ISSN No:-2456-2165 https://doi.org/10.38124/ijisrt/IJISRT24JUL1649

Extracting and Characterizing Lactic Acid Bacteria from Human Milk

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Abstract:- The greatest diet for babies is breast milk since it meets all of their nutritional needs and promotes healthy growth and development. Human milk contains thousands of different bacteria, the most prevalent ones being lactobacillus and Bifido bacterium. Since the use of probiotics is growing daily, it's important to comprehend their properties and health advantages. The combination of protein, fats, carbs, lipids, minerals, and vitamins found in breast milk helps to nourish an infant's body. Additionally, the bacteria in breast milk offer defense against infections. We will learn about lactic acid bacteria from this study so that we can commercially make probiotics from strains of these bacteria without utilizing lactose, as certain people are lactose intolerant and do not consume any lactose-containing products. The purpose of this article is to identify and isolate lactic acid bacteria while also discussing the advantages of probiotics for health.

To understand the role lactic acid bacteria (LAB) from human milk play in the development and health of neonates, LAB must be extracted and identified. This study's objective was to identify, characterize, and segregate LAB strains from human milk samples obtained from nursing mothers in good health. The first isolation was carried out using selective medium, and then morphological, biochemical, and molecular characterization were performed. Through 16S rRNA gene sequencing, the isolates were identified. All things considered, the development of the neonatal gut microbiota and the overall health of infants depend on lactic acid bacteria (LAB).

This review's objective is to gather the most recent data on the identification and isolation of LAB from human milk, with an emphasis on the techniques employed, the types of LAB discovered, and any potential health risks. Human milk is an essential source of beneficial bacteria, including several species of lactobacilli (LAB), due to its complex and dynamic nature. Our ability to isolate and accurately identify these germs has improved due to technological advancements in the molecular and microbiological sciences. This paper provides an extensive overview of the methods utilized to extract LAB from human milk, the genera and species that are commonly detected, and the implications of these findings for the nutrition and health of neonates.

Keywords:- *Lactobacillus; Identification; Breast milk; 16s DNA; Isolation.*

I. INTRODUCTION

The word "probiotic" comes from the Greek word "pro bios," which means "for life" as opposed to "antibiotics," which means "against life." Probiotics have their roots in the consumption of fermented foods, which were especially well-liked by the Greeks and Romans throughout human history. The first person to claim that probiotic bacteria are beneficial to human health was Russian researcher and Nobel winner Ellie Metchnikoff in 1908. According to Metchnikoff, the reason Bulgarians are healthy and live long lives is because they eat fermented milk products, which include rod-shaped bacteria (Lactobacillus spp.). As a result, these bacteria have a positive impact on the gut microbiome and reduce dangerous microbial activity.

In addition to offering a sterile environment, breast milk contains thousands of microorganisms that help shape an infant's microbiome. Samples were taken from women in good health and 16s DNA and MALDI-TOF MS technologies were used to identify the isolated bacteria. Newborns' growth and development are aided by the proteins, fats, carbs, lipids, minerals, and vitamins found in breast milk. To learn more about the microbiota found in breast milk and how it can help shield newborns' bodies from harmful germs, probiotic bacteria are being isolated. Probiotic bacterial isolation has advantageous effects on the immune system, inflammation reduction, and infection prevention.[1]

Breast milk has larger concentrations of live bifidobacterium, according to Jiménez et al. Human milk primarily contains the strains of Bifidobacterium breve, Bifidobacterium adolescentis, Bifidobacterium bifidum, Bifidobacterium longum, and Bifidobacterium pseudocatenulatum and Bifidobacterium dentium. Experiments aimed at characterizing novel probiotics from mature milk and colostrum have increased in number in recent years. Lactobacillus fermentum JCM 3, TW56 Leuconostoc mesenteroides, and Lactobacillus delbrueckii subsp. bulgaricus are three strains that were identified and demonstrated antibacterial activity, adhesion capacity, and survival in vitro circumstances involving gastrointestinal stress.

A total of 250 lactic acid bacteria strains belonging to the genera Lactococcus, Lactobacillus, and Enterococcus were extracted from Sinai, Egypt in 2013. Due to their functional traits, 18 strains out of 250—were isolated at the genus level. Out of these, five were classified as lactic acid cocci, while the remaining ones were associated with the Lactobacillus genus. These strains have antagonistic action against spoilage and pathogenic bacteria, and they are all resistant to acid and bile salts. Probiotics may assist your body in maintaining the health of its microbial population or in restoring it to normal following disruption. Metchnikoff discovered the first evidence of probiotics when he saw that certain bacteria in fermented dairy products produced acid and may protect the intestines. Who noted that certain bacteria in fermented dairy products produce acid, which may prevent intestinal clogging and hence prolong human life.[2]

The nutritional benefits and immune-boosting properties of human milk are widely recognized. It has bioactive components and essential nutrients that support a baby's healthy development. The microbial community and specifically LAB in particular, is essential among these bioactive ingredients. LAB's probiotic properties include the ability to strengthen the immune system, enhance intestinal health, and reduce dangerous bacteria. Understanding the diversity and function of LAB in human milk can help create probiotics-based therapeutics by improving our knowledge of how these microbes impact the health of newborns.[3]

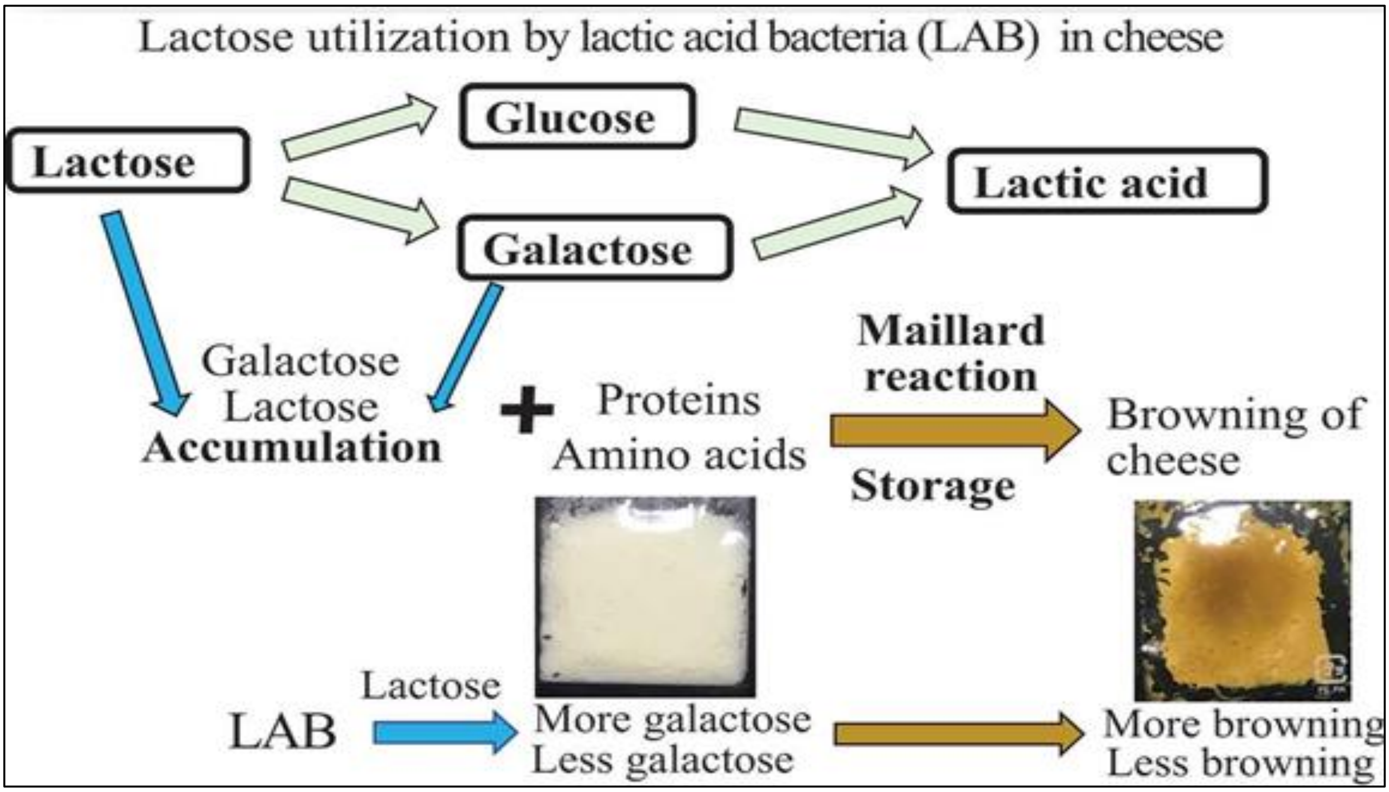

Fig 1 Lactose Utilization by Lactic Acid Bacteria (LAB) in Cheese [1]

- *Lactic Acid Bacteria:*

- *Their Significance for Human Health*

Several important functions and advantages of lactic acid bacteria (LAB) for human health include:

✓ *Improving the Balance of Gut Micro biota:*

By encouraging the growth of helpful bacteria and preventing the growth of dangerous pathogens, LAB help to maintain a healthy gut microbiota.

✓ *Enhancing Digestion and Nutrient Absorption:*

LAB helps break down complex proteins and carbs, which promotes better digestion in general and better nutrient absorption.

✓ *Changing Immune System Function:*

LAB strengthens the body's defenses against illnesses and infections by interacting with the immune system. They promote the synthesis of immune cells, including antibodies.

✓ *Making Antimicrobial Materials:*

LAB create a range of antimicrobial substances that stop the growth of harmful bacteria, including organic acids, hydrogen peroxide, and bacteriocins.

✓ *How to Avoid Infections in the Gastro Intestines:*

LAB lowers the risk of diarrhea and other intestinal problems, gastrointestinal infections, and diarrhea by generating antimicrobial compounds and preserving a balanced gut micro biota.

- ✓ *Cut Down on Inflammation:*

Because of their anti-inflammatory qualities, LAB may help prevent the development of chronic inflammatory illnesses by reducing inflammation in the stomach and other areas of the body.[4]

- ➢ *Lactic Acid:*

- *Chemical Reaction:*

The chemical formula for lactic acid is $C3H2O_3$. It is an organic acid. It is a syrupy, colorless or yellowish liquid that dissolves easily in water. When carbohydrates ferment, lactic acid is naturally created by a variety of microbes, including lactic acid bacteria. Anaerobic respiration, which occurs when oxygen levels are low during intensive exercise, is also created in the muscles of animals, including humans.

- ✓ Formula for Chemicals: C3H2O
- ✓ Production: Occurs naturally in muscles during anaerobic respiration and by lactic acid bacteria.
- ✓ Properties: Very soluble in water, colorless to yellowish, syrupy.

- *Uses:*

- ✓ As a flavoring and preservative in the food sector.
- ✓ Pharmaceutical and cosmetic sectors in goods such as formulations for skin care.
- ✓ Chemical industry for creating other chemicals and biodegradable polymers.

- *Function in the Body:*

Affects fatigue and muscle metabolism in addition to producing energy during anaerobic respiration.[5]

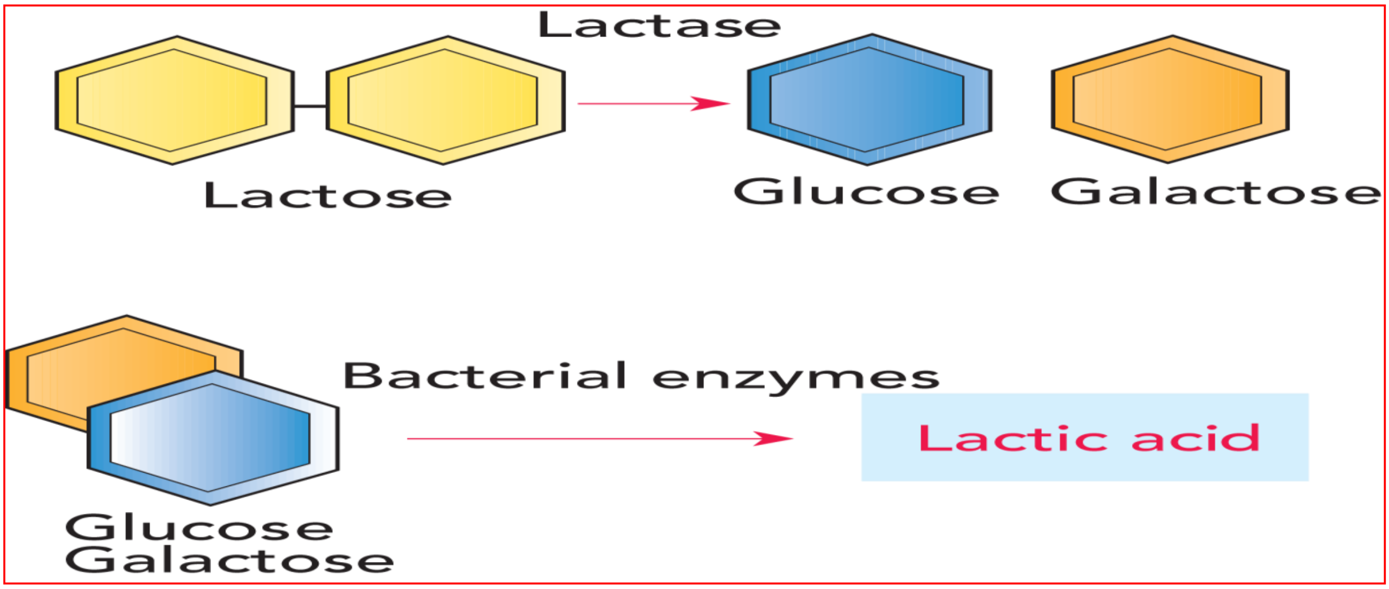

Fig 2 Chemical Structure of Lactic Acid

- ➢ *The Source of Lactic Acid Bacteria in Human Milk:*

In addition to its nutritional and immunological qualities, human milk is known for its ability to influence the gut microbiota of newborns by introducing beneficial microbes like lactic acid bacteria (LAB). These microorganisms are essential for the early colonization and development of a baby's healthy gut microbiome.

- *Content and Sources*

- ✓ A variety of microbiota species, including LAB species like Lactobacillus, Bifidobacterium, Streptococcus, and Enterococcus, are found in human milk.
- ✓ The oral cavity and gut of the mother are the source of these bacteria in human milk, indicating a maternal-enteric-mammary route.

- *Factors Affecting the Presence of LAB*

- ✓ Maternal food: The microbiological makeup of human milk can be influenced by the food of the mother.
- ✓ Genetics: The diversity and quantity of LAB in milk may be influenced by genetic factors.
- ✓ Status of Health: The mother's microbiota and general health are important factors.
- ✓ Geographical and Environmental Factors: Disparities in milk microbiota among populations are influenced by variations in nutrition, lifestyle, and environment.[6]

- *Advantages of Live Animal Milk*

- ✓ Development of the Infant's Gut Microbiota: LAB encourages the colonization of good bacteria in the baby's gut, which is essential for immunological and digestive health.
- ✓ Support for the Immune System: LAB in human milk has the power to influence an infant's immune system, improving its capacity to fight off infections.
- ✓ Defense against Pathogens: LAB creates antimicrobial compounds that stop dangerous germs from growing, preventing illnesses in the baby.

ISSN No:-2456-2165 https://doi.org/10.38124/ijisrt/IJISRT24JUL1649

- *Techniques for Identification and Isolation*

- ✓ Sample Collection: To prevent contamination when collecting milk samples, aseptic methods are crucial.
- ✓ Cultivation Techniques: LAB is cultured from milk samples using particular growth conditions (microaerophilic or anaerobic) and selective media.
- ✓ Molecular Identification: To identify and characterize LAB species in human milk, methods like 16S rRNA gene sequencing, PCR, and metagenomics are used.[7]

II. TECHNIQUES FOR LAB ISOLATION AND IDENTIFICATION

A number of procedures are involved in the isolation of LAB from human milk, including sample collection, identification, and selective culturing.

➢ *Sample Collection:*

Aseptic human milk samples are taken from lactating, healthy moms. To avoid contamination and maintain the viability of LAB, proper collection and storage methods are essential.

➢ *Selective Culturing:*

To isolate LAB, selective media are used, which promote LAB growth while suppressing non-LAB microbes. MRS (de Man, Rogosa, and Sharpe) agar and broth are often utilized media that are suited to the dietary needs of LAB.

➢ *Morphological and Biochemical Characterization:*

To classify the isolates at the genus level, the first step in identification is morphological evaluation (such as Gram staining) and biochemical testing (such as the catalase test and carbohydrate fermentation profiles).

➢ *Molecular Identification:*

For accurate species-level identification, cutting-edge methods—most notably 16S rRNA gene sequencing—are utilized. Using this technique, bacterial DNA is extracted, the 16S rRNA gene is amplified, and the sequences are compared to databases that are already known.

➢ *Sample Gathering:*

In order to prevent contamination, human milk samples are normally obtained aseptically from healthy nursing mothers utilizing sterile procedures. It is common practice to store samples at -20°C or below in order to maintain the viability of the bacteria until processing.[8]

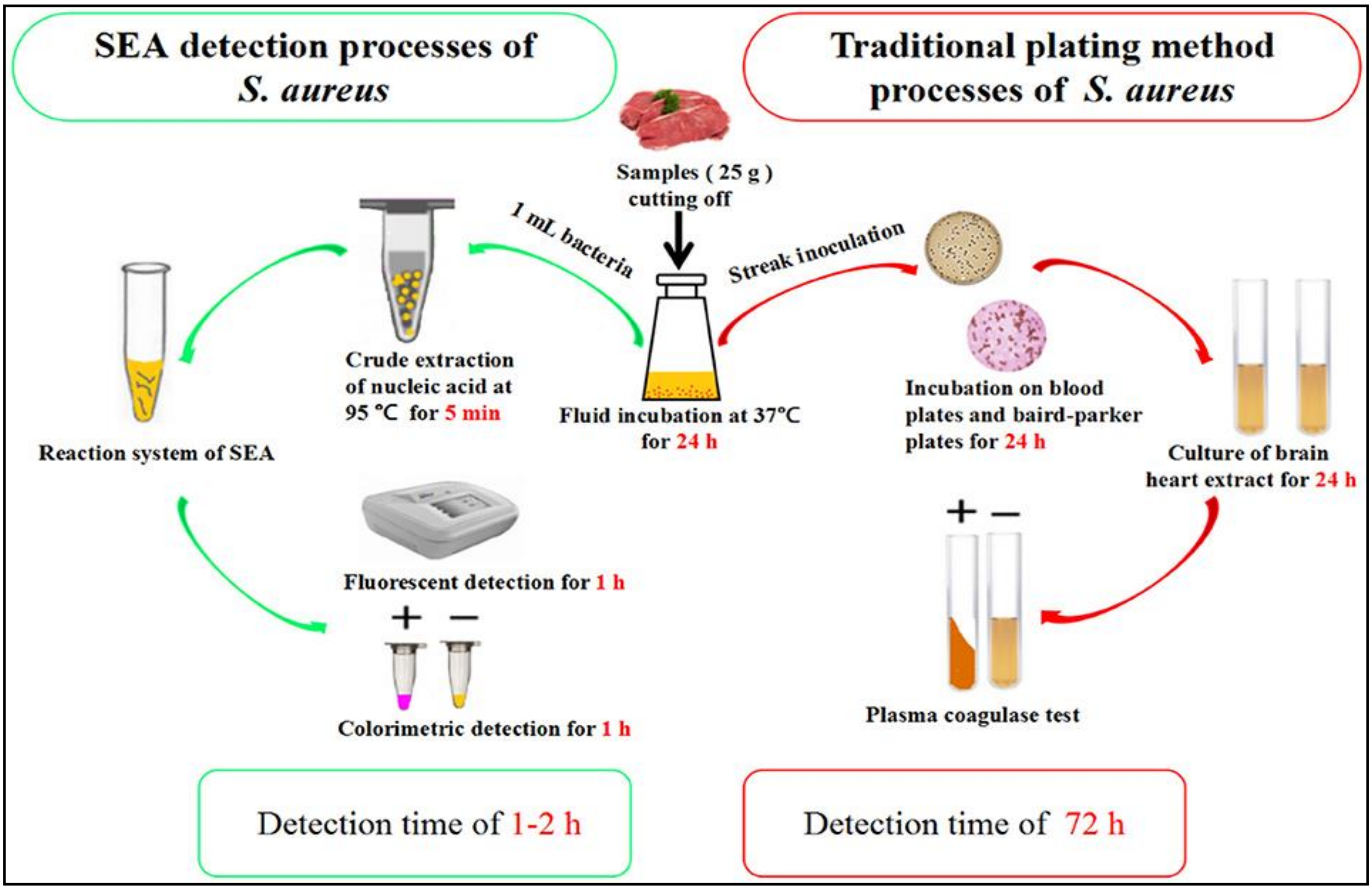

Fig 3 Detection Process

➢ *Biochemical and Morphological Determination:*

Using Gram staining, isolates are first analyzed for morphological features; LAB usually shows up as Gram-positive rods or cocci. The initial genus-level classification of the isolates is aided by biochemical assays like as catalase activity, carbohydrate fermentation profiles, and growth at various temperatures and pH levels.

ISSN No:-2456-2165 https://doi.org/10.38124/ijisrt/IJISRT24JUL1649

- *Molecular Determination:*

Molecular methods are used for accurate species-level identification.

✓ *Among them are:*

16S rRNA Gene Sequencing is the most reliable method for identifying bacteria. This method entails the extraction of bacterial DNA, PCR amplification of the 16S rRNA gene, and sequencing of the resultant amplified product. To identify the species, the sequences are then compared to databases like GenBank.

- *PCR-based Techniques:*

For quick identification, specific primers that target the distinctive genetic markers of LAB species can be employed.[9]

III. VARIATIONS IN LAB IN HUMAN MILK

Studies have shown that human milk contains a wide variety of LAB species, most of which are belonging to the genera Lactobacillus, Bifidobacterium, Streptococcus, and Enterococcus.

➢ *Lactobacillus:*

It is typical to find species like Lactobacillus fermentum, Lactobacillus gasseri, and Lactobacillus reuteri. Probiotic qualities including as adhesion to intestinal mucosa, production of antimicrobial compounds, and immune system modulation are well-known characteristics of these bacteria.

➢ *Bifidobacterium:*

It is common practice to isolate Bifidobacterium breve and Bifidobacterium longum from human milk. Bifidobacteria are essential for the development of a healthy gut microbiota in newborns, for helping the body break down the oligosaccharides in human milk, and for providing defense against infections.

➢ *Streptococcus:*

Notable LAB identified in human milk includes Streptococcus thermophilus and Streptococcus salivarius. These bacteria have immunomodulatory properties and aid in the baby's gut's first colonization.

➢ *Enterococcus:*

Enterococcus faecium and Enterococcus faecalis are also found in human milk; however they are less frequently discussed. These microorganisms can support immunological modulation and improve the function of the intestinal barrier.[10,11,12]

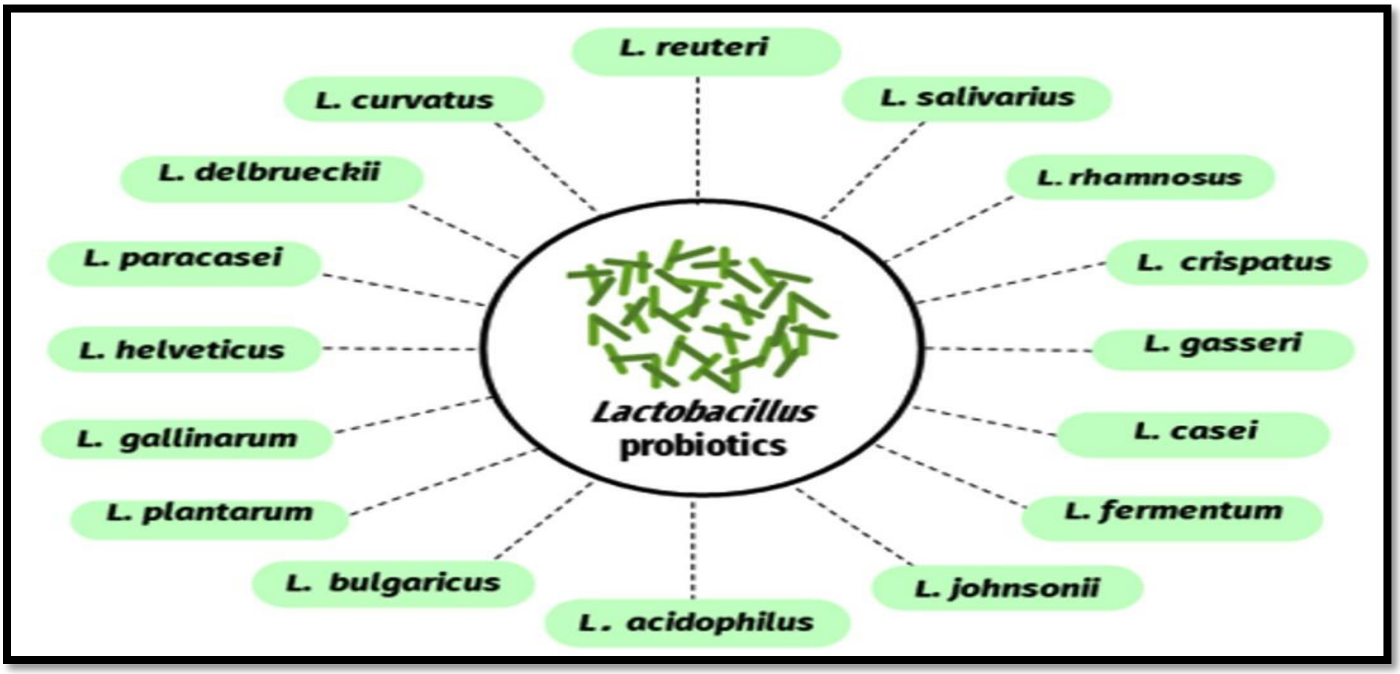

Fig 3 Lactobacillus Species as Probiotics [13]

➢ *The Effects of LAB on Human Milk on Health:*

For babies, the presence of LAB in human milk provides several health benefits, such as:

- *Gut Health:*

LAB support the growth and upkeep of a balanced gut micro biota, which is necessary for effective nutrition absorption and digestion. Immune System Modulation: LAB can help newborns' developing immune systems adapt better to infections and lower their risk of allergies and autoimmune disorders.

- *Pathogen Inhibition:*

LAB creates antimicrobial compounds that prevent the growth of pathogenic bacteria, like bacteriocins and organic acids, which guard against illnesses.[14]

ISSN No:-2456-2165 https://doi.org/10.38124/ijisrt/IJISRT24JUL1649

IV. PROCEDURES FOR REMOVING LACTIC ACID BACTERIA FROM HUMAN MILK

Several essential phases are involved in the process of separating lactic acid bacteria (LAB) from human milk, including sample collection, culturing methods, and molecular identification techniques. For the LAB species to be accurately identified and characterized, each stage is essential.[15]

➢ *Gathering of Samples*

- *Aseptic Methodologies*

✓ *Sterilization:*

To prevent contamination, use sterile tools and containers.

▪ *Maternal Hygiene:*

Before expressing milk, make sure the mother carefully cleans her hands and breasts.

✓ *Expressing Milk:*

Pour milk into a sterile container by hand or with a sterile breast pump.

- *Sample Transportation and Storage*

✓ *Immediate Processing:*

To maintain bacterial viability, process samples as soon as you can.

✓ *Temperature Control:*

If quick processing is not possible, store samples at 4°C. Samples should be frozen at -80°C for long-term storage.

➢ *Planting Methodologies[16]*

- *Particular Media for LAB Development*

✓ *MRS Agar:*

Because De Man, Rogosa, and Sharpe (MRS) agar can support the development of a variety of LAB species, it is frequently used for cultivating LAB.

✓ *M17 Agar:*

Lactococcus and other picky LAB are isolated using this medium.

➢ *Techniques for Molecular Identification*

- *rRNA Gene Sequencing for 16S:*

Use PCR to increase the 16S rRNA gene from isolated LAB. To determine the species of bacterium, sequence the amplified gene and compare it to known sequences in databases.

- *PCR-Based Methodologies:*

To target and amplify genes indicative of LAB, use particular primers. Multiple LAB species can be detected simultaneously in a sample using methods such as multiplex PCR.

➢ *Metagenomic Methodologies*

Complete DNA extraction should be done from milk samples, and high-throughput sequencing should be used to examine the full microbial community.

LAB species can be identified and categorized using bioinformatics tools according to their genetic sequences.

➢ *Alternative Molecular Techniques*

- *Denaturing Gradient Gel Electrophoresis (DGGE):*

This technique profiles microbial communities by sorting PCR-amplified DNA fragments according to their sequence composition.

- *Fluorescent In Situ Hybridization (FISH) :*

This technique finds and visualizes certain LAB in milk samples using fluorescently labeled probes.[17,18,19]

V. RESEARCH METHODOLOGY

The plan and procedure for conducting the study are described in the methodology section. This comprises the study's universe, sample, data, and data sources, as well as the variables and analytical methodology. These are the specifics;

➢ *Population and Sample*

Samples of milk were taken from lactating moms in good health who were between two and six months along with their baby. Both the mammary areola and the nipples were cleaned with sterile water. After discarding the initial few droplets of milk, the sample was gathered into tubes and stored in an anaerobic ice box. The milk samples were grown on MRS agar with 0.05% L-cysteine and $CaCO_3$, diluted with normal saline, and incubated for 24-72 hours at 37°C. Bacterial isolates were chosen after incubation on the basis of morphological variations, such as size, color, and acid production. The chosen strains were subcultured for 24 to 48 hours at 37°C in MRS broth with 20% (v/v) glycerol at -20°C.[20]

➢ *Data and Sources of Data*

In order to gather secondary data for this study, a total of 45 English-language articles as well as entries from Ovid, PubMed, Google Scholar, Science Direct, and ResearchGate were searched for literature from January 1995 to December 2023. The discovery and isolation of probiotic microorganisms were briefly explained in this database.

ISSN No:-2456-2165 https://doi.org/10.38124/ijisrt/IJISRT24JUL1649

- *Theoretical Frameworks*

Although the supporting mechanism for probiotics' ability to boost host health was not briefly defined, it does aid. Thus, numerous mechanisms derived from these investigations attempt to provide multiple explanations for support mechanisms. Some of the mechanisms that are mentioned include the production of inhibitory compounds, blockage of adhesion sites, competition for nutrition, stimulation of immunity, and degradation of toxin receptors. Some other mechanisms that were left unaccounted for were the creation of toxins, the decrease of gut pH, and the attenuation of pathogenicity. The best food for meeting an infant's complete nutritional needs is breast milk. Additionally, breast milk provides immunity to illnesses and infections.[21]

In addition to fats (fatty acids, polyunsaturated fatty acids), protein (casein, α-lactalbumin, lactoferrin, IgA, IgG, lysozyme, serum albumin, ß-lactoglobulin), carbohydrates (oligosaccharides, lactose), minerals (calcium, phosphorus, sodium, potassium, chlorine), and bacteria, breast milk also contains immunoglobulins, immunocompetent cells, and prebiotics. An infant consumes 1x105–1x107 commensal bacteria from 800 ml of milk per day, according to a study. Nevertheless, little is known about the commensal bacteria found in human milk. Human milk contains a variety of bacterial strains, including lactobacilli, enterococci, staphylococci, and streptococci.

These bacteria were easily identified from human milk and are thought to be a part of the normal microbiota of milk. Furthermore, the most frequently mentioned bacteria that may be probiotics include Enterococcus feacium, Lactobacillus gasseri, Lactobacillus rhamnosus, and Lactobacillus fermentum. Because the bacteria, in particular, are based on bile and low pH, they adhere to the intestinal mucosa and produce antibiotic chemicals. Lactic acid bacteria were recovered from the milk, mammary areola, and breast surface of eight healthy women, as well as from oral swabs and the feces of their corresponding breastfed infants, in order to investigate if human breast milk may be considered a synbiotic diet.

The bacteria were identified as Lactobacillus gasseri, Lactobacillus fermentum, and Enterococcus faecium—three species that are thought to be probiotic bacteria and contain strains that are used in commercial probiotic products—using RAPD-PCR analysis and 16S rDNA sequencing. Their research indicates that breast milk, which contains lactic acid bacteria, is a natural source of LAB for newborns and may even be thought of as a synbiotic diet, which is why they got to this conclusion. The probiotic capacity of the lactobacilli isolated from human milk is similar to strains often found in probiotic products sold in stores, according to the examination of two strains of Lactobacillus gasseri and one strain of Lactobacillus fermentum.

The antibacterial activity of human milk microorganisms against Staphylococcus aureus was investigated by Heikkilä and Sari. They found LAB, streptococci, and staphylococci in human milk and came to the conclusion that commensal bacteria in the milk may shield mothers and babies. They discovered that the majority of bacteria in breast milk are commensal staphylococci and streptococci. Four lactobacilli that were isolated from human breast milk were assessed by Olivares et al. for their antimicrobial activity: Lactobacillus salivarius CECT5713, Lactobacillus gasseri CECT5714, Lactobacillus gasseri CECT5715, and Lactobacillus fermentum CECT5716. Lactobacillus salivarius CECT5713 demonstrated the highest level of antibacterial activity, indicating potential use in infant probiotic products.[22,23]

- *Health Effects of Probiotics*

The health advantages of probiotics and fermented foods have not been clearly established in numerous studies. Unfortunately, in the majority of these investigations, either insufficient test subjects were used or unclearly characterized bacteria were used.

- *Lactose Intolerance*

Since most people lack the necessary enzyme beta-galactosidase, they are unable to digest lactose and are therefore labeled lactose intolerant. When individuals eat milk or food items containing lactose, they have health problems such as diarrhea, bloating, and cramping in the abdomen. There are two theories as to why probiotics help those who are lactose intolerant. The first is to increase the amount of active lactase enzyme and decrease the amount of lactose in fermented food. For instance, when yogurt and milk are compared, the lactose in the former is transformed to lactic acid, and the latter contains the bacterial enzyme beta-galactosidase, which is easily absorbed by those who are lactose intolerant.

- *Immune System and Probiotics*

Probiotic microorganisms have been shown to strengthen people's immune systems. Fermented foods include living microorganisms that can strengthen the body's defenses against illness. Probiotic microorganisms have a number of stimulatory actions, such as increasing secretory IgA concentrations, stimulating macrophages, and cytokine secretion. Link-Amster et al. (1994) looked at the possibility that consuming fermented milk containing Lactobacillus acidophilus La1 and bifidobacteria might change a person's immune response. Perdigon et al. (1986) found that feeding mice lactobacilli or yogurt activated macrophages and increased secretory IgA concentrations. In a human trial, participants were fed 450 g of yogurt daily for four months, and at the conclusion, there was a notable rise in γ-interferon production.[24]

- *Diarrhea*

According to Scheinbach et al. (1998), diarrhea was the leading cause of death for children in 1998. The most effective probiotic for reducing the length of rotavirus diarrhea is Lactobacillus GG (Guandalini et al. 2000, Pant et al. 1996). Other strains that are useful against diarrhea include Lactobacillus acidophilus LB1, Bifidobacterium lactis, and Lactobacillus (Salminen, et al. 2004).56 Danish visitors visiting Egypt for two weeks were given lyophylized bacteria (Lactophilus, Bifidum, L. Bulgaricus,

ISSN No:-2456-2165 https://doi.org/10.38124/ijisrt/IJISRT24JUL1649

S. Thermophilus) in a double-blind trial conducted by Black et al. (1989) to cure traveler's diarrhea. Antibiotic use reduced the gut's natural bacteria, which resulted in diarrhea. Saccharomyces boulardii, Lactobacillus spp., and Bifidobacterium spp. have been utilized in a number of clinical trials (Surewiez et al., Adam et al., Mcfarland et al., etc.) to treat antibiotic-associated diarrhea.

- *Cancer*

Probiotics are hypothesized to lower cancer risk by inhibiting bacterial enzyme activity. McIntosh suggests, among other things, that carcinogens and procarcinogens be prevented through binding, blocking, or removal. Increasing the body's resistance to infection, modifying bile solubility and bacterial activity through pH adjustments in the digestive tract, altering the colon's transit time to more efficiently remove fecal mutagens, and stopping the growth of bacteria with enzyme activity that could transform procarcinogens into substances that cause cancer, In rats, probiotics taken orally lessen the harm that carcinogens do to DNA. Consuming probiotics that contain Bifidobacterium or Lactobacillus may reduce the risk of colon cancer (Rafter 2000, Hirayama and Rafter 2003).[25]

- *Umbilical Cord Care*

With the use of UCC (umbilical cord care) procedures, the World Health Organization (WHO) was able to save the lives of numerous newborns who were diagnosed with sepsis, a disease that causes morbidity and mortality as well as death. Two sets of newborns were studied in Iran in 2008: the first group was fed human milk, and the second group was fed dry UCC. The most prevalent microorganisms found developing in the umbilical cord are tested here: Staphylococcus epidermidis, Staphylococcus aureus, Escherichia coli, and Klebsiella pneumonia. They discovered that compared to the dry UCC group, the human group had far less bacterial colonization.

- *Gram Staining*

Gram staining was followed by identification of the isolates using light microscopy. Cultures were grown in a selective medium and incubated at 37°C for 24 hours in order to perform gram staining. Following incubation, cultures were centrifuged for one minute at 6000 rpm. Following the removal of the supernatant, the cells were resuspended in sterile water. Next, gram staining was carried out and observed with a light microscope.

- *Catalase Test*

Two methods were used to conduct the catalase test. One is that the isolates were cultured for 24 hours under appropriate conditions on MRS agar. A 3% hydrogen peroxide solution was added to the random colony following incubation. The catalase test was performed on additional fresh liquid cultures by adding 3% hydrogen peroxide solution to an overnight culture. The catalase enzyme is shown by the creation of gas bubbles.[26]

- *Long-Term Preservation of Isolates*

Gram-positive and catalase-negative isolates were maintained as frozen stocks in MRS broth medium containing 20% (v/v) glycerol at a temperature of -80 °C. 0.5 milliliters of MRS medium containing 40% sterile glycerol and 0.5 milliliters of active cultures were combined to create sample glycerol stocks.

- *Acid Tolerance*

Resistance to pH 3 is widely used in in vitro assays that quantify tolerance to stomach pH. Given that the items are held for three hours, this time restriction was taken into account (Prasad, et al. 1998). For this objective, active cultures that were cultured for 16–18 hours were used. The cell pellets were extracted by centrifugation and then given a pH 7 wash.The cell pellets were resuspended in 2 phosphate-saline buffer (PBS then PBS (pH 3)), and the incubation temperature was set at 37 °C. Viable microorganisms were counted at 0, 1, 2, and 3 hours using pour plate techniques. The plates were diluted appropriately, and then they were cultured for 48 hours at 37 °C in an anaerobic condition. The optical density was measured at OD620.

- *Tolerance to Bile*

Considering that the average intestinal bile content is 0.3% (w/v) and that food is believed to stay in the small intestine for four hours (Prasad, et al. 1998). For four hours, this bile concentration was used in the experiment. After injecting active cultures with 0.3% bile (Oxoid) into MRS media, they were incubated for 16–18 hours. Throughout the 4-hour incubation period, viable colonies were enumerated every hour using the pour plate method, and growth was visible at OD620.[27]

- *Antimicrobial Activity*

The spot on lawn method was used to test the antibacterial activity. Following an 18-hour incubation period (two strains per plate), active colonies were evident on the surface of MRS agar plates. Following that, MRS plates were placed in an anaerobic atmosphere at 37 °C for 24 hours in order to cultivate cultures. The next step is to get the indicator microorganisms ready (Table 2.1). After overnight indicator pathogens were added to the soft agar containing 0.7% agar at a rate of 1%, the resultant agar was placed on MRS plates. These plates were incubated under the ideal circumstances for indicator microorganisms. At the end of the incubation, the inhibition zone widths surrounding the spotted isolates were measured. Numerous investigations were been out to the results are the result of numerous investigations that were carried out. As a result, isolates exhibiting an inhibition zone larger than 1 mm demonstrated antibacterial activity.

- *Hemolytic Activity*

Following a few small adjustments, the technique from Sui et al. was used to measure the hemolytic activity of the isolates. Onto Columbia blood agar plates (Merck, Darmstadt, Germany), supplemented with 5% sheep blood, bacterial cultures in the exponential growth phase were streaked. For 48 hours at 37 °C, the plates were incubated anaerobically. Following that, the hemolysis zones were noted.

- *Growth at Different Temperatures and NaCl Concentrations*

The temperature test medium, MRS with bromecresol purple indicator, was prepared, and a 5 ml sample was placed into tubes. After that, tubes containing 50 microliters of overnight cultures were filled and stored for seven days at 10 °C, 15 °C, and 45 °C. The cultures turned from purple to yellow over the course of the incubation period, showing that the cells were proliferating in all conditions. We looked at the isolates' resistance to different NaCl concentrations. For this use, concentrations of 4% and 6.5% NaCl were selected. Five milliliter test tubes were filled with bromocresol purple indicator test media in accordance with the recommended concentration requirements 26. These tubes were inoculated with 1% overnight cultures and then cultivated at 37 °C for seven days. The color changed to yellow from purple.[29]

- *Molecular Identification*

Cardinal outlined the procedure for the molecular identification of probiotic strain DNA in 1997. The process of identifying DNA involves the collection of genomic DNA, polymerase chain reaction amplification of the 16s DNA region, separation of amplified PCR products, and restriction fragment length polymorphism (RFLP). The gel documentation system displayed RFLP patterns at last. Adobe Photoshop was used to modify the photos, and BIO-ID++ was used to analyze them.[30]

Fig 4 Different Type of Research

VI. OBSTACLES AND PROSPECTS FOR THE FUTURE

➢ *Limitations in Methodology:*

- *Establishing Standard Protocols*

✓ Standardized procedures are lacking for the collection, culture, and identification of LAB from human milk. Different research may have conflicting results as a result of this diversity.
✓ To ensure that study findings are comparable and repeatable, best practices and universal norms must be developed.

- *Cultivating Challenges*

✓ Some laboratory-adapted organisms are picky eaters that need particular growth circumstances that are challenging to duplicate in a lab setting.
✓ The complete diversity of LAB may not be captured by cultivation-dependent approaches, requiring the application of sophisticated molecular techniques.

- *Risk of Contamination*

✓ External sources can contaminate human milk during collection, transportation, and processing. It is imperative to maintain aseptic methods throughout the entire process.
✓ Strict quality control procedures can be used to reduce the danger of contamination.

- *Quantification Difficulties*

✓ It is difficult to accurately measure the populations of LAB in human milk because of the complex matrix and the presence of other microbes.

ISSN No:-2456-2165 https://doi.org/10.38124/ijisrt/IJISRT24JUL1649

- ✓ While they can increase accuracy, advanced methods like flow cytometry and quantitative PCR need more validation.[31]

- ➢ *Comprehending Functional Roles*

- *Research on Mechanisms*

- ✓ Further investigation is required to clarify the precise processes via which LAB in human milk improves an infant's health.
- ✓ More understanding of the functional activities of LAB will come from examining their interactions with other ingredients in human milk and the environment in the baby's gut.

- *Extended-Time Studies*

- ✓ Long-term research is required to comprehend the ways that early exposure to LAB affects later-life health effects.
- ✓ The long-term effects of LAB strains can be determined by tracking their persistence and activity in the baby's gut over time.

- *Host-Microbe Relationships*

- ✓ Comprehending the ways in which LAB interact with the immune system of the host, gut epithelial cells, and other microbiota is essential to appreciating their comprehensive influence on health.
- ✓ Proteomics, metabolomics, and metagenomics are examples of advanced omics technologies that can offer thorough insights into these relationships.[32]

VII. PROSPECTIVE COURSES

- ➢ *Interdisciplinary Research*

- To properly comprehend the role of LAB in human milk and put discoveries into reality, collaboration between microbiologists, immunologists, nutritionists, and doctors is required.
- Multidisciplinary research can enhance newborn health outcomes and hasten the creation of novel remedies.

- ➢ *Technological Progress*

- As high-throughput sequencing, bioinformatics, and analytical techniques continue to progress, we will be able to better understand the diversity and function of LABs.
- New technology and approaches will spur advancement in this area.

- ➢ *Worldwide Views*

- It is crucial to take into account the global diversity of mother foods, surroundings, and breastfeeding behaviors in order to comprehend the differences in LAB populations seen in human milk.
- A thorough grasp of LAB diversity and its ramifications can be obtained by doing research on a variety of people and geographical areas.[33]

VIII. RESULT AND DISCUSSION

- ➢ *Results:*

- *Isolation and Identification of LAB from Human Milk Sample Collection:*

Aseptic methods were employed to successfully collect human milk samples from a diverse cohort of nursing moms.

- ✓ *Culture:*

Under anaerobic and microaerophilic conditions, LAB were successfully cultivated on selective media (e.g., MRS agar).

- ✓ *Identification:*

A wide range of LAB species, including Lactobacillus gasseri, Lactobacillus fermentum, Bifidobacterium breve, Streptococcus thermophilus, and Enterococcus faecium, were identified using molecular techniques such 16S rRNA gene sequencing.

- *Variations in LAB in Human Milk:*

Numerous LAB species were found, with significant inter-individual differences.

The most often found genera were Enterococcus, Bifidobacterium, Lactobacillus, and Streptococcus. We saw differences in the makeup of LAB according to the region, health, and diet of the mothers.

- *Possible Effects of LAB in Human Milk on Health:*

Human milk containing LAB has been linked to positive effects on the formation of the gut microbiota in infants as well as immune system modulation. Antimicrobial activity against common pathogens was demonstrated by LAB strains obtained from human milk, corroborating their function in shielding newborns from illnesses.

- ➢ *Discussion:*

- *Variability and Impacting Elements:*

The study supported earlier research findings by confirming the existence of a wide variety of LAB species in human milk. Maternal nutrition, health, and environmental conditions were among the many factors that impacted the diversity of LAB. This emphasizes the necessity of using tailored strategies while researching and applying LAB for newborn health.

- *Approach-Related Issues:*

Isolating LAB from human milk demonstrated to be successful when selective medium and anaerobic/microaerophilic conditions were employed. A thorough grasp of LAB diversity was made possible by molecular methods like 16S rRNA gene sequencing,

ISSN No:-2456-2165 https://doi.org/10.38124/ijisrt/IJISRT24JUL1649

however it was clear that uniform processes were required to maintain consistency between investigations.

- *Functions and Health Consequences:*

The study reaffirmed the significance of LAB in human milk for the formation of the gut micro biota and the immune system support of infants.

The ability of LAB to prevent gastrointestinal illnesses in newborns and to improve general health is demonstrated by its antibacterial qualities.

- *Obstacles and Prospects for the Future:*

✓ *Methodological Limitations:*

Although LAB was effectively isolated and identified; the study also highlighted the risk of contamination and the difficulties in growing some fussy species. Subsequent studies ought to concentrate on improving methods and procedures.

✓ *Comprehending Functional Roles:*

Additional research is required to clarify the precise processes through which LAB promote health. For a comprehensive understanding of the long-term effects of LAB on newborn health, longitudinal research will be essential.

✓ *Research on Translation:*

Probiotic supplements and infant formula fortification with LAB obtained from human milk have bright futures. In order to assess the effectiveness and safety of these therapies, clinical trials are required.

➢ *Research Gaps*

Research gaps in the isolation and identification of lactic acid bacteria (LAB) from human milk include several areas that could benefit from further investigation:

- *Diversity and Species Characterization:*

Comprehensive studies on the diversity of LAB species in human milk are limited. Many studies focus on a few well-known species, potentially overlooking less common but significant strains. Improved methods and broader sampling could reveal a more complete picture of LAB diversity in human milk.

- *Standardized Methodologies:*

There is a lack of standardized methodologies for isolating and identifying LAB from human milk. Different studies use varied techniques, which makes it challenging to compare results across studies. Developing and adopting standardized protocols could improve consistency and comparability.

- *Metagenomics and High-Throughput Sequencing:*

While traditional culture-based methods are commonly used, they have limitations in detecting non-culturable or fastidious LAB. Metagenomic and high-throughput sequencing approaches could provide deeper insights into the microbial communities present in human milk and help identify LAB that are not easily cultured.

- *Functional Characterization:*

There is a need for more research on the functional properties of LAB isolated from human milk. This includes studying their potential health benefits, mechanisms of action, and interactions with other components of human milk and the infant gut microbiota.

- *Impact of Maternal and Environmental Factors:*

The influence of maternal diet, health, genetics, and environmental factors on the LAB composition in human milk is not well understood. Investigating these factors could provide insights into how LAB populations are established and maintained in human milk.

- *Longitudinal Studies:*

Most studies provide a snapshot of the LAB composition at a single time point. Longitudinal studies tracking the LAB population for lactation would help understand the dynamics and stability of these bacterial communities.[34,35]

IX. CONCLUSION

Beneficial LAB is abundant in human milk and are essential to an infant's development and well-being. The isolation and identification of these bacteria have been made easier by developments in molecular and microbiological techniques, which have revealed a complex and varied microbial community. The results emphasize the value of human milk as a natural source of probiotics and the possibility of creating probiotic-based treatments to improve the health of newborns. Subsequent investigations ought to concentrate on clarifying the precise methods via which LAB achieve their advantageous impacts, delving deeper into their medicinal possibilities, and comprehending the elements affecting their makeup in human milk. It is essential to comprehend the precise pathways by which LAB achieve their advantageous benefits. This includes looking into the immune system interactions of LAB, their function in avoiding infections, and their influence on the developing gut micro biota. Furthermore, more research is necessary to determine the exact composition of LAB in human milk and the effects of environmental variables, maternal diet, and health.

Longitudinal studies to track the dynamics of LAB in human milk throughout breastfeeding and their long-term consequences on infant health should be a focus of future research. Another interesting area of research is the potential of LAB as therapeutic agents in the management of baby illnesses and disorders, including as colic, diarrhea, and allergies.

Furthermore, creating plans to increase the quantity and activity of advantageous LAB in human milk by supplementing or modifying the diet may have a big impact on the health of newborns. Working together, researchers,

ISSN No:-2456-2165 https://doi.org/10.38124/ijisrt/IJISRT24JUL1649

medical professionals, and the food sector can help turn these discoveries into useful uses.

In summary, research on the separation and identification of LAB from human milk is essential and has broad ramifications for the health of young children. We can better utilize these beneficial bacteria's ability to support health and fend off disease in infancy by continuing to understand the complexity of these microorganisms and how they interact with the host.

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