Study the Characteristics of Lactic Acid Bacteria Isolated from Human Milk as a Probiotic *in Vitro*

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ABSTRACT

Probiotics are live microorganism preparations that benefit their host's health. Although probiotic strains can be isolated from a variety of sources, human origin is the primary requirement for human applications. Milk and other dairy products are generally regarded as primary food sources for LAB.

After being taken from human breast milk and put through a biochemical test at Al-Zahra Teaching Hospital in Wasit Province, Iraq, a total of 50 samples produced favorable findings. The tolerance of LAB isolates in simulated gastric juice and antimicrobial activity against pathogenic bacteria (*Escherichia coli* and *Staphylococcus aureus*) were studied by using the well diffusion method. 50 positive samples for LAB isolates were identified by PCR (16S rRNA sequencing) and were catalase-negative. LAB isolates have shown tolerance for simulated gastric juice. The average viable cell count of LAB varied significantly (P≤ 0.01) among the fifty samples, falling from 4.949 ± 0.043 log cycles CFU/mL at zero time (before the incubation time) at pH 2.0 to 4.055 ± 0.087 log cycles CFU/mL after the incubation period (after 3 hours) at 37°C and pH 2.0. Additionally, the LAB isolates demonstrated a significant difference between the inhibition zone of *S. aureus* Gram positive and the inhibition zone of *E. coli* Gram negative (P≤ 0.01). LAB isolates under study show support for probiotic properties, and the value of breastfeeding over bottle feeding is demonstrated.

Keywords: lactic acid bacteria, low pH, probiotic, antibacterial,

Introduction

The World Health Organization (WHO) states that breastfeeding is the best approach for protecting a newborn baby's health in its "Global Strategy for Infant and Young Child Feeding". Thanks to mother's milk, its abiotic composition is balanced. In order to balance all the nutrients necessary for the growth of protective compounds . (Geneva et al., 2009) .Mother's milk bacteria must fulfill some basic requirements often recommended for human probiotics, such as human origin, adaptability to milk substrates, and the safe and continuous consumption of newborns (Martn et al., 2003). This makes breast milk a good source of probiotics. Several breast milk strains have been used as probiotics (D'Alessandro et al.,2022). Interestingly, these bacteria are found in sufficient quantities in breast milk at concentrations that allow colonization in the intestine of the newborn after consumption but do not stimulate an inflammatory reaction in the mammary glands. Therefore, these bacteria positively affect the intestinal microflora and reduce the toxic activity of microbes (Akir, I.C., 2003). *Lactobacilli* and group Streptococci may originate from the mother's vaginal microbiota,

while *S. aureus* strains colonizing the baby's stomach are largely derived from the skin flora of the parents. Large amounts of bacteria are found in breast milk, and similar strains of Lactobacilli, *enterococci*, and *S. aureus* have been found in both breast milk and the baby's stomach (Adlerberth 2008). During the first two years of life after birth, the structure of intestinal microorganisms undergoes important changes. When an infant is 2 years old, the human intestinal tract (GIT) transitions from an originally sterilized adult microbiome to a stable one (Wold, A. E. 2009). The benefits of breastfeeding for mothers and babies are widely recognized. Breast milk, a natural source of lactoferrin, an antimicrobial protein, and lysozyme (a natural enzyme found in milk, tears, and sweat that can convert lactose into glucose), can significantly influence the microbial composition of the gut. In addition, it provides the right nutrients for the developing baby. It has also been proposed that breast milk includes germs such as Staphylococci,*Streptococc*i, *Lactobacilli, Micrococci*, and *Bifidobacteria* and can thus be directly sourced. Ingesting milk has been shown to change the microbial makeup of the baby's stomach. In the intestines of infants, *Lactobacilli* and Bifidobacteria settle (Sinkiewicz et al. 2008).

The benefits of breastfeeding for mothers and babies are widely recognized. Breast milk, which is a natural source of lactoferrin, an antimicrobial protein, and lysozyme (a natural enzyme found in milk, tears, and sweat that can convert lactose into glucose), can also significantly affect the microbial composition of the gut. provide the right nutrients to developing babies, suggesting that breast-fed babies are more resistant to gastrointestinal infections than bottle-fed babies (Lorens-Hattingh and Viljoen, 2001b).

The most common strains belong to the genera *Bifidobacterium, Lactobacillus*, and *Streptococcus.* It has been used as a probiotic and contains *enterobacteria* (Soccol, C.R., et al., 2010). To compete with other bacteria in the environment, lactic acid bacteria (LAB), which are probiotic microorganisms, produce antibacterial peptides and small proteins known as bacteriocins. Ahrné et al. (1998) and Gill et al. (2001) found that lactic acid bacteria such as Lactobacillus species protect against many pathogenic infections in the gastrointestinal tract of humans and animals. The use of this bacterium can also be useful in the treatment and prevention of infectious diseases caused by several oral, enteric, and urogenital pathogens (Shornikova et al., 1997). The prevention of infectious diseases in the host depends on the production of antimicrobial agents such as H2O2 and lactic acid (Martn et al., 2005). Therefore, probiotics are used to treat acute diarrhea, antibiotic diarrhea, and traveler's diarrhea and have shown beneficial therapeutic effects (Akir I 2003). The low-molecular-weight bacteriocins of the gram-positive bacteria demonstrate bactericidal activity which is directed principally against certain other gram-positive bacteria . For example, the prototype lantibiotic nisin has been shown to be effective against many strains of - Gram positive bacteria, including *Staphylococci, Streptococci, Bacilli, Clostridia*, and *Mycobacteria* . However, the degree of sensitivity of these genera varies, mycobacteria being approximately 100 times less sensitive than the others. (Jack et al., 1995)

LAB, generally considered food organisms, are particularly promising for selection and use as protected crops. Since most representatives of this group participate in many foods known to people for millennia, they do not pose a threat to human health, and some of them are called "GRAS" organisms ("generally recognized as safe").

Intestinal retention is one of the most important selection factors for new probiotic strains. Their ability to survive in life can affect their tolerance to gastrointestinal (GIT) diseases (Riaz Rajoka et al., 2017).

Production of antibacterial compounds and competitive inhibition of intestinal epithelial pathogens and toxins have been identified as two mechanisms of probiotic action to maintain gastrointestinal microbial balance (Vanderpool et al., 2008). According to in vitro experimental studies, selected lactic acid strains are effective against bacteria causing diarrhea. Many strains of Lactobacillus inhibit

the growth of bacterial pathogens by producing metabolites such as acetic and lactic acid that lower pH. LAB is not a unified group.

They are not very high in G-C (Amann et al., 1995). They are also Gram-positive, facultatively anaerobic, non-spore-forming, rod-shaped (bacillus) or spherical (coccus) bacteria (Garvie 1984). Probiotic microorganisms should not only be capable of surviving passage through the digestive tract but also have the capability to proliferate in the gut. This means they must be resistant to gastric juices and be able to grow in the presence of bile under conditions in the intestines, or be consumed in a food vehicle that allows them to survive passage through the stomach and exposure to bile (FAO/WHO 2006; Pineiro and Stanton 2007).

Due to the fact that the properties of LAB isolated from breast milk the have limited this study has been prepared. It is important to mention that for a microorganism to be considered probiotic, it must survive passage through the stomach and maintain its viability and metabolic activity in the intestine. The currently available tests are not adequate to predict the functionality of probiotic microorganisms in the intestine.

The objective of this investigation is to study the antibacterial efficacy of LAB-separated breast milk against harmful bacteria (*E. coli* and *S. aureus*) and ability to the surviv of LAB in simulated gastric juice (pH 2.0).

Material and Methods

Martn, R. et al. (2009) developed a modified approach for isolating LAB from breast milk that was effective. The milk samples were triple-plated onto Man-Rogosa-Sharpe plates agar after being diluted in peptone water (MRS; Liofilchem, Italy) medium supplemented by L-cysteine (0.5%), and then incubated anaerobically by using a gas anaerobic jar (Oxoid, Basingstoke, United Kingdom) in an anaerobic workstation (Becton, Dickinson and Company, USA) at 37°C for 48-72 h. From each sample, five to seven typical colonies were chosen, grown in MRS-Cys broth for 48 hours, steeped on MRS agar for a further 48 hours, incubated anaerobically at 37°C, and then maintained at -80°C with glycerol (20% vol/vol).

Catalase test

Under a microscope, the morphology of Gram stain finishes was investigated. Hydrogen peroxide (H_2O_2), a harmful metabolic result of aerobic and facultatively anaerobic bacteria, is broken down by the enzyme catalase into non-toxic compounds such as water (H_2O) and oxygen (O_2). It is a chemical that is very reactive and damages cell systems. Therefore, bacteria that are oxygen-dependent generate the enzyme catalase, which breaks down H_2O_2 into H_2O and O_2.

$2\ H_2O_2$ ————catalase—–>$2H_2O + O_2$

To learn more about the isolates' catalase reactions, catalase assays were carried out. On MRS agar, colonies were isolated overnight under the proper circumstances. After being exposed to a 3% hydrogen peroxide solution for 24 hours, a randomly chosen colony revealed the presence of bubbles when compared to a catalase test result that was positive (Liu et al., 2020).

Molecular Identification:

Molecular Analysis

By sequencing 520-bp, (209–619)-bp, and 194-bbp and 194-bbp segments of the 16S rRNA gene with three primers and PCR (Table 1). Totally of the lactic acid bacteria separates with distinctive gram-positive and catalase-negative morphologies were documented as LAB.

Table (1) Primer castoff in this study:

gene	Primer sequences (5˚-3˚)		Product size (bp)	Reference
Bifidobacterium Spp.	F	GGGTGGTAATGCCGGATG	**520**	**Kok et al. (1996)**
	R	CCACCGTTACACCGGGAA		

Lactobacillus spp.	F	CTCAAAACTAAACAAAGTTTC	**209 - 619**	https://www.ncbi.nlm.nih.gov/tools/primer
	R	CTTGTACACACCGCCCGTCA		
Streptococcus spp.		TGAGTGCAGAAGGGGAGAGT	**194**	https://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=nucleotide&id=-1856639968
	R	CGGAAAGGATCCAACACCTA		

Preparation of Pathogenic Bacteria

E. coli and *S. aureus* are two of the harmful bacteria that make up the indicator organisms (pathogenic bacteria), which were received from Wasit University's Microbiology Laboratory in the Biology Department. The stock culture collection was kept in 20% glycerol at 4 °C. They were three times subcultured before being used in a suitable medium.

Resistance to Low pH

A slightly modified version of the method described by Isa Jawad (2017) was used to assess the transit tolerance of the simulated gastric juice. Filter-sterilized pepsin (SIGMA-AIDRICH, Germany) at 0.3% w/v and Nacl at 0.5% w/v, with pH adjustments to 2.0, made up the simulated gastric juice. Lactic acid bacteria isolates grown overnight in MRS broth were centrifuged (6,000 g, 20 min) to remove the medium. The pellets were then twice washed in a 0.85% sterile saline solution (pH 7.0). Re-suspended in 3 ml of the same buffer after that. A pH2.0 gastric solutions were used to dissolve one liter of the washed cell suspension. MRS agar was used for the total viable counts of LAB before and after 3 h of incubation at 37 °C.

Antimicrobial Activity

With a few minor tweaks, Balouiri et al.'s (2016) methodology was used to determine antimicrobial activity by looking at the antimicrobials that LAB isolates produced. A 16-hour MRS broth growth of LAB strains yielded cell-free supernatant (CFS). The cell-free supernatant was utilized after being filtered through filters (0.2 m-size cellulose acetate filter) after the cell suspension had been centrifuged at 5000 rounds per minute (rpm) for 30 minutes. The antibacterial activity of microbial extracts is evaluated using the agar-well diffusion technique. The surface of an extract nutrient agar plate is infected by evenly distributing a suspension of pathogenic bacteria (*E. coli* and *S. aureus*) made in 5 mL of normal saline solution, with the turbidity controlled to match that of a 0.5 McFarland standard inoculum. The LAB CFS is then added to wells that have been aseptically punctured with a sterile tip and have a diameter of 7 mm. Agar plates are then incubated for 24 hours at 37 °C under the proper conditions. The width of the zone of inhibition surrounding the wells was measured to evaluate the antimicrobial activity (Chopra and Mehra, 2015).

Statistical analysis

Using the Chi-Squire test and the SPSS IBM Version O20 programs, each of the findings was statistically analyzed. According to Grewal et al. (2017), a P-value ≤ 0.01 was regarded as statistically significant.

Results and Discussion

At the Biology Department in College of Science, University of Wasit in Iraq, a total of 50 samples were tested biochemically. LAB isolates from breast milk Additionally, these were demonstrated to be catalase-negative. Positive results were obtained from all 50 samples. *Streptococcus* spp strains were identified as positive in all 50 samples that had a percentage of 100% (Fig.1); *Bifidobacterium* spp strains were detected in 26 (52%) (Fig 2); and *Lactobacillus* spp strains were detected in 45 (90%) (Fig. 3).

Figure 1. Gram staining results of strepeococcus spp. isolated from human milk

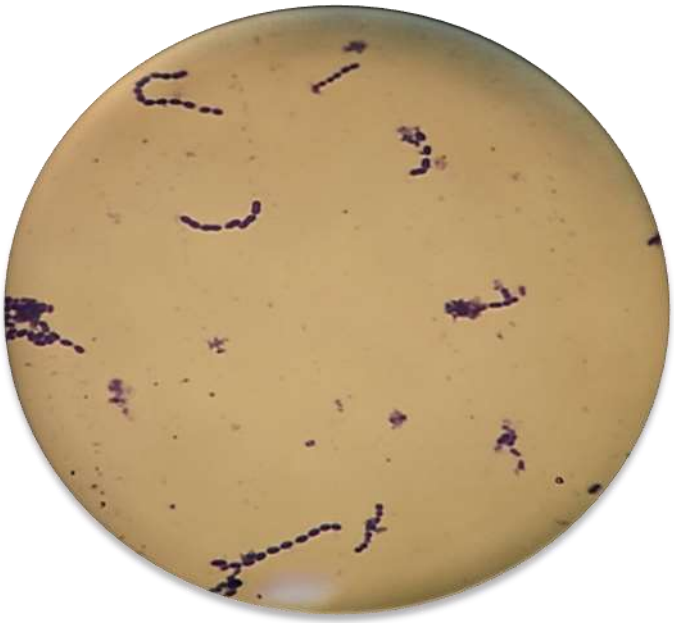

Figure 2. Gram staining results of Bifidobacterium spp. isolated from human milk

Figure 3: Gram staining results of *Lactobacillus* spp. isolated from human milk

Tolerance of lactic acid bacteria to Acidity

There are several defensive mechanisms in the human gastrointestinal tract (GIT) that protect it from invading infections. Gastritis is one of them (Amund, 2016). The production of stomach acid by the body serves as its main line of defense against the majority of ingested microorganisms. In reality, the use of proton pump inhibitors and other acid blockers, as well as gastric surgery, may encourage the colonization of the stomach by bacteria, according to Dunne et al. (2001).

A bacteria must survive in the stomach and maintain viability and metabolic activity in the intestine to qualify as a probiotic (Hyun and Shin, 1998).

Probiotics are microorganisms like *Lactobacilli* and *Bifidobacteria* that are naturally found in the gastrointestinal tract of humans and animals (Ain Arshad et al., 2018). Additionally, different species and strains of probiotic bacteria have different levels of tolerance for acidic conditions. The results of a simulation of the effect of stomach acid on lactic acid bacteria's survival are shown in Table 2. The average number of live LAB cells in 50 samples decreased from 4.949 ±0.043 log CFU ml at time zero (earlier the cultivation period) at pH 2 to 4.055 ±0.087 log CFU ml later three hours of cultivation at 37 ^{o}C pH2.0. There were significant differences ($P \leq 0.01$) between the viability counts of the LAB isolated sample for each incubation period (3 h) at 37 ^{o} C at pH 2.0 Based on our results, founded that all LAB isolates displayed the ability to grow at low (pH 2.0) after exposure for 3 hours. Significant differences ($P \leq 0.001$) were observed among the 50 LAB isolates.

Table 2. Mean standards (log CFU ml^{-1} ± SD) of tolerance of LAB. isolated from human milk to simulated stomach fluid for all sample.

Sample No.	Before incubation 0 time				After incubation (3 h at 37^{o} C)			
	Mean	±	SD		Mean	±	SD	
1	5.385	±	0.011	a	4.464	±	0.012	b
2	6.351	±	0.055	a	5.617	±	0.056	b
3	5.364	±	0.034	a	4.473	±	0.061	b
4	6.51	±	0.031	a	5.636	±	0.056	b
5	6.334	±	0.035	a	5.298	±	0.042	b
6	5.307	±	0.023	a	4.314	±	0.028	b

7	6.467	±	0.011	a	5.982	±	0.018	b
8	6.368	±	0.041	a	5.673	±	0.029	b
9	5.317	±	0.016	a	4.584	±	0.103	b
10	5.431	±	0.032	a	4.732	±	0.06	b
11	6.505	±	0.025	a	5.393	±	0.064	b
12	5.343	±	0.02	a	4.602	±	0.053	b
13	5.523	±	0.005	a	4.955	±	0.065	b
14	5.648	±	0.097	a	5.133	±	0.069	b
15	4.399	±	0.022	a	4.008	±	0.007	b
16	5.06	±	0.053	a	4.387	±	0.051	b
17	6.052	±	0.045	a	5.13	±	0.026	b
18	5.403	±	0.007	a	4.327	±	0.581	b
19	5.289	±	0.077	a	4.374	±	0.013	b
20	5.38	±	0.052	a	4.181	±	0.017	b
21	5.25	±	0.062	a	4.011	±	0.011	b
22	4.957	±	0.058	a	4.514	±	0.197	b
23	5.074	±	0.065	a	4.584	±	0.077	b
24	5.181	±	0.026	a	4.684	±	0.018	b
25	5.43	±	0.015	a	4.219	±	0.014	b
26	4.1	±	0.09	a	3.184	±	0.055	b
27	4.649	±	0.059	a	4.041	±	0.061	b
28	3.621	±	0.05	a	2.532	±	0.029	b
29	3.556	±	0.04	a	2.604	±	0.025	b
30	2.3	±	0.002	a	1.485	±	0.026	b
31	2.662	±	0.045	a	1.563	±	0.49	b
32	5.022	±	0.001	a	4.012	±	0.004	b
33	1.333	±	0.029	a	0.333	±	0.577	b
34	4.392	±	0.004	a	3.364	±	0.052	b
35	5.03	±	0.043	a	4.18	±	0.062	b
36	5.729	±	0.04	a	4.853	±	0.03	b
37	5.302	±	0.022	a	4.374	±	0.047	b
38	5.239	±	0.025	a	4.344	±	0.012	b
39	3.07	±	0.061	a	2.157	±	0.137	b
40	5.635	±	0.032	a	3.934	±	0.051	b
41	4.811	±	0.011	a	3.907	±	0.013	b
42	4.873	±	0.066	a	3.799	±	0.111	b
43	4.915	±	0.034	a	3.99	±	0.01	b
44	5.776	±	0.023	a	4.73	±	0.028	b
45	4.852	±	0.044	a	3.753	±	0.073	b
46	5.22	±	0.026	a	4.07	±	0.061	b
47	5.56	±	0.367	a	4.449	±	0.008	b
48	3.323	±	0.026	a	2.93	±	0.056	b
49	1.947	±	0.043	a	0.704	±	0.612	b
50	5.213	±	0.011	a	4.207	±	0.012	b
Mean	**4.949**	**±**	**0.043**	**a**	**4.055**	**±**	**0.087**	**b**
p-value	**0.001****							

Diverse superscript letters in the same raw characterize significant differences ($p \leq 0.01$)

When probiotic microorganisms enter the stomach with hydrochloric acid, they are subjected to severe acid stress (Liu et al., 2019). Different lactic acid bacteria strains show different acid tolerance techniques. This involves neutralizing protons in carbon dioxide generated by malolactic fermentation, producing alkaline substances through the arginine dihydrolase system to neutralize acid, and transporting protons by activating proton pumps such as F1-F0-ATPase (Microbiol et al., 2017). Multiple processes control pH homeostasis. According to Marco Gobbett et al. (2004), proton transport ATPase is the most significant enzyme in fermentative bacteria.

Internal pH control is also influenced by the plasma membrane's total proton permeability. *L. plantarum* showed the lowest membrane permeability at pH 4.0, whereas an acid-sensitive organism was seen at pH 6.0. According to Isa and Razavi (2017), proton transport ATPases appear to be essential in removing protons from cells and lowering their net permeability to protons. The capacity of luminal bacteria to increase H-ATPase production in response to low pH appeared to be associated with their acid tolerance. Therefore, it's probable that *Bifidobacterium* capacity to produce H-ATPase determines how well it can tolerate acid (Miwa et al., 2001).

Although acid was present, acid-intolerant bacteria had lower H-ATPase activity. The activity of the enzyme in different straining and types was evaluated, and strains with higher levels of tolerance developed as the environment became more acidic (Matsumoto et al., 2004). According to Bender (1987), proton transfer ATPases are thought to be primarily responsible for removing protons from cells and lowering their net permeability to protons. The F1Fo-ATPase is not the only method that Gram-positive bacteria apply to cope with acidity. The cell membrane and regulatory systems can be altered, as can different metabolic pathways and the decarboxylation of amino acids. 2003 (Cotter and Hill).

Antimicrobial Assays of LAB

The results showed significant variation between the diameter inhibitory zones of *Staphylococcus aureu*s and *E. coli*. (p≤001). The average antimicrobial effect as determined in the inhibition zone is displayed in Table 3 and Figure 4. Our results conclusions concur with those made by D. Bernard et al. (2021). Isolated LAB demonstrated inhibitory action against *S. aureus*, with inhibition zones of 18 to 22 mm in diameter, as seen in Figure 5. These recent findings are in line with those of Dayong Ren, Jianwei Zhu, et al. (2018). In addition, 54% of the 50 isolated LABs showed antibacterial activity against *E. coli*, creating inhibition zones with widths ranging from 13 to 18 mm (Figure 6).In general*, S. aureus* was more susceptible than *E. coli* to the isolated antimicrobial strains' inhibitory effects. The results demonstrated that LAB species and concentrations affected the antibacterial metabolites generated. As a result, several LAB experiments display various levels of efficacy in inhibiting harmful microorganisms. Our findings correspond with those of Liu et al. (2015); Georgieva et al. (2015); Nambundunga (2020); Ibrahim and Bezkorovany (1993); Ibrahim et al. (2003); Makras and De Vuyst (2006); and Ibrahim et al. According to Tejero-Sarinena et al. (2012), the generation of antimicrobial compounds such as hydrogen peroxides, acetic acid, lactic acid, and bacteriocins is the reason why probiotics have been shown to have antimicrobial characteristics. Although the production of organic acids and hydrogen peroxide might be attributed to the antagonistic action of gram-negative bacteria .

One possible reason is that bacteriocins have a fast acting mechanism, which forms pores in the target membrane of bacteria, even at extremely low concentrations(Perez et al., 2014). The cell wall of Gram-positive bacteria allows passage of relatively large molecules, so that there is unlikely to be a requirement for bacteriocin receptors analogous to those in the outer membranes of gram-negative cells. Anionic cell surface polymers like teichoic acid and lipoteichoic acid may be important in the initial interaction of cationic bacteriocins produced by gram-positive bacteria (Parada et al., 2007). Organic acids, fatty acids, hydrogen peroxide, and diacetyl are only a few of the antimicrobial effects of the metabolic substances that lactic acid bacteria create(Ibrahim et al., 2021) . Lactic acid is lethal to microorganisms via undissociated molecules that flow through the cell membranes and ionize inside. The acidic pH inside the cell causes deformation an damage to enzymatic activities, proteins and DNA structure, thereby damaging the extracellular membrane (Mani-Lopez et al. 2011).

However, it is evident that breast milk's natural flora aids in the child's immunity, and this may be one of the reasons why pasteurization reduces the anti-microbial power of freshly generated breast milk. Ford and others, (1977)

Table 3: Mean of diameter of Inhibition zone mm of LAB in contrast to *E. coli* and *S. aureus*

	E. coli		*S. aureus*		P-value
	Mean	**±SD**	**Mean**	**±SD**	
Diameter of Inhibition zone mm	**15.00**	**±0.05**	**20.00**	**±0.08**	**0.001****

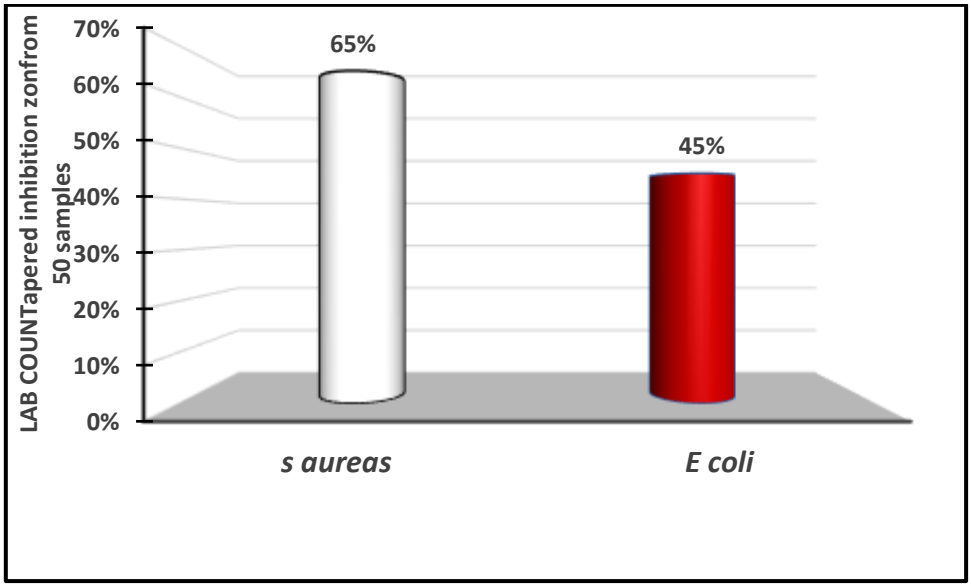

Figure 4. The number of LAB samples that showed a of inhibition zone against to *S. aureus* and *E.coli* out of fifty samples.

Figure 5: Inhibition zones of the LAB. separated from human milk counter to *S. aureus*

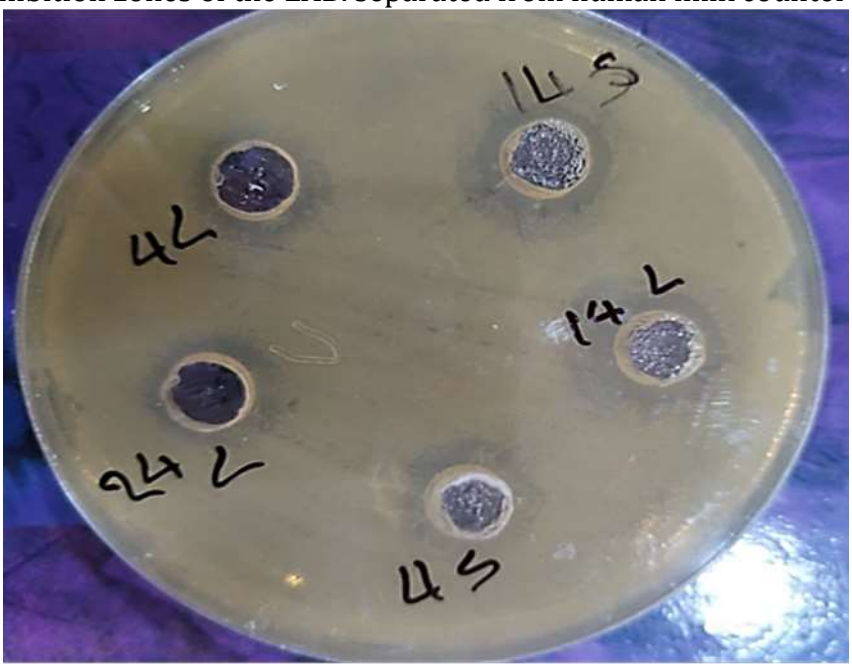

Figure 6. Inhibition zones of the LAB. separated from human milk counter to *E. coli*

Conclusions

Species that have been discovered in breast milk have been named in the current investigation. From breast milk*, Lactobacillus*, *Streptococcus,* and *Bifidobacterium* species were isolated to see if they were probiotic bacteria. This suggests that consuming breast milk may facilitate the development of a baby's gut flora. Three of the most significant functions of lactic acid bacteria are their capacity to resist low pH, combat infections, and colonize the gastrointestinal tract with pathogenic microbes. being capable of surviving in the digestive system Investigations focused on the lactic acid bacteria that were found in human breast milk. They conducted some research to examine the prerequisites for probiotic bacteria, including the capacity to endure in environments resembling those of the gastrointestinal tract and the capacity to produce antimicrobial compounds. The capacity of lactic acid bacteria to create antimicrobial organic acids, or bacteriocins, may offer a biological way of food preservation and food safety. It would be interesting to compare breastfed children to non-breastfed children to evaluate the real effect of breast milk on the gut flora. The use of such antimicrobial agents as powders or capsules with the aim of treating enteropathogenic illnesses, protecting against PPOs, particularly in infants, and as a food supplement for adults and the elderly will be another area of research in the upcoming years.

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