg	≥16	11–15	≤10
Nalicidic acid	NA	30µg	≥18	13–17	≤13
Norfoxacin	f	10µg	≥17	13 –16	≤12

Appendix 6: Acidifying capability of LAB isolates

LAB Isolate	Time (In hour)	PH	TA
		Mean±SD	Mean±SD
	6Hr	6.27± 0.00^{f}	0.15±0.00^{v}
Y-4	9Hr	6.02±0.00^{q}	0.18±0.00^{s}
	12Hr	4.55±0.00^{q}	0.75±0.00^{g}
	6Hr	6.79±0.00^{b}	0.13±0.00^{x}
Y-5	9Hr	6.03±0.00^{n}	0.16±0.00^{u}
	12Hr	4.58±0.00^{l}	0.75±0.00^{g}
	6Hr	6.17± 0.00^{s}	0.18±0.00^{s}
Y-7	9Hr	5.75± 0.00^{c}	0.31±0.00^{j}
	12Hr	4.60±0.00^{k}	0.68±0.00^{e}
	6Hr	6.79±0.00^{b}	0.15±0.00^{v}
Y-13	9Hr	6.04±0.00^{v}	0.20±0.00^{q}
	12Hr	4.02±0.00^{s}	0.81±0.00^{b}
	6Hr	6.73±0.00^{d}	0.14±0.00^{w}
Y-14	9Hr	4.70 ±0.00^{y}	0.17±0.00^{t}
	12Hr	4.30±0.00^{w}	0.78±0.00^{e}
	6Hr	6.79±0.00^{b}	0.12±0.00^{y}
Y-16	9Hr	6.26±0.00^{r}	0.17±0.00^{t}
	12Hr	4.57±0.00^{i}	0.71±0.00^{j}
	6Hr	6.79±0.00^{b}	0.14±0.00^{w}
Y-17	9Hr	6.02±0.00^{w}	0.21±0.00^{p}
	12Hr	4.61±0.00^{l}	0.36±0.00^{f}
	6Hr	6.79±0.00^{b}	0.12±0.00^{y}
Y-18	9Hr	5.81±0.00^{a}	0.22±0.00^{o}
	12Hr	4.55±0.00^{l}	0.33±0.00^{h}
	6Hr	6.79± 0.00^{b}	0.15±0.00^{v}
Y-35	9Hr	4.67±0.00^{g}	0.29±0.00^{k}
	12Hr	4.30±0.00^{y}	0.89±0.00^{c}
	6Hr	6.79±0.00^{b}	0.12±0.00^{y}
T-9	9Hr	6.31±0.00^{p}	0.17±0.00^{t}
	12Hr	5.70±0.00^{e}	0.32± 0.00^{i}
	6Hr	6.60±0.00^{h}	0.14±0.00^{w}
T-11	9Hr	4.80±0.00^{b}	0.16±0.00^{u}
	12Hr	4.08±0.00^{x}	0.81±0.00^{a}
	6Hr	6.70± 0.00^{e}	0.14±0.00^{w}
T-18	9Hr	6.52±0.00^{k}	0.15±0.00^{v}
	12Hr	4.61 ±0.00^{d}	0.29± 0.00^{k}
	6Hr	6.79±0.00^{b}	0.12±0.00^{y}
T-34	9Hr	6.35±0.00^{o}	0.19± 0.00^{r}
	12Hr	4.50±0.00^{v}	0.76±0.00^{f}
	6Hr	6.79±0.00^{b}	0.13±0.00^{x}

T-36	9Hr	6.16± 0.00^{t}	0.19± 0.00^{r}
	12Hr	4.52±0.00^{r}	0.33± 0.00^{h}
	6Hr	6.79±0.00^{b}	0.12±0.00^{y}
T-37	9Hr	6.56± 0.00^{j}	0.15±0.00^{v}
	12Hr	4.30± 0.00^{w}	0.38± 0.00^{e}
	6Hr	6.73±0.00^{d}	0.14±0.00^{w}
T-38	9Hr	5.84± 0.00^{z}	0.23±0.00^{n}
	12Hr	4.55±0.00^{j}	0.33± 0.00^{h}
	6Hr	6.80± 0.00^{a}	0.11±0.00^{z}
Control	9Hr	6.79±0.00^{b}	0.14 ±0.00^{w}
	12Hr	6.26±0.00^{r}	0.23 ± 0.00^{n}

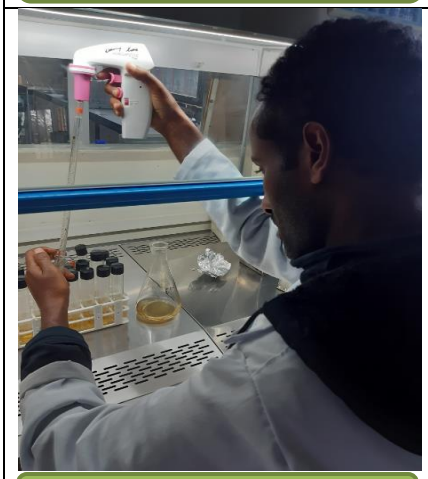
Y-4, Y-5, Y-7, Y-13, Y-14, Y-16Y-17, Y-18 and Y-35 were Ergo lactic acid bacterial isolates; T-9, T-11 T-18, T-34, T-36T-37and T-38 was Tej lactic acid bacteria isolate. All values are mean ± SD in triplicate; Values in the same column carrying the same superscript are not significantly different; S.D=standard deviation.

Appendix 7: Pictures during the Laboratory work

Sample collection

pH test

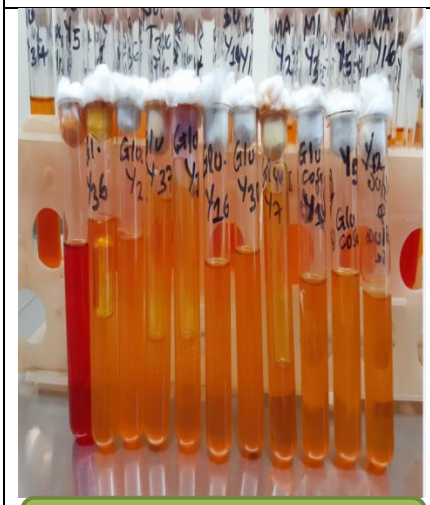
Anaerobic jar



Serial dilusion

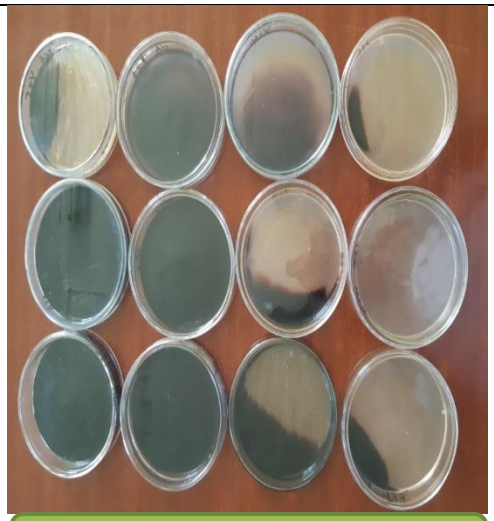
Purification of LAB

Morphological identification of LAB



LAB Sugar fermentation test

Gram staining

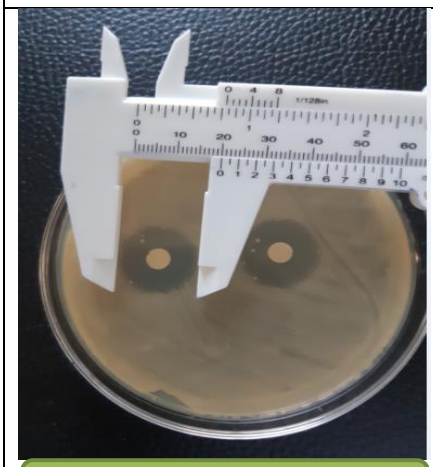


LAB Starch hydrolisis test

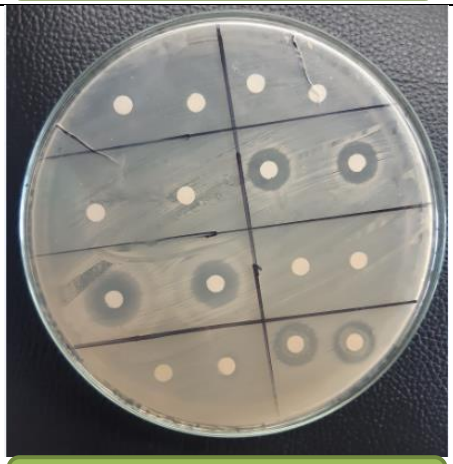
LAB Gram staining

LAB isolates

Pathogens Antibiotic susceptability test



Inhibition zone measurment



Disk diffussion

LAB Suparnatant preparation

Well diffussion assay

Colony count

Co-culture & TA