

***IN VITRO* WOUND HEALING, ANTIOXIDANT AND ANTIBACTERIAL ACTIVITY OF PROBIOTIC BACTERIA**

Submitted in partial fulfillment of the requirements for the award of
Bachelor of Technology degree in Biotechnology

By

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This is to certify that this Project Report is the bonafide work of **Y SHARON SANDRA (37230080)** who carried out the project entitled "**IN VITRO WOUND HEALING, ANTIOXIDANT AND ANTIBACTERIAL ACTIVITY OF PROBIOTIC BACTERIA ISOLATED FROM MILK AND GHEE**" under our supervision from December 2020 to March 2021

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This is to certify that this dissertation entitled "**IN VITROWOUND HEALING, ANTIOXIDANT AND ANTI BACTERIAL ACTIVITY OF PROBIOTIC BACTERIA ISOLATED FROM MILK AND GHEE**" submitted to Department of Biotechnology, SATHYABAMA INSTITUTE OF SCIENCE AND TECHNOLOGY, in partial fulfillment for the award of the degree of B.Tech. Biotechnology is a record of original work done by **SHARON SANDRA Y (37230080)** at AviGen BioTech Pvt Ltd., under my supervision and guidance from December 2020 to February 2021. This project has not been submitted to any other degree.

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DECLARATION

I, **Y SHARON SANDRA** (37230080) hereby declare that the Project Report entitled **“IN VITRO WOUND HEALING, ANTIOXIDANT AND ANTIBACTERIAL ACTIVITY OF PROBIOTIC BACTERIA ISOLATED FROM MILK AND GHEE”** done by us under the guidance of **Dr. M. BHAVANILATHA** (SATHYABAMA INSTITUTE OF SCIENCE AND TECHNOLOGY) and **DR. P. ANNAMALAI** (AVIGEN BIO TECH.PVT.LTD) at **AVIGEN BIOTECH.PVT.LTD** is submitted in partial fulfillment of the requirements for the award of Bachelor of Technology degree in Biotechnology.

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ABSTRACT

Probiotics are live microorganisms that provide health benefits when consumed. They are beneficial bacteria that provide all sorts of powerful benefits and amendments for your body and brain. *Lactobacillus plantrum* cells are rod-shaped and Gram-positive bacteria. The organism was isolated from milk and confirmed by gene sequencing method. This organism was subjected for 16s rRNA sequencing using Submission tool of NCBI and the accession number (JN043518.1) was received. The present study is investigated with *Lactobacillus plantarum* AGBT6 to execute the importance of probiotic activity. Varied lactic acid bacterial strains are isolated traditionally from milk and ghee and are analysed and assessed for probiotic properties and its antibacterial substances against some pathogenic bacteria. The inhibitory mechanism is to be investigated for antioxidant activity against wound pathogens, (Gram positive and gram negative) production. The objective is to select highly efficient probiotic bacteria, which shows optimal physio-chemical parameters for selected probiotic bacteria. Minimum inhibitory concentration is to be retained from the selected bacteria, this preliminarily work generally shows the potential cytotoxicity of the bacteria in normal cell lines by using amplification of PCR (polymerase chain reaction), sequencing and phylogenetic tree to determine the genus and species name of the selected bacteria, thus inducing in-vitro wound healing property of cell lines from the isolated probiotic bacteria.

Keywords: Probiotics, *Lactobacillus plantrum*, Antimicrobial activity, Wound healing.

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LIST OF ABBREVIATIONS AND SYMBOLS

%	Percentage
°C	Degree Celsius
bp	Base pair
g	Grams
H ₂ O ₂	Hydrogen peroxide
MEGA	Molecular Evolutionary Genetics Analysis
mg	Milligrams
mg/ml	Milligram per milliliter
MHA	Mueller-Hinton agar
min	Minute
ml	Milliliter
mm	Millimeter
MR	Methyl red
MR-VP	Methyl red- Voges-Proskauer
NCBI	National Centre for Biotechnology Information
nm	Nanometers
NR	Non redundant (proteins)
OD	Optical Density
PCR	Polymerase Chain Reaction
pH	Potential of hydrogen
RNA	Ribonucleic Acid
sp.	Species
Subsp.	Sub-species
ug/ml	Microgram per milliliter
w / v	Weight by volume

CHAPTER 1

INTRODUCTION

1.1 OVERVIEW OF PROBIOTICS:

Probiotics are live microorganisms which on ingestion in sufficient concentrations can exercise health benefits to the host. This word probiotics was coined in 2001 by the United Nations Food and Agriculture Organization (FAO) and the World Health Organization (WHO), and has been employed as the technical term of reference for science and its regulation consequently (FAO/WHO 2002). Stipulation for food containing probiotics is expanding globally due to the continuous generation of the research evidence indicating their potential health benefits to consumers.

These live bacteria and yeasts found in probiotic food that are good for you, especially your digestive system. We usually tend to think of these as germs and bacteria that cause diseases and physical illness. But your body is said to be full of bacteria, both good and bad. Probiotics are often termed "good" or "helpful" bacteria because they help keep your gut healthy. Probiotics show up to be high-quality for each generalized nervousness and nervousness associated to unique causes. Many of these microorganisms typically live in your gut, and the majority are pretty innocent. Yadav, M., Shukla, P. (2020)

It is having the right gut bacterial microorganism that is linked to numerous of health benefits, including weight loss, improved better digestion, enhanced and improved immune function, healthier skin and reduced risk of many other diseases Probiotic foods mainly include yogurt, kefir, sauerkraut, tempeh and kimchi. Further Probiotics should not be confused with prebiotics, which are dietary fibers that mostly help feed the friendly bacteria already in your gut. It manufactures and produces vitamins, including vitamin K and some of the B vitamins. These facts are said to stimulate your immune system and strengthen your gut wall well. This also prevents unwanted substances from entering your body and provoking an immune response. However, it is known that not all microorganisms in your gut are very friendly. Your gut flora is

extremely sensitive to our diet, and research show that an unbalanced gut flora is linked to many diseases and these diseases include obesity, fatigue, type 2 diabetes, and also metabolic syndrome and heart diseases including colorectal cancer, Alzheimer's and most importantly depression.

1.2 SELECTION AND IMPORTANCE OF PROBIOTICS:

Probiotics and prebiotic fibers that can help immensely to correct the balance, ensuring that the "forgotten organ" is functioning quite optimally. It is said that your gut flora consists of many hundreds of different types and forms of microorganisms. Probiotics help your gut flora perform optimally. Although probiotic treatments improve the severity of the diseases by decreasing inflammation, it did not treat the actual root cause. Moreover, there is concern of opportunistic contamination with the aid of probiotic traces as they modulate the inflammatory reputation of a subject. Thus, extra scientific trials will be wished to reveal the controversies involving the effectiveness and security troubles in order to supply higher appreciation on the manipulate mechanisms of diseases. Longer duration of studies is also required to prove the sustainability of the positive effects on human health. Probiotics have been proven to provide some new benefits for skin health. Recent studies showed that probiotic could improve atopic eczema, wound and scar healing, and help skin-rejuvenation. Probiotics used to describe live microorganisms (bacteria and/or yeasts) that expertise in beneficial effects to the humans and animals, principally by balancing of the intestinal flora.

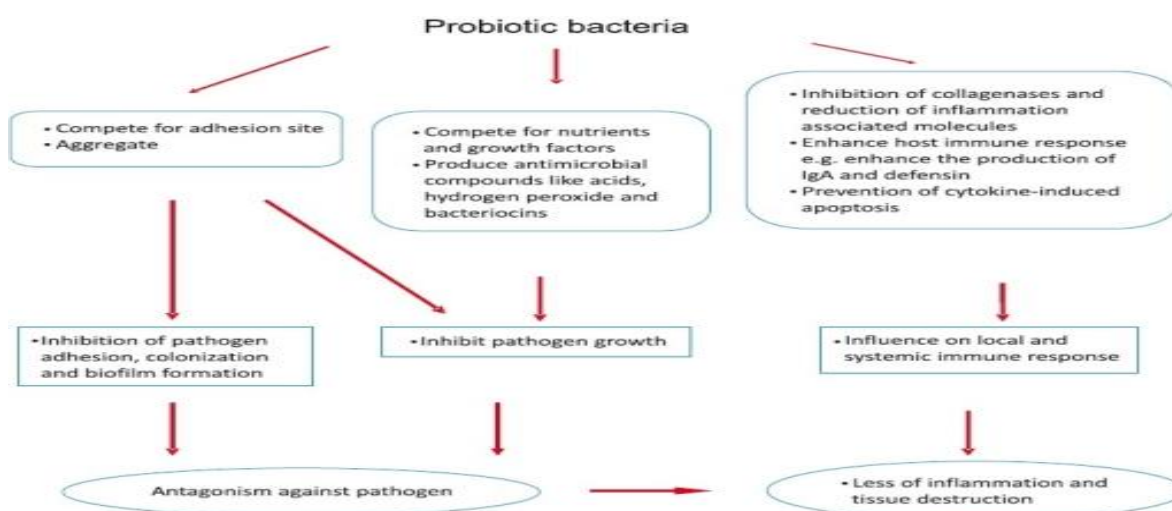


Fig 1.1 flow chart on probiotic bacteria

1.3 POTENTIAL MECHANISMS OF ACTION OF PROBIOTICS:

A huge range of possible really helpful fitness consequences have been attributed to probiotics. Claimed results vary from the alleviation of constipation to the prevention of predominant existence threatening ailments such as inflammatory bowel disease, cancer, and cardiovascular incidents. Some of these claims, such as the outcomes of probiotics on the shortening of intestinal transit time or the remedy from lactose maldigestion, are regarded well established, whilst others, such as most cancers prevention or the impact on blood ldl cholesterol levels, need similarly scientific backup (Leroy et al., 2008).

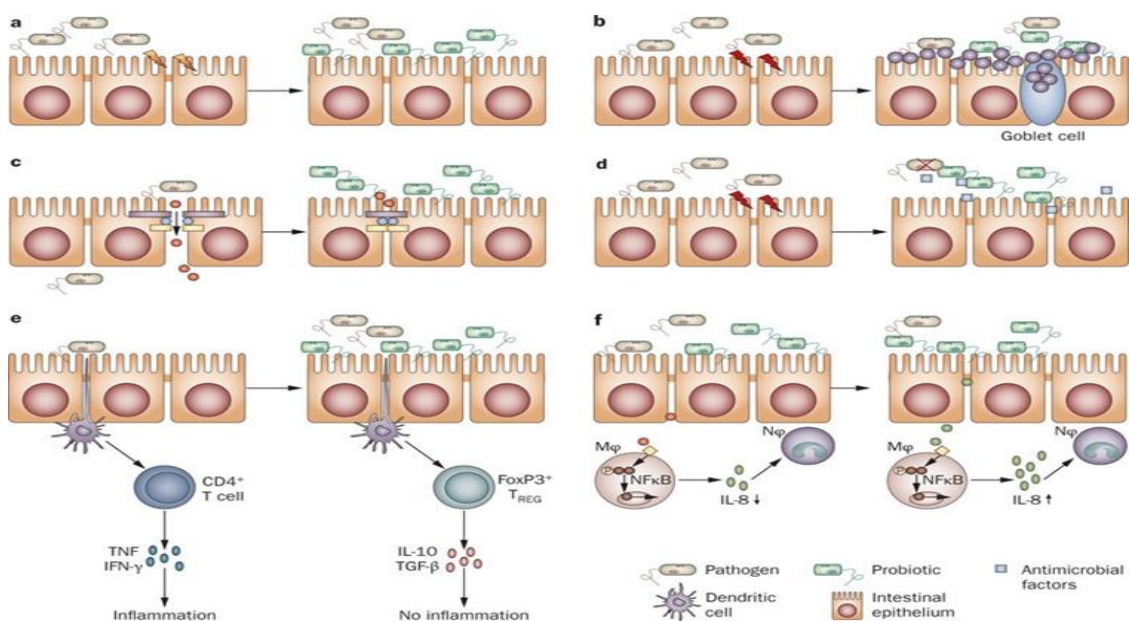


Fig 1.2 mechanism of probiotics

The mechanisms of motion might also differ from one probiotic stress to any other and are, in most cases, probable a mixture of activities, thus making the investigation of the accountable mechanisms a very tough and complex task. In general, three degrees of motion can be distinguished: probiotics can have an effect on human Probiotic organisms can supply a advisable impact on intestinal epithelial cells in several ways. a: Some lines can block pathogen entry into the epithelial cellphone through offering a bodily barrier, referred to as colonization resistance or b: create a mucus barrier by way of inflicting the launch of mucus from goblet cells. c: Other probiotics hold intestinal permeability via growing the intercellular integrity

of apical tight junctions, for example, by way of upregulating the expression of zona-occludent 1 (a tight junction protein), or by using stopping tight junction protein redistribution thereby stopping the passage of molecules into the lamina propria. d: Some probiotic traces have been proven to produce antimicrobial factors. e: Still different lines stimulate the innate immune gadget by means of signaling dendritic cells, which then travel to mesenteric lymph nodes and lead to the induction of TREG cells and the manufacturing of anti-inflammatory cytokines, which include IL-10 and TGF- β . f: Some probiotics (or their products) might also additionally stop (left-hand side) or trigger (right-hand side) an innate immune response by means of initiating TNF manufacturing by using epithelial cells and inhibiting (or activating) NF κ B in M ϕ and dampening (or priming) the host immune response with the aid of influencing the manufacturing of IL8 and subsequent recruitment of N ϕ to web sites of intestinal injury. Expansions: M ϕ , macrophage; N ϕ , neutrophil; TREG cell, regulatory T cell. Koh, T. J., DiPietro, L. A. (2011)

1.4 BENEFITS OF PROBIOTICS:

Various healing residences are stated to be identified such as immune modulation, lowering serum cholesterol, antimutagenic and anti-carcinogenic effects, alleviation of lactose intolerance symptoms, strengthening protection mechanisms, enchantment of intestinal barrier characteristic and enhancing the dietary price have been identified and attributed to the probiotics. The starting place of Probiotic microorganisms is stated to be conceded in the main of human and animal beginning and are ordinary materials of their gastrointestinal microflora. However, it is that countless probiotics have been remoted from fermented or milk foods. *Lactobacillus* spp., *Bifidobacterium* spp. and *Enterococcus* are regarded as the most frequent species of microorganisms used in manufacturing of probiotic product. Furthermore, different organisms such as *Pediococcus acidilactici*, *Enterococcus* spp. and *Saccharomyces boulardii* have been probably diagnosed as probiotics. 'Probiotic' refers to manageable microorganisms that promote or help a really useful stability of the autochthonous microbial populace of the intestine (Holzapfel and Schillinger, 2002). Strains of *Lactobacillus* spp. happen naturally in the human gut and for this reason, such intestinal lines are additionally preferentially developed for business use as probiotics. The root of the phrase probiotic comes from the Greek

phrase pro, that means "promoting," and biotic, which means "life." Probiotic is a fairly new phrase which means 'for life', which is used to title microorganisms that are related with the really helpful consequences for human beings and animals. These microorganisms make contributions to intestinal microbial stability and play a position in preserving health. Darby, I. A., Hewitson, T. D. (2007)

The discovery of probiotics got here about in the early twentieth century, when Elie Metchnikoff, recognized as the "father of probiotics," The International Scientific Association for Probiotics and Prebiotics (ISAPP) equipped an assembly of scientific and scientific specialists on probiotics (with specialties in gastroenterology, pediatrics, household medicine, intestine microbiota, microbiology of probiotic bacteria, microbial genetics, immunology, and meals science) to reexamine the idea of probiotics. They outline probiotics as "live microorganisms that, when administered in sufficient amounts, confer a fitness advantage on the host." Probiotics are an essential thought for fitness care in the twenty first century. They "should be resistant to gastric juices and be in a position to develop in the presence of bile". There is a sequence of in vitro exams such as acid and bile tolerance, antimicrobial manufacturing etc, that, though requiring similarly refinement, are commonly utilized as a first method for the determination of plausible probiotic microorganisms (FAO/WHO, 2001).

1.5 SELECTION OF LACTIC ACID BACTERIAL (LAB) STRAINS:

lactic acid microorganism (LAB) is viewed as a important team of probiotic microorganism (Collins and Gibson, 1999). An efficaciously working intestine ecosystem, the so-called microbiome (quantitative and qualitative composition of quite a number microorganisms) has a awesome influence on the person's potential to hold their health. The microflora in human intestines is the most different ecosystem on earth in phrases of species (100–1000 species). The microbiome influence's many physiological systems, consisting of immunity or intellectual state. Due to the developing attention of the function that the intestine microflora has on humans retaining their health, for over 20 years lookup work has been carried out worldwide, with regard to the probabilities of editing positively or enriching human microbiome.

The presence of LAB is critical to the intrinsic homes of dairy products. LAB have a lengthy record of protected use, mainly in the dairy industry. They play a foremost function in the manufacturing of fermented food, such as the key flavor, texture and preservative features and Recently, some of them have been definitely sequenced Therefore, comparative genomic evaluation can assist us to apprehend the genetic and organic range in LAB.

These are non-pathogenic bacteria, which technologically provide appropriate LAB for industrial processes, acid tolerance, bile tolerance and produce antimicrobial substances. The LAB is regarded commonly as the most secure (GRAS) organisms and their software as probiotics is significant as they play a main function in retaining health. Lactic acid microorganism (LAB) is stated to be a various crew of Gram-positive, non-sporulating, anaerobic or facultative cardio cocci or rods, precise genus. Lactobacillus additionally consists of nearly two hundred diagnosed species of low G + C gram-positive microorganism inside the phylum of the Firmicutes and the Clostridium-Bacillus subdivision. These are stated to produce lactic acid as one of the major dairy merchandise of the metabolism of carbohydrates ,Current Limitations and Challenges Lactic acid microorganism (LAB) are a classification of industrially distinguished microorganisms used in meals and with (Lactic Acid Bacteria) LAB can inhibit the increase of pathogenic microorganisms through a couple of mechanisms, as properly as competitively inhibiting pathogen binding, bettering the host immune system, and producing pathogen growth-inhibitory compounds such as natural acids, bacteriocins like acidophilin, acidolin, lactocidin, bulgarican, lactolin, lactobacillin and lactobrevin., and hydrogen peroxide. LAB has a lengthy record of software in the manufacturing of dairy meals due to the fact of its special physiological things to do and dietary functions. Werner, S., Grose, R. (2003)

LAB additionally has excellent conceivable as meals preservatives due to its capability of producing antimicrobial materials such as hydrogen peroxide, natural acids and bacteriocins. In addition, LAB play a vital position in keeping the integrity for immunological, digestive functions, and some are an increasing number of viewed as predominant probiotics. The human gut microbiota performs a vast

function in dietary processes. The notion of probiotics has led to huge consumption of meals preparations containing probiotic microbes such as curd and yogurt. Curd organized at domestic is fed on each day in most properties in southern India. In this find out about the home-made curd was once evaluated for lactic acid microorganism (LAB) with probiotic potential. Milk merchandise represent an appropriate supply of the lactic acid fermentation bacteria. Spontaneously fermented milk merchandise is nonetheless produced till this day in many components of the world and represent a fantastic supply of probiotic microorganisms, specifically microorganism from the genera *Lactobacillus*, *Lactococcus*, or *Streptococcus*, as nicely as yeasts. Peral, M. C., Huaman Martinez, M. A., Valdez, J. C. (2009)

Lactic acid microorganism (LAB) is a commonly widely wide-spread crew of probiotic microorganism (Naidu et al. 1999). Lactic acid microorganism (LAB) is broadly used as probiotics in the meals and feed industry. Further fitness advantages may want to be exploited for their use in the meals industry. Beneficial outcomes associated to probiotics encompass hostile effects, competition, enhancement of digestion, strengthening of the immune machine and stimulation of diet production. Some lookup has proven that lifeless cells of probiotic microorganism may also have recommended properties, such as modulation of the immune gadget and binding cancer causing agents in the host's body; then again if their impact is weaker or confined It is suggested, moreover, that the software of inactivated cells is a answer higher from the factor of view of protection of use, especially in the case of new child infants or sufferers with immunosuppression, Many of the probiotic traces have been remoted from human intestine, such as *Lb. salivarius* subsp. *Salicinius* and *Lb. acidophilus*, as properly as from human expressions, such as *B. longum* and *Lb. acidophilus*, and much less regularly from human belly such as *Lb. fermentum*, *Lb. gasseri*, *Lb. vaginalis*, *Lb. reuteri*, and *Lb. salivarius*. Koh, T. J., DiPietro, L. A. (2011)

1.6 ISOLATION AND IDENTIFICATION OF LACTIC ACID BACTERIA STRAIN:

The isolation, identification, and the characterization of the novel LAB lines have the following two advantages that has predominant structural really helpful components

for probiotics. The first is to expose the fundamental attribute taxonomy of the LAB and the 2d is to achieve promising really useful and useful probiotic LAB strain. This learn about is targeted on the significance of some of the ordinary Indian meals as a desirable supply of Probiotics. Probiotics are being abundantly used as preventive therapeutic agent for numerous diseases. For the willpower of probiotic homes of isolates these essential resolution standards have been selected: resistance to low pH, tolerance towards bile salt and the antimicrobial activity.

1.7 ANTIBACTERIAL ACTIVITY:

The administration of probiotics outcomes in the prevention of a volume of bodily stipulations and ailments and the prophylaxis and remedy of others. There are a huge range of requirements for probiotic traces in order to be protected in the composition of probiotic preparations and probiotic foods. One of the requirements for probiotic strains is to exhibit off antimicrobial activity closer to pathogenic, toxigenic and saprophytic microorganisms. The antimicrobial challenge of probiotic microorganisms is strain-specific, alternatively the mechanisms of interaction of probiotic and pathogenic microorganisms can be grouped into two groups: direct and indirect mechanisms. The direct mechanisms of interaction can be summarized in the following groups: 1) opposition for substrates and limiting resources; 2) aggressive exclusion by using opposition for adhesion sites; 3) synthesis of antimicrobial assets (organic acids, hydrogen peroxide and bacteriocins); 4) inhibition of toxin expression in pathogens. Each crew would be described in issue and referred to in the present chapter. Since all probiotic homes are strain-specific, which encompass the antimicrobial undertaking in the direction of pathogens, there is a necessity for compulsory examination of every probiotic property of every and each and every new candidate probiotic strain. Thus, a range of methods of examination of the probiotic properties of doubtlessly probiotic traces are being employed and developed, each and every strategy being characterized by way of precise techniques of examination and unravelling particular parts of records about the special mechanisms of interaction between probiotic and pathogenic microorganisms. The present-day strategies for strength of mind of the antimicrobial exercise belong to two important groups: in vivo and in vitro methods. The crew of in vitro methods for dedication of the antimicrobial recreation consists of the spot-

on backyard assay and its variants (simple spot-on backyard assay; agar spot assay; spot-on backyard assay with wells); the agar well-diffusion assay and disc diffusion assay and the approach of co-culturing. The approach of examination of each of the enlisted methods would be described, described and the information obtained would be analyzed in the cutting-edge chapter. The in vivo methods consist of examinations in animal fashions and human scientific trials. Probiotic microorganisms are moreover an open self-discipline for the enchancement of recombinant probiotics with antimicrobial peptides which grant a promising method toward pathogens with rising resistance in opposition to the currently used antibiotics and probiotics. A frequent occasion of the antibacterial pastime of lactic acid microorganism is the capacity of *Lactobacillus plantarum* to inhibit the pathogenic hobby of *Pseudomonas aeruginosa* as the presence of *Lactobacillus* prevents the wound colonization via *Pseudomonas*. Because of this antimicrobial action, *Lactobacillus plantarum* has been studied as a therapeutic agent for the topical treatment of contaminated and noninfected burns. The consequences showcase that the remedy of burns with *L. plantarum* is of related efficacy with silver sulfadiazine cure. *L. plantarum* has been eleven determined to intervene in vitro with the movement of N-acyl-homoserine lactones, of elastase and with the biofilm formation via way of the *P. aeruginosa*. In addition, the penalties from an experimental model of burns in mice tested that *L. plantarum* lead to enhancement of phagocyte venture and tissue restore.

1.8 ANTIOXIDANT PROPERTIES OF PROBIOTIC BACTERIA:

Oxidative stress defines a situation in which the prooxidant–antioxidant stability in the mobile is disturbed, ensuing in DNA hydroxylation, protein denaturation, lipid peroxidation, and apoptosis, subsequently compromising cells' viability. Probiotics have been regarded for many really useful fitness effects, and the consumption of probiotics by myself or in meals indicates that strain-specific probiotics can existing antioxidant endeavor and minimize damages induced via oxidation. However, the oxidation-resistant capability of probiotics, mainly the underling mechanisms, is now not exact understood. In this view, there is hobby to discern out the antioxidant property of probiotics and summarize the mode of motion of probiotic microorganism in antioxidation. Therefore, in the current paper, the antioxidant mechanisms of

probiotics have been reviewed in phrases of their capacity to enhance the antioxidant device and their capability to limit radical generation. Since in current years, oxidative stress has been related with an altered intestine microbiota, the outcomes of probiotics on intestinal plants composition are additionally elaborated.

1.8.1 MODES OF ACTION OF PROBIOTIC BACTERIA IN ANTIOXIDATION:

Modes of Action of Probiotic Bacteria in Antioxidation During the previous decades, research

have validated that unique probiotic microorganism lines may want to exert antioxidant capability in special ways. However, few opinions related to the foundation for the antioxidant mechanisms of probiotics have been found. Thus, the following sections supply an overview of the current understanding on the oxygen resistant mechanisms of a number of probiotic strains.

Modulation of antioxidation by way of probiotics:

1. Probiotics chelate metallic ion.
2. Probiotics possess their personal antioxidizes.
3. Probiotics produce antioxidant metabolites.
4. Probiotics up-regulate antioxidize things to do of the host.
5. Probiotics extend stages of antioxidant metabolites of the host.
6. Probiotics alter signaling pathways.
7. Probiotics down-regulate things to do of enzymes producing ROS.
8. Probiotics regulates intestinal microbiota.

1.9 ANTIBIOTIC SENSITIVITY OF *lactobacilli* AND TEST PATHOGENS:

Antibiotic susceptibility check of distinct lactobacilli and check pathogens was once completed through 'Kirby-Bauer's technique (1966). For this assay, inoculum of all the 116 lactobacilli and check pathogens have been organized and processed. The turbidity of the cellphone suspensions was once matched to 0.5% MacFarland answer to make certain uniformity. Further, the inoculum of the man or woman isolate used to be evenly unfold on separate Mueller–Hinton agar plates. The plates have been incubated for 15 to 20 min and then antibiotic discs of kanamycin,

penicillin, rifamycin, cefotaxime, gentamycin, amoxicillin, methicillin, vancomycin, and tetracycline (Hi Media Pvt. Ltd., Mumbai, India) had been utilized on seeded agar plates in triplicates. Plates have been incubated at 37 ± 1 C for 24 h and diameter of zones used to be measured in every case to verify sensitivity, susceptibility or resistance in opposition to the antibiotic used.

1.10 *Lactobacillus plantarum*:

This study is aimed to find out and elucidate the goals and mechanisms of anti-staphylococcal outcomes from bioactive metabolites produced through lactic acid bacteria. We aimed to higher apprehend the protection and efficacy of these bioactive metabolites in in vivo systems, generally at topical sites. The cell-free supernatant and protein-rich fraction from *Lactobacillus plantarum* USM8613 inhibited staphyloxanthin biosynthesis, decreased ($p < 0.05$) the telephone quantity of *Staphylococcus aureus* by using 106 CFU/mL and decreased biofilm thickness by using 55% in *S. aureus*-infected porcine skins. Genome-wide evaluation and gene expression evaluation illustrated the manufacturing of various plantaricins, in particular the plantaricins EF and JK that more advantageous the anti-staphylococcal results of *L. plantarum* USM8613. In vivo information the usage of rats confirmed that the protein-rich fraction from *L. plantarum* USM8613 exerted wound recovery residences by using direct inhibition of *S. aureus* and promoted innate immunity, in which the expression of β -defensin was once considerably ($p < 0.05$) upregulated by using 3.8-fold. The protein fraction from *L. plantarum* USM8613 additionally appreciably more desirable ($p < 0.05$) the manufacturing of cytokines and chemokines thru a variety of degrees of wound recovery. Using Δ atl *S. aureus*, the protein-rich fraction from *L. plantarum* USM8613 exerted inhibitory pastime with the aid of concentrated on the atl gene in *S. aureus*. Taken altogether, our current learn about illustrates the workable of *L. plantarum* USM8613 in assisting wound healing, suppressing of *S. aureus* contamination at wound web sites and advertising host innate immunity. Wound recuperation is a manner in pores and pores and skin or specific organs that is occurs after injury. It is a body's natural method of regenerating dermal and epidermal tissue in pores and pores and skin and is a complicated technique involving inflammation, cell proliferation, and tissue redesigning which is regulated by using a cascade of inflammatory mediators,

collectively with cytokines and growth factors. Scientists are though concerned in wound healing, especially with regard to factors that prolong or restrict this process. A fibrous scar with collagen is the quit product of this process. Collagen offers electrical energy and integrity to dermis Although quite a few amplify factors are worried in the process of wound healing, reworking boom factor-beta (TGF- β) exerts the satisfactory fibro genic impact at some point of repair. Complications would possibly additionally show up in the wound restoration approach in victims with underlying metabolic disorders. As an instant, in every experimental and scientific diabetes disease, troubles such as compromises in cellphone migration, vascular proliferation, and extracellular matrix redesigning make terrible impaction tensile strength and wound restoration manner.

1.11 APPLICATIONS OF LACTIC ACID BACTERIA (LAB) IN PROBIOTICS:

Application of bacteriocins in ingredients may also be accomplished both via inoculation of the meals with bacteriocin producing LAB or with the aid of direct addition of the purified or semi purified bacteriocin. Once in the food, LABs are believed to be protected for consumption barring any chance for human beings. Consumption of dairy merchandise such as probiotic curd, milk, ice-cream, and yoghurt is the most convincing strategy to supply these advisable antibacterial proteins in the human gut. The use of probiotics to forestall and deal with a large range of illnesses has received taste in the previous decades. There are growing reviews of the efficacy of probiotics in the remedy of illnesses such as pouchitis. As one hundred trillion-character microorganism of one hundred extraordinary kinds inhabit the intestine, it is difficult for probiotic lines to get set up as gastrointestinal microflora. Thus, an organism that is to be used as a probiotic pressure should compete with present organisms and inhibit or kill the pathogenic microorganism in the intestinal milieu.

Additionally, the probiotic pressure is supposed to inhibit any pathogenic counterpart that is both already existing there or may additionally invade later. These days the questioning of sustenance has modified from nourishment giving vitality to our physique to consuming methodologies that supply physiological advantages in administration and counteractive action of infections. Insurance of prosperity for the

purchaser requests adjusted nourishing admission for the digestion laptop of the human physique to counteract lack or overabundance of special segments, and the probability of practical sustenance was once as soon as lengthy previous in the past than from the aforementioned origination. These propensities are the vital bringing about troubles of non-transmissible ceaseless degenerative sicknesses. Thus, to limit the danger of such ailment, the improvement of new sustenance devices that encompass organically dynamic factors has been proposed and pro-bonification of nourishments is one of the strategies used to furnish such new advisable sustenance.

Probiotics really helpful sustenance, which are characterized as continue to be microbial meals that presents an intestinal scientific reap to the host. Various in vitro and in vivo lookup are on hand which demonstrates the advantages of probiotic nourishments for human beings through the usage of keeping up or bettering their intestinal microflora. These small-scale living beings have a variety of well-being advancing impacts, for example, enhances lactose digestion system, anticipates intestinal tract ailments and enhance safety, lessens serum ldl cholesterol stage animates calcium retention, mixture of dietary nutritional vitamins (vitamin B, nicotinic corrosive, and folic corrosive), enhances protein digestibility and neutralizes the outcomes of sustenance borne pathogens.

Probiotic objects have to be made on hand for purchasers who trip the sick penalties of sustenance related concern like lactose slim mindedness, so they do now not desire to desolate tract advantages of probiotics. This prefer has caused enchantment of probiotic devices from gorgeous nourishment networks consisting of leafy foods. Products of the soil have been endorsed as sensible media for increase of probiotics due to the truth they inalienably include key supplements; immoderate measure of vitamins, mineral and polyphenolic mixes, free from allergens and correctly accessible with appealing appear and taste. The historical backdrop of probiotics can be determined to the main utilization of cheddar and aged devices that had been except a doubt understood to the Greeks and Romans who advised their utilization.

CHAPTER 2

LITERATURE SURVEY

2.1 ANTI-INFLAMMATORY, ANTI-PATHOGENIC AND PROTEOMIC EFFECTS OF LACTIC ACID BACTERIA:

Probiotics that are potentially defined as live microorganisms which when directed in adequate amounts deliberate a health benefit on the host. The objective of this study is to investigate the diversity of the selected commercial and potential probiotic lactic acid bacteria using common in vitro screening assays. The aim of this study is to analyze probiotic properties of isolated lactic acid bacteria from milk sample specifically from a certain breed of native cow known as kangeyam it is said the term probiotic is derived from the Latin preposition “pro,” which means “for” and the Greek word “biotic” meaning “bios” or “life”. Evolution of this definition was coined by Parker in 1974, who proposed that “probiotics are organisms and the substances which contribute to intestinal microbial balance“ Probiotics have been known for many beneficial health effects, and the consumption of probiotics alone or in food shows that strain-specific probiotics can present antioxidant activity and reduce damages caused by oxidation Recently, several studies have reported that Lactobacillus strains can exhibit antibacterial activity through several mechanisms such as the production of antimicrobial substances or metabolites (hydrogen peroxide, lactic acid, bacteriocin), competition for nutrients, inhibition of bacterial adhesion to the mucosa, and enhancement of the immune response. The products milk and ghee are Obtained from a certain native breed cow (Kangeyam). Cows of native breed of Indian origin, can sustain and grow well in their own habitat. Unlike the huge sized foreign breed of cows grown by commercial dairies, native cows are well suited for our weather, soil and water Native cows have natural resistance against diseases and have good immune system it is also said to have the formula as similar to that of mother’s milk hence it is also known as A2 milk. Brandi, J., Cheri, S., Manfredi, M., Di Carlo, C., Vanella, V. V, Federici, F., Bombiero, E., Bazaj, A., Rizzi, E., Manna, L. (2020).

2.2 PROBIOTIC PROPERTIES OF NON-CONVENTIONAL LACTIC ACID BACTERIA:

Varied lactic acid bacterial strains are isolated traditionally from dairy foods (milk and ghee) and are analyzed and assessed for probiotic properties. A cow's milk sample was cultured on MRS Agar media with appropriate dilution for the isolation of potential probiotic strains and pure cultures were obtained by continuous sub-culturing. Identification of lactic acid bacterial (LAB) was done by Gram's staining and catalase test and further confirmation was based on morphological, cultural, physiological and different biochemical test and its antibacterial substances against some pathogenic bacteria. The inhibitory mechanism is to be investigated for antioxidant activity against wound pathogens, (Gram positive and gram negative) production. (Marie LeFevre et al., 2017) proved the objective is to select highly efficient probiotic bacteria, which shows optimal physio-chemical parameters for selected probiotic bacteria. Minimum inhibitory concentration is to be retained from the selected bacteria, this preliminary work generally shows the potential cytotoxicity of the bacteria in normal cell lines by using amplification of PCR (polymerase chain reaction), (Hong et al., 2008) examined Further analysis by 16S rDNA sequencing revealed that the selected isolates belong to Lactobacillus species and phylogenetic tree to determine the genus and species name of the selected bacteria, thus inducing in-vitro wound healing property of cell lines from the isolated probiotic bacteria. Foligné, B., Dewulf, J., Breton, J., Claisse, O., Lonvaud-Funel, A., Pot, B. (2010)

2.3 PROBIOTIC BACTERIA IN WOUND HEALING; AN IN-VIVO STUDY:

This particular bacterium known as the lactic acid bacteria plays a critical function in meals production and health maintenance. There is an increasing interest in these species to reveal the many possible fitness advantages related with them. The movements of LAB are species and strain specific, and depend on the number of bacteria reachable in the gastrointestinal tract. (Guo M et al., 2017) Consumers are very worried of chemical preservatives and processed foods. However, merchandise with or processed with LAB are popular as an herbal way to retain food and promote health. Probiotic lines can be remoted from many sources, for human

purposes one of the essential standards is being mammal origin. Cow milk is a vital nutrient supply for human. Lot of research confirmed that this fluid has recommended outcomes on the fitness of human. One cause of being recommended is explaining through the microflora of cow milk consisting of recommended lactic acid bacteria. In this lookup work, 4 strains isolated have been identified as lactic acid microorganism by way of biochemical characterization and in addition studied for probiotic activity. Among them 1 lactic acid microorganism with high inhibition concentration was the one possessing active probiotic activity. Sinha, A., Sagar, S., Osborne, J. (2019)

2.4 SKIN MICROBIOTA, CHRONIC WOUNDS, AND PROBIOTICS FROM LAB STRAINS:

Lactic acid microorganism (LAB). It plays a crucial function in food, agricultural, and medical applications. The quick developing traits of LAB and their metabolic pastime have been the key in most functions which include meals production, agricultural industry, and probiotics. However, the biochemical and biophysical environments have considerable impact on the increase and metabolic exercise of LAB. EMAN H. ISMAIL et.al., (2015), While the biochemical prerequisites are most probably established, controlling and optimizing of biochemical prerequisites have many obstacles and challenges. In addition to deciding on the proper strain, appropriate metabolic approaches required optimizing and controlling the accessible vitamins inclusive of sugars, peptides, free amino acids, minerals, and nutritional vitamins in addition to buffering agents. Thus, a lot of lookup used to be performed to apprehend the influence of accessible vitamins on the boom and metabolic things to do of LAB. However, solely a few dietary parameters ought to be managed at a time whilst maintaining different parameters constant. Wong, V. W., Martindale, R. G., Longaker, M. T., Gurtner, G. C. (2013)

2.5 HEALTH AND NUTRITIONAL PROPERTIES OF PROBIOTICS IN FOOD INCLUDING POWDER MILK WITH LIVE LACTIC ACID BACTERIA:

The dietary parameters might also additionally engage with every different ensuing in erroneous results. Characteristics of LAB such as fastidiousness in their dietary

requirements, potential to produce acid and antimicrobial compounds, and versions in the dietary necessities amongst lines have brought extra obstacles and challenges in this regard. (Adak A et al.,2013). Fermentation of a variety of meals stuffs by means of lactic acid micro bacteria is one of the oldest types of bio preservation practiced via mankind. Bacterial antagonism has been diagnosed for over a century however in current years this phenomenon has obtained greater scientific interest, mainly in the use of more than a few lines of lactic acid microorganism One essential attribute of many LAB is their capability to produce antimicrobial compounds referred to as bacteriocins. In current years hobby in these compounds has grown appreciably due to their possible usefulness as herbal alternative for chemical meals preservatives in the manufacturing of meals with stronger shelf lifestyles and / or safety. (Hong et al.,2008). There is growing patron consciousness of the hyperlink between weight loss program and health. Recent scientific proof helps the position of probiotic LAB in mediating many effective fitness effects. Traditional probiotic dairy lines of lactic acid microorganism have a lengthy record of protected use and most traces are regarded commensal microorganisms with no pathogenic potential. (Ulrich et al., 2018) The main purpose for using the lactic acid microorganism is to find its probiotic property from the four strains observed and to analyze its wound healing property. Over the previous a hundred years, adjustments in society and technology have led to life-style adjustments and enchantment of many basic life wants globally. Simultaneously, these adjustments have resulted in new ailments and amendment of present dermatoses. Hotel, A. C. P., Cordoba, A. (2001)

2.6 INFLUENCE OF LACTOBACILLUS ACIDOPHILUS AND LACTOBACILLUS PLANTARUM ON WOUND HEALING:

The discovery and great use of antibiotics has generated various antibiotic-resistant variants, giving upward push to new health risks. Staphylococcus aureus is a transient opportunistic skin pathogen of people and different animals. S. aureus is well equipped with more than a few virulence elements that motive moderate to severe infections, ranging from cutaneous to systemic infections. (Guo M et al.,2017) In addition to virulence factors, S. aureus additionally contains several floor elements such as Protein A and extracellular adhesion proteins which facilitate S.

aureus to steer clear of the inhibitory movements of antibiotics and cognizance by way of the host immune device. The emergence of antibiotic-resistant strains has in addition decreased therapy and therapeutic options. Hence, there is a want for herbal choice compounds against. Gudadappanavar, A. M., Hombal, P. R., Timashetti, S. S., Javali, S. (2017)

2.7 EFFICACY OF USING PROBIOTICS WITH ANTIBACTERIAL ACTIVITY AGAINST PATHOGENS OF WOUND INFECTIONS:

Lactic acid microorganism (LAB) is Gram-positive, nonsporulating, microaerophilic microorganism that produce lactic acid as the important cease product of carbohydrate fermentation. , Madar Z (2005) LAB have a lengthy record of use in the meals industry due to their potential to exert a variety of advisable outcomes In addition to its additive impact on meals content, the intake of LAB additionally confers fitness advantages to the host via an elevated intestine ecosystem, decreased serum cholesterol and better host immune system. Among them, individuals of the genera *Lactobacillus* are the most commonly and commercially used. Despite the long-term use of these advisable lactobacilli in the meals industry, it was not till these days that the use of lactobacilli used to be extended to enhance dermal health. Several researches have suggested the use of these really useful LAB to preserve cutaneous homeostasis and enhance law of the pores and skin immune system. Fijan, S., Frauwallner, A., Langerholc, T., Krebs, B., ter Haar née Younes, J. A., Heschl, A., Mičetić Turk, D., Rogelj, I. (2019)

CHAPTER 3

AIM AND SCOPE

AIM:

The aim of this study is to provide a better understanding on the properties of probiotic bacteria and its potential in wound healing properties from milk and ghee. This study mainly determines the evaluation of antibacterial, antioxidant and *in vitro* wound healing properties and its effects from isolated lactobacilli present in milk and ghee.

SCOPE OF THE PRESENT INVESTIGATION:

the probiotics are microbes recommended to the host. Based on the proof so far, probiotics can positively affect the wound healing system with the aid of accelerating the wound healing. More specifically, a latest meta evaluation of animal research confirmed that the administration of probiotics is an effective pharmacological cure of cutaneous wounds, as probiotic remedy accelerates the wound recuperation processes.

- To Isolate the sample organisms from the source using serial dilution
- Identification of the isolated organisms from the pure culture
- Antibacterial activity against wound pathogens and antioxidant activity are done on the sample
- Optimization of physio chemical parameters (Ph and temperature) on the selected probiotic bacteria.
- Minimum inhibitory concentration for the selected probiotic bacteria
- Cytotoxicity assay by using normal cell lines
- Sequencing of the isolated probiotic bacteria using 16s rRNA method.
- Analyzing the bacteria's effect on the *in vitro* wound healing property.

CHAPTER 4

MATERIALS AND METHODS

4.1 METHODOLOGY:

The materials that are used for the process are

4.1.1 EQUIPMENTS:

- Conical flasks
- Measuring cylinder
- Petri plates
- Test tubes
- Funnel
- Inoculation loop
- L-rod
- Cork borer
- swab
- Micro pipette
- Glass pipette
- Nonabsorbent cotton
- Cotton plugs
- Scraper
- Aluminum foil

4.1.2 REAGENTS AND CHEMICALS:

- Sodium chloride
- Tryptone
- Yeast extract
- Agar agar
- Hcl
- Naoh
- Lactobacillus MRS broth granulated

- 2,2-Diphenyl-1-picrylhydrazyl (DPPH)
- Saline

4.1.3 DEVICES:

- Centrifuge
- Incubator
- Hot air oven
- Autoclave
- Laminar air flow chamber
- UV- spectroscopy

4.2 SAMPLE COLLECTION:

The pattern is amassed with the aid of culturing milk and ghee in nutrient agar media. A hundred ml of distilled water and about 50 ml of nutrient agar media alongside with petri plates had been sterilized in the autoclave for about 15 -20 minutes at 121 C, 15 psi. The media is immediately poured into the plate's interior in a laminar air glide chamber. The milk and ghee are diluted by using serial dilution, 0.1 aliquots from 10⁻¹ to 10⁻⁵ dilution and it had been unfolded on the nutrient agar media containing petri plates. They have been allowed to solidify and then incubated for 24 hrs. The microorganism had been determined for increase after the incubation time.



Fig 4.1 milk



Fig 4.2 ghee

4.3 SERIAL DILUTION:

The obtained sample is subjected to serial dilution. 6 test tubes for milk is taken and 1 ml of the milk sample is added to the test tube using a dropper then 9ml of saline water is added to the sample and from the initial test tube serial dilution is done and further the isolation of the probiotic bacteria is done.

4.3.1 MRS MEDIUM:

De Man, Rogosa and Sharpe agar, regularly abbreviated to MRS, is a selective traditional culture medium designed to favor the luxuriant growth increase of Lactobacilli for lab study.

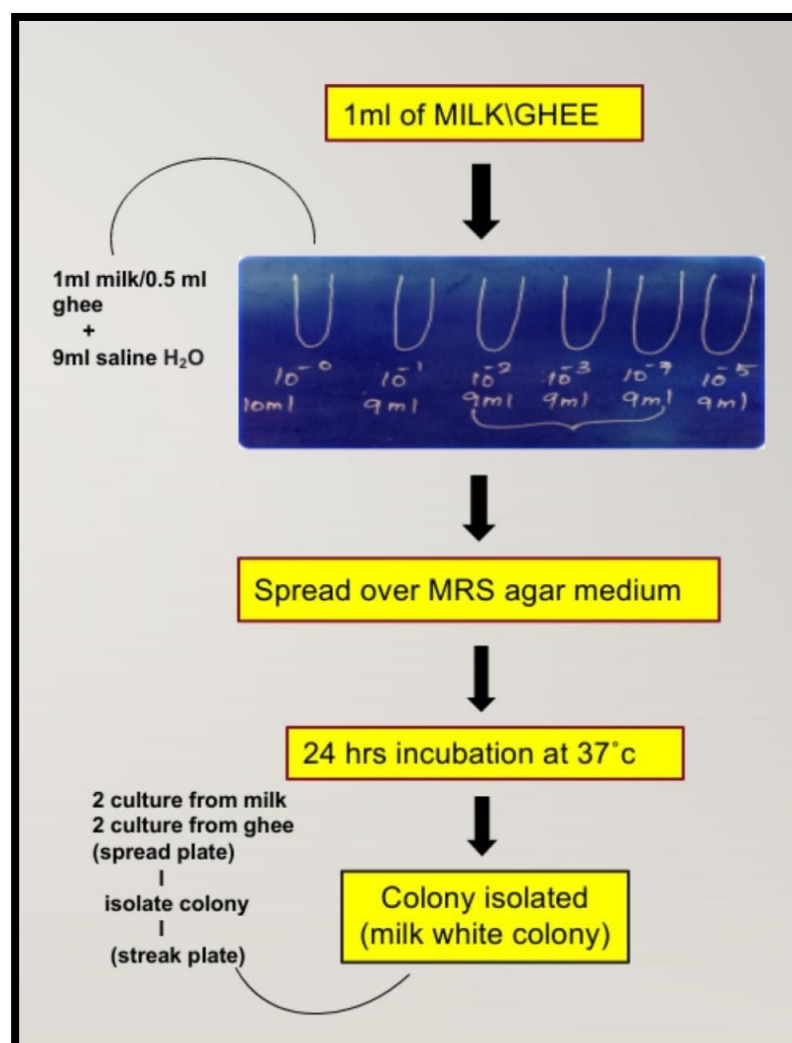


Fig 4.3 serial dilution

4.3.2 AUTOCLAVE:

As mentioned in Fig 4.3 after the sample is subjected to serial dilution MRS agar medium is prepared then 100 ml MRS media (agar plates) is used, a set of four petri plates is taken and is autoclaved then the Medium is poured on to the plates, and then it's allowed to solidify.

4.3.3 SPREAD PLATE:

50µL of the isolated bacteria is spread in each petri plates for milk and ghee using the L rod at -3, -4 dilution factor. Then the petri plates are incubated at 37 degree Celsius in the incubator for 24 hours. Then the plates were observed and the colonies are selected.

4.4 SCREENING OF BACTERIA:

Gram staining is a four-part procedure, which makes use of sure dyes to make a bacterial cell stand out in opposition to its background. The specimen must be established and constant on a slide earlier than you proceed to stain it. The reagents you will want to effectively operate this operation are:

- Crystal Violet (the Primary Stain)
- Iodine Solution (the Mordant)
- Decolourizer (ethanol is a good choice)
- Safranin (the Counterstain)
- Water (preferably in a squirt bottle)

Before starting, make sure that all reagents, as well as the squirt-bottle of water, are easily accessible because you won't have time to go get them during the staining procedure. Also, make very sure that you are doing this near a sink as it can get really messy. Wear a lab coat whenever you enter a lab

STEP 1: Place your slide on a slide holder. Flood (cover completely) the whole slide with crystal violet. Let the crystal violet stand for about 60 seconds. When the time has elapsed, wash your slide for 5 seconds with water. The specimen has to show up blue-violet when determined with the bare eye.

STEP 2: Now, flood your slide with the iodine solution. Let it stand about a minute as well. When time has expired, rinse the slide with water for 5 seconds and right away proceed to step three. At this point, the specimen must nevertheless be blue-violet.

STEP 3: This step includes addition of the decolourizer, ethanol. Step three is particularly subjective due to the fact the use of too a lot decolourizer may want to end result in a false Gram (-) result. Likewise, now not the use of sufficient decolourizer can also yield a false Gram (+) results. To be safe, add the ethanol dropwise till the blue-violet colour is no longer emitted from your specimen. As in the preceding steps, rinse with the water for 5 seconds.

STEP 4: The closing step entails making use of the counterstain, safranin. Flood the slide with the dye as you did in steps 1 and two Let this stand for about a minute to enable the micro-organism to include the safranin. Gram effective cells will contain little or no counterstain and will stay blue-violet in appearance. Gram bad bacteria, however, take on a purple shade and are without difficulty distinguishable from the Gram positives. Again, rinse with water for 5 seconds to dispose of any extra of dye. After you have performed steps 1 thru 4, you must blot the slide gently with bibulous paper or enable it to air dry earlier than viewing it underneath the microscope.

4.5 ISOLATION AND CULTURE OF THE BACTERIA:

After the screening the sample bacteria, it is isolated and inoculated into a sterile slant with fresh nutrient agar culture medium using streaking technique, it is then incubated for around 24hrs and taken as the sample for further testing.



Fig 4.4 Streak plate



Fig 4.5 Streak plate (milk and ghee)

4.6 ANTIBACTERIAL ACTIVITY:

The Assay used to be carried out by using agar well diffusion method. Antibacterial exercise of sample was once decided by using well diffusion technique on Muller Hinton agar (MHA) medium. The Muller Hinton agar medium used to be weighed as 3.8 gms and dissolved in 100ml of distilled water and add 1gm of agar. Then the medium is stored for sterilization. After sterilization the media used to be poured into sterile Petri plates and have been allowed to solidify for 1hr. After the medium was once solidified, the inoculums had been unfolding on the stable plates with sterile swab moistened with the bacterial suspension. Wells had been made the use of cork borer. Samples of respective concentrations (10 ,25, 50 and one hundred µl) have been loaded in respective wells. These plates have been incubated for 24 hrs at 37°C. Then the microbial increase used to be decided by means of measuring the diameter of area of inhibition.

4.6.1 SCREENING OF *lactobacillus* BY ANTIBACTERIAL ACTIVITY:

These gram bacteria are to react with the probiotic bacterial strain that is to be isolated from (milk, ghee), thereby to demonstrate the in vitro wound healing property of the probiotic bacteria that is to be isolated.

Two-gram negative Bac

Two-gram positive Bac

- *Staphylococcus aureus*
- *Bacillus subtilis*
- *Pseudomonas aeruginosa*
- *E. coli*

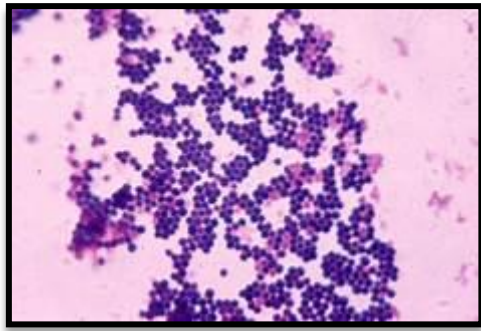


Fig 4.6

Staphylococcus aureus
(Gram+ve)

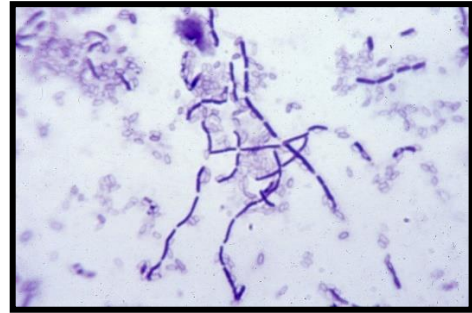


Fig 4.7

Bacillus subtilis
(Gram+ve)

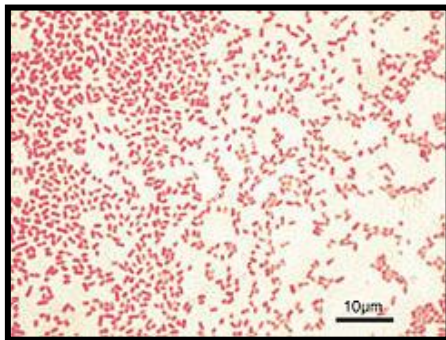


Fig 4.8

Pseudomonas aeruginosa
(Gram-ve)



Fig 4.9

E. coli
(Gram-ve)

4.6.2 ANTIBACTERIAL ACTIVITY OF ISOLATED STRAINS AGAINST WOUND PATHOGENS:

Stock cultures were maintained at 4°C on slant of nutrient agar. Active cultures are necessary for experiments were prepared by the transferring of a loop full of cells from the stock cultures to the test tubes of the nutrient broth for bacteria that were incubated at 24hrs at 37° C. Both disk diffusion and MIC methods employ the phenotypic identification of susceptibility, and therefore, requires the following process by (Bayot ML, Bragg BN,2020).

- Preparation of a standardized inoculum from a bacterial culture:
- Choosing well-isolated colonies
- Creating a bacterial suspension (inoculum)
- Standardizing the bacterial suspension using McFarland standards
- Dilution of bacterial suspension (only for MIC method)
- Inoculation of bacterial suspension to one of the following
- A particular growth medium (e.g., Mueller Hinton Agar, MHA for disk diffusion)
- Addition of antimicrobial disks (only for disk diffusion)
- Incubation of plates (disk diffusion)
- Measuring the zone of inhibition
- Interpretation of AST results

5ml of mrs broth in four test tubes is taken and the isolated strains obtained is (M1, M2, G1, G2) inoculated and grown for 24hrs. After 24hrs, the 2ml of grown cultures were centrifuged at 5000rpm for 5mins. After centrifugation, the supernatant was collected. The freshly cultured bacterial suspensions *Bacillus subtilis*, *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* (100 µl) were swabbed on Nutrient agar plates aseptically Wells were made with the help of a sterile cork borer. The wells were loaded with 50 µg/mL of the bacterial suspensions and 20 µg/mL of amoxicillin as positive control in a single well. The plates were incubated at 37 °C for 24 hours Zone of inhibition (ZOI; mm) appearing around the wells was recorded.

4.6.3 ZONE OF INHIBITION AGAINST WOUND PATHOGENS:

S. No.	Name of the microorganism	Zone of inhibition (mm in diameter)				
		Positive control (Amoxicillin)	Samples			
			M1	M2	G1	G2
1	<i>B. subtilis</i>	22	20	-	-	-
2	<i>S. aureus</i>	25	16	-	-	09
3	<i>E. coli</i>	26	18	-	-	08
4	<i>P. aeruginosa</i>	24	20	-	-	10

Table Fig 4.1 Zone of inhibition against wound pathogens

Only the strain m1 shows highest inhibition against the wound pathogens thus the m1 strain (isolated probiotic bacteria) is taken for further use.

4.7 ANTIOXIDANT ACTIVITY:

Probiotics are regarded for their fitness advisable outcomes and are hooked up as dietary adjuncts. Probiotics have been recognized for many recommended fitness effects. In this view, there is pastime to locate the attainable probiotic traces that can showcase antioxidant residences alongside with fitness benefits. In vitro and in vivo research point out that probiotics show off antioxidant potential. In this view, consumption of probiotics by myself or meals supplemented with probiotics may additionally decrease oxidative damage, free radical scavenging rate, and change in pastime of vital antioxidative enzymes in human cells. Incorporation of probiotics in ingredients can furnish a exact approach to provide dietary antioxidants, however greater research are wished to standardize strategies and consider antioxidant residences of probiotics earlier than they can be advocated for antioxidant potential. In this paper, the literature associated to acknowledged antioxidant manageable of probiotics and proposing future views to behaviour such research has been reviewed.

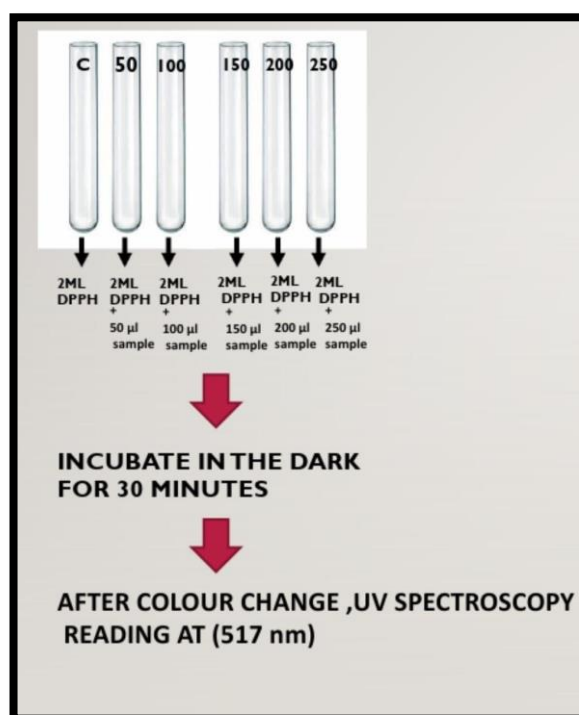


Fig 4.10 DPPH assay

4.7.1 DPPH ASSAY (2,2-diphenyl-1-picrylhydrazyl):

The DPPH free radical scavenging assay was once stated to be carried out for the comparison of the antioxidant activity. This assay measures the free radical scavenging ability of unique concentrations of extracts. DPPH is a molecule containing a steady free radical. In the presence of an antioxidant, which can donate an electron to DPPH, the red coloration standard for free DPPH radical decays, and the absorbance trade at 517 nm is measured. This check presents data on the capability of a compound to donate a hydrogen atom, on the range of electrons a given molecule can donate, and on the mechanism of antioxidant action. After 30 min of incubation at 25°C, the reduce in absorbance used to be measured at 517 nm.

- Six clean test tubes are taken washed and autoclaved for about 15 minutes after autoclaving the test tubes, the test tubes are placed in a test tube holder with the use of a marker the test tubes are marked as C, 50, 100, 150, 200 and 250 after marking them place them back in the test tube holder.
- 2 ml of DPPH solution is added to each of the six test tubes.
- Leaving the control untreated, in the other test tubes containing the dpph is then treated with the sample simultaneously with the following concentrations (50 µl, 100 µl, 150 µl, 200 µl, 250 µl).
- The sample is then incubated for about 30 minutes.
- The morphological changes of untreated (control) and the treated cells were observed and then uv- spectroscopy readings are taken after 30 minutes and photographed and the graph is plotted to view its inhibition concentration.

4.8 OPTIMIZATION OF PH AND TEMPERATURE:

Freshly inoculated cultures were taken and put in different test tubes containing varying pH from 4 to 8. The cultures were then kept for incubation for around 24 hrs and the growth was observed. In another 3 test tubes the inoculated cultures were taken and kept in 3 different temperatures 27°C, 32°C, 37°C, 42°C, 47°C. The cultures were allowed to grow for 24-36 hrs in media. A graph was plotted to observe and differentiate the growth of the organism.

4.9 MINIMUM INHIBITORY CONCENTRATION:

Minimum inhibitory concentrations (MICs) are said to be described as the lowest awareness of an antimicrobial that is to inhibit the increase of a microorganism after a single day incubation, and the minimal bactericidal concentrations at the lowest concentration of the antimicrobial that will stop the increase of an organism after subculture on to an antibiotic-free media. MICs are used via diagnostic laboratories commonly to verify resistance, however most regularly as a lookup device to decide the in vitro activity of new antimicrobials, and statistics from such research have been used to decide MIC breakpoints. MIC is additionally described as the lowest concentration (expressed as mg/L or $\mu\text{g}/\mu\text{L}$) of an antimicrobial or antibacterial agent that inhibits the seen in-vitro increase of the microorganisms. The MIC check additionally determines the antibacterial undertaking of a test agent in opposition to a unique bacterium. E-test, tube dilution method, and agar dilution strategies are employed to decide MIC value.

- As the test results proved only M1 strain to showcase highest inhibition against wound pathogens it is thus used further for determining the minimum inhibitory concentration for the isolated M1 probiotic bacteria.
- 50ml of mrs broth in four test tubes is taken and the isolated strain obtained (M1) is inoculated and grown for 24hrs in it.
- After 24hrs, the 2ml of grown cultures were centrifuged at 5000rpm for 5mins.
- After centrifugation, the supernatant was collected.
- The freshly cultured bacterial suspensions ***Bacillus subtilis***, ***Escherichia coli***, ***Staphylococcus aureus***, ***Pseudomonas aeruginosa*** (100 μl) were swabbed on Nutrient agar plates aseptically
- Wells were made with the help of a sterile cork borer.
- The wells were loaded with the supernatant collected in different concentrations and 20 $\mu\text{g}/\text{mL}$ of amoxicillin as positive control in a single well.
- The plates were incubated at 37 °C for 24 hours
- Zone of inhibition (ZOI; mm) appearing around the wells was recorded.

4.10 CYTOTOXICITY ASSAY USING NORMAL CELL LINES:

4.10.1 CELL MAINTAINENCE:

VERO normal epithelial cell line was obtained from the National Centre for Cell Sciences (NCCS), Pune, India. Cells were maintained in the logarithmic phase of growth in Dulbecco's modified eagle medium (DMEM) supplemented with 10% (v/v) heat inactivated fetal bovine serum (FBS), 100 U/mL penicillin, 100 µg/mL streptomycin. They were maintained at 37°C with 5% CO₂ in 95% air humidified incubator

4.10.2 CYTOTOXICITY EFFECT OF CELL LINE:

The cytotoxicity effect of the sample was tested against VERO cell line by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (Mossman, 1983). The cells were seeded in 96-well microplates (1 x 10⁶ cells/well) and incubated at 37°C for 48 h in 5% CO₂ incubator and allowed to grow 70-80% confluence. Then the medium was replaced and the cells were treated with different concentrations (20, 40, 60, 80, 100 µg/mL) of sample and incubated for 24 h. The morphological changes of untreated (control) and the treated cells were observed under inverted microscope (20X magnification) after 24 h and photographed. The cells were then washed with phosphate-buffer saline (PBS, pH-7.4) and 20 µL of (MTT) solution (5 mg/mL in PBS) was added to each well. The plates were then stand at 37°C in the dark for 2 h. The formazan crystals are dissolved in 100 µL DMSO and the absorbance was taken spectrometrically at 570 nm. The percentage of cell viability was calculated using the given formula:

Cell viability (%) = (Absorbance of sample/Absorbance of control) X 100.

Graph was plotted using the cell viability (%) at Y-axis and concentration of the sample in X-axis.

4.11 16srRNA SEQUENCE ANALYSIS:

The amplified DNA product of 500bp were given for sequencing for Sanger dideoxy sequencing. The sequence that showed >97% sequence identity were retrieved and saved in FASTA format. The FASTA file was opened and multiple sequence alignment was done using the ClustalW tool (Larkin et al., 2007) using Bio Edit software. The multiple sequence alignment was saved in *.meg format and loaded in MEGA X for the construction of phylogenetic tree. The phylogenetic tree was constructed with default parameters using maximum parsimony (character-based method), minimum evolution and neighbor joining (distance-based method) algorithm. A bootstrap value of 1000 replicates were used to construct a consensus dendrogram. The strain was identified and classified based on the percentage of identity (%), i.e., the sequence showing >97% sequence identity.

4.12 PHYLOGENETIC ANALYSIS:

Multiple sequence alignments were produced using CLUSTAL W algorithm available in MEGA X (Kumar S et al., 2016). Phylogenetic relationships were inferred using the neighbor joining method (Saitou et al., 1987) with bootstrapping (Felsenstein J, 1985). Evolutionary distances were computed using the P-distance method (Nei et al., 2000) using MEGA X Bootstrap values (1000 iterations) were calculated based on >70% majority rule and the confidence limits were said to be placed at the major nodes of the tree.

4.13 IN-VITRO WOUND HEALING USING *Lactobacillus plantarum*:

Lactobacillus plantarum is a broadly distributed and versatile lactic acid bacterium. It represents phase of the microbiota of many ingredients and feeds, which includes dairy, meat, fish, vegetable fermented merchandise (e.g., must, sauerkraut, pickled vegetables, sourdoughs), and silage; it is additionally a herbal inhabitant of the human and animal mucosa (oral cavity, gastrointestinal tract, vagina, etc.). *Lactobacillus plantarum*, derived from the milk is used in wound healing assay, it is normally related with suited residences in many wound assays, and is clearly introduced to a range of them in order to beautify their satisfactory or

This describes the taxonomy, genetics, metabolism, and technological and health-related houses of *Lb. plantarum* and its applications, with one of a kind focal point from dairy products.

4.13.1 CELL MAINTAINENCE:

3T3 mouse fibroblast normal cell line was obtained from the National Centre for Cell Sciences (NCCS), Pune, India. Cells were maintained in the logarithmic phase of growth in Dulbecco's modified eagle medium (DMEM) supplemented with 10% (v/v) heat inactivated fetal bovine serum (FBS), 100 U/mL penicillin, 100 µg/mL streptomycin. They were maintained at 37°C with 5% CO₂ in 95% air humidified incubator.

4.13.2 IN- VITRO SCRACH WOUND HEALING ASSAY:

3T3 cells were seeded in 6-well plates (8×10^5 cells/well) and grown until reached a confluence of 85-90%, in the optimum culture conditions. In the middle of cell monolayer, a scratch was made by a P10 pipette tip, to mimic a wound, and cell debris were removed by washing with fresh medium. The wound was exposed for 30 µg/mL of samples for 24-72 hrs at 37°C in a humidified atmosphere of 5% CO₂. The cell grown in extract-free medium were used as control. Scratch wound closure was analyzed under the inverted microscope equipped with a digital SLR camera, by acquiring digital images at different time 0th day (T0), 24hr (T1), 48hr (T2) (static imaging). In the static imaging modality, the closure of the scratch was quantified, as recently described (Felice *et al.*, 2015), by measuring the difference between the wound width at T0 and T1/T2, using the ImageJ processing.

CHAPTER 5

RESULTS AND DISCUSSION

5.1 SAMPLE COLLECTION:

After 24 hrs. the bacteria were observed to have grown without any contamination on the nutrient agar media.

5.2 SERIAL DILUTION:

the dilution factor remains constant for each dilution, resulting in an exponential decrease in concentration hence the isolation of probiotic bacteria.



Fig 5.1 milk



Fig 5.2 ghee

5.3 ISOLATION AND CULTURE OF THE SAMPLE:

The isolated bacteria were streaked on sterile petri plates with nutrient agar and grown for 24 hrs using spread plate method. Then the plates were observed and the colonies were selected.

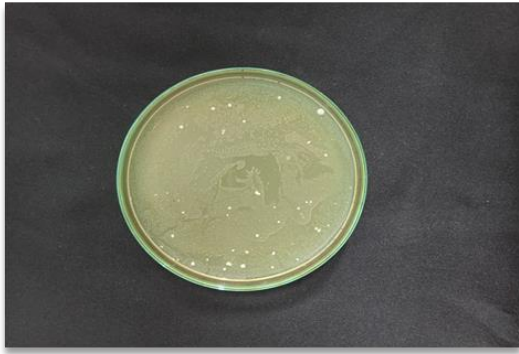


Fig 5.3 (milk 10^{-4})



Fig 5.4 (milk 10^{-3})



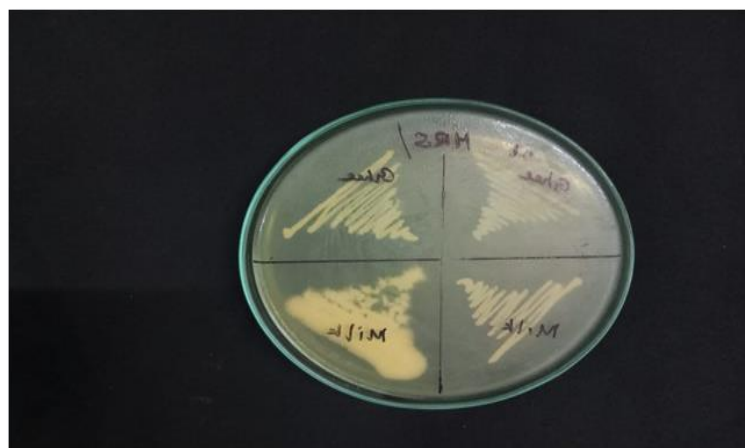
Fig 5.5 (ghee 10^{-4})



Fig 5.6 (ghee 10^{-3})



Fig 5.7 streak plate with isolated bacterial colonies



***Fig 5.8 pure culture with isolated bacteria (milk and ghee)
(streak plate method)***

5.4 ANTIBACTERIAL ACTIVITY:

The sample bacteria were added in different concentrations in the wells via wound pathogens swabbed on the nutrient agar medium and the zone of inhibition was observed after 24hrs.

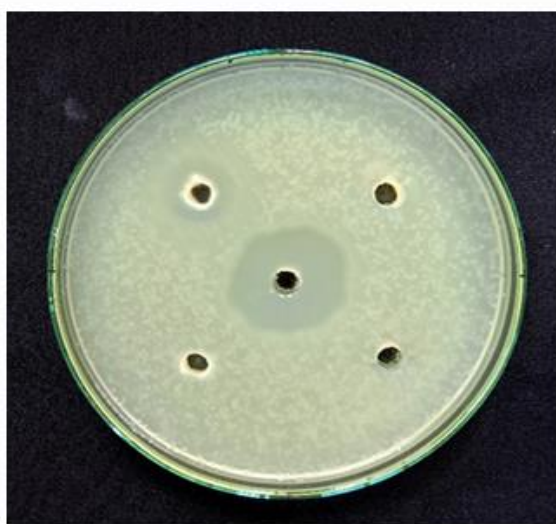


Fig 5.9 bacillus subtilis

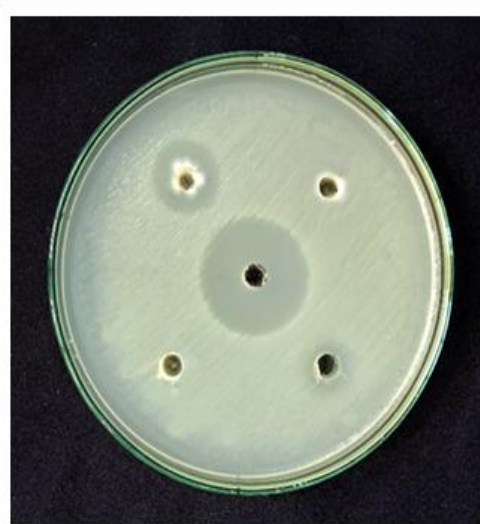


Fig 5.10 staphylococcus aureus

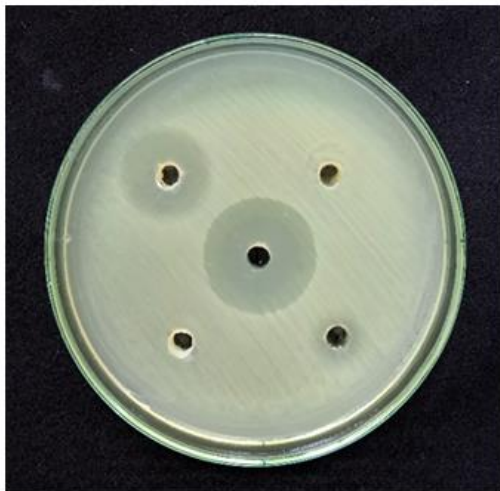


Fig 5.11 *Escherichia coli*



Fig 5.12 *pseudomonas aeruginosa*

Sample of concentrations (50 μ l) were added to the wells of the plates with the respective bacterial suspension on each plate.

5.4.1 ZONE OF INHIBITION AGAINST WOUND PATHOGENS:

S. No.	Name of the microorganism	Zone of inhibition (mm in diameter)				
		Positive control (Amoxicillin)	Samples			
			M1	M2	G1	G2
1	<i>B. subtilis</i>	22	20	-	-	-
2	<i>S. aureus</i>	25	16	-	-	09
3	<i>E. coli</i>	26	18	-	-	08
4	<i>P. aeruginosa</i>	24	20	-	-	10

Table Fig 5.1 zone of inhibition against wound pathogens

Results:

Only strain m1 shows highest inhibition against the wound pathogens thus only m1 strain will be subjected in the future tests.

5.5 SCREENING OF PROBIOTIC BACTERIA FOR ANTIOXIDANT ACTIVITY:

DPPH molecule contains a stable free radical. In the presence of an antioxidant, which can donate an electron to DPPH, the purple color typical for free DPPH radical decays, after 30 min of incubation at 25°C, the decrease in the absorbance is measured at 517 nm.

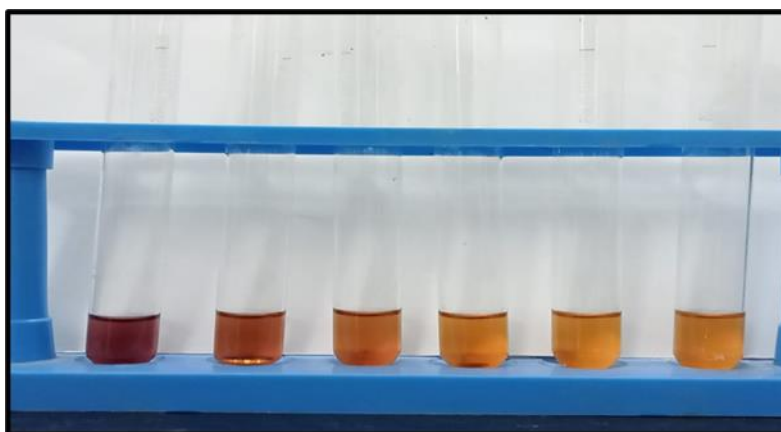


Fig 5.13 DPPH assay

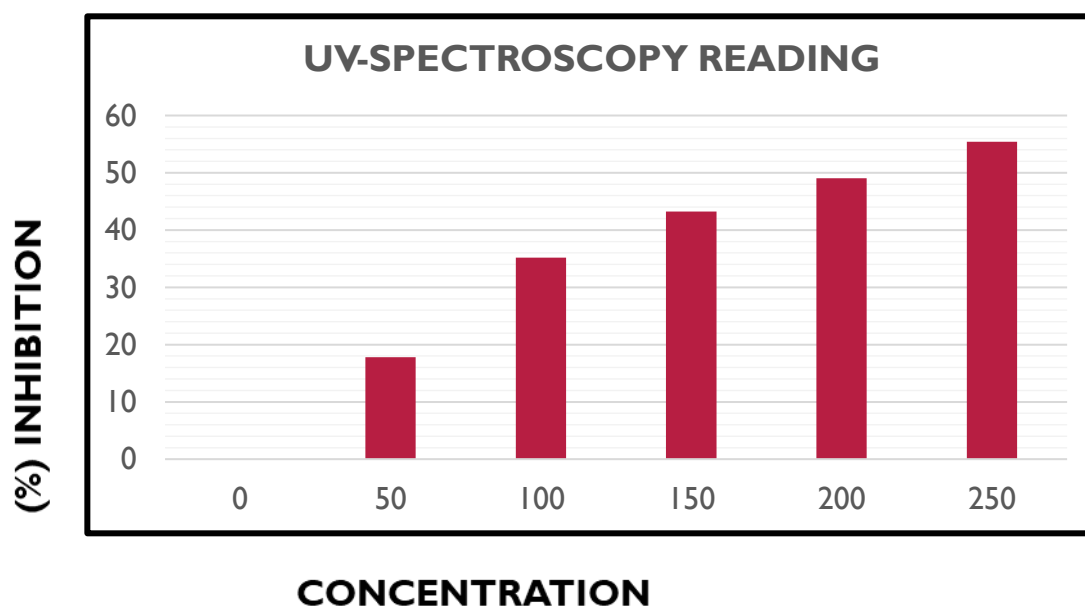


Fig 5.14 Antioxidant activity

This check affords facts on the capability of a compound to donate a hydrogen atom, the variety of electrons a given molecule can donate, and on the mechanism of antioxidant action. The radical scavenging recreation was once calculated from the equation:

$$\% \text{ scavenging activity} = \frac{\text{Abs control} - \text{Abs sample}}{\text{Abs control}} \times 100$$

Results - Percentage of inhibition is 55.4 % at 250 μ l.

5.6 OPTIMIZATION OF PHYSIOCHEMICAL GROWTH PARAMETERS FOR SELECTED PROBIOTIC BACTERIA:

5.6.1 PH OPTIMIZATION:

The pH OD readings were taken at 600 nm after the bacteria was grown for 24hrs

PH	Absorbance
5	0.041
6	0.025
7	0.06
8	0.058
9	0.051
10	0.048

Table 5.2 pH OD readings of the sample

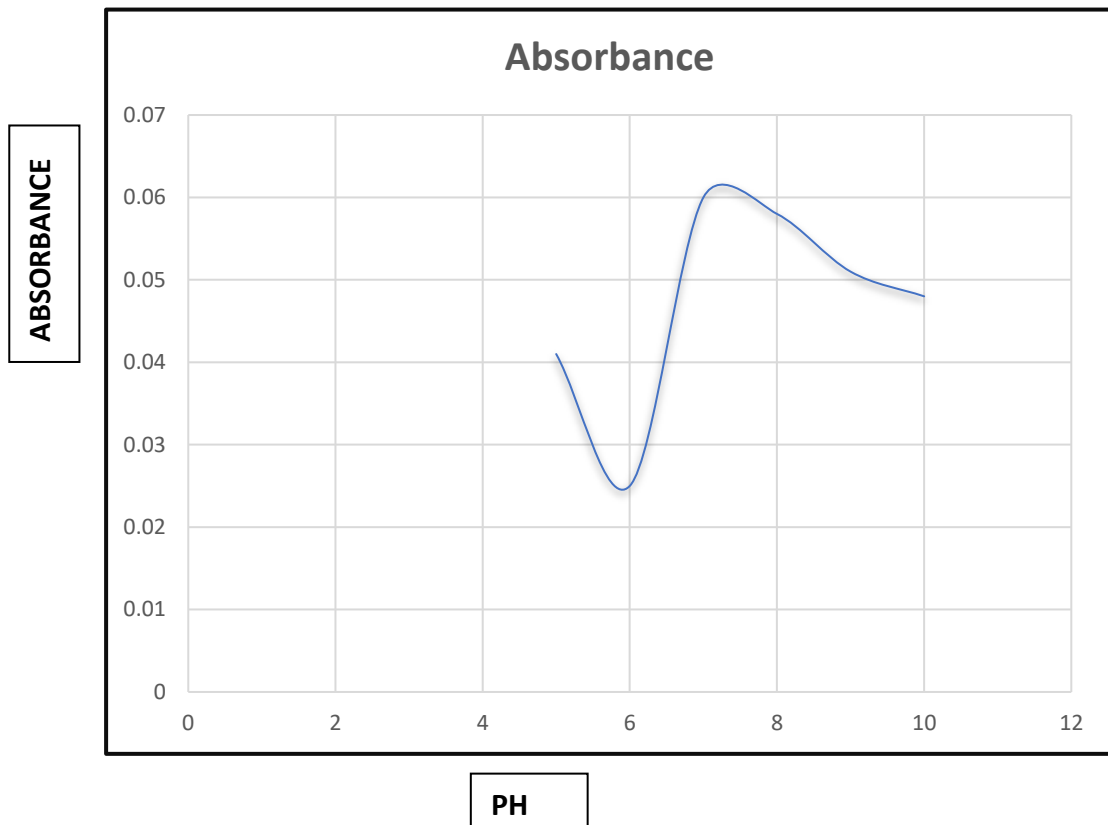


Fig 5.15 Graph of optimal pH

The optimal pH was the growth of the sample was found at a pH of 8. The bacterium seems to grow at a maximum rate when it's in an environment with an almost alkaline ph.

5.6.2 TEMPERATURE OPTIMIZATION:

The readings were taken after the bacteria was allowed to grow for 24hrs in a controlled environment with different temperatures.

Temperature (°C)	Absorbance
27	0.093
32	0.069
37	0.111
42	0.08
47	0.065

Table 5.3 OD readings of sample at different temperature

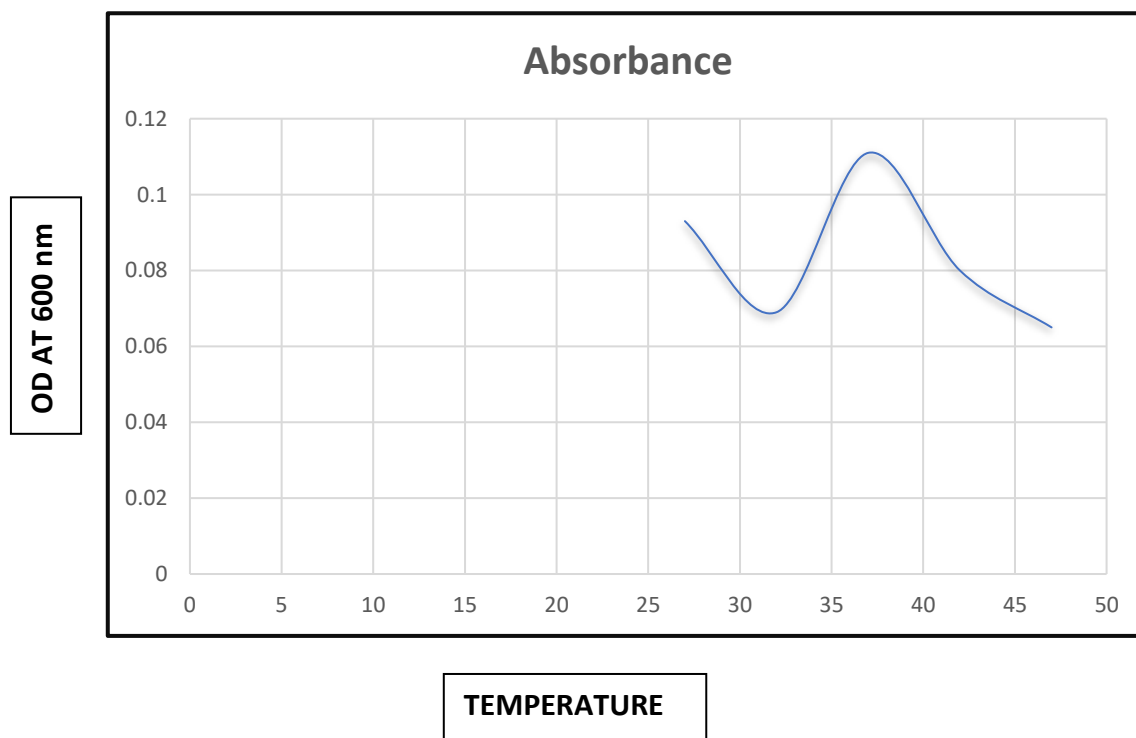


Fig 5.16 Graph of temperature tolerance

The maximum growth was seen at 37°C. Although the bacterium can tolerate high temperatures, the maximum growth with 24 hrs. was found to be at a moderate temperature.

5.7 MINIMUM INHIBITORY CONCENTRATION:

The sample bacteria were added in different concentrations in the wells via wound pathogens swabbed on the nutrient agar medium and the zone of inhibition was observed after 24hrs.

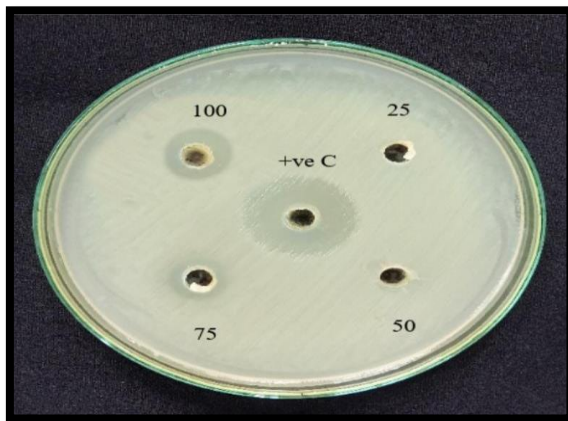


Fig 5.17 *Bacillus subtilis*

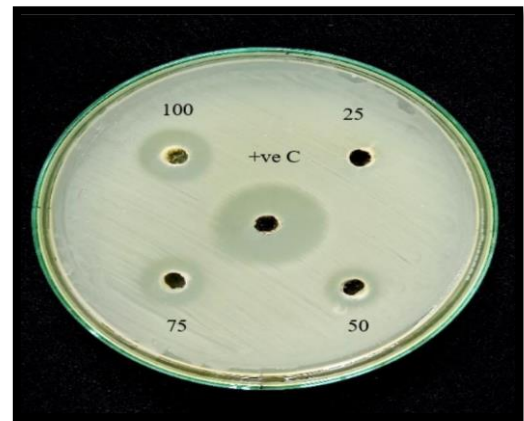


Fig 5.18 *Staphylococcus aureus*

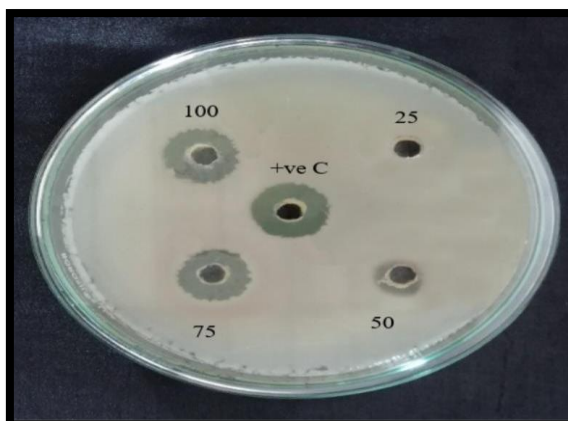


Fig 5.19 *Escherichia coli*



Fig 5.20 *Pseudomonas aeruginosa*

5.7.1 ZONE OF INHIBITION OF THE ISOLATED M1 STRAIN AGAINST WOUND PATHOGENS:

S. No.	Name of the microorganism	Zone of inhibition (mm in diameter)				
		Positive control (Amoxicillin)	M1 (µl)			
			25	50	75	100
1	<i>B. subtilis</i>	22	-	-	08	12
2	<i>S. aureus</i>	26	-	08	12	18
3	<i>E. coli</i>	18	-	07	13	17
4	<i>P. aeruginosa</i>	21	-	-	13	18

Table Fig 5.4 zone of inhibition against wound pathogens

Results:

Minimum Inhibitory Concentration (MIC) is the lowest concentration (expressed as mg/L or µg/µL) of an antimicrobial agent that is to inhibit the visible in-vitro growth of microorganisms. Thus, MIC for *S. aureus*, *E. coli* was found to be 50 µg and *B. Subtilis*, *P. aeruginosa* was found to be 75 µg.

5.8 CYTOTOXICITY ASSAY BY USING NORMAL CELL LINES:

The morphological changes of untreated (control) and the treated cells were observed under inverted microscope (20X magnification) after 24 h and photographed. The cells were then washed with phosphate-buffer saline (PBS, pH-7.4) and 20 μ L of (MTT) solution (5 mg/mL in PBS) was added to each well. The plates were then placed at 37°C in the dark for 2 h. The formazan crystals are dissolved in 100 μ L DMSO and then the absorbance was taken spectrometrically at 570 nm. Percentage of cell viability was calculated using the formula, Cell viability (%) = (Absorbance of sample/Absorbance of control) X 100. Graph was plotted using the cell viability (%) at Y-axis and concentration of the sample in X-axis.

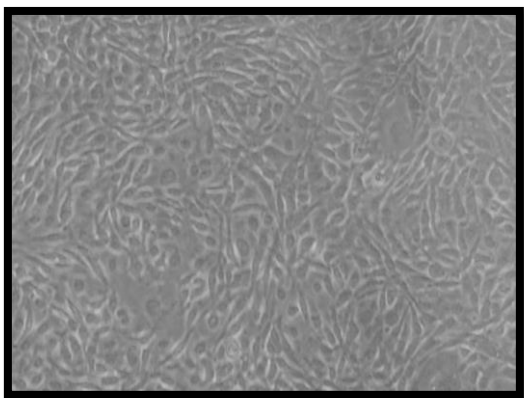


Fig 5.21 Control

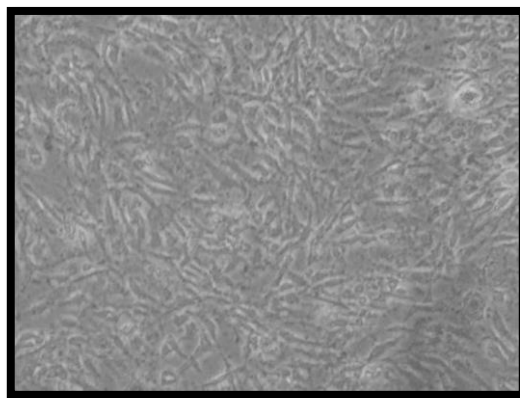


Fig 5.22 (20 μ g/ML)

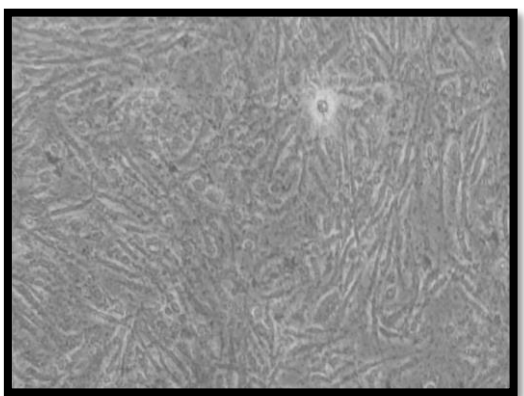


Fig 5.23 (40 μ g/ML)

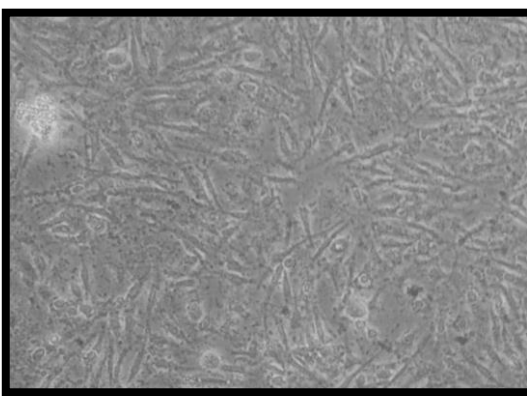


Fig 5.24 (60 μ g/ML)

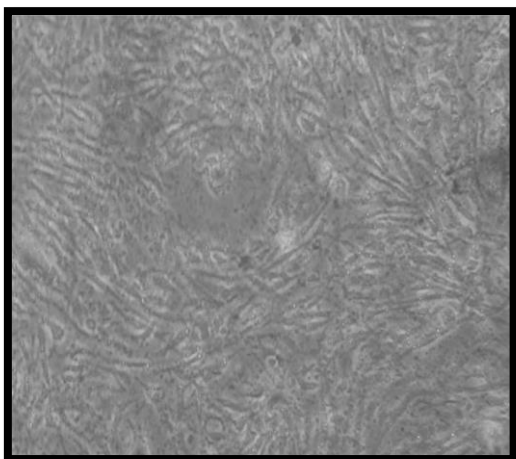


Fig 5.25 (80 µg/ML)

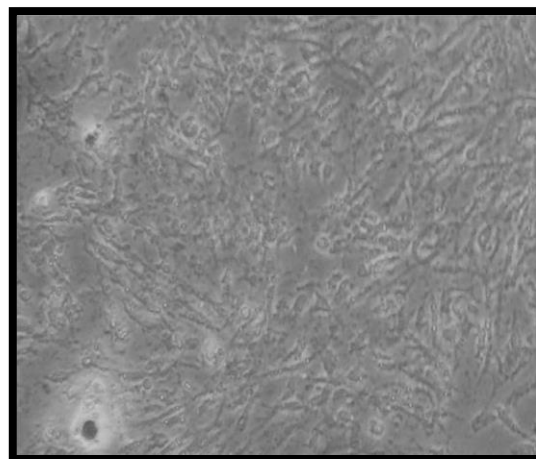


Fig 5.26 (100 µg/ML)

5.8.1 CELL VIABILITY IN CYTOTOXICITY ASSAY:

Concentrations (µg/mL)	Absorbance		Average	Cell viability (%)
	I	II		
Control	0.875	0.882	0.8785	100
20	0.831	0.84	0.8355	95.10529311
40	0.758	0.765	0.7615	86.68184405
60	0.677	0.671	0.674	76.72168469
80	0.6	0.594	0.597	67.95674445
100	0.435	0.427	0.431	49.06089926

Table Fig 5.5 Cell viability in cytotoxicity assay

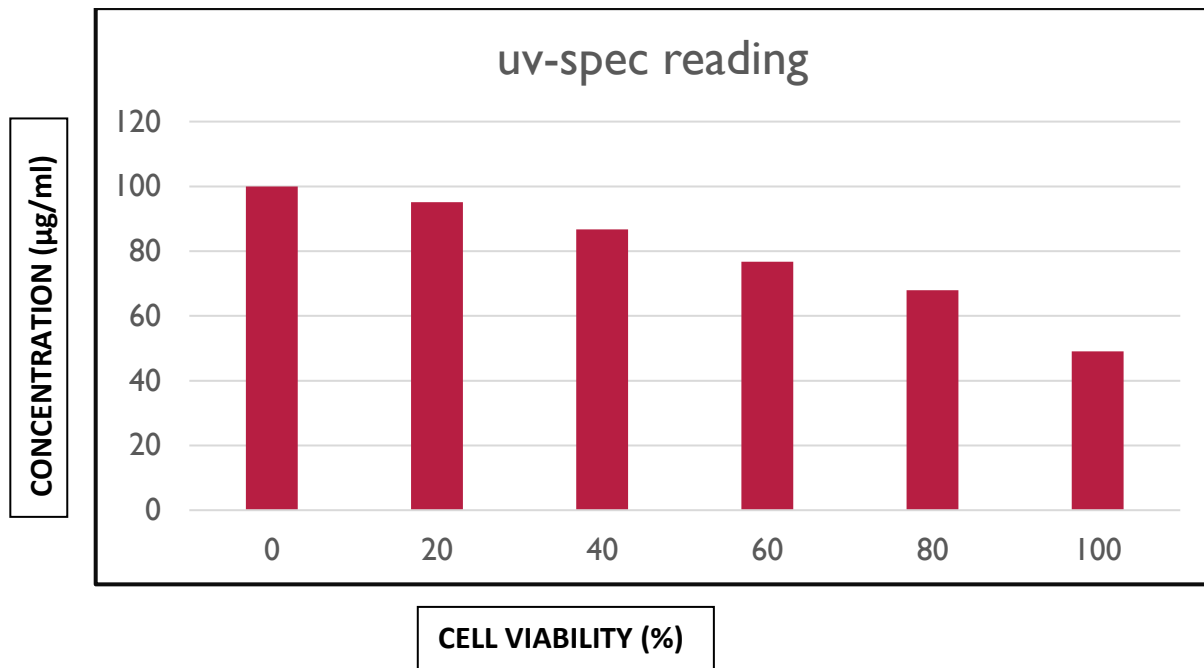


Fig 5.27 Cell viability in cytotoxicity assay

Measurements for cell viability are used to evaluate the death or life of cancerous cells, the rejection of implanted organs, or the effectiveness of a drug candidate. Cell viability assays are also often useful to determine optimal growth conditions of cell populations maintained in culture.

5.9 16srRNA SEQUENCING:

- DNA used to be isolated and extent has once measured the usage of NanoDrop Spectrophotometer and the high-quality was once determined using 2% agarose gel.
- A single band of high-molecular-weight DNA has been observed.
- 16S rRNA gene used to be amplified using 16SrRNAF and 16SrRNAR primers. A single discrete PCR amplicon band of 698 bp was once determined when resolved on Agarose gel.

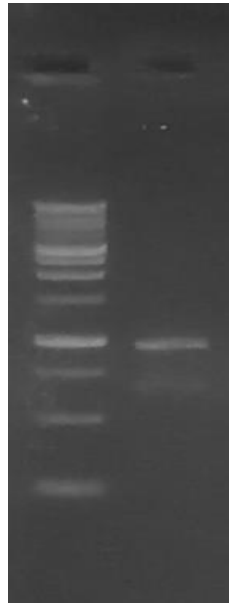


Fig 5.28 gel picture

- The PCR amplicon was once purified to eliminate contaminants.
- Forward and reverse DNA sequencing response of PCR amplicon was once carried out with forwarding primer and reverse primers the usage of BDT v3.1 Cycle sequencing package on ABI 3730xl Genetic Analyzer.
- Consensus sequence of 16S rRNA gene was once generated from ahead and reverse sequence

5.9.1 SEQUENCE ANALYSIS 16S rRNA GENE PARTIAL SEQUENCE (~698bp):

GGCGGCCTAATACATGCAAGTCGAACGAACTCTGGGTATTGATTGGTGCTTGC
 ATCATGATTTACATTTGTGTGAGTGGCGAACTGGTGAGTAACACGTGGTGAAA
 CCTGCCCCGAAGCGGGGGATAACACCTGGAAACAGATGCTAATACCGCATAA
 CAACTTGGACCGCATGGTCCGAGTTTGAAAGATGGCTTCGGCTATCACTTTTAG
 GATGGTCCCGCGGCGTATTATGCTAGATGGTACAGGTAACGGCTCACCATGGC
 AATGATACGTACGCCGACCTGAGAGGGTAATCGGCCACATTGGGACTGAGACA
 CGGCCCAAACCTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGA
 AAGTCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGGTTTCGGCTCGTAAAAC

TCTGTTGTTAAAGAAGAACATATCTGAGAGTAACTGTTTCAGGTATTGACGGTA
TTTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAG
GTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTTTT
TAAGTCTGATGTGAAAGCCTTCGGCTCAACCGAAGAAGTGCATCGGAAACTGG
GAAACTTGAGTGCAGAAGAGGACAGTGGAAGTCCATGTGTAGCGGTGAAAAA
CCGTAGAAAAA

- 16S rRNA gene sequence was once used to carry out BLAST with the database of NCBI GenBank database. Based on most identification rating first ten sequences have been chosen and aligned the use of more than one alignment software program application Clustal W. Distance matrix was once generated and the phylogenetic tree used to be developed via the usage of MEGA 7.

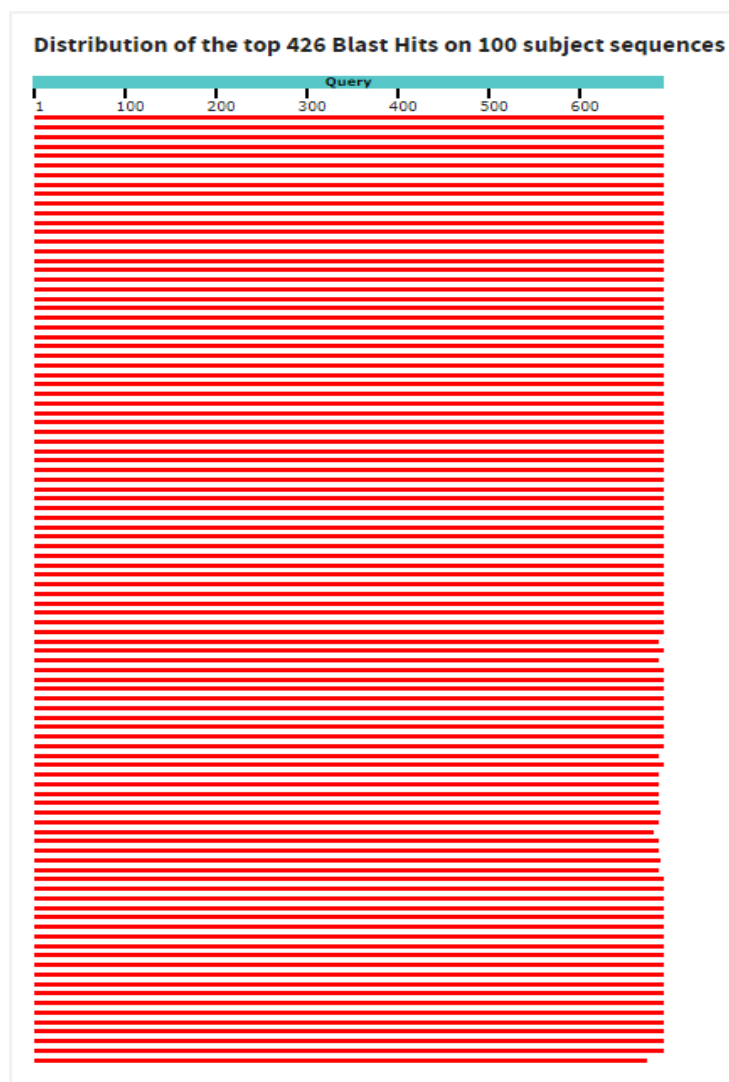


Fig 5.29 blast results

Table 5.6 NCBI BLAST top 10 Scores

S.No.	Description	Max Score	Total Score	Query Cover	E value	Per. ident	Accession
1	Lactobacillus plantarum strain cczz3 16S ribosomal RNA gene, partial sequence	1206	1206	97%	0	98.54	JN043518.1
2	Lactobacillus plantarum subsp. plantarum strain HBUAS56153 16S ribosomal RNA gene, partial sequence	1205	1205	97%	0	98.54	MT231775.1
3	Lactobacillus pentosus strain HBUAS56244 16S ribosomal RNA gene, partial sequence	1205	1205	97%	0	98.54	MT229656.1
4	Lactobacillus plantarum strain HBUAS56043 16S ribosomal RNA gene, partial sequence	1205	1205	97%	0	98.54	MN994357.1
5	Lactobacillus plantarum strain TB5a 16S ribosomal RNA gene, partial sequence	1205	1205	97%	0	98.54	MN242002.1
6	Lactobacillus plantarum Shonai-SU4 gene for 16S rRNA, partial sequence	1205	1205	97%	0	98.54	LC512751.1
7	Lactobacillus plantarum Tosa-SU1 gene for 16S ribosomal RNA, partial sequence	1205	1205	97%	0	98.54	LC500349.1
8	Lactobacillus plantarum strain A7034 16S ribosomal RNA gene, partial sequence	1205	1205	97%	0	98.54	MN439952.1
9	Lactobacillus plantarum Shinshu N-07 gene for 16S ribosomal RNA, partial sequence	1205	1205	97%	0	98.54	LC494014.1

10	Lactobacillus plantarum strain SK3 16S ribosomal RNA gene, partial sequence	1205	1205	97%	0	98.54	MK246167.1
11	Lactobacillus plantarum Kinko-SU4 gene for 16S ribosomal RNA, partial sequence	1205	1205	97%	0	98.54	LC428213.1
12	Lactobacillus pentosus strain S1 16S ribosomal RNA gene, partial sequence	1205	1205	97%	0	98.54	MF443879.1
13	Lactobacillus plantarum Wakasa-SU2 gene for 16S ribosomal RNA, partial sequence	1205	1205	97%	0	98.54	LC333204.1
14	Lactobacillus pentosus strain NGI17 16S ribosomal RNA gene, partial sequence	1205	1205	97%	0	98.54	MT919355.1
15	Lactobacillus plantarum strain P5 16S ribosomal RNA gene, partial sequence	1201	1201	97%	0	98.4	MF098787.1

After getting the sequence it was then analyzed with BLAST (Basic Local Alignment Search tool) The application compares nucleotide or protein sequences to sequence databases and calculates the statistical value of matches. BLAST can be used to infer purposeful and evolutionary relationships between sequences as nicely as assist become aware of participants of gene families. It used to be located that the sequence which was once uploaded has 98.54% similarity with Lactobacillus plantarum AGBT6. Plantarum strain.

5.10 PHYLOGENETIC TREE:

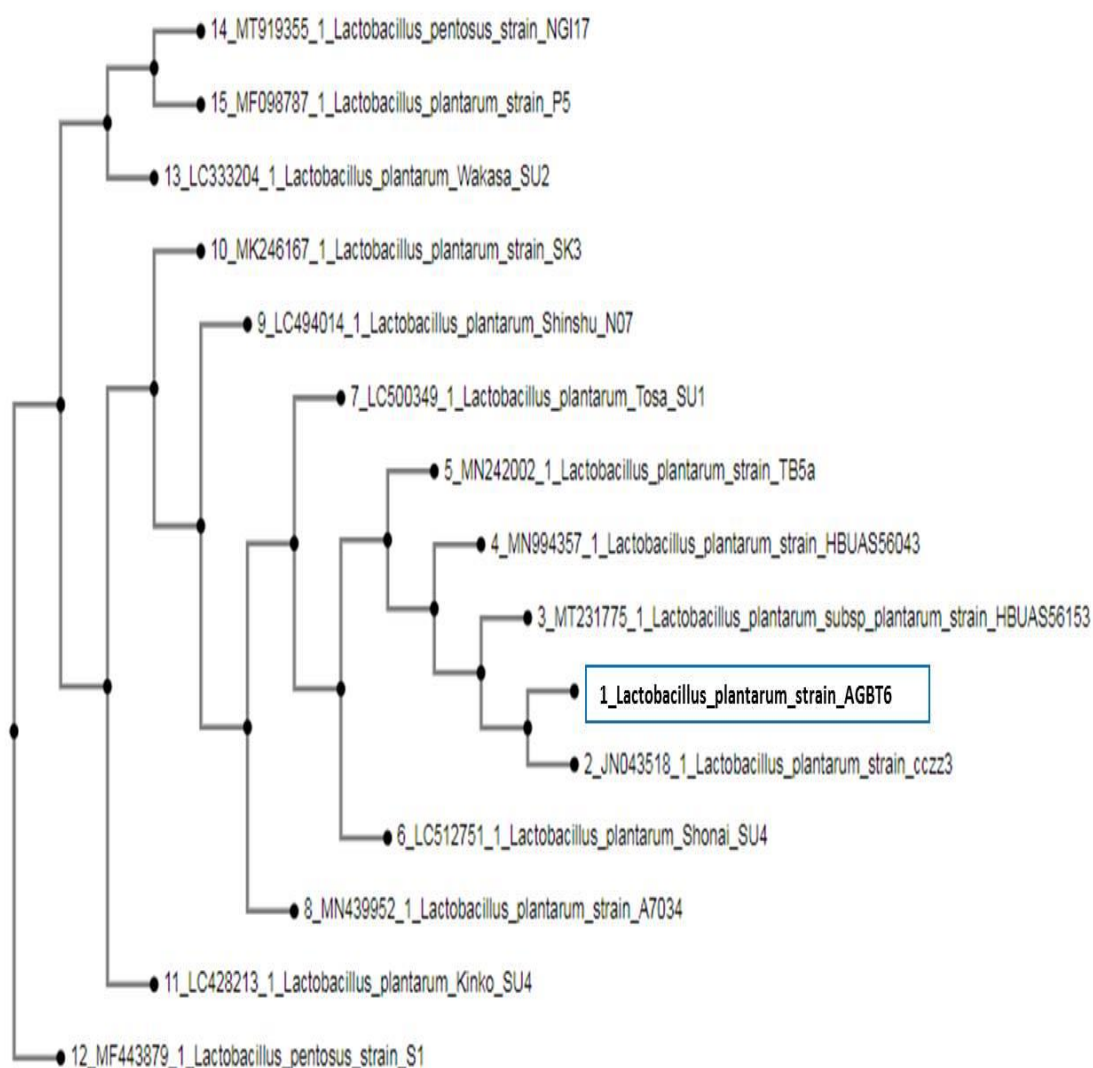


Fig: 5.30 Evolutionary relationships of taxa

The evolutionary records were once inferred the usage of the Neighbor-Joining method. The gold standard tree with the sum of department size = 0.21980934 is shown. The proportion of replicate timber in which the related taxa clustered collectively in the bootstrap check (1000 replicates) are proven subsequent to the branches. The evolutionary distances have been computed the use of the Maximum Composite Likelihood approach and are in the devices of the variety of base

substitutions per site. Phylogenetic tree generated from the 16S rRNA gene sequence records the use of neighbor-joining tree constructing algorithm. The colored identify suggests the sequence generated from the current study. The evaluation worried 15 nucleotide sequences. Codon positions covered have been 1st+2nd+3rd+Noncoding. All positions containing gaps and lacking statistics had been eliminated. There was once a complete of 956 positions in the closing dataset. Evolutionary analyses have been performed in MEGA7.

5.11 *IN-VITRO* WOUND HEALING ASSAY:

Scratch wound closure was analyzed under the inverted microscope equipped with a digital SLR camera, by acquiring digital images at different time 0th day (T0), 24hr (T1), 48hr (T2) (static imaging). In the static imaging modality.

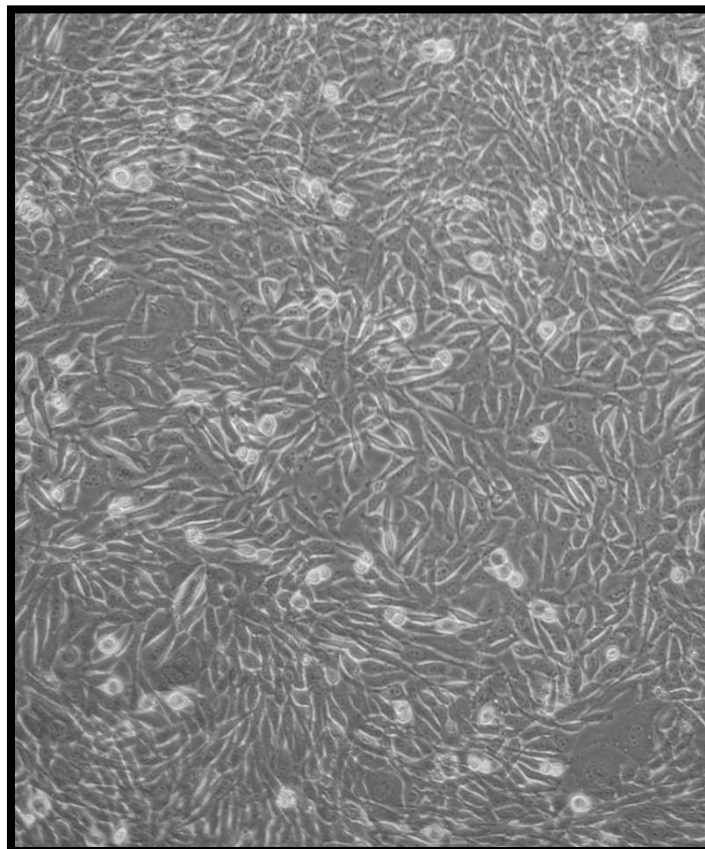


Fig 5.31 Normal cells

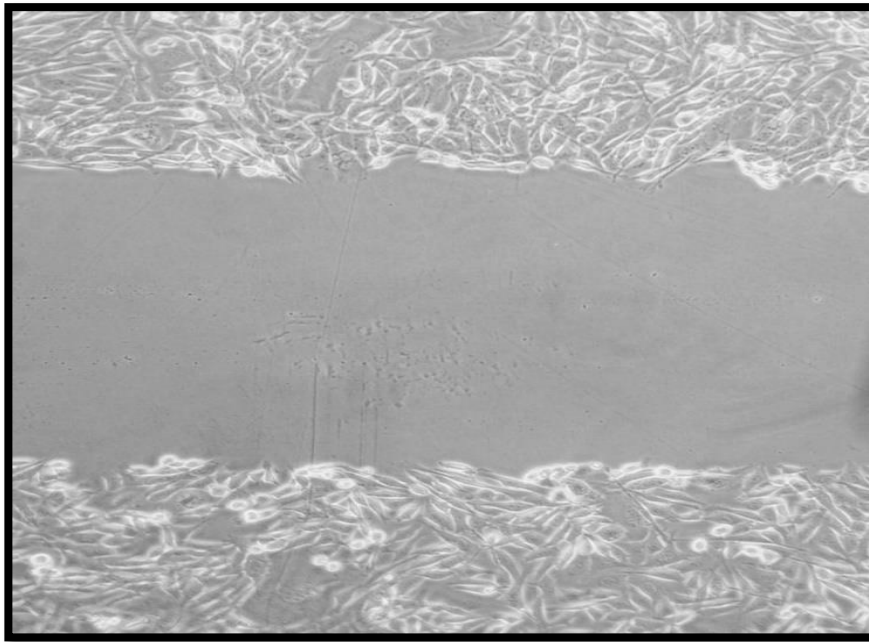


Fig 5.32 Wounded cells

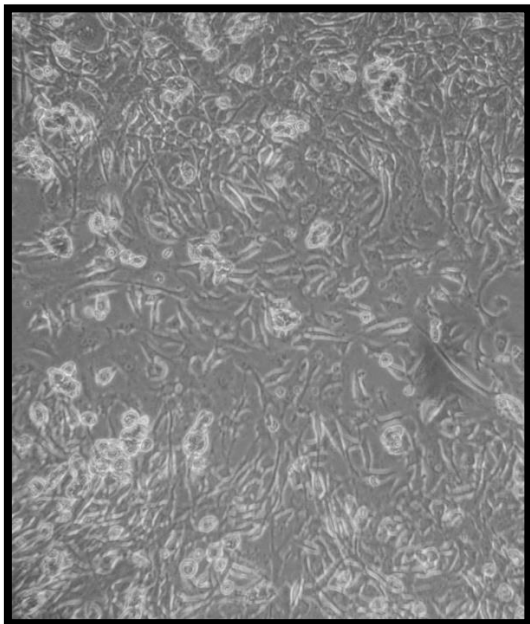


Fig 5.33 at 24 hrs 45.89%

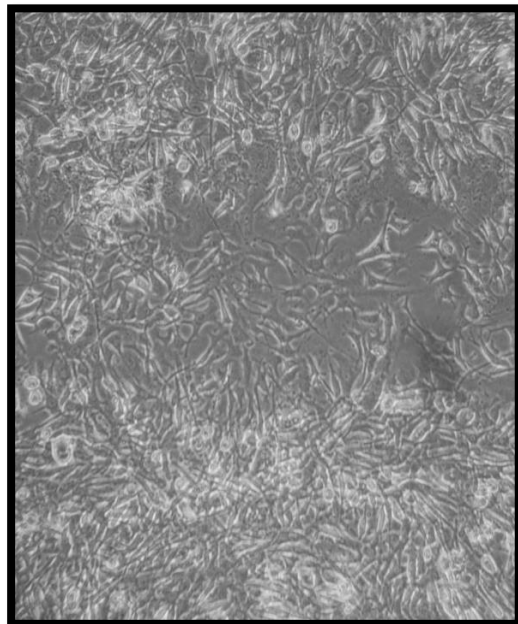


Fig 5.34 at 48 hrs 78.41%

RESULTS:

the closure of the scratch was quantified, by measuring the difference between the wound width at T0 and T1/T2, using the ImageJ processing software.

CHAPTER 6

SUMMARY AND CONCLUSIONS

6.1 SUMMARY:

The work is based on the analysis of *Lactobacillus plantarum*, importance and applications in *In-vitro* wound healing process. Having the proper intestine microorganism is even linked to several fitness benefits, which include weight loss, multiplied digestion, stronger immune function, more healthy pores and skin and a decreased hazard of many diseases, and easy healing of wounds inside and outside of the body. *Lactobacillus plantarum* is Gram positive, bacilli formed bacterium. *L. plantarum* cells are said to be rod shaped with rounded ends, straight and usually 0.9–1.2 μm extensive and is 3–8 μm long, going on singly and in pairs or in short chains. It has been extensively and widely used in the livestock and poultry industries as important antibiotic alternatives and also for wound healing techniques. *Lactobacillus* spp. produce antimicrobial metabolites that can be used as a substitute to the use of synthetic chemicals or as a supplement to the use of bio-edible, and also wound healing properties. Many studies have shown to support this theory.

The organism was isolated by culturing milk and ghee in a MRS agar media, particularly used for the growth of the *Lactobacillus* bacteria and it was inoculated into 4 fresh petri plates containing the culture medium. Identification of the bacteria was done by screening tests like gram staining. Using streak plate method inoculated m1 and m2 strains also known as milk strains were streaked on a plate and g1 and g2 ghee strains were streaked on the plate coherently 50 μL of the isolated bacteria is spread in each petri plates for milk and ghee using the L rod at -3, -4 dilution factor. Then the petri plates are incubated at 37 degree Celsius in the incubator for 24 hours. Then the plates were observed and the colonies are selected. The isolation of pure culture was done by in a petri plate, 2g of milk and ghee isolated and selected colony is taken streaked on four divided parts of the medium using an inoculation loop. MRS agar medium is used. The bacteria were tested for optimal pH and it was found to have maximum growth at 8 ph. It was also

tested for ideal temperature for growth and it was observed to have significant growth at 37°C. Antibacterial tests were conducted with four wound pathogens *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* but the bacteria seem to be susceptible to all the 4 pathogens other than the m1 probiotic strain only m1 strain showed zone of inhibition at the maximum of 20, hence the m1 strain was only used for the wound healing assay and other tests. Antioxidant assay was conducted with dpph assay, DPPH is a molecule containing an atmot stable free radical. In the presence of an antioxidant, which is to donate an electron to the DPPH molecule, the purple color which is typical for free DPPH radical decays, after 30 min of incubating it at 25°C, the decrease in absorbance is measured at 517 nm, hence the Percentage of inhibition is 55.4 % at 250µl. Minimum inhibitory concentration was conducted with the m1 strain, adjusting its ph to 8 and its temperature at 32°C to determine the lowest concentration of an antimicrobial agent that inhibits the visible in-vitro growth of microorganisms, Thus MIC for *S. aureus*, *E.coli* was found to be 50µg and *B.subtilis* and *P.aeruginosa* was found to be 75 µg showing its zone of inhibition with the m1 strain were the other two remained susceptible against the m1 strain showing no zone of inhibition. Cytotoxicity assay using normal cell lines and cell viability was also demonstrated to determine optimal growth conditions of cell populations maintained in culture.

The isolated sample bacteria were then sequenced using the 16srRNA method and it was found to be a *Lactobacillus plantarum* AGBT6. In-vitro wound healing was also done using 3T3 cells, they were seeded in 6-well plates (8×10^5 cells/well) and grown until reached a confluence of 85-90%, in the optimum culture conditions. In the middle of cell monolayer, a scratch was made by a P10 pipette tip, to mimic a wound, and cell debris were removed by washing with fresh medium. The wound was exposed for 30 µg/mL of samples for 24-72 hrs at 37°C in a humidified atmosphere of 5% CO₂. The cell grown in extract-free medium were used as control. Scratch wound closure was analyzed under the inverted microscope. Test sample bacteria (*Lactobacillus plantarum*) were observed to have more significant growth than control cells. Taken altogether the present study illustrates the potential of *Lactobacillus plantarum* AGBT6 aiding wound healing and suppressing infections at wound sites and promoting host innate immunity.

6.2 CONCLUSION:

In conclusion, *Lactobacillus plantarum* AGBT6 is non-pathogenic and is now widely used as a probiotic bacterium because of its effect on helping the gut flora to stay healthy although it should be used after several safety assessments since some strains are known for causing harmful side effects. Its known to produce a lot of enzymes and secrete antimicrobial substances which makes it an ideal choice for medical field especially pharmaceuticals and also against defining illness such as AIDS. Probiotics are nonpathogenic bacterial traces that exert really helpful outcomes on the host and topical use of some traces of probiotic microorganism have true consequences on the restoration of cutaneous wounds. Hence from this study lactobacillus plantarum was seen to determine the closure of the scratch wound Thus, the employment of these bacterial strains can be considered as new therapeutic strategies for the treatment of wounds. This was quantified, by measuring the difference between the wound width at T0 and T1/T2, using the ImageJ processing software.

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