ISOLATION, BIOCHEMICAL CHARACTERIZATION AND MOLECULAR IDENTIFICATION OF RIBOFLAVIN OVERPRODUCING LACTIC ACID BACTERIA FROM DAIRY PRODUCTS

By

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Certificate

The Thesis submitted by Imran Khan to PARC Institute of Advance Studies in Agriculture, Quaid-i-Azam University, Islamabad, Pakistan, is accepted in its current form. This thesis fulfills all the requirement for facilitating him with Degree of Master of Philosophy in Animal Genomics and Biotechnology.

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DECLARATION

I would like to declare that the data presented in this thesis "Isolation, Biochemical Characterization and Molecular Identification of Riboflavin Overproducing Lactic Acid Bacteria from Dairy Products" is generated by myself from original research work under the supervision of **Dr. Haider Khan,** at Department of Animal Genomics and Biotechnology, PARC Institute of Advanced Studies in Agriculture National Agricultural Research Centre, Islamabad, Pakistan. The results and material used in this thesis have never been presented anywhere else earlier.

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Imran Khan

Dedication

This research is lovingly dedicated to my Ammi and Abu,
Who have been my constant source of inspiration
They have given me the drive and discipline
to tackle any task with enthusiast and
determination. Without their love
and support this project
would have not
been made
possible
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CONTENTS

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ABSTRACT

Background: Vitamins are daily need of every individual and their deficiency can result in serious illness. Riboflavin (vitamin B2) is one of the essential vitamins required for normal functioning of human body. Some strains of Lactic Acid Bacteria (LAB) belonging to the genus *Lactobacillus and Lactococcus* have the potential to produce riboflavin in excess. Since LAB are generally regarded as safe (GRAS), therefore, could be used for the production of riboflavin enriched products.

Methods: To estimate riboflavin concentration in market milk and yogurt, 20 samples were collected and riboflavin contents were determined spectrophotometrically. For isolation of potential riboflavin overproducers, 20 milk and yogurt samples were collected from four major cities of Pakistan and cultured on MRS agar. Gram positive and catalase negative colonies were selected for further studies. DNA was extracted from these isolates and 16S rDNA gene was amplified by PCR followed by DNA sequencing for identification at species level. The isolates identified as belonging to genus *Lactobacillus* or *Lactococcus* were then grown on Riboflavin Assay Medium (RAM), to test their riboflavin overproducing ability. The potential riboflavin overproducers were then tested for their safety in terms of hemolysis and antibiotic resistance. Finally, potential riboflavin overproducers found to be safe were then used for yogurt preparation. The yogurt thus obtained was then tested for riboflavin contents and comparison was done with market yogurt samples.

Results: The mean riboflavin concentration observed in the market milk and yogurt samples was 2.515 ± 0.65 mg/L and 2.54 ± 0.573 mg/L respectively. To isolate potential riboflavin overproducers, 20 samples were spread on MRS agar. From the colonies thus obtained, 78 isolates were selected for Gram staining and catalase reaction. The results showed that 19 isolates were Gram positive and catalase negative (a typical characteristic of Lactic Acid Bacteria). Extracted DNA and PCR showed clear bands on gel. The sequencing results confirmed 7 LAB strains. Safety tests confirmed that these strains are safe and could be used for production of edible products. Four strains were found suitable for yogurt preparation and the highest riboflavin content of the prepared yogurt was 6.3 mg/L.

Conclusion: Riboflavin overproducing LAB can be isolated from dairy products. They have the potential to double the content of riboflavin in the edible products to fulfill the necessary daily requirements of human body.

Chapter 1

INTRODUCTION

1.1 Background

Livestock is a substantial part of agriculture, contributing for about 58.6% of agricultural value added and 11.6 percent of Pakistan's overall GDP. Milk is the most valuable and major product in the livestock sector. Pakistan is currently the world's fourth largest milk producer, producing approximately 62 billion liters in 2020-21. (Government of Pakistan, 2021). However, a large volume of milk is traded as raw or is processed by conventional means. It has been assessed that approximately 5% of total milk produced is processed for various products, and thus there is huge potential for value addition in dairy products, which can bring higher revenues to livestock farmers.

1.2 Vitamins

Vitamins are complex biological molecules that an organism need in trace amounts to fulfil regular activities. Vitamins are vital nutrients that the human body cannot produce. Thiamine, riboflavin, niacin, pyridoxine, pantothenic acid, biotin, folate, and cyanocobalamin are among the B vitamins, which are water soluble. These vitamins are necessary for cellular metabolism and must be included in one's diet.

1.3 Riboflavin and its Significance

Riboflavin (RF) is one of essential vitamins that is soluble in water. It can be derived from plants and microbes. It is required for human development and reproduction (Revuelta *et al.,* 2016). It is a water-soluble heat stable compound, that was first isolated from milk whey in 1879. Its natural sources include calf liver, fish, milk, egg, fruits, nuts, mushrooms, legumes, wild rice, cheese, vegetables and beer. In humans, RF works as a precursor for the coenzymes FMN and FAD, which both participate in oxidation and reduction pathways. These flavo-coenzymes metabolize carbohydrates, ketone bodies, lipids and proteins that provide energy to living organisms (Buehler, 2011).

Because of the restricted absorption of RF in humans, Zhang *et al.,* (2010) found that vertebrates preserved the least amount of it. Orally given RF is recommended to avoid ariboflavinosis (which produces a sore tongue, cheilitis, and a rough patch on the vulva or scrotum). RF is not hazardous since it is eliminated in the urine rather than being kept in the body when consumed in large quantities.

RF is phosphorylated to FMN (Flavin Mono Nucleotide), which is then transformed to FAD (Flavin Adenine Dinucleotide) intracellularly. FMN and FAD are necessary for co-enzyme functioning in a variety of redox reactions in all forms of life and are key cofactors in the breakdown of energy. RF also aids in iron mobilization and the conversion of tryptophan to niacin, as well as the active forms of vitamins B6 and B9. (Revuelta *et al.,* 2016; Cisternas *et al.,* 2018; EFSA FEEDAP Panel, 2018).

It is an essential vitamin to improve the conventional therapies for the treatment of various diseases such as cisplatin-induced apoptosis of intestinal epithelial cells and *Staphylococcus aureus* infections (Mal *et al*., 2013).

1.4 Riboflavin producing microorganisms

Riboflavin biosynthesis has been reported from many Gram-positive and Gram-negative bacteria. *Clostridium acetobutylicum* synthesizes riboflavin as a useful product during acetone-butanol fermentation, making it the first commercial synthesis of RF using microbes (Leviton, 1946).

Many fungal species, including *Eremothecium ashbyii*, *Pichia guilliermondii*, *Ashbya gossypii*, *Candida famata* and others, have since been identified as naturally flavinogenic microorganisms (MO's) capable of synthesizing riboflavin from two major precursors: guanosine triphosphate and ribulose 5-phosphate (Sabry *et al.,* 1993; Leathers and Gupta, 1997; Suryadi *et al.,* 2000; Babyak *et al.,* 2002; Abbas and Sibirny, 2011) The MOs' RF accumulation, on the other hand, was moderate and low in concentration, indicating that they gathered RF at very low concentration, which was inadequate for industrial RF synthesis (Ohara *et al.,* 2016).

Based on Liu *et al.,* (2020) work, RF biosynthesis begins with two key components, GTP and Ribu5P, which are generated from the pentose pathway and/or, purine biosynthesis and then proceeds through seven enzymatic steps to produce the

end product. According to a research on RF biosynthesis, many enzymes and steps involved in the RF synthesis are mostly same across prokaryotes and plants, however fungi use a different pathway and enzymes.

Abbas and Sibirny, (2011) reported that *C. famata* and *B. subtilis* make GTP and Ribu5P precursors from glucose, whereas *A. gossypii* prefers fatty acids. For two major industrial producers, the Gram-positive bacteria *B. subtilis* and the fungus *A. gossypii*, many insights on riboflavin biosynthesis have been obtained.

1.5 Riboflavin producing lactic acid bacteria

Lactic acid bacteria (LAB) are industrially significant MO's utilized in the food and dairy industry because of their fermentative properties. LAB are Generally Regarded as Safe (GRAS) organisms, and their use as probiotics is widespread because they play an important role in maintaining health (Stamer, 1979).

LAB have a completely fermentative metabolism, with lactic acid as the primary end product following sugar fermentation, (Stamer, 1979). LAB may be found in a range of environments, particularly foods and animals and humans' gastrointestinal and urogenital tracts. Although most vitamins are not synthesized by humans or animals, bacteria have the genetic ability to synthesize those metabolites (LeBlanc *et al*., 2011). Many studies have shown that LAB can confer beneficial characteristics on their hosts, particularly members of the genus *Lactobacillus*, and as a result, they are the most extensively used probiotic MO's (LeBlanc *et al.,* 2011). Apart from their technological significance in food production, these MO's also have a variety of additional benefits, such as the ability to synthesize B group vitamins, particularly riboflavin, to produce fermented bio enriched food (Capozzi *et al*., 2011; Laino *et al*., 2012; Vaesken *et al*., 2012). LAB are commonly utilized in the dairy industry, and adding riboflavin-producing bacteria to fermented dairy products such as fermented milks, yoghurt, and cheeses enhance RF levels in an easy and cost-effective manner. (LeBlanc *et al*., 2005a).

Customers are getting more health conscious and selective in their food selections as a result of today's lifestyle. (Burgess *et al*., 2004). In current scenario, LAB are used to replace chemical processes in the manufacturing of RF while also

boosting the nutritional value of food (LeBlanc *et al*., 2012). Microbial fermentation process for riboflavin production is economical and environmental friendly as well. Other elements that contribute to the benefits of LAB include the utilization of renewable resources and higher final product quality.

Many LAB strains in dairy products have the potential to create B group vitamins such as RF and niacin, as well as folic acid (Michaelidoua and Steijnsb, 2006). The use of vitamin-producing microorganisms is more cost-effective replacement to fortification utilizing chemically produced pseudo-vitamins, and would allow for the manufacture of foods with greater vitamin concentrations which are less likely to trigger unwanted side effects.

Probiotics have a variety of health benefits, including promoting a healthy microbiota, lowering blood cholesterol, preventing food allergies and infectious diseases, stabilizing the intestinal mucosal barrier, improving anticarcinogenic activity, improving body immune adjuvant characteristics, alleviating symptoms of intestinal bowel disease, and improving lactose digestion in hosts who are lactose intolerant (Ouwehand *et al.,* 2002; Deshpande *et al.,* 2011; Soccol *et al.,* 2011)

LAB provide fermented foods a better texture, flavor (by producing aromatic compounds), and nutritional value by modifying or synthesizing proteins, exopolysaccharides, and essential nutrients such as vitamins, in addition to producing lactic acid (Wood and Holzapfel, 1995; Hugenholtz, 2008). Because of their beneficial characteristics, LAB like *Streptococcus* and *Lactobacillus* are frequently employed in the food and pharmaceutical sectors (Leroy and Vuyst, 2004; van Hylckama Vlieg and Hugenholtz, 2007; Hugenholtz, 2008)

Various strains of LAB can generate, release, and/or increase specific beneficial compounds in foods. These bioactive substances are commonly referred to as nutraceuticals, a word used by Stephen DeFelice in 1989 that describe "food that provides medical or health advantages, such as disease prevention or therapy."

The appropriate choice and use of nutraceutical producing MO's is an intriguing technique for manufacturing innovative products with increased nutritional and/or health-promoting properties (Hugenholtz and Smid, 2002). Indonesian scientists isolated indigenous LAB from fermented products and other sources (Pramono, 2008; Antara, 2009; Lawalata, 2011; Suhartatik *et al.,* 2014). LAB having probiotic properties

have been claimed to be advantageous to one's health (Purwandhani and Rahayu, 2003; Rahayu *et al.,* 2015), for example, producers of β-glucosidase (Suhartatik *et al.,* 2014; Djafaar *et al.,* 2013), angiotensin-converting enzyme (ACE) inhibitor produced by this enzyme (Wikandari *et al.,*2012); antioxidant and isoflavone activities (Djafaar *et al.,* 2013a).

Human RF deficiency is common in both poor and developed nations, despite the abundance of most vitamins in diet (O'Brien *et al*., 2001; Blanck *et al*., 2002) owing to a lack of food or an imbalanced diet (LeBlanc *et al*., 2011).

Therefore, bacterial strains belonging to the LAB group have been researched for RF synthesis, and numerous LAB strains that can overproduce riboflavin have recently been found (Thakur and Tomar, 2015; Thakur and Tomar, 2016, Thakur *et al*., 2016). As a result, during the fermentation process, the product is fortified with this vitamin. Making neutraceutical yoghurt enhanced with RF will be a beneficial alternative to using fortified foods or pharmaceutical formulations to adjust for RF deficiency, because fermented foods such as yoghurt are not further processed after fermentation.

1.6 Riboflavin producing genes

Many studies have looked at the mechanism of RF biosynthesis. In most bacterial species, riboflavin production is controlled by four genes grouped in an operon rib (A, B, G, and H). These genes encode the transcription and translation of the enzymes required for the biosynthesis of riboflavin from Guanosine Triphosphate (GTP). The transcription among these genes is controlled by the ribP1 promoters and regulatory region, which is located at the 5' terminus of the operon (Perkins *et al*., 1999).

The bacterial strains which possess all the genes for RF production have the capability to produce RF during growth; however, the concentration varies from strain to strain.

According to Pedrolli *et al*., 2015, the metabolic pathway carried out from the rib operon in *B*. *subtilis* is made up of five genes: rib (D, G, B, H, and T). The DNA of *A. gossypii* is arranged on seven chromosomes and genes involved in RB biosynthesis, rather than being aggregated as in bacteria. The six riboflavin biosynthetic genes in *A.*

gossypii are RI (B1, B2, B3, B4, B5, B7), and their regulation are very analogous to those of *Saccharomcyes. cerevisiae*, that has become a widely used model for the development biology of fungi.

1.7 Production of riboflavin through genetic engineering

Several researchers have reported successfully creating genetically modified strains of organisms such as *E. coli*, *Corynebacterium ammoniagenes*, *Candida spp*. and *B. subtilis* utilizing metabolic engineering approaches in previous years. Most of the time, these genetic engineering techniques result in overexpression of structure and regulating genes responsible for the synthesis of RF and its precursor, leading in increased yield of the industrial fermented product (Koizumi *et al.,* 2000; Taniguchi and Wendisch, 2015; Wang *et al.,* 2015).

There are, nevertheless, some unsolved questions, which are generated by a number of non-specific processes in RF biosynthesis that aren't well known.

The most recent scientific investigations have looked into increasing the production of vitamin B2 and its precursors by microorganisms utilizing various tactics such as medium constituent optimization, genetic engineering, screening, mutations and biocatalyst conversion.

1.8 Production of Riboflavin at industrial level

At Industrial level RF can be produced by both chemical and fermentation process. Fermentation produces vitamin B2 in a single step, which is a cost-effective way, but the chemical synthesis is a multi-step procedure that is also costly. During the past 15 years, the chemical synthesis has been replaced by fermentative production due to its economic and ecological feasibility and the riboflavin produced by fermentation was almost doubled (from 4000 t a^{-1} to 9000 t a^{-1}) for human and animal consumption (Schwechheimer *et al.,* 2016).

Currently, 70 percent RF available in the market are mostly employed as a feed supplement manufactured by fermentation using genetically engineered strains (Revuelta *et al.,* 2016).

Selection of appropriate RF overproducing strains is essential for preparation of yogurt enriched with this vitamin. A simple method for this purpose is the utilization of roseoflavin, a toxic RF analogue as selective agent in the growth medium. Because of changes in the rib operon's regulatory region, it has been discovered that resilience to this harmful substance commonly correlates with RF excessive production in strains of bacteria (Burgess *et al*., 2004).

1.9 Consequences of riboflavin deficiency

Vitamins and their precursors can be found in a wide range of foods. However, the Vitamin deficiencies still occur in the most of the developing countries like Pakistan due to the imbalanced diet consumed by people. In developing countries where diet lacks meat and dairy products riboflavin deficiency persists (Combs, 1992; Rohner *et al*., 2007). Young females in the UK and Republic of Ireland also have been shown to have a high frequency of low RF levels (O'Brien *et al*., 2001; Powers, 2003). RF deficiency results in poor eyesight, slowed development, and excessive homocysteine levels, all of which increase the risk of heart disease (Moat *et al*., 2003), pre-eclampsia (Wacker *et al*., 2000), oxidative stress (Ashoori and Saedisomeolia, 2014) anaemia (Shi *et al*., 2014), and hair loss (Wood and Holzapfel, 1995). Its deficiency also results in liver and skin damages, as well as changes in brain glucose metabolism (LeBlanc *et al*., 2011). The symptoms of riboflavin deficiency include sore throat, hyperemia, oedema of the oral and mucosal membranes, cheilosis, and glossitis (Wilson, 1983). Furthermore, RF is also required for activation of folic acid and pyridoxine to their respective co-enzyme forms. Therefore, deficiency of riboflavin can also result in the disorders related to pyridoxine and folic acid deficiencies (Massey, 2000). As a result, many countries have passed legislation requiring the enrichment of diets with riboflavin. However, the researchers are now looking for natural and economically viable alternatives, and utilization of riboflavin producing bacteria is being looked as one of such alternatives (Le Blanc *et al.*, 2011).

1.10 Dietary requirements of riboflavin

The Recommended Daily Allowance (RDA) for RF is 1.6 mg per day (Wood and Holzapfel, 1995). Despite the reality that riboflavin may be found in a variety of

foods, levels were lower in many parts of the world, especially in impoverished nations (Hugenholtz, 2008). A riboflavin supplement or the use of RF enriched fermented foods like cheese and yoghurt can assist to recover for the deficiency. Chemical methods of producing riboflavin were previously used, but microbial fermentation technologies have subsequently made their mark (Leroy and Vuyst, 2004).

1.11 Beneficial health effects of riboflavin

1.11.1 Reperfusion Oxidative Injury

According to Sanches *et al.,* (2014), reperfusion oxidative injuries relate to tissue damage produced by an increase in free radicals and inflammatory cytokines following ischemia. Due to its capacity to scavenge free radicals and hence prevent re-oxygenation damage, RF can reduce oxidative injuries. Reactive oxygen species (ROS) are thought to have a pivotal role in the pathogenesis of ischemia/reperfusion illness, according to new study.

1.11.2 Cancer disease

RF deficiency has been implicated as a health risk for cancer illness. According to an investigation, higher folate supplementation may lower the breast cancer risk in Chinese women (Shrubsole *et al*., 2011).

1.12 *In-situ* food enrichment with riboflavin

RF was formerly produced chemically for dietary and fodder enrichment, but commercial microbial processes have boosted its production in recent years (Stahmann *et al*., 2000). The fact that enrichment happens *in-situ* is a significant advantage of vitamin-producing LAB. Because of their ability to ferment, and also their biosynthesis ability and metabolic variety, LAB are strong contenders for *in-situ* riboflavin biosynthesis in food (Arena *et al*., 2014b). Folates, biotin, pantothenic acid, nicotinic acid, riboflavin, pyridoxine, and thiamine are among the water-soluble B vitamins produced by gut commensal microbes. (Hill, 1997). This study focuses on the LAB and their genetic potential for RF biosynthesis.

1.13 Why to study

The current project has been planned for the preparation of a novel dairy product, i.e. nutraceutical yogurt.

1.14 Research Objectives

The objectives of the current research study are:

1) Estimation of RF in dairy products i.e. milk and yogurt.
2) Isolation and biochemical characterization of RF producing LAB.
3) Molecular identification of riboflavin producing LAB.
4) Screening of riboflavin overproducing LAB.
5) Application of riboflavin producing strains in yogurt preparation.

Chapter 2

MATERIALS AND METHODS

2.1 Preparation of riboflavin standard curve

A stock solution of riboflavin was prepared by dissolving 0.0528 g of riboflavin (Sigma, Pcode:1001439702) in 1000 mL of distilled water to obtain concentration of 1.403×10^{-4} M. The stock solution was used to prepare serial dilutions ranging from 2×10^{-5} to 5×10^{-6} M. Finally, the riboflavin estimation was determined using a spectrophotometer at 440 nm, which demonstrated to be precise, repeatable, and accurate. A total of 8 dilutions were prepared for preparation of standard curve.

2.1.1 Sample collection and riboflavin estimation in market yogurt and milk samples

For the determination of riboflavin concentration, yogurt and milk samples were collected from local market of Islamabad, Rawalpindi, Mardan and Peshawar. The content of riboflavin was determined by filtering the supernatant of yogurt and curdled milk with Whatman's No.1 filter paper. Spectrophotometric determination was done at 440 nm. The riboflavin concentration in the samples was determined using the standard curve.

2.2 Sample collection for isolation of Lactic Acid Bacteria

Yogurt and raw milk samples were collected from major retailers in Islamabad, Rawalpindi, Peshawar and Mardan. The samples were collected in sterile vials and brought on ice as quickly as possible to the laboratory of **Animal Products Improvement Program, Animal Sciences Institute, National Agriculture Research Center** for further processing. The samples were kept at 4˚C till further processing.

2.3 Preparation of serial dilutions

2.3.1 Milk

One mL raw milk was diluted in nine mL physiological saline to make a serial dilution of milk. From this first dilution, 1mL of the aliquot was pipetted out into another vial containing nine ml of physiological saline. In this way the final dilution was prepared up to 10^{-3}. One mL of the aliquot was used for inoculation on MRS agar.

2.3.2 Yogurt

The yogurt samples were first homogenized with a whisk to remove any lumps in yogurt. After homogenization, one gram of yogurt was measured and poured into vials containing the physiological saline to make the first yogurt dilution. From this yogurt dilution, the serial dilution was carried out as explained above and final dilution was set to 10^{-3}. From each dilution 1 mL of dilution was used for inoculation.

2.4 Inoculation

MRS (Man, Rogosa and Sharpe) agar media was used for inoculation (culturing and sub-culturing).

2.4.1 Isolation and culturing of LAB from raw milk and yogurt

The samples were cultured using the spread plate method. 1 mL of the aliquot was spread onto MRS (Man, Rogosa and Sharpe) agar using a glass spreader. Following that, the plates were incubated for 24 hours at 37 ℃.

2.4.2 Sub-culturing

The well isolated colonies from the initial incubation were sub-cultured on agar plates for 3-4 times using the streak plate method for further identification of bacteria. The colony shape, catalase response, and Gram's reaction of the purified isolates were then investigated.

2.5 Biochemical identification of LAB

The LAB were initially identified using the differentiation method published by Schillinger and Lucke (1987), which comprised of Gram's staining and the catalase test.

2.5.1 Gram's Staining

Vincent described the Gram's staining of bacterial isolates (1970). Appendix I lists the staining reagents. Briefly, a drop of distilled water was placed on a clean glass slide and a single isolated colony from fresh bacterial culture was chosen and mixed with water droplet to form a smear. Heat was used to fix the smear.

After the heat fixed smear was prepared, it was proceeded with Gram staining steps. First, crystal violet was used to stain the slide. After thirty seconds, the slide was rinsed with distilled water, and iodine solution was applied to the smear, and the slide was washed with distilled water. After washing, the smear was decolorized for a few seconds with 75 percent alcohol. After washing the bacterial smear, it was stained with safranin. Cover slips were put on top of glass slides. that had been rinsed with distilled water. The slides were then examined under a microscope at magnification of 1000X. Gram's positive colonies with a purple color were tested for catalase activity, whereas Gram's negative colonies with pink color were discarded.

2.5.2 Catalase activity

Catalase activity of bacterial strains was determined by using hydrogen peroxide (H_2O_2). For this purpose, MRS agar plates were streaked with Gram positive bacterial strains and incubated for 24 hours at 37 °C. A single bacterial colony was placed on a glass slide, along with 2 drops of 3% hydrogen peroxide. (H_2O_2). After ten seconds, bubble production indicated positive result and no bubble formation indicated negative results (Montgomerie, 1966; Macfaddin, 2000).

The Gram's positive bacterial colonies (cocci and bacilli) showing negative catalase activity were selected as presumptive LAB and preserved for further studies.

2.6 Identification of the LAB using Polymerase Chain Reaction (PCR)

2.6.2 DNA extraction

DNA was extracted from 24-hour fresh bacterial colonies (grown on MRS agar) using the phenol chloroform technique. Well isolated bacterial colonies were dissolved in PBS. The material was then dissolved in 700 µl of High Salt lysis buffer (10 mM Tris HCl, 50 mM EDTA, 100 mM NaCl) and kept at room temperature for 20-30 minutes followed by addition of 13 µl of 20% SDS (Sodium Dodecyl Sulphate) and 25 µl of proteinase K. The tubes were then incubated at 56 °C for 3 hours.

After incubation, the samples were treated with 500 µl of phenol, chloroform, and isoamyl alcohol to ensure full lysis. For gradual and comprehensive mixing, the suspended solution was centrifuged for ten minutes at 13000 rpm. For purification and separation of DNA, the aqueous phase was moved to another tube. 500 µl of chloroform and isoamyl alcohol were used to treat the supernatant layer and centrifuged again for 10 minutes at 13000 rpm. Then 55 µl of 3M sodium acetate and 500 µl of cold isopropanol were added to the aqueous layer in a 1.5 ml centrifuge tube. The tubes were kept at -20°C for 45 minutes to ensure their preservation, followed by centrifugation at 13000 rpm for 10 minutes. The supernatant was removed, and the pellet was processed with 500 µl of 70% ethanol and centrifuged for 5 min at 7500 rpm to eliminate any contaminants. The pellet was maintained, and the supernatant was removed and air dried. The DNA pellet was resuspended in 200 µl of TE Buffer (Tris EDTA buffer) and kept at 4 ℃.

2.6.3 DNA quantification

The quality and amount of DNA were determined using the Nanodrop plates (Skanit RE 4.1, Thermoscientific), absorbances were measured at 260, 280 and 320 nm.

2.6.4 Agarose gel electrophoresis

One percent agarose gel was prepared by mixing 1 g of agarose in 100 ml of 1X TAE (Tris Acetic Acid Ethylene Diamine Tetra Acetic Acid) buffer. A clear liquid was produced after heating. The DNA intercalating dye ethidium bromide solution was

added to agarose gel solution (7 µl). Agarose gel solution was poured into the gel tray. The solidified gel caster was put in the gel tank having 1X TAE carefully and gently the combs were removed. Two µl DNA and 2 µl of 6X bromophenol blue dye (loading dye) were mixed by pipetting. It was then loaded in wells. The gel was run for 60 min at 500 mA of current and 75 Volts.

2.6.5 PCR (Polymerase Chain Reaction) amplification

In molecular biology, PCR is being used to amplify DNA. Two sets of primers were used to amplify the DNA of bacterial samples. Table 2.1 shows the forward primer and reverse primer sequences.

Table 2.1 16S rDNA gene primers

1492R	GGTTACCTTGTTACGACTT
27F / 8F	AGAGTTTGATCCTGGCTCA

The following chemicals were utilized at the specified concentrations:

- DNA template
- Forward primer and Reverse Primer (Macrogen, Korea)
- Enzyme Taq polymerse 5U/ µL (Thermo scientific 01047431)
- PCR buffer ((Thermo scientific 01047431)
- $MgCl_2$ (Thermo scientific 01047431)
- dNTPs (Thermo Scientific 01044193)
- PCR grade water (Invitrogen, AM-9935)

Table 2.2 PCR Reaction Mixture

Reagents	Stock	Working	Vol
DNA template	-	-	1 µl
$P\text{-}_F$	10 µM	0.2 µM	0.4 µl

P-$_R$	10 µM	0.2 µM	0.4 µl
dNTPs.	10 mM	0.2 mM	0.4 µl
PCR Buffer.	10X	1X	2 µl
$MgCl_2$.	25 mM	2.5 mM	2 µl
Taq-Polymerase.	5U/ µl	1.5U	0.3 µl
PCR grade H_2O			13.5 µl
Final Volume			20 µl

A Galaxy XP Thermal Cycler was used to run PCR (BIOER, PRC).

Table 2.3 Optimized conditions for PCR

	Steps	Temperature	Time	cycles
PCR-Cycles.	Initial-denaturation	95 ˚C	10 min	1
	Denaturation.	95 ˚C	60 sec	40
	Primer-annealing	56 ˚C *	60 sec	
	Primer-extension	72 ˚C	60 sec	
Final-extension		72 ˚C	10 min	1
Hold.		04 ˚C	for ∞	1

2.6.5.2 Gel electrophoresis

Two percent agarose gel was used to observe the amplified product under UV light.

2.6.6 DNA sequencing

The PCR products were sequenced by using Sanger sequencing technique and for this purpose the amplified strains were sent to Macrogen (South Korea).

2.7 Preservation of LAB

For further examination, the LAB strains were preserved in 20% (v/w) glycerol and stored at -80 °C. To create glycerol stock solutions, 0.5 ml active cultures were combined with 0.5 ml MRS broth and 0.5 ml glycerol in Eppendorf tubes. In the end, the final solution had 20% glycerol. The frozen samples were prepared in triplicate to

avoid contamination or loss of activity, and only one batch was used for the experiments.

2.8 Bioinformatics analysis

For bacteria, BLAST analysis for closest family members was carried out through EzTaxon-e identification service (http://www.ezbiocloud.net/identify) following recension of the gene sequence files using ChromasPro software (http:// www.technelysium.com.au/ Chromas Pro.html) (Chun *et al.,* 2007).

2.9 Riboflavin test and screening of Lactic Acid Bacteria

2.9.1 Screening of riboflavin production by the bacterial isolates

LAB's capability to synthesize RF differs within species and even among strains. Growth on a riboflavin assay medium in the presence or absence of the RF, was the most accurate way to determine this. For this purpose Riboflavin Assay Medium (RAM) was prepared which was devoid of riboflavin. About 1% of the various cultures' overnight inoculums were introduced into the sterile medium and incubated at 37 ºC in static environment. After 24 hours of growth, RF was detected in cultured samples.

2.10 Growth on Riboflavin Assay Medium

Identified lactic acid bacterial strains were cultured on RAM (BD Difco). The media was deprived of riboflavin content. Only potential riboflavin producers can grow on RAM medium.

2.11 The safety tests of LAB isolates

2.11.1 Hemolytic activity

Bacteria were cultivated on a blood agar basis (Oxoid, Thermo Fisher Scientific, USA) enriched with 5% (v/v) sheep blood to determine their capacity to generate different types of hemolysins. Plates were incubated with gas-generating kits in anaerobic jars (Oxoid, Thermo Fisher Scientific, USA) at 37 °C. The data was collected

after 24-hour and 72-hour incubation periods. The existence of a clear zone on the blood agar plates signified a positive result.

2.11.2 Antibiotic susceptibility

To determine the antimicrobial properties of the isolated LAB, the agar disc diffusion method was applied. Nine commercially prepared antibiotics such as vancomycin, chloramphenicol, gentamicin, tetracycline, clindamycin, erythromycin, streptomycin, kanamycin and ampicillin (Oxoid, Thermo Fisher Scientific, USA) were chosen to represent the several groups of therapeutically significant antibiotics. LAB isolates (10^8 CFU/mL) were swabbed onto Muller-Hinton agar plates, and then antibiotic discs were placed aseptically using sterile forceps and incubated at 37 °C for 24 hours. Zones of inhibition were observed on the MHA plates. The diameter of the inhibition zones (mm) was used to measure the antibiotics' inhibitory efficacy.

2.12 Application of the bacterial strain

2.12.1 Yogurt manufacturing

Yogurt was prepared from skimmed milk powder (SMP). For this purpose 12% skimmed milk was prepared from SMP. The milk was inoculated with bacterial strains and then incubated for 6 hours at 37 °C, until the pH level reached about 4. The containers containing the fermented milk were then quickly refrigerated to 15 °C. The experiment was replicated thrice.

2.12.2 Determination of riboflavin in yogurt samples

The content of riboflavin was determined by filtering the supernatant of yogurt with Whatman’s No.1 filter paper. Spectrophotometric analysis was done at 440 nm. The riboflavin concentration was obtained from the standard curve.

2.13 Statistical analysis

The results were determined as the mean and standard deviation (Mean±SD) of three separate experiments.

Chapter 3

RESULTS

3.1 Estimation of riboflavin in samples

3.1.1 Standard curve preparation

To check the absorbance of riboflavin of various samples of yogurt and milk, a standard curve was prepared. For this purpose, eight dilutions with known concentrations were taken (as shown in the Table 3.2) and their absorbance was obtained on spectrophotometer at 440 nm.

Table 3.1: Riboflavin concentration for standard curve preparation

S. No	Conc (M)	mg/L	Abs (440 nm)
1.	0.00002	5.52	0.294
2.	0.000015	4.00	0.23
3.	0.00001	2.76	0.177
4.	0.000009	2.484	0.17
5.	0.000008	2.208	0.151
6.	0.000007	1.932	0.14
7.	0.000006	1.656	0.121
8.	0.000005	1.38	0.116

The riboflavin absorbance of different (milk and yogurt) samples was then estimated from standard curve. Figure 3.1 shows the standard curve of absorbance of the known solutions.

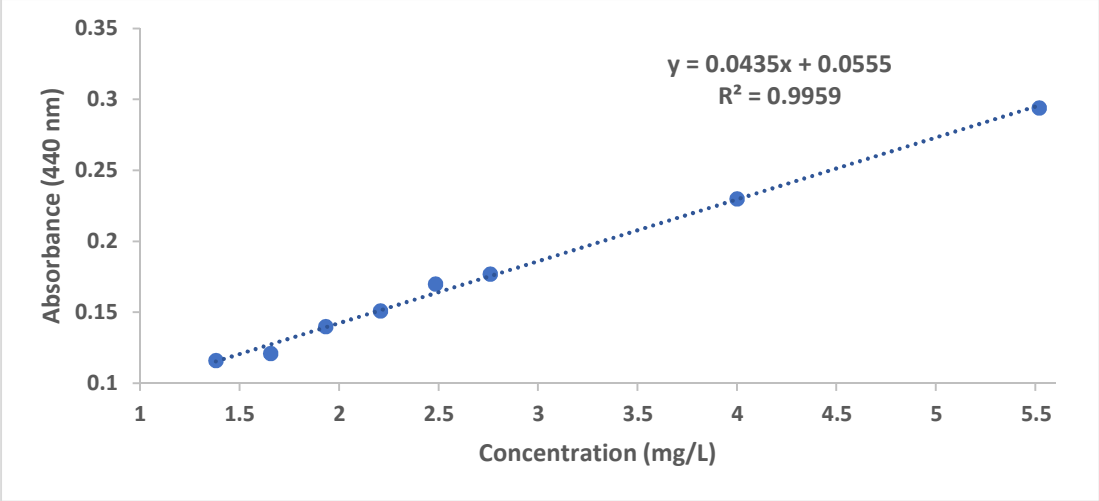

Fig 3.1: The standard curve for riboflavin estimation

3.1.2 Riboflavin estimation in market milk samples

The supernatant of the curdled milk was taken and absorbance was obtained by using spectrophotometer. The absorbance was compared to the standard curve as discussed above. The concentration was obtained accordingly and depicted in Table 3.2. The highest riboflavin concentration in milk was 3.48 mg/L and the lowest was 1.9 mg/L. The average value obtained was 2.515 ± 0.65 mg/L.

Table 3.2: Riboflavin concentration in market milk samples

S. No	Sample ID	Absorption	Conc. (mg/L)
1.	MR-1	0.171	2.65
2.	MR-2	0.191	3.11
3.	MR-3	0.158	2.36
4.	Mgoat-1	0.189	3.07
5.	ML-1	0.162	2.45
6.	ML-2	0.188	3.04
7.	MI-1	0.159	2.38
8.	MI-2	0.202	3.36
9.	MI-3	0.153	2.24
10.	Mcow-1	0.151	2.2
11.	Mbuff-1	0.189	3.07
12.	MR-4	0.121	1.51
13.	MR-5	0.127	1.65
Mean			**2.545385**
Standard Deviation			**0.573405**

3.1,3 Riboflavin estimation in market yogurt samples

The supernatant of the yogurt was taken and absorbance on spectrophotometer was obtained. The absorbance was compared to the standard curve. The concentration was obtained accordingly and depicted in Table 3.3. The highest riboflavin concentration in yogurt was 3.11 mg/L and the lowest was 1.51 mg/L. The average value obtained was 2.54 ± 0.573 mg/L.

Table 3.3: Riboflavin concentration in market yogurt samples

S. No	Sample ID	Absorption	Conc. (mg/L)
1.	YR-1	0.179	2.84
2.	YR-2	0.144	2.04
3.	YR-3	0.182	2.91
4.	YL-1	0.207	3.48
5.	YI-1	0.138	1.9
6.	YI-2	0.139	1.92
		Mean	**2.515**
		Standard Deviation	**0.655858**

3.2 Morphological and biochemical tests

A total of seventy-eight bacterial strains were isolated from 20 samples. These isolates were biochemically and morphologically characterized under compound microscope (Figure 3.2 and Figure 3.3) following Gram staining. Then catalase activity was also analyzed by catalase test (Figure 3.4). It was noticed that 18 strains were Gram positive and catalase positive, 20 isolates were Gram negative and catalase positive, 21 strains were Gram negative and only 19 isolates were Gram positive and catalase negative. The percentage of these isolates si depicted in figure 3.5. The 19 Gram positive and catalase negative isolates were proceeded for further studies. Table 3.5 shows complete description of results.

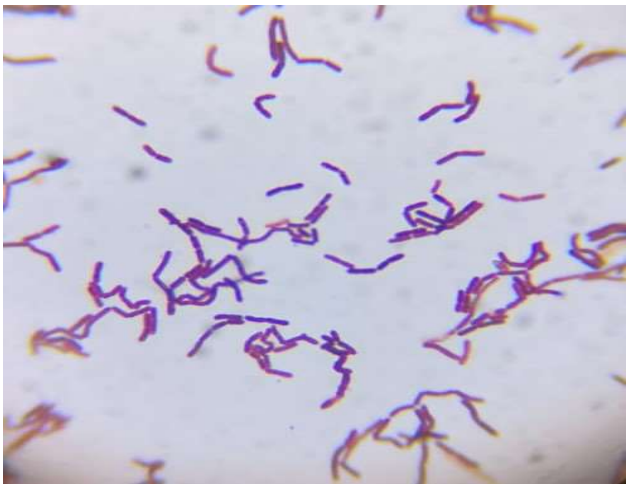

Fig 3.2: Lactobacillus strain as observed under compound microscope (1000x)

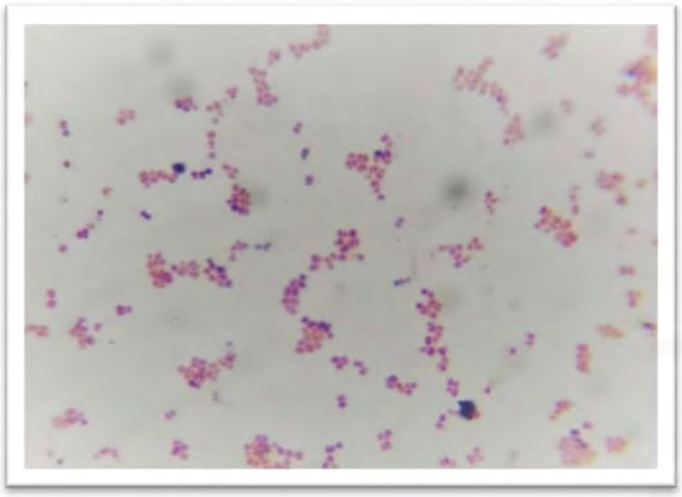

Fig 3.3: Lactococcus strain as observed under compound microscope (1000x)

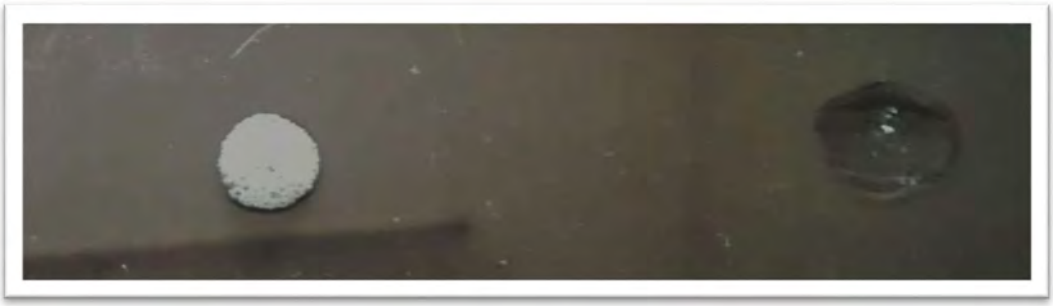

Fig 3.4: Catalase test: the left side of the slide shows bubble clearly indicates positive catalase reaction while right side with no bubbles indicates catalase negative reaction.

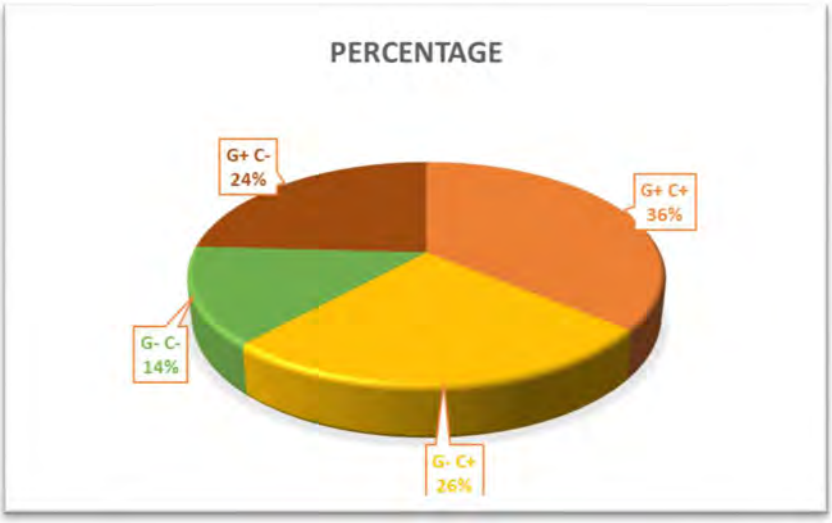

Fig 3.5 The percentages of the strains with the results of biochemical tests

Table 3.4 Biochemical Characteristics of Isolated Strains

Gram+ Cat +	Gram- Cat +	Gram- Cat -	Gram+ Cat -
28	20	11	19

3.3 Molecular identification

3.3.1 DNA extraction

The DNA of 19 strains was extracted which were catalase negative and Gram positive. The DNA was visualized Under the UV Trans-Illuminator bio Doc Analyzer. Clear bands on agarose gel confirmed presence of DNA in Figure 3.6.

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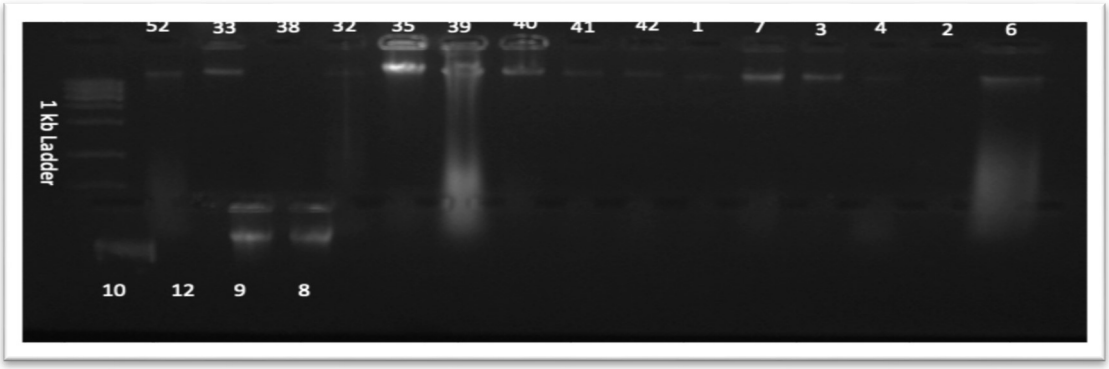
Fig 3.6 DNA bands on gel with comparison to 1KB Ladder



3.3.2 DNA quantification

The DNA of the 19 strains was quantified. The 260/280 ratio was between 1.7 and 2.0, indicating excellent quality DNA, while the DNA amount was between 800 and 1200 ng/L. The 260/280 ratio and the DNA concentration of each sample are shown in the Table 3.5.

Table 3.5 DNA concentration of bacterial isolates

Sr. No	Sample ID	260/280	Quantity (ng/ µL)
1	52	1.603	344.6
2	33	1.662	281.8
3	38	1.416	275.6
4	32	1.446	106.1
5	35	1.643	332.8
6	39	1.584	367.2
7	40	1.656	951.4
8	41	1.368	243.8
9	42	1.366	427.1
10	1	1.341	334.1
11	7	1.0271	81.30
12	3	1.615	256.8
13	4	1.512	253.2
14	2	1.321	690.6
15	6	1.656	310.1
16	10	1.332	108.8
17	12	1.446	205.6
18	9	1.601	200.4
19	8	1.666	97.30

3.3.3 16S rDNA amplification

The DNA of 19 samples were amplified using 1492R and 27F/8F primers. The amplified PCR product was visualized using 100 bp gene ruler ladder PLUS (Thermo scientific). Fig 3.7 shows clear bands of rDNA of 19 bacterial strains.

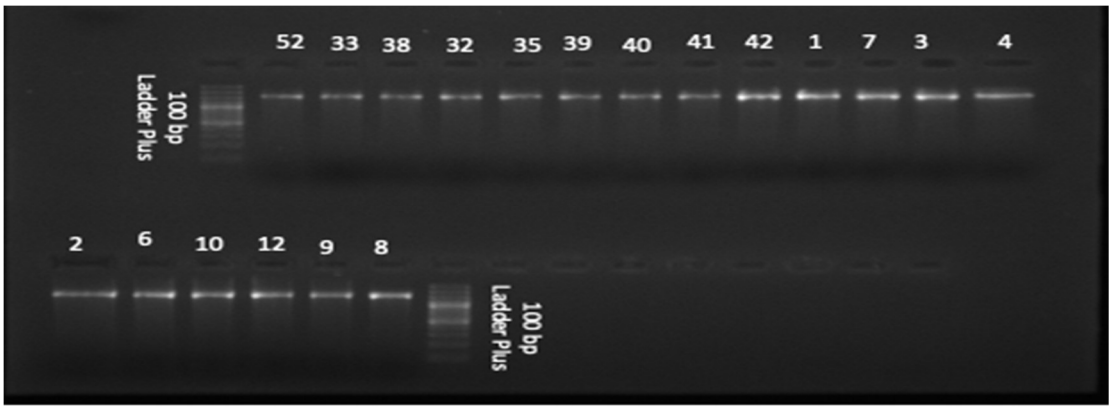


Fig 3.7: DNA bands on gel with comparison to 100bp Ladder.

3.3.4 DNA Sequencing

The DNA was opened in chromas software. The noisy peaks were trimmed to obtain a clear chromatogram (figure3.8). The file was saved in ab1 format.

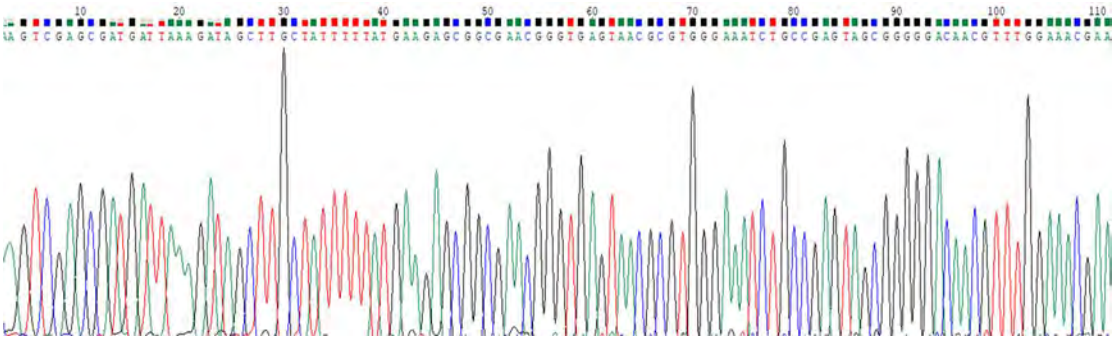


Fig 3.8: Chromatogram of sequenced strain

3.3.4.1 Trimmed file

The trimmed file was then exported to the FASTA format (Figure 3.9), which can be further used for similarity check using Basic Local Alignment Search Tool (BLAST).

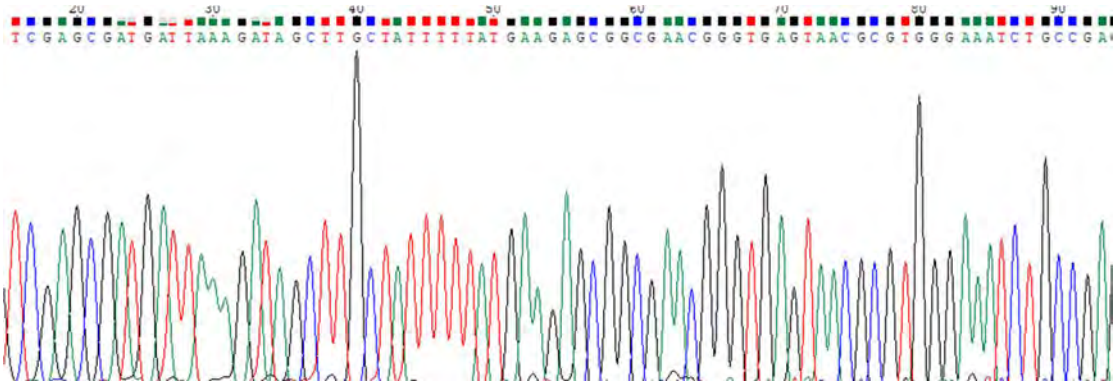


Fig 3.9: Trimmed file of sequence

3.3.4.2 BLAST

The sequences were aligned in BLAST tool in NCBI (Figure 3.10). After BLAST of sequence to nucleotide sequences in NCBI, bacterial strains showed similarity to the nearest sequence (figure 3.11). Out of 19 samples, only 7 bacterial strains were found to be Lactic Acid Bacteria. 10 nearest sequences were downloaded for phylogenetic analysis.

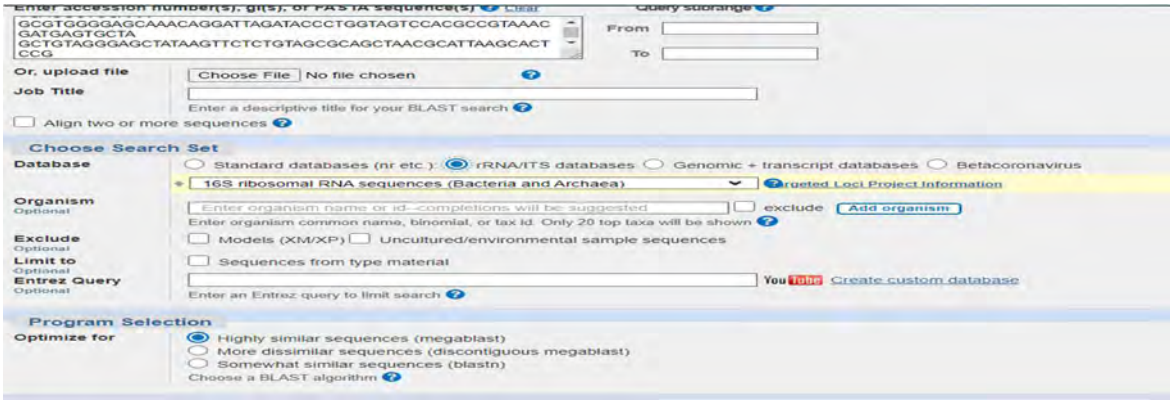


Fig 3.10: The Online Preview of the BLAST at NCBI.

Fig 3.11: Similar Sequences to Lactobacillus Plantarum (LAB-38)

3.3.4.3 Lactic Acid Bacteria (LAB) strains identified

The BLAST results show the similarity of the seven bacterial strains. Following table 3.8 showing list of LAB strains.

Table 3.6: List of LAB strains identified after 16S rDNA sequencing

S. No	Strain ID	Species identified	Seq % similarity	Seq length	Seq. of the strain
1.	LAB-01	*Lactococcus garvieae*	99.89%	847 bp	GCAAGT……CACTCCG
2.	LAB-03	*Lactococcus garvieae*	99.88%	855 bp	CTATAC ……. ACTCCG
3.	LAB-04	*Lactococcus garvieae*	99.76%	853 bp	GCTATA ……. GCACTCC
4.	LAB-07	*Lacgticoccus lactic*	99.76%	846 bp	TGCAGT...……TCCGCC
5.	LAB-09	*Lactobacillus paracaei*	99.06%	865 bp	CTATA.…...…CATTAAGC
6.	LAB-10	*Lactococcus garvieae*	99.64%	856 bp	CTATAC ……...ACTCCGC
7.	LAB-38	*Lactobacillus plantarum*	99.77%	856 bp	CTATAC ……. AGCATT

3.4 Phylogenetic analysis:

Multiple sequence alignment from10 similar sequences of NCBI database were done with Mega-11 software. The downloaded similar sequences from NCBI were retrieved in the alignment file. The 16s rDNA sequences of the seven isolates were aligned in the alignment file. Figure 3.12 clearly indicates the sequence alignment. The alignment file was saved in FASTA format for phylogenetic analysis.

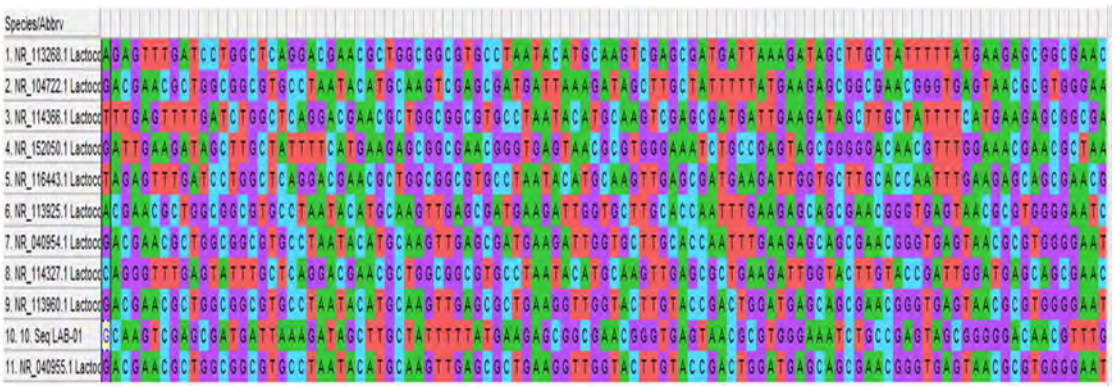


Figure 3.12 Multple Sequence Alignment (MSA) preview of sequence LAB-01

3.4.1 Phylogenetic tree

The phylogenetic tree of 7 strains was constructed using Mega-11 software. The data for phylogeny was retrieved from MSA saved file in fasta format.

3.4.1.1 Sequence LAB-01

The neighbor joining method was used to construct the phylogenetic tree. Sequence LAB-01 shows close similarity with *Lactococcus formosensis* strain 516 16S ribosomal partial sequence (figure 3.13). The sequence is also closely related to *Lactococcus lactis* species. The sequence is distantly related to *Lactococcus taiwanensis* strain 0905C15 16S rDNA partial sequence.

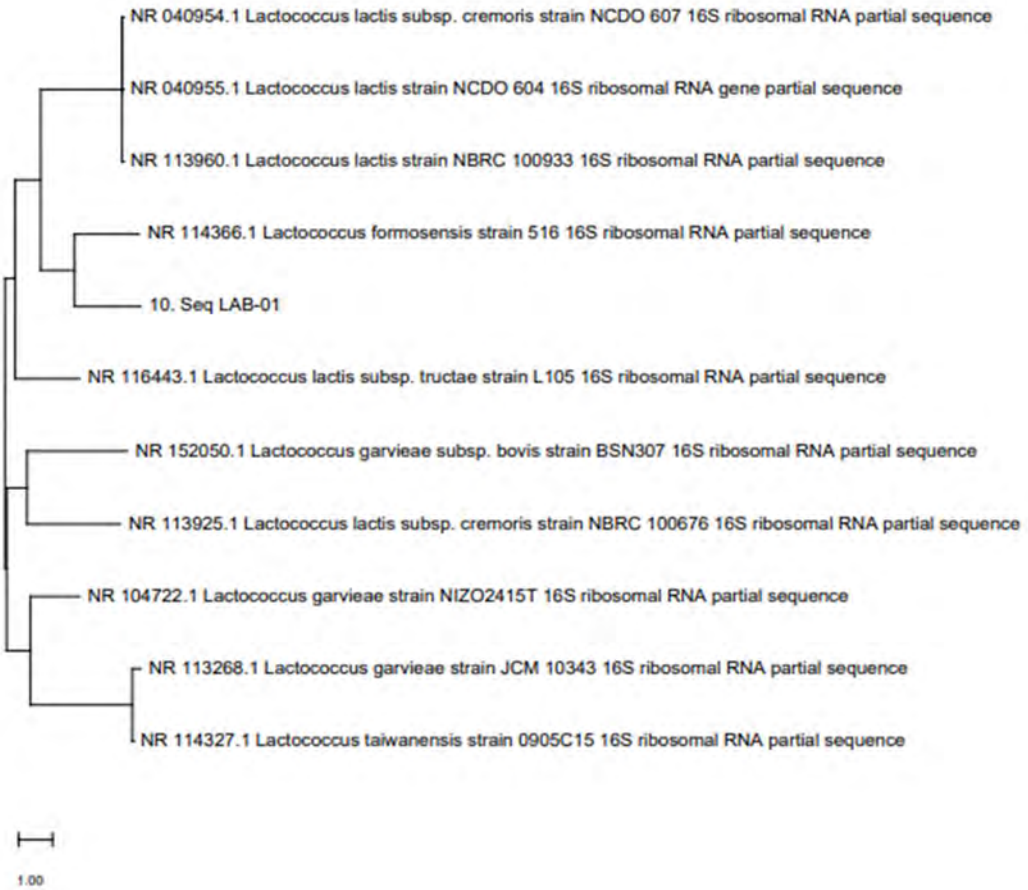


Fig 3.13 Phylogenetic tree of Sequence LAB-01

3.4.1.2 Sequence LAB-03

The neighbor joining method was used to construct the phylogenetic tree. Sequence LAB-03 shows close similarity with *Lactococcus formosensis* strain 516 16S ribosomal partial sequence. The sequence is closely related to *Lactococcus garvieae* sub-strain *bovis* BSN 307 16S rDNA partial sequence (figure 3.14). The sequence is linked to the

Lactococcus garvieae JCM 10343 16S rDNA partial sequence and the *Lactococcus taiwanensis* strain 0905C15 16S rDNA partial sequence.

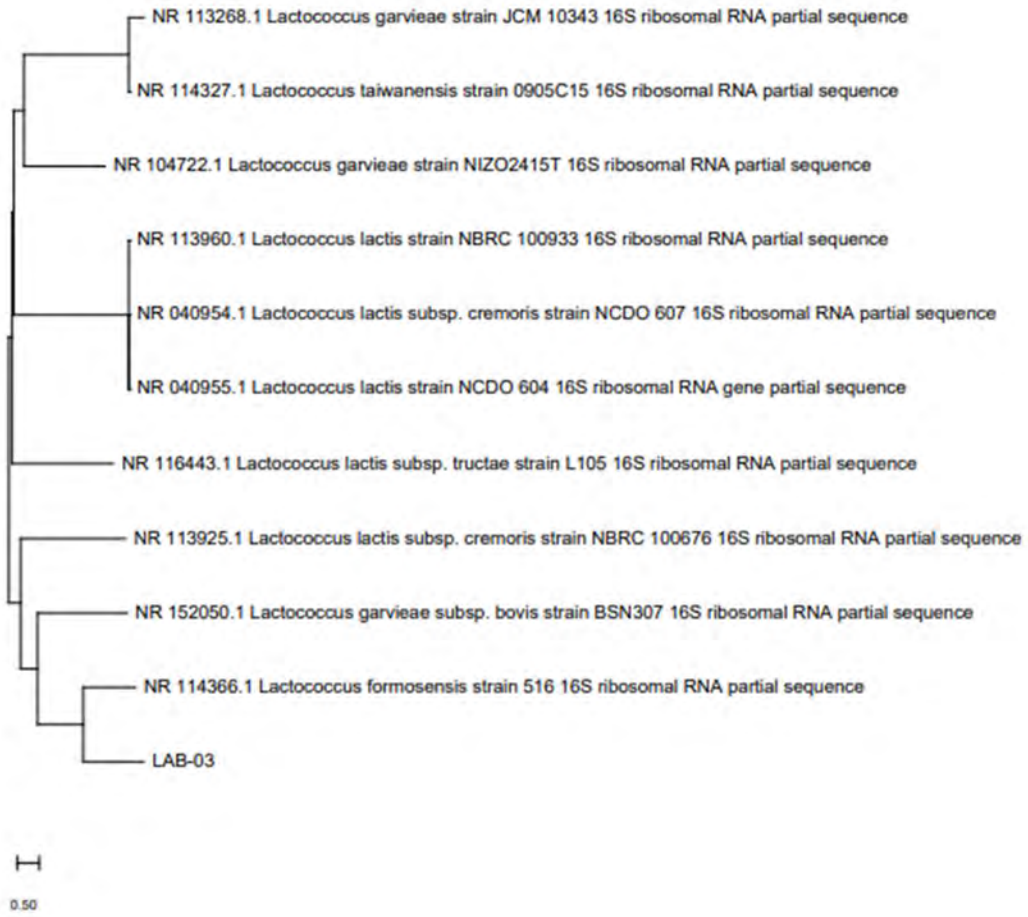


Fig 3.14 Phylogenetic tree of Sequence LAB-03

3.4.1.3 Sequence LAB-04

The neighbor joining method was used to construct the phylogenetic tree. Sequence LAB-04 showing close similarity with *Lactococcus formosensis* strain 516 16S rDNA partial sequence (figure 3.15). The sequence is also closely related to *Lactococcus garvieae* species. The sequence is distantly related to *Lactococcus taiwanensis* strain 0905C15 16S rDNA partial sequence.

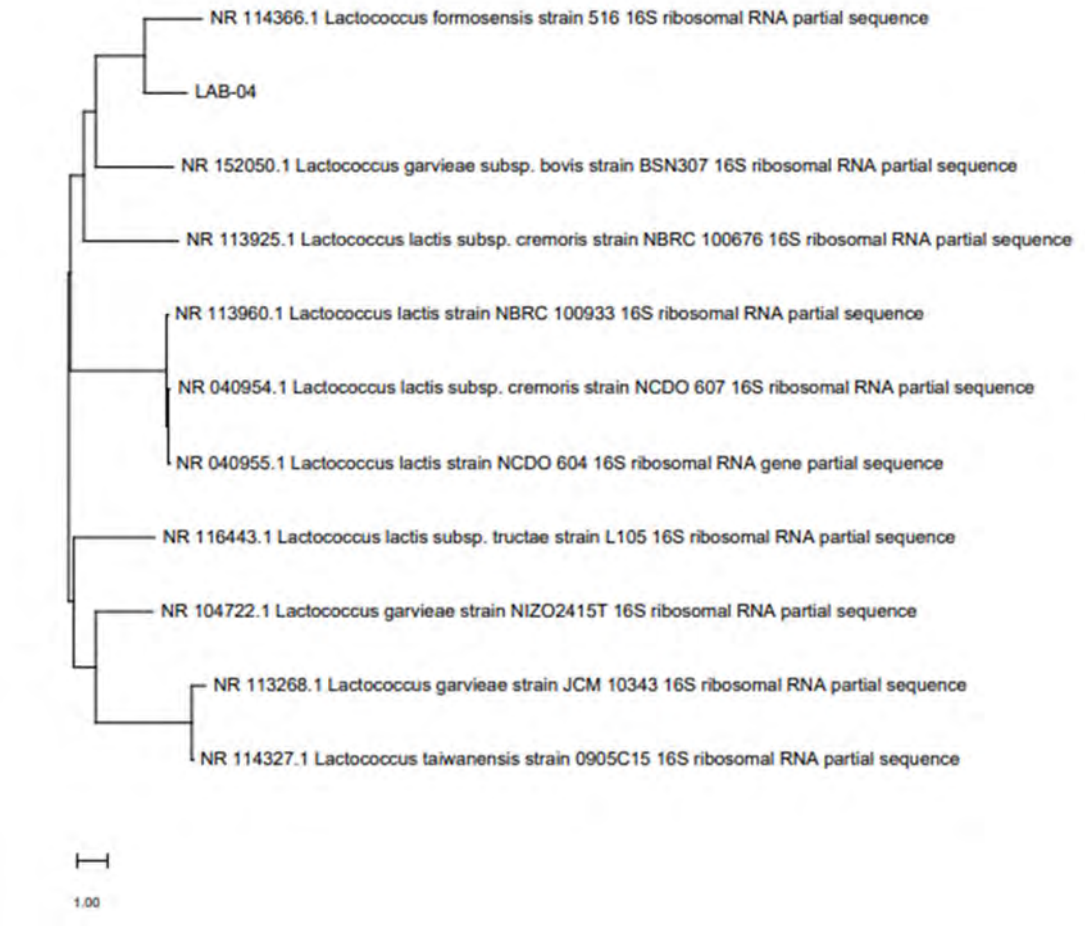


Fig 3.15 Phylogenetic tree of Sequence LAB-04

3.4.1.4 Sequence LAB-07

The neighbor joining method was used to construct the phylogenetic tree. Sequence LAB-07 shows close relationship with *Lactococcus lactis* L105 strain 16S rDNA partial sequence (figure 3.16). The sequence is likewise comparable to the partial 16S rDNA sequence of *Lactococcus taiwanensis* 0905C15. The sequence is distantly linked to the 16S rDNA partial sequence of *Lactococcus lactis* strain NBRC 100933.

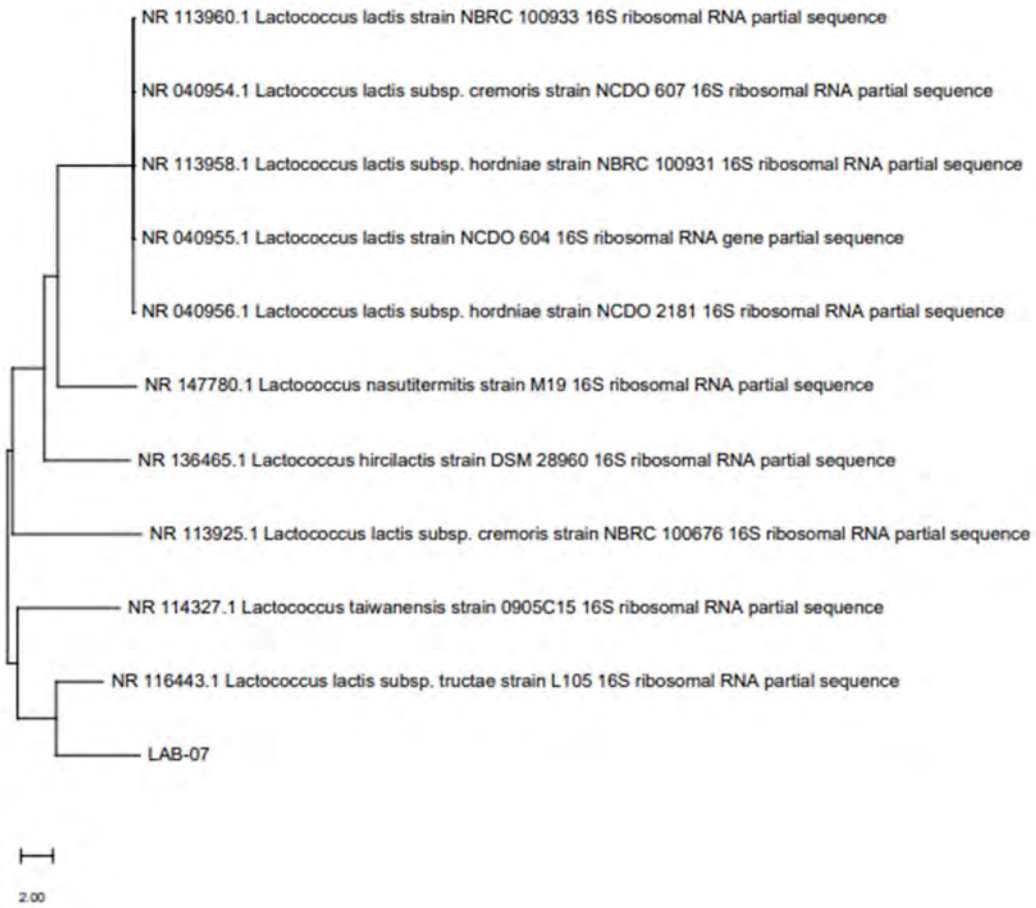


Fig 3.16 Phylogenetic tree of Sequence LAB-07

3.4.1.5 Sequence LAB-09

The neighbor joining method was used to construct the phylogenetic tree. Sequence LAB-09 shows close relationship with *Lactobacillus* genus. The tree shows close similarity of strain to the *Lactobacillus paracase*, *Lactobacillus zeae, Lactobacillus rhamnosus* (figure 3.17). There is no distantly related strain found.

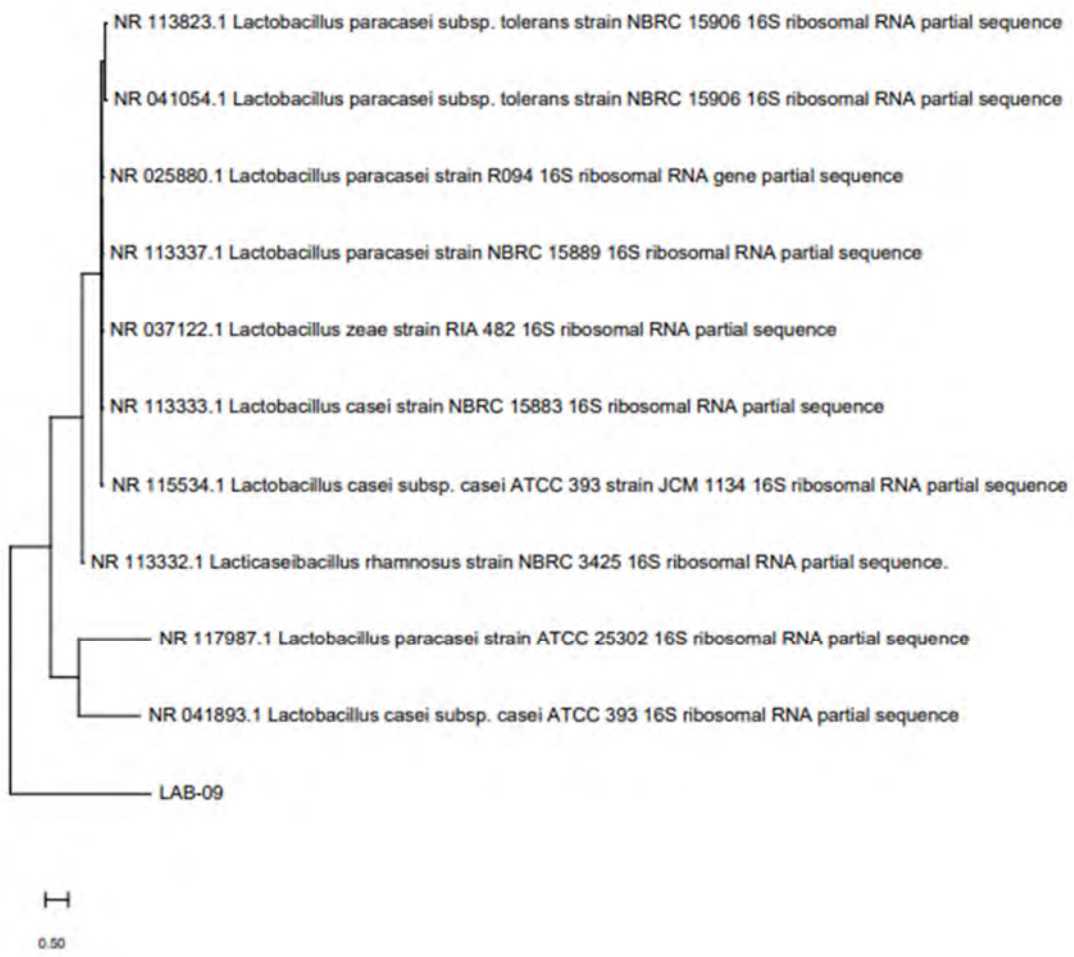


Fig 3.17 Phylogenetic tree of Sequence LAB-09

3.4.1.6 Sequence LAB-10

The neighbor joining method was used to construct the phylogenetic tree. Sequence LAB-10 shows close similarity with *Lactococcus formosensis* strain 516 16S ribosomal partial sequence (figure 3.18). The sequence is closely related to *Lactococcus lactis* species. The sequence is distantly related to *Lactococcus taiwanensis* strain 0905C15 16S ribosomal RNA partial sequence.

Fig 3.18 Phylogenetic tree of Sequence LAB-10

3.4.1.7 Sequence LAB-38

The neighbor joining method was used to construct the phylogenetic tree. Sequence LAB-38 shows close relationship with *Lactobacillus plantarum* and *paraplantarum*. There is no distantly related strain found (Figure 3.19).

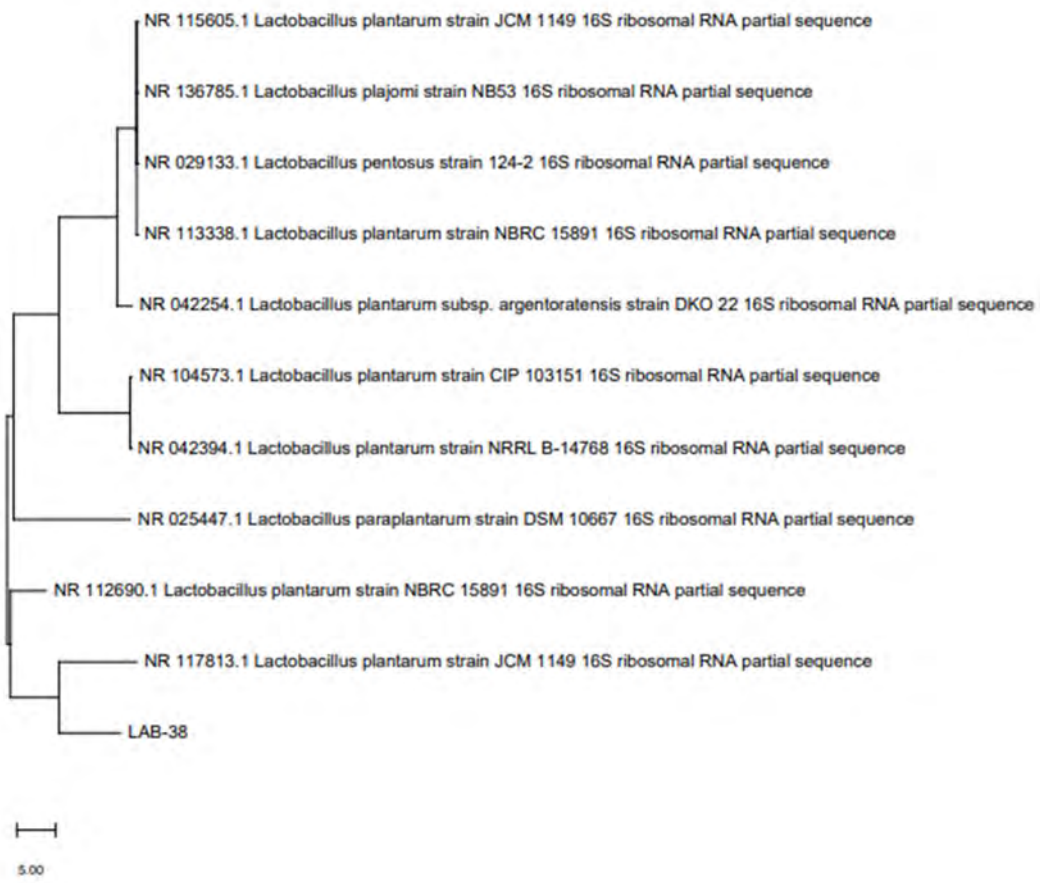


Fig 3.19 Phylogenetic tree of Sequence LAB-38

3.4.2 Sequence similarity among bacterial isolates

The phylogenetic tree is divided into two branches. Upper branch contains two strains while lower branch contains five strains (figure 3.20). Strains 1 and 10 are closely related in the upper branch. The lower branch having similarity between 03 and 04. The lower branch also having similarity between strain 9 and 38. Strain 7 having similarity with strain 3, 4, 9, and 38.

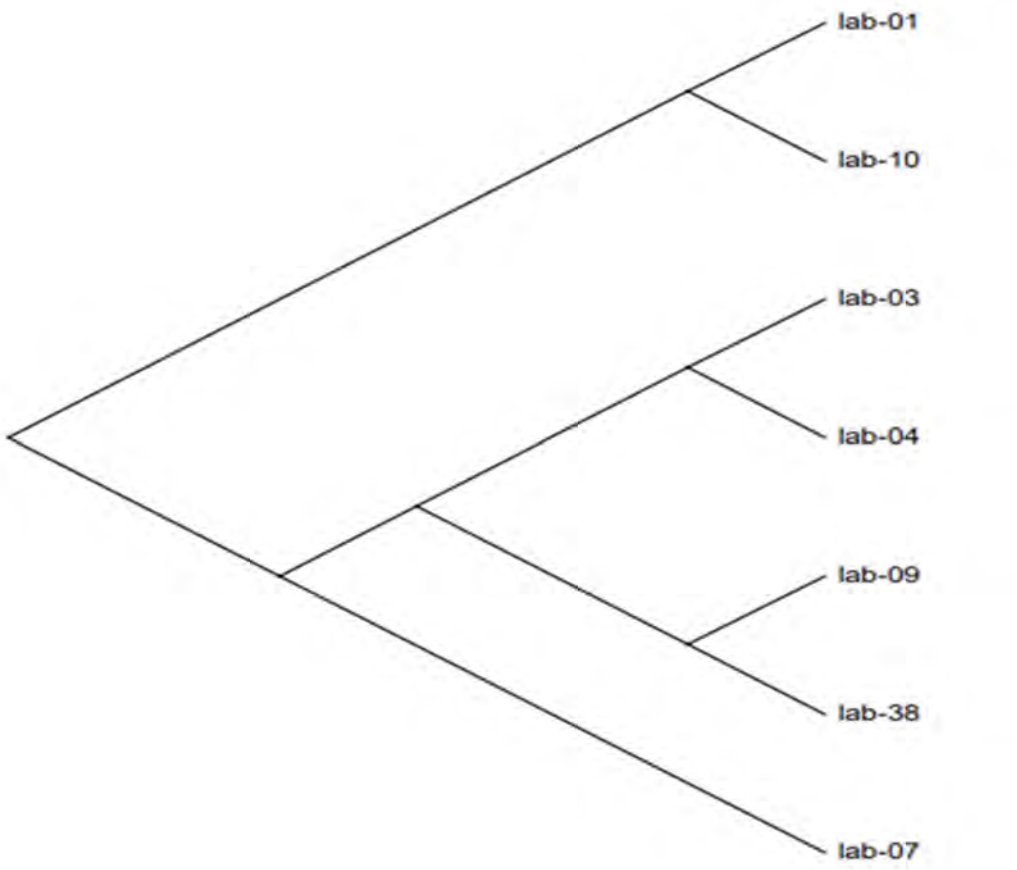


Fig 3.20 Phylogenetic tree depicting intra strain similarity

3.5 Riboflavin Assay Medium

Among the seven isolates only four strains were able to grow on RAM and showed clear colonies which was indication that these strains produced riboflavin. Riboflavin producing strains were further taken for application in dairy industry. Before using these strains as yogurt culture, safety tests such antibiotic sensitivity and hemolytic tests were performed.

3.6 Hemolytic test

Lactic Acid Bacteria showed no hemolysis on blood agar (figure 3.21). The grey color clearly indicated that the RBC haven't lysed by LAB. The positive control clearly showed gamma hemolysis. This clearly indicates that the LAB strains are safe and can be used in dairy industry.

Fig 3.21: γ-hemolysis on blood agar.

3.7 Antibiotic sensitivity test

The Kirby Bauer disk diffusion test was performed to check the antibiotic susceptibility pattern of bacteria. In this test, nine different commercially available disk such as, vancomycin, Chloramphenicol, Gentamicin, Tetracycline, Clindamycin, Erythromycin, Streptomycin, Kanamycin and ampicillin were used as shown in the Table No. 3.9 and shown no resistance to the mentioned antibiotics.

Table 3.7 Antibiogram profiling of the selected isolates

S. No	Antibiotics	Strain lab-04	Strain lab-07	Strain lab-10	Strain Lab-38
1.	Ampicillin	33 mm	24 mm	28 mm	34mm
2.	Chloramphenicol	31mm	30 mm	24 mm	33 mm
3.	Clindamycin	29 mm	26 mm	31 mm	28 mm
4.	Erythromycin	24 mm	28 mm	36 mm	34 mm
5.	Gentamycin	28 mm	25 mm	37 mm	29 mm
6.	Kanamycin	25 mm	22 mm	24 mm	26 mm
7.	Streptomycin	25 mm	27 mm	23 mm	30 mm

8.	Tetracycline	36 mm	34 mm	31 mm	27 mm
9.	Vancomycin	37 mm	35 mm	38 mm	36 mm

3.8 Yogurt preparation from skimmed milk

The four strains were streaked on MRS agar and colonies were obtained after 24 hours of incubation. The colonies were inoculated in MRS broth and incubated in shaking incubator for 48 hours. The obtained cells were centrifuged and washed thrice with sterile normal saline. The 12% skimmed milk was inoculated with these cells for 6 hours.

3.9 Estimation of riboflavin in yogurt

The supernatant of the yogurt prepared from riboflavin overproducers was checked for riboflavin concentration. The sample were taken in triplicate. The average of the values was taken shown in the following table 3.10. The standard deviation of the concentration was calculated using Microsoft excel.

Table 3.8 Estimation of riboflavin in yogurt samples prepared from riboflavin overproducing strains

Sample ID	Absorbance	Riboflavin concentration (mg/L)	Mean (mg/L)
LAB -04	0.2	6.62	6.38 ± 0.20
	0.195	6.40	
	0.189	6.12	
LAB-07	0.171	5.3	5.33 ± 0.14
	0.168	5.16	
	0.176	5.52	
LAB-10	0.153	4.48	4.70 ± 0.18
	0.158	4.70	
	0.163	4.94	
LAB-38	0.155	4.56	4.71 ± 0.11
	0.159	4.74	
	0.161	4.84	

Chapter 4

DISCUSSION

Livestock is an important sector of agriculture contributing approximately 58.6% towards agricultural value added (more than combined share of crops, fisheries and forestry sectors) and 11.6% to the overall GDP of Pakistan. Milk is the most significant product in the livestock sector and Pakistan is the fourth-largest producer of milk in the world., with an annual production of about 62 billion liters during the year 2020-21 (Government of Pakistan, 2021). As Pakistan is the fourth largest producer in the world, it will be a great opportunity to produce dairy related products.

Riboflavin serves as a metabolic precursor for the coenzymes FAD and FMN, which are involved in redox reactions in all organisms. Proteins, ketone bodies, lipids and carbohydrates are metabolized by flavo-coenzymes, which provide the majority of energy to living organisms. (Buehler, 2011). The study shows that riboflavin is very important and needed on daily basis for the normal functioning of our daily metabolic processes. Zhang *et al.,* (2010) described that vertebrates stored RF in least amount due to its limited absorption in humans. It is taken orally to meet daily requirements and to prevent ariboflavinosis, which produces scaly rashes on the genital organs, cheilitis, and sore tongue. My aim of study was to prepare riboflavin enriched yogurt that may and fulfill daily need of a human. It is an cost effective approach and can used by anyone around the globe. The first objective was to estimate the level of riboflavin in the dairy products available in the market. The highest level of riboflavin found was 3.48 mg/L.

LAB can be found in a variety of ecological environments, including foods and animals' and humans' gastrointestinal and urogenital tracts. Despite the fact that animals and humans are unable to manufacture most vitamins, bacteria have the genetic ability to produce those metabolites. (LeBlanc *et al*., 2011). As this study reports that riboflavin can be produced by Lactic Acid Bacteria (LAB), so the idea was to isolate LAB for potential production of riboflavin. In the current study a total of 20 samples were taken and 78 isolates were initially selected for the study. Out of 78 isolates, 19 were found Gram positive and catalase negative, which is a typical characteristic of LAB differentiating them from other bacterial species. The 16S rDNA of the 19 strains were extracted and sequenced. Sequencing results showed that only 7 strains belonged to

LAB group. Specific species of the genus *Lactobacillus* can transfer favorable characteristics to their hosts, and because of this characteristic, bacteria are the most usually utilized as probiotic microorganisms. (LeBlanc *et al.,* 2011). To provide fermented bio-enriched food, these bacteria may synthesize B group vitamins, particularly riboflavin. (Capozzi *et al*., 2011; Laino *et al*., 2012; Vaesken *et al*., 2012).

My study found that some LAB isolates can grow on riboflavin deficient media. The media was deprived of riboflavin but the lactic bacterial strains still had the potential to produce own riboflavin using their biosynthetic pathway. LAB are widely used in the dairy sector, and adding riboflavin-producing bacteriaLAB to fermented dairy products such as fermented milks, yoghurt, and cheeses boosts riboflavin concentrations in a simple and cost-effective way. (LeBlanc *et al*., 2005a). LAB have long been used to make fermented milk products like yoghurt and cheese, and they are Generally Regarded As Safe (GRAS). However to confirm safety of the isolates in the current study, two safety tests were performed i.e. testing for hemolysis and antibiotic sensitivity. The bacterial strains showed no hemolysis on blood agar strains and no resistance to antibiotics. This clearly indicated that these strains could be used for preparation of edible products. The lactic acid bacterial strains were tested for yogurt production and it was found that four out of seven bacterial strains had curdled the milk. The riboflavin overproduction was confirmed by estimating the riboflavin content in supernatant of the yogurt. All the four strains were found to produce higher riboflavin contents than the market yogurt and milk samples. However, the bacterial strain Lab-04 (identified as *Lactococcus garvieae*) was found to produce highest riboflavin concentration of 6.38 mg/L which was more than double the riboflavin present in yogurt and milk available in the market. This strain, therefore, has the potential to be used for preparation of riboflavin enriched dairy products as well as for industrial production of riboflavin.

CONCLUSIONS

From the study conducted over 12 months it can be concluded that the dairy products available in the market don't contain sufficient quantities of riboflavin. However, the riboflavin overproducing lactic acid bacterial strains can be isolated from the dairy products. In the current study four selected strains showed riboflavin overproduction potential upto the maximum level of 6.38 mg/L. These *Lactobacilli* and *Lactococci* strains can be used for the nutraceutical yogurt production and also have the potential to be used for riboflavin production at large scale in industry.

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APPENDIX: 1

Composition of MRS agar

S. No	Composition	g/L
1	Peptone	10.0
2	Lab lemco	10.0
3	Yeast extract	5.0
4	D-Glucose	20.0
5	Tween 80	1.00
6	Dipotassium hydrogen phosphate	2.00
7	Sodium acetate	5.00
8	Tri-ammonium citrate	2.00
9	Magnesium sulphate, hydrated	0.20
10	Manganese sulphate, hydrated	0.05
11	Bacterial agar	15.0

All the components of MRS media were dissolved in enough distilled water until the final volume of the media become 1 litre. The pH of the medium was adjusted to 5.55 and autoclaved at 121 ˚C for 15 minutes.

APPENDIX: 2

Composition of Mueller Hinton Agar

S. No	Composition	(g/L)
1	Acid hydrolysate of Casein	17.50
2	Beef Extract	2.00
3	Starch	1.50
4	Agar	15

All the components of MHA media were dissolved in enough distilled water until the final volume of the media become one liter. The pH of the medium was adjusted to 7.3 and autoclaved at 121 ˚C for 15 minutes.

APPENDIX: 03

Gram's staining reagents preparation

Crystal violet stain

S. No	Composition	Quantity
1	Crystal violet	5g
2	Ammonium Oxalate	2g
3	Ethanol	50ml
4	Distilled water	200ml

Gram Iodine

S. No	Composition	Quantity
1	Iodine	1g
2	Potassium Iodide	2g`
3	Ethanol	25ml
4	Distilled water	10ml

Safranin

S. No	Composition	Quantity
1	Safranin	2.5g
2	Ethanol	10ml
3	Distilled water	100ml

These are the Gram's staining reagents and can prepared in normal laboratory conditions.

APPENDIX: 04

Preparation of TE buffer

S. No	Composition	Quantity
1	EDTA (0.5 M)	2 ml
2	Tris-HCL	10 ml
3	Distilled water	1000 ml

Usually I M Tris-HCl and 0.5 M EDTA is used for the preparation of TE buffer

- Mix 2 ml of 0.5 M EDTA and 10 ml of Tris-HCl buffer to make solution.
- Add distilled water to make 1000 ml of solution.