ISOLATION AND CHARACTERIZATION OF POTENTIAL PROBIOTIC LACTIC ACID BACTERIA FROM THE GASTROINTESTINAL TRACT (GIT) OF POULTRY

MSc THESIS

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I hereby certify that I have read and evaluated this thesis entitled Isolation and Characterization of Potential Probiotic Lactic Acid Bacteria from the Gastrointestinal Tract (GIT) of Poultry prepared under my guidance by Ashenafi Teklay. I recommend that it be submitted as fulfilling the thesis requirement.

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BIOGRAPHICAL SKETCH

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ACRONYMS AND ABBREVIATIONS

BP	Base pair
CFU	Colony forming unit
MRS	De Man, Rogosa, and Sharpe
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamine tetra acetic acid
FAO	Food and Agriculture Organization
GIT	Gastrointestinal tract
LAB	Lactic acid bacteria
OD	Optical density
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
RFLP	Restriction fragment length polymorphism
rRNA	Ribosomal ribonucleic acid
SDS	Sodium dodecyl sulfate
SPSS	Statistical Package for Social Science
TAE	Tris acetate EDTA
TE	Tris EDTA
WHO	World Health Organization

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ABSTRACT

Lactic acid bacteria are one of the most important groups of microorganisms useful in animal and human health as probiotics. Probiotics are the health promoting living microorganisms and produces antimicrobial compounds such as organic acids, H_2O_2 and bacteriocins. Now a day, the use of antibiotics as growth promoters is questionable. In this case, probiotics have a potential to solve this problem as one of the alternatives to antibiotic growth promoters. The aim of this study was to isolate LAB from GIT of poultry and examine their potential uses as probiotic strains in vitro. For this purpose, 20 samples were collected from 4 parts of GIT obtained from 5 poultry (hens). In the present study physiological, biochemical, probiotic property and their safety status were done. Isolates were identified using molecular and Biolog methods at genus and species level respectively. In the present result, a total of 104 LAB isolates were isolated and screened for their antimicrobial activity against Escherichia coli ATCC25922, Salmonella typhimurium ATCC13311 and Staphylococcus aureus ATCC25923. Out of the 104, ten isolates of LAB were selected according to their levels of antimicrobial activities for further analysis of physiological, biochemical, probiotic and molecular characteristics. The results indicates that selected LAB isolates were exhibited excellent potential probiotic characteristics including homofermentative metabolic activity, ability to ferment different sugars, tolerance to different temperatures and salt concentrations. In addition, the hydrophobicity test indicated that some of the isolates exhibited higher percent (51-100%) of hydrophobicity towards chloroform. Furthermore, identification at molecular level was shown that the isolates belonged to the group of LAB with genera specific primers that produced a PCR product of about 350 bp. Finally, LAB isolates were identified at species level using Biolog method. In general, these selected LAB isolates have beneficial probiotic properties that make them safe to use in different applications. This study provides baseline information for further investigations of LAB isolates in vivo.

Key words: Antimicrobial activity, Biolog, Lactic acid bacteria, Poultry, Probiotic

1. INTRODUCTION

Lactic acid bacteria are a broad group of gram positive, non-spore forming, catalase negative, anaerobic or facultative aerobic cocci or rods and produce lactic acid as one of the main fermentation products of the metabolism of carbohydrates (Quinto *et al*., 2014). They are the most widespread in foods (dairy products, fermented meat, vegetable, sour dough and silage or animal feed), soil, beverages (including wine), in plants, sewage, in the genital and gastrointestinal tract (GIT) of humans and animals and also makeup the intestinal micro biota of humans and animals (Shakoor *et al*., 2017; Wambugu, 2015; Estifanos, 2016). Lactic acid bacteria can also ferment different carbohydrates and generate lactic acid that has a beneficial effect on formation of texture, aroma and flavor in different milk products (Forhad *et al*., 2015). Lactic acid bacteria have been well known for thousands of years for their important role in the food industry due to their fermentative ability (Torshizi *et al.,* 2008). In addition, lactic acid bacteria have been used as food and feed preservatives for thousands of years, and they are used to produce bacteriocins that could replace chemical preservatives to protect bacterial spoilage and the growth of pathogenic bacteria in food products (Chen *et al*., 2010).

Lactic acid bacteria have been receiving recently a considerable attention as probiotics because of their innate ability to exert antimicrobial activity against pathogenic and spoilage organisms (Chowdhury and Islam, 2016). A probiotic is defined as "a live microbial feed supplement, which beneficially affects the host animal by improving its normal flora and keeping the balance of the intestinal flora" (Shokryazdan *et al*., 2014). Some of the physiological importance of probiotic organisms includes removal of carcinogens, lowering of plasma cholesterol, immune stimulation and allergy lowering effect, synthesis and enhancing the bioavailability of nutrients and alleviation of lactose intolerance (Shuhadha *et al*., 2017). Most probiotic organisms are LAB which comprise of a wide range of genera including *Lactobacillus*, *Bifidobacterium*, *Streptococcus*, *Lactococcus* and *Enterococcus* (Gogineni *et al*., 2013). These bacteria are the main components of the starter culture used in fermentations and exert a beneficial effect by inhibiting the growth of pathogenic bacteria such as *E.coli* O157: H7, *Salmonella* sp and *Staphylococcus aureus*. These probiotic bacteria are also found normally in the gastrointestinal tract of poultry species (Jannah *et al.,* 2014; Shuhadha *et al*., 2017). Within the LAB group, the genus *Lactobacillus* is the most widely employed as

probiotics, because it displays numerous antimicrobial activities. This is mainly due to the production of antimicrobial metabolites including organic acids, hydrogen peroxide and bacteriocins. Bacteriocins generally exert their antimicrobial actions by interfering with the cell wall or the membrane of target organisms, either by inhibiting cell wall biosynthesis or causing pore formation, subsequently resulting in death (Tambekar and Bhutada, 2010). As a result, such lactic acid bacteria are used to produce antimicrobials that inhibit the growth of pathogenic bacteria in the digestive tract and ultimately serve as probiotics. For instance, *Lactobacillus* is often found in the gastrointestinal tract of cattle and can be used as probiotics in livestock to increase the productivity of livestock (Alipin and Safitri, 2016).

Potential probiotic lactic acid bacteria must have the ability to survive in the condition of the digestive system in order to exert their beneficial effect within the host organism, because the digestive system of humans and animals contains digestive enzymes, HCl and bile acid, which make it a harsh environment and affect the life of probiotic strains to exert their beneficial properties. Therefore, bacterial strains must have some special properties to be considered as a potential probiotic candidates. The main criteria for selection of probiotic bacteria are: antimicrobial activity against enteric pathogens, lysozyme resistance, adherence to intestinal cell lines, acid tolerance, bile tolerance, phenotypic and genotypic stability, patterns of carbohydrate utilization and antibiotic resistance (Astuti, 2016). Low pH, gastric enzymes and bile salts are examples of barriers of the gastrointestinal tract in which probiotic bacteria need to be resist after being ingested (Bakari *et al*., 2011). The production of antimicrobial compounds, especially organic acids and bacteriocins by LAB has provided these microorganisms with a competitive advantage over the other species as excellent probiotic candidates (Noohi *et al*., 2014).

Probiotics are becoming increasingly popular as one of the alternatives to antibiotic growth promoters due to the presence of antibiotic resistant microbes and the increased interest for organic products. The purpose of using probiotics in animal feed is to maintain and improve the performance (productivity and growth) of the animal and to prevent and control against invading pathogenic microorganisms (Bajagai *et al*., 2016). However, several studies have reported that, there is a misidentification or mislabeling of probiotic species in addition to the presence of unspecified species in many commercial probiotic products. In Ethiopia, one of

the problems expected to be encountered in the use of probiotics as an alternative to antibiotic growth promoters by poultry farmers is the lack of extensive studies on the potential probiotic properties of bacterial strains (Shokryazdan *et al*., 2014). This is because probiotic properties are strain specific and cannot be extrapolated to the whole genus or species. Therefore, the purpose of the present study is to isolate LAB from GIT of poultry and examine their potential uses as probiotic strains. In addition, this study is important as a preliminary step for selection of potential probiotics for further *in vivo* experiments.

General objective

The major objective of this study is to isolate and characterize potential probiotic lactic acid bacteria from the gastro intestinal tract (GIT) of poultry

Specific objectives

- To isolate and characterize LAB from GIT of poultry
- To screen LAB isolates for selected potential probiotic properties
- To assess the safety status of LAB isolates
- To identify LAB isolates using molecular and Biolog methods

2. LITERATURE REVIEW

2.1. The History and Definition of Probiotics

There are several species of microorganisms surrounding us in which some are beneficial and some are harmful to our health. The history of probiotics began with the history of man by consuming fermented foods that is well known in Greeks and Romans. Small and large intestines of animal and human also naturally contain bacteria, often referred to as 'normal flora'. Normal flora contains more than 400 species of bacteria that endow with many beneficial functions. Most of the probiotic bacteria are belong to the group of lactic acid bacteria and among them *Lactobacillus* and *Bifidobacterium* play a great role in maintaining the intestinal balance and in stimulating the immune system of the host (Forhad *et al*., 2015).

Microorganisms from the genera of *Lactobacillus* and *Bifidobacterium* mainly, and some other species such as *Streptococcus* has been used as probiotics for hundreds of years in food manufactured and therapeutic applications. Many reports have shown that selected strains of Lactobacilli and Bifidobacteria increasingly being introduced into various food products because they are considered to be non-pathogenic and safe (Makete *et al*., 2016).

The term 'probiotic' firstly used by Lilly and Stillwell (1965) to describe unknown growth promoting substances produced by a ciliate protozoan that stimulated the growth of another ciliate. After this year, the definition of 'probiotic' was used in different meaning according to its mechanism and beneficial effects on human and animal health. The meaning was improved to the closest one we use today by Parker (1974). Parker defined 'probiotic' as 'substances and organisms which contribute to intestinal microbial balance'. The meaning used today was improved by Fuller (1989). Thus, probiotic is a live microbial supplement which affects host's health positively by improving its intestinal microbial balance (Bajagai *et al*., 2016). According to The joint Food and Agriculture Organization of the United Nations (FAO) and World Health Organization (WHO) definition of probiotics as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" is the most widely accepted and adopted by the International Scientific Association for Probiotics and Prebiotics (Hill *et al.*, 2014).

2.2. Microorganisms Used as Probiotics

Probiotic is used to name microorganisms that are associated with the beneficial effects for humans and animals. These microorganisms contribute to intestinal microbial balance and play a role in maintaining health (Soccol *et al*., 2010). Microbes from many different genera are being used as probiotics (Table.1). Most of the probiotic microbes are members of the heterogeneous group of lactic acid bacteria belong to the genera of *Lactobacillus, Bifidobacterium, Enterococcus, Lactococcus, Streptococcus* and *Leuoconostoc.* However, some *Bacillus* spp and fungi such as *Saccharomyces* spp and *Aspergillus* spp are also regarded as probiotics. *Lactobacillus* and *Bifidobacterium* species are the most popular in the manufacture of probiotic products. It is generally accepted that with the exception of some *Streptococci* and *Enterococci,* LAB are rarely pathogenic to humans and animals (Mokoena *et al*., 2016; Makete, 2016).

Lactic acid bacteria are present in the Microbiota of mammals and birds, and those originating in the intestine have undergone intensive study for their potential probiotic properties and their rapid establishment as bacterial communities for the prevention of colonization with pathogenic bacteria (Nazef *et al*., 2008). Different studies aimed to identify the Microbiota of the gastrointestinal tract (GIT) of poultry. The most commonly identified *Lactobacillus* species from GIT of poultry are *L. crispatus*, *L. reuteri,* and *L. salivarius*. In addition, a great deal of diversity could be found in lesser reported including *Bacillus, Enterococcus, Streptococcus, Pediococcus* and *Bifidobacterium* (Noohi *et al*., 2014).

Probiotics are used for long period of time in food ingredients for human and also to feed the animals without any side effects and are acceptable because of being naturally in intestinal tract of healthy animal (Yavuzdurmaz, 2007). Probiotics which are used to feed both man and animals are as described in Table.1 below.

Table.1.Microorganisms used as probiotics

Lactobacillus species	*Bifidobacterium* species	Others species
L. acidophilus	*B. bifidum*	*Enterococcus faecalis*
L. rhamnosus	*B. animalis*	*Enterococcus faecium*
L. gasseri	*B. breve*	*Streptococcus salivarus* subsp. *thermophilus*
L. casei	*B. infantis*	*Lactococcus lactis* subsp. *lactis*
L. reuteri	*B. longum*	*Lactococcus lactis* subsp. *cremoris*
L.delbrueckii subsp. *bulgaricus*	*B. lactis*	*Propionibacterium freudenreichii*
L. crispatus	*B. adolascentis*	*Pediococcus acidilactici*
L. plantarum	*B. essensis*	*Saccharomyces boulardii*
L. salivarus	*B. laterosporus*	*Leuoconostoc mesenteroides*
L. johnsonii		*B. subtilis*
L. gallinarum		*B. coagulant*
L. paracasei		*B. cereus*.
L. fermentum		
L. helveticus		
L. lactis		

Adopted from Yavuzdurmaz (2007), Makete *et al*., (2016)

2.3. Mechanism of Probiotic Action

Different probiotics exert their beneficial effects on health of human and animal through various mechanisms; even closely related strains may differ in their mode of action. The major different mechanisms of action proposed for probiotics are as described in the following sections (Hassanein and Soliman, 2010; Fajardo *et al.,* 2012).

2.3.1. Increase in digestion and absorption of nutrients

The gut microenvironment has an effect on the nutrition, feed conversion and maintaining the microbial ecology of the gut. It has many microorganisms that could potentially function as probiotics, of which *Lactobacillus* and *Bifidobacterium* species are the most commonly used probiotics. These probiotic bacteria can affect the host beneficially either direct or indirect, such as enhancement of digestion and absorption of food, alteration of the intestinal microflora and exclusion of pathogens (Hemaiswarya *et al.,* 2013). The improvement in productivity of animals due to probiotic bacteria can be associated with an increase in digestion and absorption of nutrients. In one study, the response in broiler chickens to dietary supplementation with *L. bulgaricus* varied with level of probiotic provided. At a rate of 2×10^6

cfu/g, there was no significant effect on digestibility of crude protein and fat, but at 6 $\times 10^6$ cfu/g and 8 $\times 10^6$ cfu/g there was a significant increase, ranging from 7 to 11% for protein and 6.5 to 13.4% for fat, with 7.9 to 11.7% increase in weight gain (Apata, 2008).

Increased digestibility of nutrients in diet may be due to increased enzyme activity in the intestine by the probiotic bacteria. *Lactobacillus* species are the most important probiotic bacteria, which involved in the alteration of digestive enzyme activity within GIT of poultry and pigs. Amylase activity in poultry species increased by 42% in response to *L. acidophilus* supplied at a rate of 2 $\times 10^6$ cfu/g of maize soybean based diet (Mombelli and Gismondo, 2000). This improvement in amylase activity was associated with a 4.6% increase in body weight gain and 5% improvement in feed use efficiency. Similarly, lactase and amylase but not peptidase activity in the small intestine of pre-weaned pigs increased in response to a commercial probiotics contained *L. plantarum, L. acidophilus, L. casei and E. faecium* (Bajagai *et al*., 2016).

2.3.2. Production of antimicrobial substances

One of the major probiotic mechanisms of action is production of anti-microorganism substances, such as organic acids and bacteriocins. 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 activity of potential probiotic against pathogens (Makras *et al*., 2006). The undissociated form of the organic acid enters the bacterial cell and dissociates inside its cytoplasm. Eventually lowering of the intracellular pH or the intracellular accumulation of the ionized form of the organic acid can lead to the death of the pathogen (Bermudez-Brito *et al*., 2012). In addition to this, many bacterial species, including lactic acid bacteria (LAB) can produce several types of thermostable bacteriocins which have antimicrobial activity against a range of potential pathogens of animals including *Bacillus, Staphylococcus, Enterococcus, Listeria*, and *Salmonella* species. Bacteriocins produced by LAB (for example Nisin) can destruct the target pathogen by pore formation and/or inhibition of cell wall synthesis (Hassan *et al*., 2012).

Intestinal probiotic bacteria can produce health promoting fatty acids and De-conjugated bile acids that show a stronger antimicrobial activity. In addition to this, certain strains of intestinal

Bifidobacteria and Lactobacilli have been shown to produce conjugated linoleic acid (CLA), a potent anti-carcinogenic agent. It is well known that some strains of probiotics produce metabolites that inhibit the growth of fungi and other species of bacteria. Some researchers have reported that *Lactobacillus* can produce antifungal substances, such as benzoic acid, methylhydantoin, mevalonolactone and short chain fatty acids (Bermudez-Brito *et al*., 2012).

2.3.3. Stimulation of the immune response

It is well known that probiotic bacteria can exert an immunomodulatory effect. However, the mechanism is not well understood. Human studies have shown that probiotic bacteria can have positive effects on the immune system of their hosts. These bacteria have the ability to interact with both innate and adaptive immunity. This interaction can affect the immune system in different ways such as; producing cytokines, stimulating macrophages, increasing secretory IgA concentrations (Mombelli and Gismondo, 2000; Bajagai *et al*., 2016).

Epithelial cells in the gastro intestinal mucosa create a selectively permeable barrier between the intestinal lumen (which contains harmful substances such as foreign antigens, microorganisms and toxic materials, as well as beneficial nutrients) and the internal environment of the body. This barrier is the first line of defense against the microbes in the GIT. When pathogens disrupt this barrier, they are inducing inflammation of the intestinal wall, and intestinal damage (Groschwitz and Hogan, 2009; Peterson and Artis, 2014). However, the use of probiotics has been effective in enhancing the mucosal barrier to pathogens and antigen presentation. Recent study reported that known probiotics (*Lactobacillus* strains) could stimulate up regulation of mucous genes in intestinal cells. Furthermore, the effect of these probiotics on the activation and secretion of mucus in the intestine was directly correlated with the inhibition of pathogenic attachment and damage to the intestinal tract (Teitelbuam and Walker, 2002).

Several studies have demonstrated immunostimulatory effects of probiotics such as; *L. fermentum* and *S. cerevisiae* are probiotic bacterial strains which stimulated the intestinal T-cell immune system, indicated by increased production of $CD3^+$, $CD4^+$ and $CD8^+$ T-lymphocytes in the GIT of broiler chickens (Bai *et al.*,2013). The expression of $CD3^+$, IL-2 and IFN-γ genes was significantly greater in the small intestine of neonatal chicks (day 3 and

7) fed with probiotics *L. jensenii* TL2937 and *L. gasseri* TL2919 than in the control without probiotics (Sato *et al.*, 2009). Similar studies indicates that effects of probiotics on the intestinal immune system of broiler chickens treated with a commercial probiotic product contained *L. acidophilus, L. casei, E. faecium* and *B. bifidum* being an increased population of intestinal intraepithelial lymphocytes (IEL) compared with control birds not given the probiotic. Similarly, administration of probiotic *E. faecium* to broiler chickens also challenged with *E. coli* resulted in increased concentrations of cytokines (IL-4) and IgA in the small intestinal mucosa (Bajagai *et al*., 2016).

Probiotics also play a great role for increase serum immunoglobulin levels. Multi-strain probiotic contained *L. acidophilus, B. subtilis* and *C. butyricum* can increase serum levels of IgA and IgM in chickens (Zhang and Kim, 2014).

2.3.4. Increase in colonization resistance

Protection of the host intestines from exogenous pathogens with beneficial bacteria a phenomenon termed colonization resistance was described more than five decades ago and was thought to result from microorganism mediated direct inhibition (Buffie and Pamer, 2013). On the other hand, the beneficial effects of probiotic bacteria are related to the competition for attachment sites, or competitive exclusion. The potential probiotic bacteria attach to the intestinal mucosa, thus forming a physical barrier that prevents the attachment of pathogenic bacteria (Ribeiro *et al*., 2007).

Hydrophobicity is one of the important factors involved in the adhesion of probiotic bacterial cells to epithelial cells in order to colonize human and animal gastrointestinal tract and exert their beneficial effects. Certain strains of *Lactobacillus* and *Bifidobacterium* possess hydrophobic surface layer proteins which help the bacteria to nonspecifically adhere to the animal cell surface. Such adhesion of probiotic bacteria to the intestinal epithelium covers the receptor binding sites, preventing pathogenic microorganisms from attaching to the epithelium cells (Johnson-Henry *et al.*, 2007). As compared to hydrophilic bacterial strains, hydrophobic lactobacilli are adhered better to intestinal epithelial cells (Karimi Torshizi *et al.*, 2008). The ability of probiotic strains to adhere to host epithelial cells was previously investigated *in vitro*

using cell surface hydrophobicity towards chloroform, O-xylene and ethyl acetate (Sharma *et al*., 2017).

2.4. Selection Criteria for Probiotic Potential

Before bacteria can be considered for use as probiotics, it is recommended that they meet certain selection criteria and possess a number of intrinsic physico-chemical characteristics outlined in a joint report by the FAO and WHO in 2002 (Culligan *et al*., 2009). It was recommended that *in vitro* tests should be carried out before any subsequent *in vivo* trials were initiated. These bacteria used as potential probiotics are selected on the basis of their survival in the gastrointestinal tract environmental condition (ability to withstand low pH and high concentrations of bile acids). The ability to tolerate an acidic environment and bile varies among strains of probiotic bacteria. The major desirable characteristics to be selected as probiotic bacteria are the ability to adhere to the intestinal epithelium, enabling the probiotic strain(s) to colonize the intestine, safe to use for food and clinical, bile salt hydrolase activity, reduction of pathogen adherence to surfaces and antimicrobial activity against pathogenic bacteria (Bajagai *et al*., 2016; Yavuzdurmaz, 2007). Some of the major selection criteria for potential probiotic bacteria are discussed in detail below.

2.4.1. Acid, bile and phenol tolerance

Potential probiotic bacteria are mix together with a food system and moving in to the lower intestinal tract through the mouth. In this stage probiotic strains should have the ability to resist the digestion processes, such as resistance to lysozyme enzyme in the oral cavity (Yavuzdurmaz, 2007). The environmental condition of stomach may highly affect the survival of bacterial strains, in which probiotic bacteria need to be tolerating this condition to be exerting a beneficial effect in our stomach (Sahadeva *et al.,* 2011). Therefore, probiotic bacteria are expected to have the ability to resist the stressful acidic condition of the stomach, with pH between 1.5 and 3.0. However, a significant decrease in the viability of bacterial strains is often observed at pH 2 and below (Yavuzdurmaz, 2007). In chickens also, the intraluminal pH of the crop is relatively basic as compared to proventriculus and gizzard pH range from 2.5 to 4.74 (Shokryazdan *et al.,* 2014*)*. Generally, a good potential probiotic organism should withstand at least pH 3 (Sahadeva *et al.,* 2011).

In the intestine also, bile perform a beneficial role in emulsifying lipids and enables lipolysis and absorptions of lipids. The bile salt tends to damage the cell membrane structure of microbes, because they contain lipids and fatty acids on their cell membrane. However, some bacteria are able to resist bile salts by their ability to hydrolyze bile salts through the production of bile salt hydrolase enzyme (BSH) (Wambugu, 2015). Therefore, bile tolerance is an important factor in addition to acid tolerance for survival and growth of potential probiotic in the gastrointestinal tract (Rajoka *et al*., 2018) and this is why such property is considered as one of the criteria for colonization and metabolic activity of probiotic bacteria in the small intestine of the host organism (Sharma *et al*., 2017). Although the bile concentration in the gastrointestinal tract varies, the mean intestinal bile concentration is believed to be 0.3% and the time of its residence in the small intestine is suggested to be 4 hours (Torshizi *et al.,* 2008*;* Makete, 2015).

Lactobacillus spp is the most important potential probiotic strains found in the poultry of GIT. Noohi *et al* (2014), tried to isolate acid and bile resistant variants of *Lactobacillus* spp. According to the results, 96 *Lactobacillus* isolates were examined for resistance to acid and bile salts. Out of which, 31 isolates were identified as resistant to low pH and bile salts. Only 24 *L. brevis,* two *L. vaginali,* three *L. plantarum* and two *L. reuteri* strains showed resistance to acid and bile salts (Noohi *et al.,* 2014*).*

Phenol is a toxic metabolite produced by deamination of some amino acids during disintegration by intestinal bacteria (Forhad *et al*., 2015). The effect of phenol is due to the activity on the bacterial cell wall, by denaturing and coagulating the protein within the cell wall structure of bacteria (Wen, 2009). Therefore, resistance to phenolic compounds is one of the criteria to select potential probiotic bacteria that would be potentially capable of performing effectively in human and animal gastro intestinal tract.

2.4.2. Antimicrobial activity

Antimicrobial activity is one of the most important selection criteria for probiotics. Antimicrobial activities of potential probiotic strains are mainly used to prevent and control the enteric pathogens into the host of organism. Lactic acid bacteria are among the most important antimicrobial compound producer of potential probiotic bacteria. Antimicrobial

effects of lactic acid bacteria are through the production of antimicrobial compounds, such as organic acids (lactic acid, acetic acid and propionic acids), carbon dioxide, hydrogen peroxide, diacetyl, low molecular weight antimicrobial substances and bacteriocins (Yavuzdurmaz, 2007).

In one study, 20 isolates of lactic acid bacteria strains were isolated from chicken gastrointestinal digestive tract. They were examined for their antimicrobial activity against *Escherichia coli*, *Salmonella* sp, and *Staphylococcus aureus* using the agar diffusion method. According to the results, all 20 isolates showed the antimicrobial activity against *Escherichia coli* (with an inhibition zone 8-25 mm in diameter), *Salmonella* sp. (13-40 mm) and *Staphylococcus aureus* (6-24 mm). The antibacterial activity of LAB may often due to the production of organic acids (Musikasang *et al.*, 2009).

2.4.3. Safety status of probiotics

Traditional diary probiotic strains of Lactic acid bacteria mainly some species of *Lactobacillus* and *Bifidobacterium* have a long history safe use without any obvious effect. These strains are commonly recognized in traditional diary product as safe organism (Wu, 2006). With the growing interest of consumers, new and more specific microorganisms of potential probiotic strains induced into food products for humans and feed additive products for animals. However, these novel probiotic microorganisms cannot be assumed to share the historical safety of these traditional strains. Therefore, before the microorganisms to be used as potential probiotic candidates in food and pharmaceutical industry, the strains should be assessed and tested for their safety status and effectiveness of proposed use. Before studying the probiotic microorganisms *in vivo*, some of the important *in vitro* assays used to assess the safety status of new probiotic strains are antibiotic resistance test, haemolytic activity and some enzymes such as Gelatinase and DNase production (Gupta and Sharma, 2017; Wu, 2006). In general, new probiotic bacteria should be fulfill the following safety requirements; they have no history of relationship with disease like gastro intestinal tract disorders, non-pathogenic, should not carry transferable antibiotic resistance genes and strains for human use are preferred to be human origin and isolated from healthy human (Yavuzdurmaz, 2007).

Haemolytic activity, Gelatinase and DNase enzyme production are an important criterion to assess the virulence potential status of probiotic bacteria. Gelatinase is a metallo endopetidase able to hydrolyzing insulin, casein, hemoglobin, fibrinogen, collagen and gelatin, which affecting the membrane integrity (Gupta and Sharma, 2015). DNase is the enzyme which helps the growth of pathogens by enlarging the pool of available nucleotides by DNA hydrolysis in the host and also aids the evasion of the innate immune response by degrading neutrophil extracellular traps (NETs) (Sharma *et al*., 2017). Haemolysis activity would break down the epithelial layer and blood cells (Singroha *et al*., 2017). Therefore, microorganisms possess any Haemolytic activity, Gelatinase and DNase enzymes production could not be used as potential probiotic in any pharmaceutical and food or feed industries.

Antibiotics are an important tools used by the medical and pharmacological industries in order to protect pathogenic bacteria. Therefore, to be used LAB as a potential probiotic must exhibit the ability to resist various antibiotics (Lim and Im, 2009). Beneficial antibiotic resistant probiotic bacteria can be co-administered with antibiotics for disease treatment. However, transferable of resistance gene between probiotic and pathogenic bacteria are possible in the gastro intestinal tract, if the resistance gene is existed on the plasmid of the bacteria. If the gene is located on the chromosome of the bacteria, it is not transferable gene (Petsuriyawong and Khunajakr, 2011). According to the suggestion of safety considerations in the probiotic study that potential probiotic strains does not contain transferable antibiotic resistance gene (Singroha *et al*., 2017).

2.5. Molecular Identification of Probiotic Strains

Before the expansion of technology in microbiology the distribution, characteristics and benefits of lactic acid bacteria to human beings and animals were not clearly identified. Through time technology expansion microbiologists developed different methods used for detection and characterization of lactic acid bacteria according to physiological and biochemical tests. Although, these traditional methods have limitations, they are used for identification along with the newly developed technology for molecular typing. This technology is getting more reliable in the identification and differentiation of many microorganisms. Molecular identification methods are a powerful to classify taxonomically

even between closely related species. There are also a number of alternative taxonomic classification methods including hybridization with species specific probes. Polymerase chain reaction based methods such as amplification fragment length polymorphism (AFLP), polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) and random amplification polymorphism DNA (RAPD) are mainly used as molecular tools and the most powerful and accurate one is sequencing (Yavuzdurmaz, 2007).

In one study, a molecular technique (comparative sequence analysis of the 16S rRNA gene) was used to identify potential probiotic *Lactobacillus* strains isolated from chickens of intestinal tissues. The isolates selected for their good acid and bile tolerance were identified to species level. 16S rRNA gene was amplified by PCR and each purified PCR product was cloned into *E.coli* plasmid. Then, DNA sequence analysis was carried out for plasmid with the unique insert. According to the study, the results of the 16S rRNA gene sequencing showed that all the three isolates were 99% similar to *L. salivarius* DQ444477.1 (Shokryazdan *et al*., 2014).

In another study, a PCR amplified ribosomal DNA restriction analysis (ARDRA) technique was used to identify potential probiotic *Lactobacillus* species isolated from bovine vagina. 16S rRNA gene was amplified by PCR and products were digested with four restriction enzymes (*Sau* 3AI, HinfI, *Hinc* II and *Dra* I). Most of the digestion profiles obtained from the amplified 16S rDNA gene of these strains agreed with the theoretical profile matching with *Lactobacillus fermentum*. Among all strains, four homofermentative lactobacilli showed a restriction profile that matched with *Lactobacillus gasseri* and a facultative heterofermentative strain was identified as *Lactobacillus rhamnosus* (Otero *et al*., 2006).

3. MATERIALS AND METHODS

3.1. Description of the Study Area

The present study was conducted in northern part of Ethiopia, Mekelle University which is located 780 km north of Addis Ababa in Tigray Regional State. Its geographical location is $13^{o}32^{'}$ N altitude and $39^{o}33^{'}$ E longitude. It has an average altitude of 2200 meters above sea level with a mean minimum, mean maximum and mean average monthly temperatures of 8.7, 26.8 and 17.60, respectively (Getachew, 2015). The study period was conducted from August 2017 to July 2018.

3.2. Research Design

The study involved a laboratory based experimental design. Twenty samples from 4 part of GIT content were collected aseptically from 5 healthy poultry birds (hen) and cultured on sterile de Man, Rogosa, and Sharpe (MRS) and Medium 17 (M17) agar medium using a streak plate method. Isolated colonies were characterized for their ability to produce CO_2 from glucose, to ferment different sugars, to grow at different temperatures and NaCl concentrations. To select good candidates of potential probiotic from among LAB isolates further *in vitro* investigation was conducted to evaluate their tolerance to bile (0.3%), tolerance to phenol (0.1, 0.2, 0.3 and 0.4%), tolerance to low pH (2.0, 3.0 and 4.0), antibacterial activity against *Escherichia coli* ATCC25922, *Salmonella typhimurium* ATCC13311 and *Staphylococcus aureus* ATCC25923, cell surface hydrophobicity and safety status (haemolytic activity, antibiotic activity, Gelatinase and DNase production). Finally, the selected potential probiotic isolates of LAB were identified at genus and species level using molecular and Biolog method of identification respectively.

3.3. Sample Collection

A total of 20 samples were collected aseptically from 4 part of GIT (small intestine, ceca, colon and gizzard) of 5 healthy poultry birds (hen). Healthy poultry birds (hen) were selected randomly and samples were collected at the four month of age from the poultry farm of Mekelle University, College of Veterinary Medicine in August 2017. After collection, samples were kept aseptically in sterile test tubes contained normal saline solution (0.85% NaCl) and

stored at 4°C in a refrigerator at microbiology laboratory of Mekelle University to protect from deterioration and contamination until used for further study.

3.4. Isolation of Lactic Acid Bacteria

Lactic acid bacteria (LAB) were isolated from gastro intestinal tract content of healthy poultry birds (hen) using MRS and M17 agar medium using streak plate method. One gram of each sample was taken and homogenized in 9 ml of a sterile normal saline solution (0.85% NaCl) using vortex (Estifanos *et al*., 2016). Serial dilution was carried out by adding 1ml of each homogenized sample to 9 ml of saline solution. Then 0.1ml of the fourth dilution (10^{-4} dilution) was streak plated onto MRS and M17 agar and incubated anaerobically in anaerobic jar using candle for 48 hours (h) at 37°C and 30°C, respectively. Different colony sizes (large, medium and small) were randomly taken from the plates and more purified by sub-culture continuously on MRS and M17 agar. After purification, colonies were further characterized morphologically using gram stain method and biochemical test.

3.4.1. Catalase and oxidase test

Single overnight isolated colony was used to perform catalase test. One drop of 3% hydrogen peroxide was placed onto a glass slide and mixed aseptically with one colony that was taken by sterile inoculating loop. Formation of gas bubbles indicates the presence of catalase enzyme that breaks down hydrogen peroxide into water and oxygen. Therefore, production of oxygen indicated the positive response of the bacteria to catalase test.

Oxidase test was performed using an oxidase reagent of tetra methyl-p-phenylene diamine dihydrochloride. This test was used to identify for the presence of cytochrome oxidase in LAB isolates that catalyze the transport of electrons between electron donors and the redox dye. Oxidase reagent was prepared as 1% solution of tetra methyl-p-phenylene diamine dihydrochloride. Filter paper was placed in a sterile petri dish to which three drops of freshly prepared oxidase reagent was added. A colony from an overnight culture was streaked on the filter paper contained the reagent. Finally, oxidase positive and negative isolates of LAB producing blue color and no color change respectively, were observed within 10 second.

3.4.2. Endospore staining and motility test

Pure isolates of LAB were examined for the formation of endospore using malachite green (primary stain) as described by Shuhadha *et al*. (2017). Bacterial smears were prepared on microscopic slides and heat fixed. Smears were flooded with malachite green reagent and heat fixed for a few minutes. Slides were removed from Bunsen burner and washed with water until the water ran clear. Then, slide was covered with counter stain (safranin) for 30 second and washed with water. Finally, slide was air dried and observed under the light microscope.

Motility of isolated LAB was examined by using SIM medium (Sulphide Indole Motility medium). This medium has a very soft consistency that allows motile bacteria to migrate readily through them causing cloudiness. Sulphide Indole Motility medium was prepared in sterile test tubes and inoculated with one colony of overnight culture. Inoculated test tubes were incubated at 37°C and 30°C for 48h. Negative results were indicated by growth only on the inoculated region whereas positive results were indicated by growth along the inoculated region and tend to swim out away from the inoculated region.

Non-spore forming, non-motile, gram positive, oxidase negative, catalase negative and growth under anaerobic condition, coccus and bacillus shaped isolates were considered as LAB (Powthong and Suntornthiticharoen, 2013; Ribeiro *et al*.,2013). These pure isolates of LAB were preserved in MRS and M17 broth contained 20% (v/v) sterile glycerol at -20°C deep freeze until used for further characterization.

3.5. Physiological and Biochemical Characterization

3.5.1. Growth at different temperatures

Ability of the isolates to grow on various temperatures was tested according to the method described by Pundir *et al*. (2013) and Patel and Parikh (2016). Sterile MRS agar was streak plated with one fresh colony of LAB isolates and incubated anaerobically in anaerobic jar at 10°C, 15°C, 25°C, 30°C, 37°C and 45°C for 48 h. Growths of LAB isolates were evaluated by observing their colony grown on MRS agar. Growth on MRS agar plats were used to designate isolates as temperature tolerant. The test was carried out in triplicates.

3.5.2. Growth at different NaCl concentrations

Isolates were examined for their tolerance against different NaCl concentrations according to the method described by Forhad *et al*. (2015). For this purpose 1-10% of NaCl concentrations were selected. De Man, Rogosa, and Sharpe (MRS) agar medium were prepared by adjusted with 1-10% of NaCl concentrations and without NaCl concentrations used as positive control. After sterilization, each petri plate was inoculated with fresh colony of LAB isolates and incubated anaerobically at 37°C for 48 h. Growth was evaluated by streak plated on MRS agar and compared their growth after 48 h incubation time with positive control.

3.5.3. CO_2 production from Glucose

Gas productions from glucose were examined to determine the homofermentative and heterofermentative characteristics of LAB isolates, as described by the method of Makete (2015) and Yavuzdurmaz (2007) with slight modification. Of basal medium contained glucose and inverted Durham tubes were prepared and inoculated with overnight fresh cultures of LAB isolates. Finally, the test tubes were incubated anaerobically in anaerobic jar at 37°C for 5 days. Gas formation in Durham tubes after 5 days incubation was used as evidence for CO_2 production from glucose.

3.5.4. Determination of sugar fermentation

Ability of sugar fermentation of LAB isolates were performed as described by Forhad *et al*. (2015) with slight modifications. Eight different sugars (glucose, fructose, sucrose, galactose, maltose, glycerol, sorbitol and xylose) were used for characterization of isolated LAB by their ability to ferment the different carbohydrate. All sugar solutions were prepared at a final concentration of 5% (w/v) and sterilized with 0.22μm pore diameter of filter membrane. Sterilized 10 ml of basal medium (containing Bromo thymol blue) in test tubes were prepared. One ml of the different sugar solutions was transferred into different test tubes containing sterile basal medium and inoculated with one fresh colony of LAB isolates. Inoculated test tubes were incubated anaerobically at 37°C for 72 h and sugar fermentation was observed, as the acid production changed the color of the medium from its original color to yellow. Sterile basal medium without the addition of any sugar solution was used as a negative control.

3.6. Potential Probiotic Properties of LAB Isolates

3.6.1. *In vitro* test for acid tolerance

Lactic acid bacterial isolates were examined for resistance to acid according to the combined methods described by Wambugu (2015) and Estifanos (2016) with slight modifications. Isolates of LAB were grown in MRS broth anaerobically at 37°C for 24 h. Aliquot of one milliliter of the 24 h culture was centrifuged at 500 rpm for 5 min at 4°C and the resulting pellet was washed once with phosphate buffer saline (PBS, pH 7.0). Then cell pellets were inoculated into 10 ml MRS broth whose pH was adjusted to 2.0, 3.0, and 4.0 using 1N HCl and incubated anaerobically at 37°C for 3 h. After 0, 1, 2, and 3 h of incubation, appropriate dilutions were done and petri plates inoculated with spread plate technique. The inoculated plates were then incubated anaerobically in anaerobic jar at 37°C for 48 h. This was followed by viable counts which were performed by counting the number of colonies from petri dishes containing 10 to 300 colonies at 0, 1, 2, and 3 h of incubation time and expressed in colony forming unite per milliliter (log cfu/ml). In addition to this, % of survivor cells were calculated according to the following equation described by Rajoka *et al*. (2018): % of survivor cells = final after 3 h colony counted (log cfu/ml) / initial at 0 h of colony counted (log cfu/ml) x 100.

3.6.2. *In vitro* test for Bile tolerance

Lactic acid bacteria isolates those survived in acidic condition were selected for bile tolerance test. De Man, Rogosa, and Sharpe (MRS) broth containing 0.3% bile concentration and no bile were prepared and autoclaved for sterilization. One ml of 18 h fresh culture of LAB isolates were centrifuged at 500 rpm for 5 minute at 4°C and the pellet was washed once with phosphate buffer saline (PBS, pH 7.0). Then cell pellets were inoculated aseptically into 10 ml of MRS broth containing 0.3% bile and incubated under anaerobic condition at 37°C for 4 h (Shuhadha *et al.,* 2017). After 0, 2, and 4 h of incubation, appropriate dilutions were done and petri plates inoculated with spread plate technique. The inoculated plates were then incubated anaerobically in anaerobic jar at 37°C for 48 h. Then viable colonies were counted from petri plates containing 10 to 300 colonies at 0, 2 and 4 h of incubation time and expressed in log colony forming unite per milliliter (log cfu/ml) (Yavuzdurmaz, 2007 and Wambugu, 2015). Finally, the % of survivor cells was calculated according to the following equation described

by Rajoka *et al*. (2018): % of survivor cells = final after 4 h of colony counted (log cfu/ml) / initial at 0 h of colony counted (log cfu/ml) x 100.

3.6.3. *In vitro* test for phenol tolerance

Phenol tolerance test was performed as described by the method of Forhad *et al*. (2015) with slight modification. De Man, Rogosa, and Sharpe (MRS) agar medium were prepared and adjusted with different concentrations (0.1%, 0.2%, 0.3% and 0.4%) concentration of phenol and without phenol concentration as positive control. After sterilization, petri plates were inoculated with overnight culture of LAB isolates and incubated at 37°C for 48 h. After 48 hours of incubation their growths were determined by comparing the cultures with a positive control.

3.6.4. Cell surface hydrophobicity test

Bacterial adhesion to hydrocarbon method was used to determine the degree of hydrophobicity as a measure of their adherence to the epithelial cells in the gut (Sharma *et al*., 2017). Hydrophobicity potential of LAB isolates were determined according to the method described by Ilavenil *et al.* (2015) using chloroform. Fresh LAB isolates grown in MRS broth were centrifuged at 1200xg for 10 min. After removing the supernatant, pellet was washed two times with phosphate buffer saline (PBS). Then the pellet was re-suspended in 5 ml of PBS. Three milliliter of the suspension and 1ml of chloroform were mixed by vortex and incubated at 37°C for 1h for phase separations. Aqueous phase was taken and measured for its absorbance at 600 nm. Finally, the % of hydrophobicity was calculated using the following formula: % of hydrophobicity = [(OD_{600} before adding with chloroform − OD_{600} after adding with chloroform) / OD_{600} before adding with chloroform] × 100.

3.6.5. Antimicrobial activity

Antimicrobial activity of LAB isolates were studied *in vitro* by the agar diffusion method as described by Estifanos (2016) with slight modification. Isolates of LAB were evaluated for their potential antimicrobial activity against pathogenic bacteria of chicken (*Escherichia coli* ATCC25922 and *Salmonella typhimurium* ATCC13311) and humans (*Staphylococcus aureus* ATCC25923) (Musikasang *et al*., 2009). These pathogens were obtained from Tigray regional

laboratory. Each pure isolate of LAB was inoculated into 10 ml of MRS and M17 broth and incubated anaerobically at 37°C and 30°C for 72 h respectively. Two milliliter cell-free supernatant from the cultures of MRS and M17 broth media were obtained by centrifugation at 8000 rpm for 20 minutes at 4°C. Overnight culture of test pathogens were also grown in their respective appropriate media at 37°C and diluted to a turbidity equivalent of 0.5 McFarland standard (equivalent to $1x10^8$ cfu/ml) and inoculated onto sterile Mueller Hinton agar medium using spread plate method. Then cell-free supernatant was loaded into a diameter of 6 mm wells that were prepared on the Mueller Hinton Agar. After diffusion, plates were incubated at 37°C for 24 h. Finally, diameters of inhibition zones were measured using a caliper in millimeters (mm).

3.7. Safety Assessment of Potential Probiotic LAB

3.7.1. Gelatinase test

Gelatinase enzyme production of LAB isolates were studied using MRS agar medium supplemented with 3% gelatin. Petri plates containing agar medium with 3% gelatin were spot inoculated from fresh 18 h cultures of LAB isolates and incubated anaerobically in anaerobic jar at 37°C for 72 h. Then petri plates were flooded with saturated ammonium sulfate solution. Development of clear zones around the spots against the opaque background indicated a positive reaction (Sharma *et al*., 2017; Singroha *et al*., 2017).

3.7.2. DNase activity

The ability of LAB isolates to produce DNase was determined by line streak inoculated fresh overnight culture on DNase agar as described by Shuhadha *et al*. (2017). Plates were incubated at 37°C for 48 h in anaerobic jar. After incubation, DNase agar plates were flooded with 1N HCl acid and left for a few minutes in order to absorb the reagent into the plates. Excess HCl acid was removed and then observed within 5 min for the development of clear zones around line streaked culture. Strain of *S.aureus* ATCC25923 was used as positive control.

3.7.3. Haemolytic activity

Gelatinase and DNase negative LAB isolates were screened for their haemolytic activity on blood agar base plates. Fresh sheep blood was collected using test-tube containing ethylene diamine tetra acetic acid (EDTA) that used as anticoagulant and transported to the laboratory of Mekelle University. Then blood agar was prepared by adding 7% fresh sheep-blood into sterile blood agar base at 45°C. Overnight cultures of LAB isolates were line streak inoculated onto sterile blood agar plates. Plates were incubated anaerobically at 37°C for 48 h and evaluated for signs of β-hemolysis (clear zone of hydrolysis around the colonies), α-hemolysis (a partial hydrolysis and greening zone) and γ-hemolysis (no clear zones around colonies) on blood agar plates. *S.aureus* ATCC25923 was used as positive control (Anas *et al*., 2014; Singroha *et al*., 2017).

3.7.4. Antibiotic resistance test

Antibiotic resistance ability of LAB isolates were evaluated using the disc diffusion method according to the method described by Colombo (2017) and Makete (2015). The following 10 different antibiotics were used: streptomycin (10 μg/disc), chloramphenicol (30 μg/disc), ciprofloxacin (5 μg/disc), penicillin-G (10 U/disc), ampicillin (10 μg/disc), vancomycin (30 μg/disc), tetracycline (30 μg/disc), gentamicin (10 μg/disc), erythromycin (15 μg/disc) and rifampicin (5 μg/disc). Overnight cultures of LAB isolates were diluted using 0.85% (w/v) of NaCl until their turbidity reached similar to 0.5 McFarland standards (equivalent to $1x10^8$ cfu/ml). Hundred micro liter suspensions of LAB isolates were spread plated onto the surface of sterile MRS agar plates using sterile swab. Then different discs of antibiotics were placed aseptically onto the surface of plated MRS agar and left over for 10 min within safety cabinet for diffusion of antibiotics. Finally, the plates were incubated under anaerobic condition in anaerobic jar at 37°C for 48 h. After incubation times, zone of inhibition was measured using calipers in millimeter (mm) and results were described as antibiotic resistant (no inhibition zones), moderately susceptible (≤ 10 mm) and susceptible (> 10 mm).

3.8. Molecular Identification of Probiotic Bacteria

3.8.1. Genomic DNA extraction

Genomic DNA of LAB isolates were extracted according to the method described by Lopez *et al*. (2003) with some modifications. Cells were harvested from the overnight grown culture by centrifugation. After washed cells twice with normal saline (0.85% NaCl), the pellet was resuspended in 200µl of lysis buffer (50 mM Tris, 100 mM EDTA, 1% SDS, pH 8) and mixed gently. Then 10 µl of RNase A (10mg/ml) was added and incubated at 37°C for 1h. After incubation, 10 µl of proteinase K (20mg/ml) was added and incubated at 50°C for 3 h in a water bath. Following the incubation time, 200 µl of phenol-chloroform-isoamyl alcohol (25:24:1) was added and mixed gently by hand for 2-3 min. After mixed, it was centrifuged at 12000 rpm for 15 min at room temperature and removed the aqueous phase to another new eppendorf tube. Cold 500 µl of 97% ethanol was added into aqueous phase and left it at -20°C for 12 h to precipitate the DNA and then centrifuged at 12,000 rpm for 15 min at 4°C. Pellet was washed with cold 500 µl of 70% ethanol and centrifuged at 12,000 rpm for 5 min at 4°C. Then, pellet was dried and resuspended with 100 µl of TE buffer. Finally, the quality of DNA was analyzed on 0.8% agarose gel electrophoresis and stored at -20°C until used for PCR amplification.

3.8.2. Amplification of the target 16s rRNA gene

Extracted genomic DNA was subjected to PCR (TIANLONG®) amplification according to the method described by Camul *et al*. (2007) with slight modifications. To amplify V3-V4 region of the 16S rRNA gene from species of LAB, genera specific primers of LAC1 (5′-AGCAGTAGGAATCTTCCA-3′) and LAC2 (5′-ATTTCACCGCTACACATG-3′) were used. Amplification reaction was performed in a final volume of 25 µl, contained 1x reaction buffer, 2.5 mM of $MgCl_2$, 200 µM of dNTP mix, 0.3 µM of each primer, 0.05 U/µl of FIREPol® DNA polymerase and 5 µl of DNA template. Negative control was used that contained all the above except DNA template. Amplification conditions were consisted of an initial denaturation step at 95°C for 5 min (one cycle) followed by denaturation at 95°C for 1 min, annealing at 48°C for 1 min and extension at 72°C for 1 min (each of 40 cycles), and final extension at 72°C for

7 min performed at the end of the final cycle. Finally, amplification reaction was cooled to 4°C and stored the PCR product at -20°C until analyzed by agarose gel electrophoresis.

3.8.3. Separation of amplified PCR products

Agarose gel was prepared at concentration of 1.5%. 1.5 g of agarose was dissolved in 100 ml of 1x TAE buffer by boiling. After cooled the prepared agarose gel, 3µl of ethidium bromide solution was added and poured onto the gel tray stand having a comb. Then combs were removed after 20 min from solidified gel and produced wells. Twenty µl of amplified PCR products and 4 µl of loading dye were mixed and loaded into each wells. Hundred bp (base pair) DNA ladder (marker) was loaded into the first well to determine the size of DNA fragments. Finally, the PCR products were electrophoresed on agarose gel at 400 mA and 92 V for 60 min. and visualized under UV light.

3.9. Identification of LAB Isolates Using Biolog

Pure cultures of LAB isolates were sent to National Animal Health Diagnostic and Investigation Center (NAHDIC) at Sebeta to identify at species level using biolog method of identification. Identification at species level was carried out using Biolog method according to the manufacturer's instructions. Pure isolates of LAB were inoculated onto sterile Biolog Universal Growth (BUG) agar plate and incubated under anaerobic condition at 37°C for 48 h. Fresh LAB isolates were inoculated into each well of the micro-plate contained 96 wells with 95 different carbon sources plus a water control on a 96 well plate and incubated under anaerobic condition in anaerobic jar at 37°C for 48 h. Finally, the micro-plate was read with the Biolog Micro Station system consisted of an automated plate reader coupled with a computer, which interprets the results and compares them with the resident Biolog database to identify the LAB isolates at species level (Al-Dhabaan and Bakhali, 2017).

3.10. Data Analysis

The experimental data were summarized using Microsoft Excel spreadsheets. Then, the results were expressed and interpreted using descriptive statistical methods. Statistical analyses were performed using Statistical Package for Social Sciences (SPSS) version 20.

4. RESULTS AND DISCUSSION

4.1. Isolation and Characterization of LAB

A total of 104 LAB isolates were isolated from GIT (small intestine, large intestine, ceca and gizzard) of poultry birds (Hen) using a streak plate method on MRS and M17 agar medium. Of them 64 and 40 LAB isolates were obtained from MRS and M17 agar medium, respectively. All isolates were selected aseptically based on their colony size (small, medium and large) from the culture of MRS and M17 agar medium. The cell morphologies of the 104 isolates were observed using light microscopy and showed that 60 and 44 of them were rod and spherical shaped respectively. All of isolates were found gram positive and arranged singly, pairs and short or long chains. Catalase test showed that of the 104 isolates, 82 and 22 isolates were catalase negative and catalase positive respectively. In the present study, all of 82 LAB isolates were found non-spore forming, non-motile, oxidase negative and grown well under anaerobic condition. Out of 82 isolates, a total of 10 LAB isolates were selected for further analysis according to their potential antimicrobial activity against selected pathogens.

All of selected 10 isolates were also characterized further using physiological and biochemical tests. As shown in the results, all selected isolates of LAB did not produced CO_2 from glucose. This was contradict with the results of Yavuzdurmaz (2007) who reported that, AS17 and AS83 LAB isolates isolated from human milk were able to produce CO_2 from glucose. Based on this characteristic, they were classified as homofermentative LAB isolates. Homofermentative LAB produces only lactic acid as a by-product via glycolytic pathway from fermentation of glucose without gas production (Makete, 2015). One of the criteria to identify the isolates was the ability to grow at different temperatures. The results of 48 h growth show that, 4 isolates were able to grow at 10°C, 15°C, 25°C, 30°C, 37°C and 45°C, whereas isolates of MU10, MU9, MU8, MU1 and MU3 grow only at 37°C and 45°C (Table.1). This was similar to the reports of Estifanos (2016) who indicates that, all LAB isolated from the head of cabbage were grown at 37°C and 45°C. In the present study, the reason for selecting this temperature was to examine whether the isolates of LAB were able to grow within range of body temperature or not. If the isolates were not able to grow within the range of body temperature then they would not have chance to continue to exist in the gut of humans and animals (Shakoor *et al*., 2017; Pundir *et al*., 2013) and in the environment industrial

processing, which is an important requirement for probiotics production and application in industries. Regarding growth at different NaCl concentrations, all the 10 isolates were unable to tolerate higher percentage of salt concentration (6-10% w/v NaCl). The inability of the isolates to grow in a medium containing 8% and 10% NaCl observed in this study was not similar to the finding of Powthong and Suntornthiticharoen (2013), where all LAB isolated from chicken intestine, entrails of swine, and soil were able to grow at 8% and 10% NaCl. As indicated in Table.1, 4 isolates were able to grow at 1%, 2%, 3%, 4% and 5% w/v NaCl concentration containing MRS agar medium. This finding is however similar to the previous finding reported by Patel and Parikh (2016). The present result also showed that isolate MU3 tolerated only 1% NaCl, whereas MU2 was able to grow only at 1% and 2% NaCl concentrations (Table.1). Sodium chloride is an inhibitory substance which can limit the growth of various types of bacteria (Shakoor *et al*., 2017). If the isolates of LAB do not survive in different NaCl concentrations then it would not be able to claim their activity in the presence of NaCl concentrations.

Monosaccharide that occurs in gastro intestinal tract will affect the life of microbes in the GIT (Makete, 2015). One of the beneficial potential probiotic characteristics of LAB isolates is the ability to ferment different sugars. Therefore, fermentation of different sugars was observed, by detecting changes in the original color of the medium into yellow color because of acid production. It was found that, all isolates were not able to ferment xylose (Table.1). As shown in the table.1, all the isolates of LAB except MU10 were able to ferment fructose. Isolates MU5 and MU6 were able to ferment glycerol partially which all the other 8 isolates could not ferment. Furthermore, isolates MU10, MU7, MU1 and MU3 were unable to ferment sucrose and sorbitol while the remaining 6 isolates were able to ferment them. In general, 4 isolates of LAB were able to ferment fructose, galactose, sucrose, glucose, maltose and sorbitol. This result is in agreement with the results of Forhad *et al*. (2015) who reported that all the isolates of LAB from buffalo milk were able to ferment fructose, sucrose, dextrose, maltose and sorbitol.

Table.2. Physiological and biochemical characterization of LAB isolates

Characteristics	Lactic acid bacteria isolates									
	MU 1	MU 2	MU 3	MU 4	MU 5	MU 6	MU 7	MU 8	MU 9	MU 10
Growth at										
10°C	-	+	-	+	+	+	-	-	-	-
15°C	-	+	-	+	+	+	+	-	-	-
25°C	-	+	-	+	+	+	+	-	-	-
30°C	-	+	-	+	+	+	+	-	+/-	-
37°C	+	+	+	+	+	+	+	+	+	+
45°C	+	+/-	+	+	+	+	+/-	+	+	+
Gram stain	+	+	+	+	+	+	+	+	+	+
Motility test	-	-	-	-	-	-	-	-	-	-
Catalase test	-	-	-	-	-	-	-	-	-	-
Oxidase test	-	-	-	-	-	-	-	-	-	-
Spore forming	-	-	-	-	-	-	-	-	-	-
Cell shape	bacilli	bacilli	bacilli	cocci	bacilli	bacilli	bacilli	bacilli	bacilli	bacilli
Gas from glucose	-	-	-	-	-	-	-	-	-	-
Growth at different NaCl										
1%	+	+	+	+	+	+	+	+	+	+
2%	+	+	-	+	+	+	+	+	+	+
3%	+	-	-	+	+	+	+	+	+	+
4%	-	-	-	+	+	+	+	-	-	-
5%	-	-	-	+	+	+	+	-	-	-
6-10%	-	-	-	-	-	-	-	-	-	-
Fermentation of:										
Fructose	+	+	+	+	+	+	+	+	+/-	-
Glucose	+	+	-	+	+	+	+/-	-	+	+/-
Sucrose	-	+	-	+	+	+	-	+	+	-
Galactose	-	+	-	+	+	+	+	+	-	+
Maltose	-	+	+/-	+	+	+	+	+/-	-	+/-
Glycerol	-	-	-	-	+/-	+/-	-	-	-	-
Sorbitol	-	+	-	+/-	+	+	-	+	+/-	-
Xylose	-	-	-	-	-	-	-	-	-	-

Note: '+' indicates positive reaction; '+/-' indicates moderate reaction; '-' indicates negative reaction; MU represents Mekelle University; MU1-MU10 represents isolates of LAB

4.2. Potential Probiotic Properties of LAB Isolates

4.2.1. *In vitro* test for acid tolerance

In the present study, all selected isolates of LAB were evaluated *in vitro* for different pH resistance (pH 2.0, 3.0 and 4.0). The time that takes for the digestion of a single meal in the stomach is 3 hours depending on the feed size of the food (Yavuzdurmaz, 2007). Therefore, all the isolates were evaluated their resistance to low pH during 3 h of the incubation times. The results are shown in Table.3 in terms of both log cfu/ml and percent of survivor cells. As shown in the result, all selected isolates were resistant to pH 3.0 and 4.0 with relative reduction in viable count of log cfu/ml values after 3 h of incubation times. However, all selected isolates were survived with decrease in viable count of log cfu/ml values after 2 hours of incubation times at pH 2.0 (Table.3). Overall, at pH 3.0 and pH 4.0 the percent of survivor cells were higher as compared to pH 2 for all tested isolates of LAB. This result is similar to the findings of Rajoka *et al*. (2018) who reported that, the isolated strains of LAB from intestinal tract of poultry showed higher percentage survival at pH 3.0 than at pH 2.0. In general, the data in Table.3 indicated that, all tested isolates showed above 60% and 88% of survivor cells after 3 hours of incubation at pH 3.0 and pH 4.0 respectively, which means these isolates of LAB were able to survive and perform their beneficial effects under these stressful pH conditions.

Table.3. Tolerance of LAB isolates to different pH

			LAB isolates									
pH	T	SC and % SC	MU 1	MU 2	MU 3	MU 4	MU 5	MU 6	MU 7	MU 8	MU 9	MU 10
pH 2	0h	SC	2.08	1.95	2.12	2.12	2.03	1.99	2.05	2.00	2.12	2.10
	1h	SC	2.00	1.53	2.08	1.90	1.76	1.63	1.97	1.85	1.99	1.88
		% SC	96	78.5	98.1	89.6	86.7	81.9	96.1	92.5	93.9	89.5
	2h	SC	1.81	1.23	1.88	1.11	1.78	1.08	1.40	1.36	1.46	1.28
		% SC	87	63	88.7	52.4	87.7	54.3	68.3	68	68.9	60.9
	3h	SC	1.32	0.00	1.68	0.00	0.00	0.00	0.00	0.00	1.04	0.00
		% SC	63	0.00	79.3	0.00	0.00	0.00	0.00	0.00	49.0	0.00
pH 3	0h	SC	2.18	2.03	2.30	2.04	2.10	1.99	2.26	2.07	2.16	2.23
	1h	SC	2.05	1.85	2.14	1.90	2.00	1.83	2.10	1.88	2.04	1.99
		% SC	94	91	93	93.1	95.2	91.9	92.9	90.8	94.4	89.2
	2h	SC	1.75	1.45	1.98	1.72	1.80	1.53	1.76	1.58	1.69	1.64
		% SC	80	71	86	84.3	85.7	76.8	77.9	76.3	78.2	73.5
	3h	SC	1.68	1.25	1.81	1.23	1.61	1.21	1.59	1.38	1.52	1.56
		% SC	77	61.6	78.7	60.3	76.7	60.8	70.4	66.7	70.4	69.9
pH 4	0h	SC	2.31	2.21	2.34	2.20	2.29	2.10	2.32	2.16	2.21	2.24
	1h	SC	2.29	2.16	2.33	2.18	2.17	2.04	2.28	2.10	2.18	2.16
		% SC	99	97.7	99.6	99.1	94.8	97.1	98.3	97.2	98.6	96.4
	2h	SC	2.26	2.07	2.30	2.16	2.13	1.95	2.20	2.00	2.18	2.10
		% SC	97.8	90.9	98.3	98.2	93	92.9	94.8	92.6	98.6	93.7
	3h	SC	2.24	1.95	2.28	2.13	2.05	1.87	2.09	1.92	2.08	1.99
		% SC	96.9	88.2	97.4	96.8	89.5	89	90	88.9	94.1	88.8

Note: SC= survivor cells (log cfu/ml); % SC= % of survivor cells; T= incubation times in hours; MU= Mekelle University where LAB isolated

4.2.2. *In vitro* test for bile tolerance

The bile tolerance of LAB isolates were examined in MRS broth containing 0.3% bile after 4 hours of incubation. In the present result indicates that, all of selected isolates were resistant to 0.3% bile concentration and the majority, increased in viable count (log cfu/ml) while all of the isolates showed above 97% survivor cells after 4 hours incubation times (Table.4). The present results in agreement with the findings of Yavuzdurmaz (2007) who reported that, all of the isolates of LAB from human milk were resistant to 0.3% bile salt. The current result indicates that all of the isolates of LAB are able to grow and exert their beneficial effects in 0.3% bile concentration.

Table.4. Bile tolerance (0.3%) of LAB isolates

BC	T	SC and % SC	LAB isolates									
			MU 1	MU 2	MU 3	MU 4	MU 5	MU 6	MU 7	MU 8	MU 9	MU 10
0.3%	0h	SC	2.13	2.04	2.00	2.02	2.04	2.05	2.12	2.07	2.09	2.00
	2h	SC	2.15	1.92	1.92	2.00	1.90	1.86	2.08	2.00	2.05	2.01
		%SC	101	94.1	96.0	99.0	93.1	90.7	98.1	96.6	98.0	101
	4h	SC	2.27	2.08	2.16	2.12	2.08	2.00	2.18	2.06	2.21	2.15
		%SC	107	102	108	105	102	97.5	103	99.5	106	108

Note: BC= Bile concentration; SC= survivor cells (log cfu/ml); % SC= % of survivor cells; T= incubation times in hours; MU= Mekelle University where LAB isolated

4.2.3. *In vitro* test for phenol tolerance

Probiotic candidates of LAB isolates were screened for their ability to tolerate phenolic compounds. The results of the present study indicate that all the isolates of LAB except MU5, MU6 and MU2 were able to tolerate from 0.1-0.4% concentration of phenolic compounds (Table.5). This is similar with the findings of Forhad (2015) who reported that, all probiotic isolates of LAB were competent to tolerate from 0.1% to 0.4% phenol concentrations.

Table.5. Phenol tolerance of LAB isolates

Isolates of LAB	Concentrations of phenol			
	0.10%	0.20%	0.30%	0.40%
MU1	+	+	+	+
MU2	+	+	+	-
MU3	+	+	+	+
MU4	+	+	+	+
MU5	+	+	+	-
MU6	+	+	+	-
MU7	+	+	+	+
MU8	+	+	+	+
MU9	+	+	+	+
MU10	+	+	+	+

Note: '+' indicates positive reaction; '-' indicates negative reaction; MU1-MU10 represents isolates of LAB

4.2.4. Cell surface hydrophobicity test

In the present study, cell surface hydrophobicity was evaluated using chloroform. The results indicates that isolates of MU9 (75.34%), MU10 (51.02%), MU8 (93.27%), MU1 (90.73%) and MU3 (62.18%) exhibited high percentage of cell surface hydrophobicity towards chloroform

as compared to the remaining LAB isolates (Figure.1). According to Serrano-Nino *et al.* (2016), the percentage of hydrophobicity was classified as low from 0 to 29%, medium from 30 to50% and high from 51 to 100%. Accordingly, these five isolates of LAB showed high hydrophobicity (51-100%). The results are also in agreement with the findings of Ilavenil *et al.* (2015) who reported that all isolates of lactobacilli isolated from animal manure showed significant hydrophobicity with xylene and chloroform. As suggested by Singroha *et al.* (2017), Torshizi *et al.* (2008), the high percentage of hydrophobicity of isolated strains could indicate their potential to adhere to the epithelial cell of the intestine, resist the movement of digested materials and enhance their beneficial properties in the competitive exclusion of pathogens.

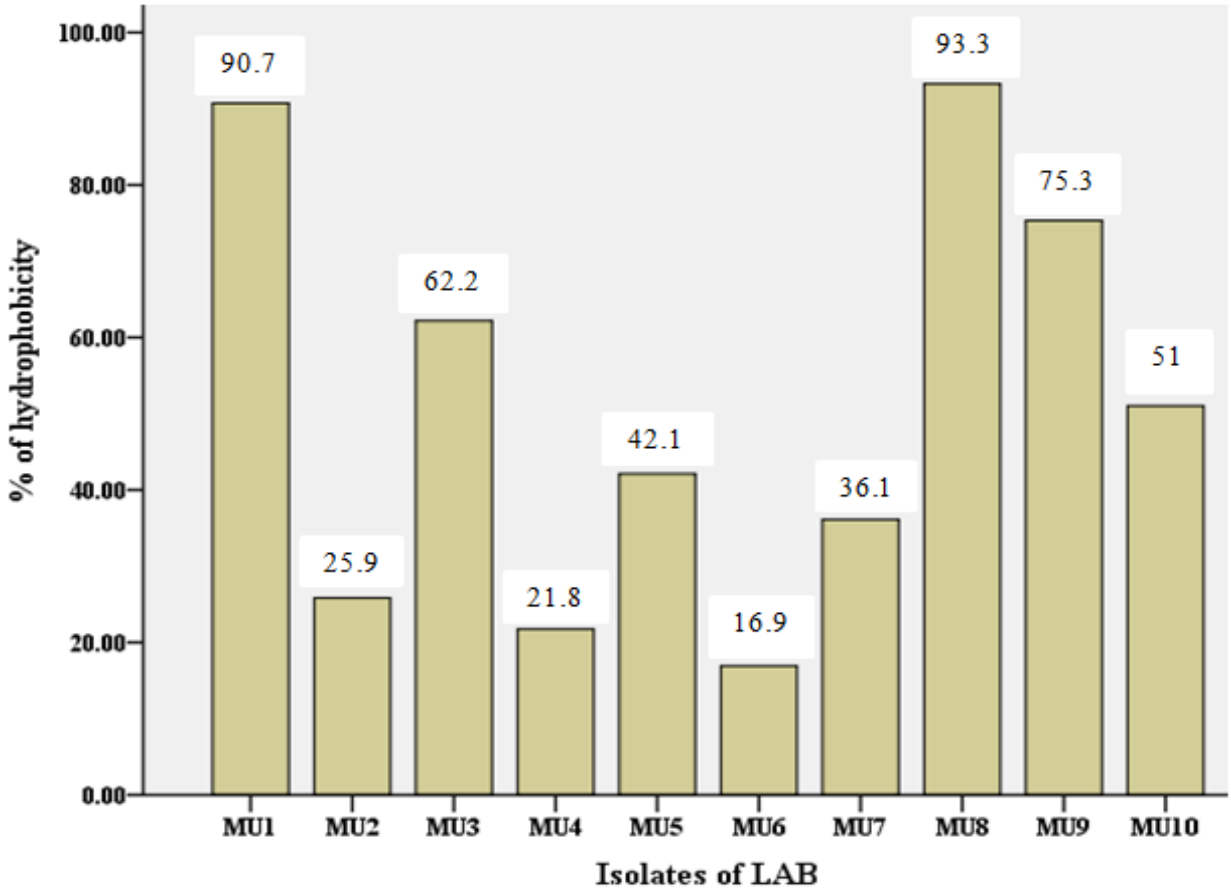

Figure.1. Hydrophobicity ability of LAB isolates using chloroform

4.2.5. Antimicrobial activity

Antimicrobial activity of the cell free supernatants obtained from culture of LAB isolates were examined by the agar well diffusion method against three pathogens: *Escherichia coli*

ATCC25922, *Salmonella* typhimurium ATCC13311 and *Staphylococcus aureus* ATCC25923. The tests were conducted twice and the averages of inhibition zones were taken. The present results indicating that, MU4, MU5, MU6 and MU7 isolates were able to inhibit all three indicator pathogens. The present results in agreement with the findings of Chowdhury and Islam (2016) who reported that LAB isolated from local crud were produced antimicrobial activity against *Escherichia coli* ATCC25922, *Staphylococcus aureus* ATCC25923. Furthermore isolates of MU10, MU3 and MU1 exhibited antibacterial activity against *Escherichia coli* ATCC25922 and *Staphylococcus aureus* ATCC25923 (Table.6). As shown in the results isolates of MU9 and MU8 able to inhibited only the growth of *Escherichia coli* ATCC25922 and *Staphylococcus aureus* ATCC25923, respectively whereas isolate MU2 was inhibited against the growth of both *Staphylococcus aureus* ATCC25923 and *Salmonella* typhimurium ATCC13311 (Table.6).

Table.6. Antimicrobial activity of LAB isolates

	Mean ± SD diameter of inhibition zone (mm)		
LAB isolates	*S. aureus*	*E. coli*	*S. typhimurium*
MU1	14 ± 0.67	12 ± 1.30	0
MU2	12 ± 1.00	0	12 ± 1.00
MU3	13 ± 1.30	13 ± 0.40	0
MU4	15 ± 0.53	17 ± 0.16	13 ± 1.00
MU5	17 ± 0.80	16 ± 1.67	13 ± 0.40
MU6	14 ± 1.30	12 ± 0.05	11 ± 1.00
MU7	13 ± 0.23	11 ± 0.32	12 ± 0.23
MU8	12 ± 0.52	0	0
MU9	0	14 ± 0.40	0
MU10	16 ± 0.70	16 ± 0.32	0

Note: Each value in the table indicates the mean value ± standard deviation (SD) for two independent measurements

4.3. Safety Assessment of Potential Probiotic LAB

4.3.1. Haemolytic, Gelatinase and DNase activity

In the present study none of the tested LAB isolates were found to possess virulent potential as pathogenicity factors and considered safe to use as a probiotic for future application (Appendix figure.4 and 5). The present results similar with the findings of Ribeiro *et al*. (2013) and Wambugu (2015), who reported that all of the LAB isolates were found a negative result for any Haemolytic activity, Gelatinase and DNase enzymes production.

4.3.2. Antibiotic resistance test

In the present study LAB isolates were assayed for their antibiotic resistance to 10 antibiotics, using the disk diffusion method. The present results indicating that all selected LAB isolates were susceptible to chloramphenicol, penicillin-G, erythromycin and rifampicin, whereas resistant to streptomycin, ciprofloxacin and vancomycin antibiotics (Table.7). The present results in agreement with the findings of Nallala and Jeevaratnam (2015) who reported that all the six isolates of LAB isolated from chicken gastrointestinal tract were susceptible to chloramphenicol, penicillin-G and erythromycin and resistant to streptomycin and ciprofloxacin. On the other hand, in contradiction findings were reported that none of the LAB isolates were resistant to vancomycin antibiotic (Ribeiro *et al*., 2014).

Table.7. Antibiotic resistance of LAB isolates

Antibiotics	Dose	Lactic acid bacteria isolates									
		MU 1	MU 2	MU 3	MU 4	MU 5	MU 6	MU 7	MU 8	MU 9	MU 10
Streptomycin	10 µg/disc	R	R	R	R	R	R	R	R	R	R
Chloramphenicol	30 µg/disc	S	S	S	S	S	S	S	S	S	S
Ciprofloxacin	5 µg/disc	R	R	R	R	R	R	R	R	MS	R
Penicillin-G	10 U/disc	S	S	S	S	S	S	S	S	S	S
Ampicillin	10 µg/disc	R	S	R	S	S	S	R	R	S	R
Vancomycin	30 µg/disc	R	R	R	R	R	R	R	R	R	R
Tetracycline	30 µg/disc	S	S	S	S	S	MS	R	S	R	S
Gentamicin	10 µg/disc	S	R	S	S	S	S	R	MS	R	R
Erythromycin	15 µg/disc	S	S	S	S	S	S	S	S	S	S
Rifampicin	5 µg/disc	S	S	S	S	S	S	S	S	S	S

Note: R= antibiotic resistant (no inhibition zones); M= moderately susceptible (≤ 10 mm); S= susceptible (> 10 mm); MU1-MU10 represents for the isolates of LAB

4.4. Molecular Identification

The present results indicating that, 8 isolates of LAB produced a PCR product of about 350 bp (Figure.2). The present results in agreement with the findings of Porcellato *et al*. (2012) who reported that the genera specific primers (LAC1 and LAC2) amplified the 16S rRNA gene and resulted in a PCR product of about 350 bp. As indicated in the result also, isolates of MU1 and MU6 have characteristics of probiotic properties of LAB but not amplified using the genera specific primer (LAC1 and LAC2). This indicating that, the primer pair used in this study may be not specific for MU1 and MU6 isolates because LAC1 and LAC2 amplifies from species of

lactic acid bacteria but not all species of LAB. In general the present result indicated that, all these 8 isolates were confirmed within the group of LAB species at molecular level.

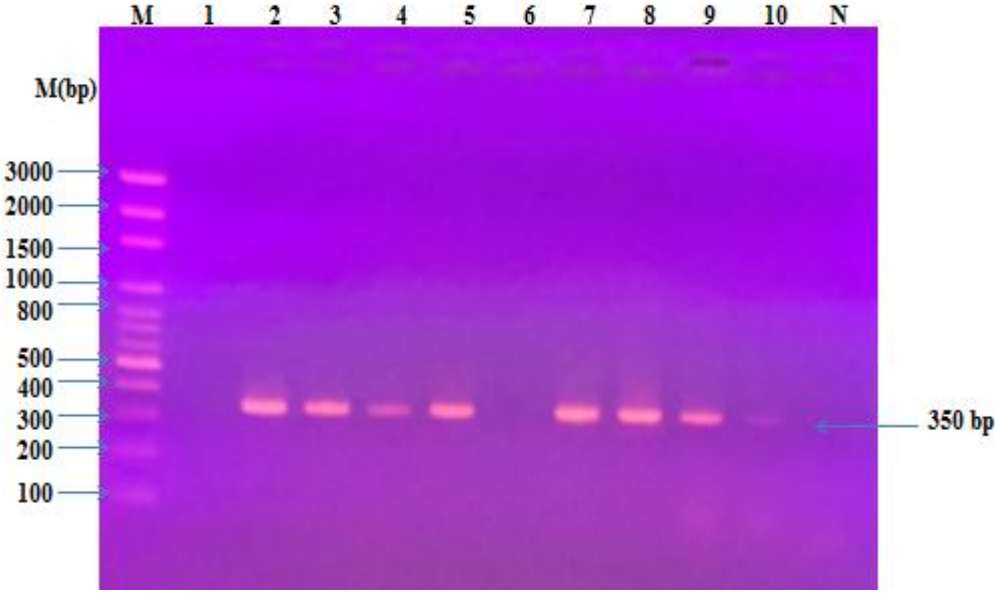

Figure.2. Amplified PCR product of 16S rRNA gene on 1.5% agarose gel

Note: Lane M =100bp DNA ladder (100-3000bp); lane N = negative control; lanes 1-10 = LAB isolates of MU1, MU2, MU3, MU4, MU5, MU6, MU7, MU8, MU9 and MU10 respectively.

4.5. Identification of LAB Isolates Using Biolog

Biolog micro plates were originally developed for the rapid identification of bacterial isolates according to their potential utilization of 95 different carbon sources. When these carbon sources oxidized, a purple dye develops visible patterns of positive (purple) and negative (clear) which provide an identifiable metabolic fingerprint of individual species (Al-Dhabaan and Bakhali, 2017). The present results of Biolog indicating that four and three LAB isolates were identified as *Lactobacillus mali* and *Lactobacillus reuteri* respectively (Table.8). However, isolate MU4 was not identified satisfactorily because although it had a spherical shaped structure as shown in table.1, it was identified as *Lactobacillus mali*, which is rode shaped. As shown the results in Table.8, isolates of MU1, MU9 and MU10 were not identified at species level indicating that these isolates may be out of the database, because in the present study the resident Biolog database containing only 40 species of LAB (Lactobacillus, *Leuoconostoc*, *Pediococcus* and *Lactococcus*) but not all types of LAB species.

Table.8. Identified species of LAB isolates using Biolog

LAB isolates	Types of species
MU1	No ID
MU2	*Lactobacillus mali*
MU3	*Lactobacillus reuteri*
MU4	*Lactobacillus mali*
MU5	*Lactobacillus mali*
MU6	*Lactobacillus mali*
MU7	*Lactobacillus reuteri*
MU8	*Lactobacillus reuteri*
MU9	No ID
MU10	No ID

Note: No ID = means the metabolic reactions of LAB isolates could not match to any species within the resident Biolog database

5. SUMMARY, CONCLUSION AND RECOMMENDATIONS

Summary

Lactic acid bacteria (LAB) have been well known for thousands of years for their important role in the food industry due to their fermentative ability. They are one of the most important groups of microorganisms useful in animal and human health as probiotics. Probiotics are the health promoting living microorganisms that exhibit beneficial effects on the health of animals and humans by improving the intestinal microbial balance. Now a day, the use of antibiotics as growth promoter is questionable because of the presence antibiotic resistant pathogens and the increase interest for organic products have led to search for alternatives. In this case, a potential probiotic bacteria can be used an excellent alternative growth promoter to antibiotics to enhance the performance of better animal productive and health. Therefore, Isolation and Characterization of potential probiotic properties of lactic acid bacteria (LAB) isolated from gastrointestinal tract (GIT) of poultry was the aim of this study.

To determine the probiotic properties of LAB isolates different tests were conducted such as cell surface hydrophobicity test, resistance to low pH and bile concentration, tolerance to phenolic environment, determine the safety status of isolates and antimicrobial activity. Isolates of LAB were characterized by phenotypic and molecular methods. For the phenotypic characterization, morphological study, tolerance to different temperatures and NaCl concentrations, CO_2 production from glucose and different sugar fermentations were performed. In the present results indicated that, all tested isolates were exhibited high potential to resist the stressful of gastrointestinal tract environmental condition (tolerant to phenol, 0.3% of bile, pH 2.0, pH 3.0 and pH 4.0) and antimicrobial activity against *Escherichia coli* ATCC25922, *Salmonella* typhimurium ATCC13311 and *Staphylococcus aureus* ATCC25923 pathogens. Antimicrobial compounds produced by LAB isolates could be made them as good candidates for potential application as probiotic bacteria in humans and animals. Concerning the safety status, none of the tested isolates of LAB were showed Haemolysis activity, Gelatinase and DNase enzyme production. Furthermore, some of the LAB isolates were showed higher percent of hydrophobicity towards chloroform and this is make them to adhere the epithelial cells of the host with a strong hydrophobic interaction potential. Regarding to molecular characterization, isolates of LAB were confirmed if they were belonged in the

group of LAB using genera specific primer and produced a PCR product of about 350 bp. According to the Biolog results 4 and 3 LAB isolates were identified as *Lactobacillus mali* and *Lactobacillus reuteri* respectively.

Conclusions

Gastrointestinal tract of poultry is a good source of lactic acid bacteria with a good potential probiotic property. In this study, 10 LAB isolates having potential probiotic properties were isolated from GIT of healthy poultry birds (Hens). Isolates of MU1, MU3, MU8 and MU9 had high potential to adhere and pass through the harsh environmental condition within GIT. Overall, isolates of MU4, MU5, MU6 and MU7 had comparatively higher potential to be select as potential probiotic indifferent application due to they possess high ability of antimicrobial activity, tolerance the environmental condition of GIT and have no relationship with haemolytic, Gelatinase and DNase activity. In general, this present study provides baseline information for further investigations of LAB isolates *in vivo* in order to be used isolates as potential probiotic bacteria in health, food and pharmaceutical industry.

Recommendations

Based on this present study it is recommended that:

- Further *in vitro* and *in vivo* investigations are still needed to use LAB isolates reliably in different application.
- Identification of LAB isolates at strains level should be required using 16S rRNA sequences.
- Further work can also be required to investigate the transferable antibiotic resistance gene at molecular level.

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7. APPENDICES

Appendix A

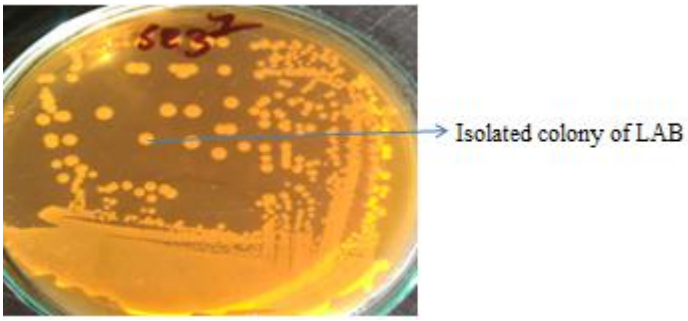
Appendix Table.1. Designation of LAB isolates name and its source of GIT

Name of LAB isolates	Source of isolates
MU1	Small intestine
MU2	Gizzard
MU3	Small intestine
MU4	Large intestine (Colon)
MU5	Small intestine
MU6	Large intestine (Colon)
MU7	Small intestine
MU8	Small intestine
MU9	Gizzard
MU10	Cecum (first part of large intestine)

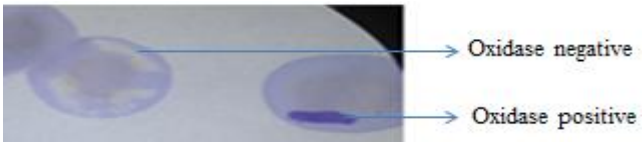
Appendix Table.2. Recommended PCR reaction mix

Component	Volume per 100 µl	Volume per 25 µl	Final concentration
FIREPol® DNA polymerase (5U/ µl)	1 µl	0.25 µl	0.05U/ µl
10X Buffer BD	10 µl	2.5 µl	1X
25Mm $MgCl_2$	10 µl	2.5 µl	2.5mM
20mM DNTP mix	1 µl	0.25 µl	200 µM
Primer forward (10pml/µl)	3 µl	0.75 µl	0.3 µM
Primer reverse (10pml/µl)	3 µl	0.75 µl	0.3 µM
DNA template	20 µl	5 µl	10ng/ µl
H_2O PCR grade	52 µl	13 µl	
Total	100 µl	25 µl	

Appendix figure.1. Growth of LAB isolates on MRS agar



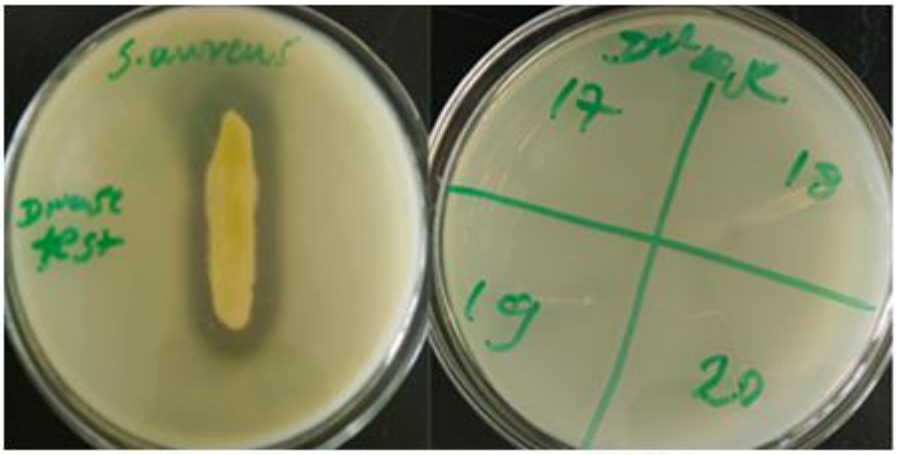
Appendix figure.2. Oxidase test of LAB isolates



Appendix figure.3. Different sugar fermentation ability of LAB isolates

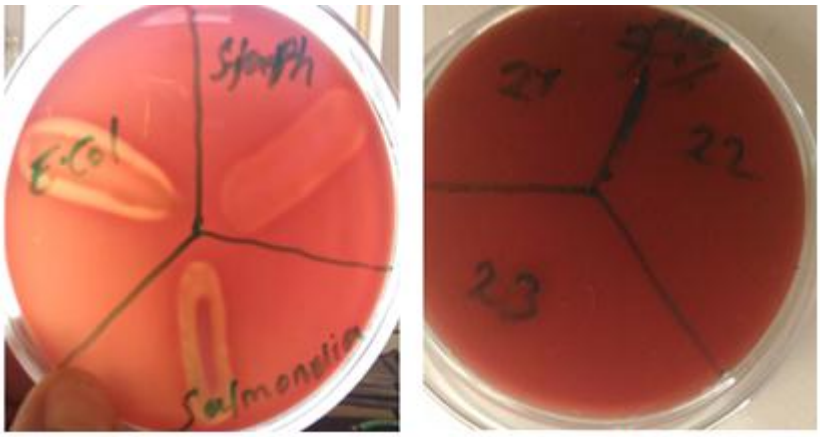
Fermentation of different sugars Negative control with out any sugar

Appendix figure.4. DNase test of LAB isolates and *S.aureus* as control



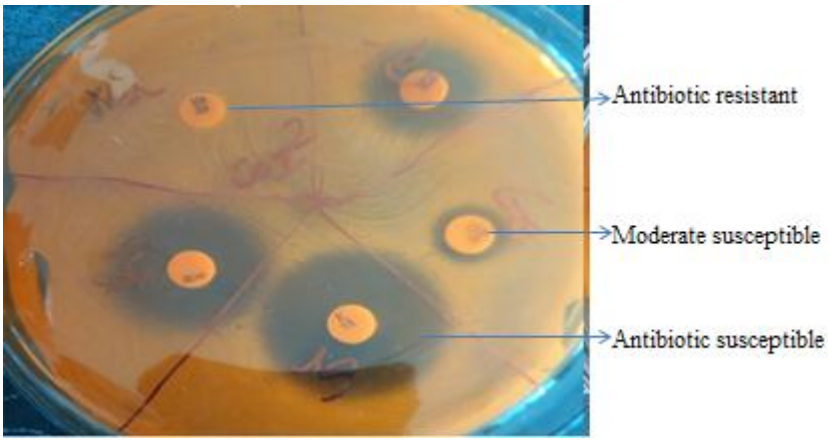
Positive control LAB isolates

Appendix figure.5. Haemolysis activity of LAB isolates and *E.coli, S. typhimurium* and *S. aureus* as control.

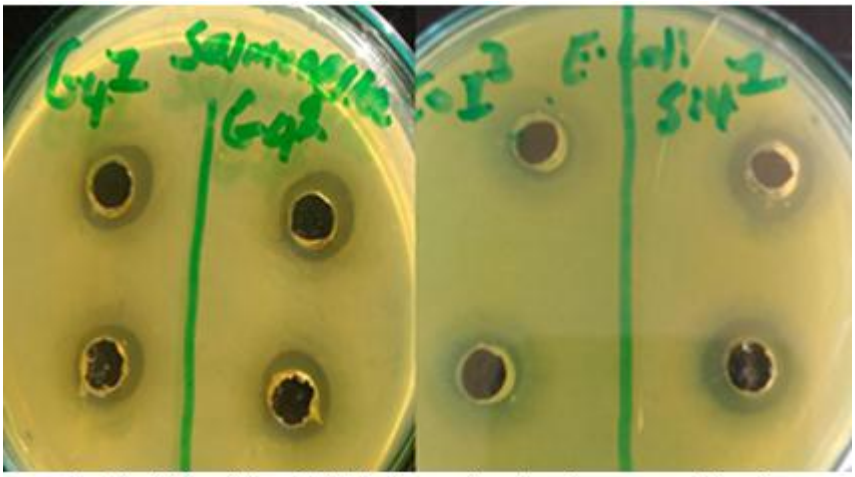


Positive control LAB isolates

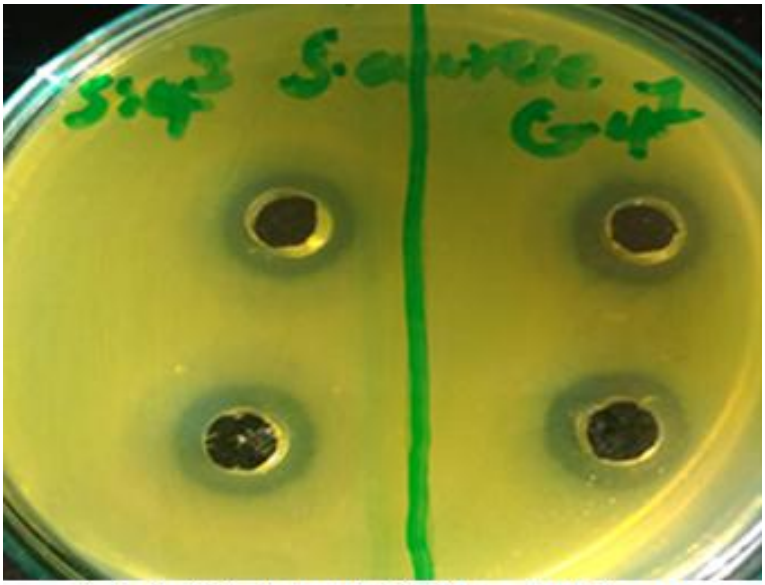
Appendix figure.6. Antibiotic test of LAB isolates



Appendix figure.7. Antimicrobial activity of LAB isolates against selected pathogens



Antimicrobial activity of LAB isolates against *S. typhimurium and E. coli*



Antimicrobial activity of LAB isolates against *S. aureus*

Appendix B: Culture Medium

1. MRS agar

Proteose peptone	10.00 g
Beef extract	10.00 g
Yeast extract	5.00 g
Dextrose	20.00 g
Polysorbate80	1.00 g
Ammonium citrate	2.00 g
Sodium acetate	5.00 g
Magnesium sulphate	0.10 g
Manganese sulphate	0.05 g
Dipotassium phosphate	2.00 g
Agar	12.00 g

Final pH (at 25^0c) 6.5±0.2

Suspend 67.15grams in 1000 ml distilled water. Sterilize by autoclaving at 15Ibs pressure (121^0c) for 15 minutes. Cool to 45-50^0c. Mix well and pour into sterile petri plates.

2. MRS broth

Proteose peptone	10.00 g
Meat extract B#	10.00 g
Yeast extract	5.00 g
Dextrose	20.00 g
Polysorbate80	1.00 g
Ammonium citrate	2.00 g
Sodium acetate	5.00 g
Magnesium sulphate	0.10 g
Manganese sulphate	0.05 g
Dipotassium phosphate	2.00 g

Final pH (at 25^0c) 6.5±0.2

Suspend 55.15grams in 1000 ml distilled water. Sterilize by autoclaving at 15Ibs pressure (121^0c) for 15 minutes. Cool to 45-50^0c. Mix well and pour into sterile test tubes.

3. Of basal medium

Casein enzymic hydrolysate	2.00 g
Sodium chloride	5.00 g
Dipotassium phosphate	0.30 g
Bromo thymol blue	0.08 g
Agar	2.00 g

Final pH (at 25^0c) 6.8±0.2

Suspend 9.4 grams in 1000 ml distilled water. Sterilize by autoclaving at 15Ibs pressure (121^0c) for 15 minutes.

4. SIM medium

Beef extract	3.00 g
Peptic digest of animal tissue	30.00 g
Peptonized iron	0.20 g
Sodium thiosulphate	0.025 g
Agar	3.00 g

Final pH at 25^0c 7.3±0.2

Suspend 36.23 grams in 1000 ml distilled water. Sterilize by autoclaving at 15Ibs pressure (121^0c) for 15 minutes.

5. Blood agar (base)

Meet extract	10.00 g
Peptone	10.00 g
Sodium chloride	5.00 g
Agar	15.00 g

Final pH at 25^0c 7.3±0.2

Suspend 40.00 grams in 1000 ml distilled water. Sterilize by autoclaving at 121^0c for 15 minutes. For blood agar, cool to 45-50^0c and add aseptically 7% of fresh sheep blood. This is a non-selective medium for the isolation and cultivation of many pathogenic and non-pathogenic microorganisms.

6. M17 broth

Casein enzymic hydrolysate	2.50 g
Peptone	2.50 g
Soya peptone	5.00 g
Yeast extract	2.50 g
Beef extract	5.00 g
Ascorbic acid	0.50 g
Magnesium sulphate	0.25 g
Lactose	5.00 g
Disodium-β-glycerophosphate	9.00 g

Final pH at 25^0c 7.1±0.1

Suspend 42.25 grams in 1000 ml distilled water. Sterilize by autoclaving at 121^0c for 15 minutes.

Appendix C: Buffers and Solutions

1. 1x Phosphate buffered saline (PBS)

NaCl	8.00 g
KCl	0.20 g
Na_2HPO_4	1.44 g
KH_2PO_4	0.24 g

Dissolve the reagents listed above in 800 ml of distilled water. Adjust to pH 7.4 with HCl, and then add distilled water to 1000 ml. Sterilize by autoclaving at 121^oc for 15 minute and store PBS at room temperature.

2. 10x TAE buffer (200 ml) stock solution

9.68g of Tris was dissolved in 100 ml of distilled water. 2.28 ml of glacial acetic acid and 4 ml of 0.5 M EDTA (pH 8) were added to the solution. Finally, volume was adjusted to 200 ml with distilled water and store at room temperature. Dilute it to desire concentration before use (ie.1x).

3. 10x TE buffer (100 ml, pH 8) stock solution

0.12g Tris was dissolved in 80 ml of distilled water and added 0.2 ml of 0.5 M EDTA (pH 8). Finally, volume was adjusted to 100 ml with distilled water and sterilized by autoclaving. It often used to elute DNA or to dilute primers and helps to prevent degradation of DNA. This is also often used for long term storage of DNA to prevent degradation of DNA. Dilute it to desire concentration before use (ie.1x).

4. 1x TE buffer (100 ml, pH 8) working solution

10 ml of 10x TE buffer was taken and the volume was adjusted to 100 ml with distilled water. It was sterilized by autoclaving and store at room temperature.

5. 0.5M EDTA stock solution pH 8 (1L)

EDTA stock solution was prepared by dissolving 186.1g disodium EDTA in 800 ml of distilled water. It was mixed well by heating gently. The pH was adjusted to 8.0 with 10N NaOH (or 20g of NaOH pellets). Volume was adjusted to 1L with distilled water and sterilized by autoclaving.

6. Ethidium bromide (10 mg/ml)

Ethidium bromide was prepared by dissolving 1g of EB in 100 ml of distilled water. It was stirred for several hours to ensure that the dye was completely dissolved. Wrap the container contained the solution by aluminum foil and stored at 4°C. Ethidium bromide is a mutagen and toxic. Therefore, wear gloves when working with EB and a mask when weighing the powder.

7. Phenol-chloroform-isoamyl alcohol (25:24:4)

100 ml of phenol and 96 ml of chloroform were mixed with 4 ml of isoamyl alcohol.

8. Agarose gel preparation

Agarose gel was prepared at 1.5% of agarose. 1.5 g of agarose was dissolved in 100 ml of 1x TAE buffer working solution (contained 10 ml of 10x TAE buffer and 90 ml of distilled water) by heating. After cooling to 60°C, 3µl of ethidium bromide was added into the dissolved agarose and mixed gently. Finally, it was poured onto gel tray to produce solidified agarose gel.