JMBFS
Journal of Microbiology, Biotechnology and Food Sciences
International peer-reviewed scientific online journal

Published by Faculty of Biotechnology and Food Sciences

IN VITRO AND *IN VIVO* INVESTIGATION OF PROBIOTICS ISOLATED FROM HUMAN MILK

Manikandan Kathirvel[1], Sangeetha Menon[1], Saranya P[2], Kabilan G Mariappan[2]*

Address(es): Dr. Sangeetha Menon,
[1] Department of Life Sciences, Kristu Jayanti College (Autonomous), Bengaluru-560077, Karnataka, India
[2] Department of Biotechnology, Sri Kaliswari College, Sivakasi-626189, Tamil Nadu, India.

*Corresponding author: sangeethamenon@kristujayanti.com

https://doi.org/10.55251/jmbfs.9587

ARTICLE INFO

Received 30. 10. 2022
Revised 24. 7. 2024
Accepted 16. 9. 2024
Published 1. 10. 2024

Regular article

OPEN ACCESS

ABSTRACT

People all over the world are embracing probiotics as a result of growing awareness of the health advantages connected to ingesting living bacteria. Proper administration of probiotics has been shown to provide numerous health advantages to the host. The Lactobacillus genus, which naturally resides in the mucosal linings of humans and animals, and it is essential for stabilizing the gastrointestinal tract and protecting intestinal health from infections. There has been a great deal of interest in their potential uses as probiotics, mucosal vaccine vectors, and agents regulating metabolic activities. The present study aimed at isolating and characterizing novel probiotics from human milk and functionally assessing them *in vivo* for their physiological, enzymatic and antimicrobial activity. Probiotic isolates from human milk were amylolytic, proteolytic and lipolytic and were found to be tolerant to low pH of 3.5, 10% salt and 0.3% bile salt. Primary method of cross streaking and secondary streaking by agar diffusion method revealed antagonistic activity of the isolates against tests pathogens of humans *viz., S. aureus Newman, P. fluorescens, S. epidermis* and *S. aureus.* Antibiofilm activity of probiotic isolates *in vivo* against *P. fluorescens* and *S. aureus Newman* indicated that the metabolites present in the Cell Free Supernatant (CFS) of the isolates may have the ability to inhibit the formation of biofilm of pathogenic strains. The most efficient probiotic isolates was identified as *Weissella confusa* and the NCBI GenBank accession number was obtained. The infected mice administered with probiotic isolate *Weissella confusa* PS17 showed reduced mortality rate than the mice infected with pathogenic strain *S. aureus Newman*, thus indicating that probiotic strain boosted the immune response of the mice. The Serum uric acid levels was normalised after the infected mice was treated with *W. confusa* PS17 and this in turn enhanced the survival rate of mice. The present study indicates that isolate *W. confusa* PS17 can be a promising candidate in improving the condition of health.

Keywords: Probiotics, human milk, *Weissella confuse,* Serum uric acid

INTRODUCTION

In addition to meeting all of an infant's nutritional needs, human milk (HM) has been shown to contain a range of immunoprotective components, such as IgA, lysozyme, lactoferrin, and others **(Newburg 2005),** as well as intestinal microbiota, which acts as a disease-prevention mechanism **(Gonzalez *et al.*, 2013; Morrow and Rangel, 2004; Hanson and Korotkova, 2002; Wright *et al.*, 1998).** HM's microbial population is influenced by the bacteria in the mother's bowel, which are carried to the mammary glands through the lymphatic system by macrophages and dendritic cells **(Jeurink et al., 2013).** Human milk has been found to contain the genera Lactobacillus, Staphylococcus, Bifidobacterium, Streptococcus, Leuconostoc and Enterococcus **(Matsumiya *et al.*, 2002; Heikkila and Saris, 2003; Martin *et al.*, 2003; Martın *et al.*, 2012; Jeurink *et al.*, 2013)** that are potential probiotics for the bowel of infants **(Martın *et al.*, 2007).** The World Health Organisation (WHO) and the Food and Agriculture Organisation (FAO) both classify probiotics as live bacteria that, when ingested in sufficient quantities, have positive effects on host health **(Grajek et al., 2005).** Human milk contains probiotic lactic acid bacteria (LAB), such as *Enterococcus faecium, Lactobacillus gasseri, Lactobacillus rhamnosus, Lactobacillus plantarum, and Lactobacillus fermentum*. Research indicates that LABs have the ability to produce antimicrobial chemicals such as hydrogen peroxide, organic acids, bacteriocins and others and they can compete with a variety of newborn pathogenic bacteria to exhibit antimicrobial activity and probiotic qualities **(Olivares *et al.*, 2006; Martin *et al.*, 2005; Beasley and Saris, 2004; Heikkila and Saris, 2003).**

MATERIAL AND METHODS

Collection of human milk samples and isolation of probiotic bacteria

Five human milk samples were taken at Cloud Nine Hospital, HRBR layout, Bangalore, India, from five healthy nursing moms who had a full-term pregnancy and nursed their babies at 10 months postpartum. Before being sent to the lab, the samples were gathered in sterile glass bottles and refrigerated. After being collected, the samples were processed in two hours. Nursing staff members from the hospital collected the samples aseptically. Samples of milk were obtained after the nipples and mammary areola were cleaned with soap, sterile water, and disinfectant. Following a serial dilution in 0.1% peptone water, the milk samples were spread out on Man Rogosa Sharpe (MRS) agar with 0.5% calcium carbonate and 0.004% bromocresol purple (de Man et al., 1960) and cultured for 48 h at 37°C. For later usage, the pure colonies were separated, kept alive on MRS agar slants, and kept cold (4°C).

Preliminary identification of probiotic bacteria

By examining the physical and biochemical traits, the isolates were initially identified (Klein, 2003).

Screening for enzymatic activity

Enzymatic activity tests were performed on the isolated colonies. Specifically, lipolytic activity was assessed using tributyrin agar medium **(Verma and Sharma, 2014),** amylolytic activity was assessed using starch agar medium **(Anteneh and Prapulla, 2015),** and proteolytic activity was assessed using skim milk agar **(Desai, 2008)**. For 24 to 48 h, the plates were kept in an inverted orientation at 37°C.

Gastrointestinal tolerance assay (Hoque *et al.*, 2010)

By inoculating 1% of an overnight culture of each isolated species in MRS broth with varied pH levels (3.5-8.5), adjusted with 99% pure acetic acid and 5N NaOH, the gastrointestinal tolerance of the chosen isolated species was assessed. The broths that had been inoculated were kept at 37ºC for overnight incubation. 1% of the overnight grown isolates in MRS broth were tested with different concentrations of NaCl (2–10%), and the isolates with salt tolerance were ascertained. The broths that had been inoculated were kept at 37ºC for 24 h. The isolated strains were cultured in MRS broth containing 0.3%, 0.6%, and 0.8% (w/v) of bile salts for 3h at 37°C in order to assess the bacterial resistance to bile salts.

Antibiotic susceptibility test

Using Muller Hinton agar (MHA) plates, the disc diffusion method was used to assess antibiotic susceptibility. Soft MHA (containing 0.7% agar) was spread over MHA plates and inoculated at 0.5 McFarland with isolates **(Bauer et al., 1966)**. The agar plates were placed with commercially available antibiotic discs (Oxide) containing Ampicillin (25µg), Amikacin (30µg), Tetracycline (10µg), Carbenicillin (30µg), Tobramycin (30µg), and Kanamycin (30µg). The inhibition zone diameters surrounding the discs were measured after a 24h incubation period at 37°C. Based on these measurements, the LAB isolates were classified as resistant (R), intermediate resistance (I), or sensitive (S) in accordance with the CLSI **(Clinical and Laboratory Standards Institute, 2012)** standards.

***In vitro* antagonistic activity of selected isolates**

Target pathogens

Four pathogenic strains *viz., Staphylococcus aureus, Pseudomonas fluorescens, Streptococcus epidermidis* and *Staphylococcus aureus* Newman were used in the study. *Staphylococcus aureus* strain Newman, a clinical isolate was generously gifted by Prof. Simon Foster, University of Sheffield, UK. All the other strains were maintained in Department of Life Sciences, Kristu Jayanti College, Bengaluru, India.

Cross Streaking method

The antagonistic activity potential of ten selected probiotic isolates was studied *in vitro* to analyse the growth inhibition of the target pathogenic bacteria. Overnight broth of probiotic strains were streaked vertically in the centre of nutrient agar plate under aseptic condition. Overnight nutrient broth of the pathogenic strains was streaked horizontally alongside of the probiotic bacteria and, the plates were kept in the incubator for 24 to 48 h at 37°C. The probiotic isolates inhibited the pathogenic bacterial isolates, resulting in the absence of growth **(Lu et al., 2018).**

Agar Well Diffusion Method

Probiotic isolates' antibacterial efficacy against the specified pathogens *viz., P. fluorescens, S. aureus Newman, S. epidermis* was assessed through secondary screening using traditional method of diffusion method based on **Balouiri *et al.* (2016).** Overnight cultures of the probiotic isolates in MRS was centrifuged (6500g, 10 min,4°C) and a 0.2 µm membrane syringe filter was used to gather and sterilise the Cell-Free Supernatant (CFS). Overnight cultures of pathogenic strains were standardized to 2 x 10^8 CFU/ml using 0.5 McFarland standard and was aseptically swabbed on sterile Mueller Hinton Agar (MHA) plates (HiMedia Laboratories Pvt. Limited, India). Following inoculation, the seeded agar plates were kept ideally for 15 min in order to facilitate the inoculum's absorption into the substrate. Using a sterile cork borer with an eight mm diameter, wells were cut, and 100 µl of CFS of each probiotic strain was added. The wells were then sealed and left to incubate for 24h at 37°C. Using a Vernier calliper, the inhibition zone surrounding the wells was measured following incubation. The experiment was performed in triplicates.

Antibiofilm activity

Selected probiotic strains' antibiofilm activity was investigated in a 96-well micro titre plate utilising a semi-quantitative adherence assay. A 96-well micro titre plate was seeded with 100 microliters of an overnight-grown culture (10^7–10^8 CFU/ml) of the test pathogen, and it was incubated at 37°C for 24 h to promote the production of biofilms. Planktonic cells were eliminated from the microtitre plates by three PBS washes following incubation. The wells were incubated for 18h at 37°C with 200 µl of fixed Cell Free Supernatant (CFS) of specific isolated probiotic strains. Following the incubation period, the plates underwent three rounds of washing with phosphate-buffered saline, were air dried, stained for 45 minutes with 1% crystal violet, and then suspended in 200 µl of 33% glacial acetic acid. The absorbance was then measured at OD_{570}. Untreated pathogenic strain was utilised as negative control and chlorhexidine as the positive control. Four duplicates of the experiment were conducted **(Sharma et al., 2015).**

Molecular Characterization of Efficient Strains

Using 16S rRNA sequencing, the most efficient bacterial isolate was molecularly characterised. The protocol for extracting and purifying DNA from a bacterial isolate was followed by **Sambrook and Russell (2006).** Using the universal bacterial forward primer 5'GAGTTTGATCMTGGCTCAG3' and reverse primer 5'TACGGYTACCTTGTTACGACTT3', the target bacterial isolate's 16S rRNA gene was obtained and sequenced at Yaazh Genomics, Coimbatore. Using MEGA 4.0, a phylogenetic tree was built, and the isolate was located. After the sequence was acquired, it was added to the NCBI database and an accession number was generated.

***In vivo* studies in animal model**

Animal experiments were carried out with the permission granted from Institutional Animal Ethical Committee (IAEC), in the registered Animal House Facility (Reg.1086/PO/Re/S/07/CPCSEA), of Sri Kaliswari College, Sivakasi, India.

Experimental Design

Swiss Albino mice of eight-week old, weighing of 18-22g and standard mouse pellets were obtained from Biogen Animal laboratory, Bangalore. Three groups of mice (n = 8 per group) were created. Group A was considered as control mice (Untreated), Group B mice infected with *S. aureus* Newman and untreated and Group C mice was challenged with pathogenic Newman strain and treated with the selected isolate of probiotic bacterial strain.

Administration of pathogenic strain into mice

Staphylococcus aureus strain Newman, was grown to exponential growth phase (A_{600} = 1), extracted the cells at 10,000 rpm,10 min, 4ºC, cleaned with PBS and again suspended in 0.2 M $NaHCO_3$ buffer with 2% glucose. Mice were challenged intravenously with a virulent strain of *S. aureus* strain Newman. After 5-6 days of infection, the mice were treated with the selected probiotic strain.

Administration of probiotic bacterial isolate into mice

The selected probiotic isolate was grown overnight and spun at 10,000 rpm, 10 min, 4ºC and the pellet was air dried. Fifty milligrams of the pelleted cells of selected isolate was resuspended in bicarbonate buffer and 25µl was administered orally to the mice. Oral administration was continued for nine days with an interval of three days **(Castillo *et al.*, 2011).**

Monitoring of clinical parameters

For each group, the clinical indicators and symptoms as well as the death/survival ratio were recorded. Faecal samples was collected, resuspended and mechanically homogenized in saline. On the chosen media, dilutions were plated and cultured for two days at 37°C. The growth of animals supplemented with isolated Probiotic strains was compared with the control. The differences in body weight gain of different groups of mice are monitored. Serum uric acid was also measured for each group.

RESULT AND DISCUSSION

Isolation and preliminary identification of probiotic isolates

Using MRS agar media, probiotic bacteria were identified from human breast milk. Twenty two isolates were obtained, purified and maintained in agar slants. The isolates were given codes as PS1, PS2, etc. The isolates were assigned to the genus based on phenotypic tests. Phenotypic testing indicated that the majority of the isolates fell within the genus *Lactobacillus* (10/22), genus *Enterococus* (8/22), *Pediococcus* (2/22) and *Weisella* (2/22).

Table 1 Screening for enzymatic activity

Isolates	Protease activity	Amylase activity	Lipase activity
PS1	+	+	+
PS2	-	-	-
PS3	-	-	-
PS4	-	-	-
PS5	-	-	-
PS6	-	-	-
PS7	-	-	-
PS8	-	-	-
PS9	-	-	-
PS10	+	+	-
PS11	-	-	-
PS12	-	-	-
PS13	-	-	-
PS14	-	-	-
PS15	-	-	-
PS16	-	+	-
PS17	+	+	+
PS18	-	-	-
PS19	-	-	-
PS20	-	-	-
PS21	-	-	-
PS22	-	-	-

Legend: '+' = Positive test, '-' = Negative test

Screening for enzymatic activity

Enzymatic activity tests were performed on the isolated colonies using skim milk agar, starch agar medium for amylolytic activity, and tributyrin agar medium for lipolytic activity. A clean zone encircling the colony suggested that the proteolytic activity had responded favourably. Isolates PS1, PS10, PS16, PS17 exhibited proteolytic activity. Amylase activity was indicated by the colourless zone surrounding colonies producing extracellular amylase after flooding the plates with iodine. Isolates PS1, PS10 and PS17 indicated amylase enzyme activity. The isolates were also screened for lipase activity where a positive reaction was indicated by clear zone surrounding the colony. The isolates PS1 and PS17 showed signs of lipolytic activity (Table 1).

Gastrointestinal tolerance assay

Since probiotic strains must withstand the harsh circumstances of the stomach in order to enter the small intestine, resistance to low pH is a crucial criteria. While the stomach's pH can drop as low as 1.0 **(Conway et al., 1987),** pH 3.0 has been found to be optimal in the majority of in vitro tests **(Ehrmann et al., 2002).** The isolated probiotic microbes were cultured for 24h at 37°C in MRS broth that had been pH-adjusted to range from 3.5 to 8.5. The isolates PS1 and PS17 were the only ones that could withstand a low pH of 3.5 (Figure 1).

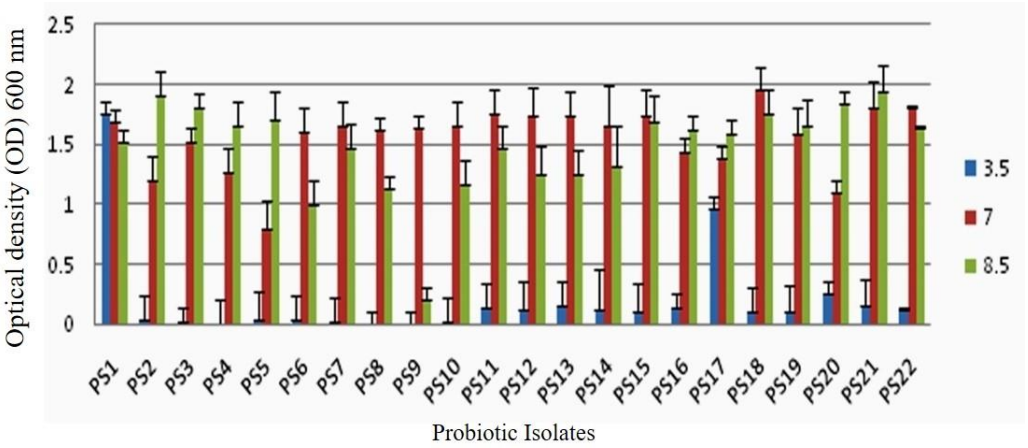


Figure 1 pH tolerance of the isolates

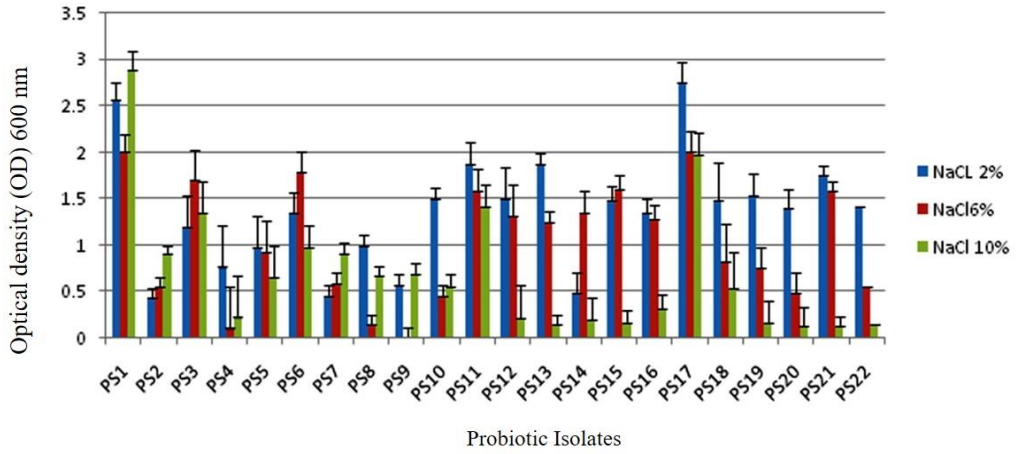


Figure 2 Salt tolerance of the isolates

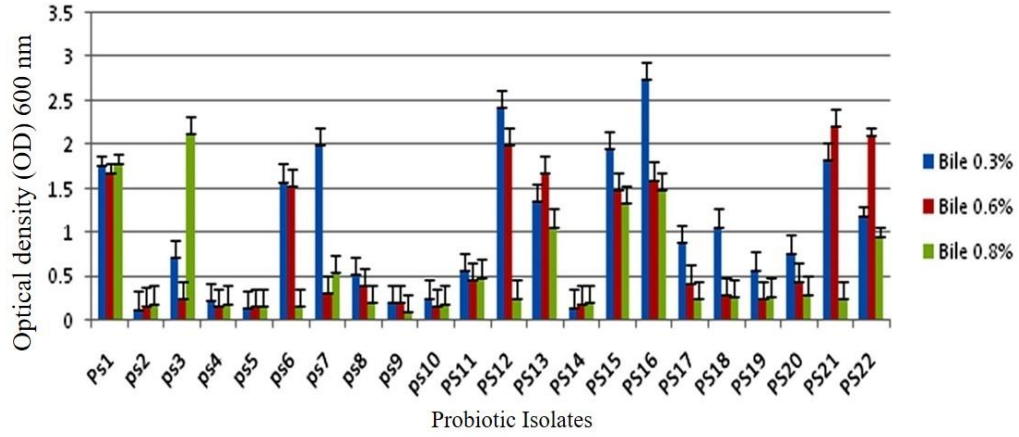


Figure 3 Bile salt tolerance of the isolates

The isolated probiotic strains were grown at different concentrations of salt (2-10% NaCl) in MRS broth at 37ºC for 48h. The isolates PS1 and PS17 were able to grow at high salt concentration (10% NaCl) whereas the rest of the isolates could tolerate only a maximum of 6% NaCl (Figure 2).

The strains were screened for their tolerance to the bile salt at 0.3%, 0.6% and 0.8%. The average intestinal bile concentration in the human gastrointestinal tract is likely to be 0.3% w/v, with an anticipated 4h stay. However, the bile concentration in the tract varies **(Sahadeva *et al.*, 2011).** Figure 3 indicates that the

isolates PS1, PS12, PS15, PS16 and PS17 were tolerant to 0.3%. The capacity to endure the harsh circumstances such as bile salts and stomach acidity in the intestine is an essential attribute for probiotic strains **(Koll *et al.*, 2008).** While the pH plays a major role in creating the harsh conditions of the gastric environment, other digestive and enzymatic substances present in the gastric fluid, food, and the probiotic bacteria's delivery matrix can also have an impact on the cells' ability to survive during gastrointestinal transit **(Fredua-Agyeman and Gaisford, 2015).** The examined isolates could be able to survive passage through the gastrointestinal tract, according to the data; still, more biorelevant testing might be needed. The isolates' resistance was superior to what has been previously documented **(Shokryazdan et al., 2014**). These days, a lot of study is being done on how to safeguard probiotic cultures during manufacturing, storage, and transit through the digestive system, including adding probiotics to certain food ingredients **(Argyri *et al.*, 2013).**

Antibiotic susceptibility test

Probiotics containing lactic acid bacteria (LAB) have the potential to harbour antibiotic resistance genes, which can subsequently be passed on to pathogenic bacteria **(Prittesh and Vrutika, 2015).** Using the disc diffusion method, an antibiotic susceptibility assay was performed on a subset of isolates for six different antibiotics (Table 2). Antimicrobial-resistant bacteria have become more prevalent due to the overuse of antibiotics in the treatment of human diseases and as a growth booster in livestock production **(Herreros et al., 2005).** It is believed that the primary pathway for resistant bacteria to spread to humans is the food chain. Therefore, probiotic strains having genes for resistance that can be transferred to harmful bacteria shouldn't be added to meals **(Klare *et al.*, 2007).**

Table 2 Antibiotic susceptibility of Probiotic isolates

Isolates	Antibiotics					
	Carbenicillin (30 µg)	Tetracycline (10 µg)	Amikacin (30 µg)	Kanamycin (30 µg)	Ampicillin (25 µg)	Tobramycin (30 µg)
PS1	R	R	R	R	R	R
PS3	R	R	R	R	R	R
PS4	R	S	I	R	R	R
PS5	R	S	I	R	R	R
PS6	R	R	R	R	R	R
PS11	R	R	R	R	R	R
PS12	R	R	R	R	R	R
PS13	R	R	R	R	R	R
PS14	R	R	R	R	R	R
PS15	R	R	R	R	R	R
PS16	R	R	R	R	R	R
PS17	S	S	R	S	S	R

Legend: R - Resistant, I - Intermediate resistant, S - Sensitive

***In vitro* antagonistic activity of selected isolates**

Cross Streaking Method

The antagonistic effect of selected strains by cross streaking method was tested against the microbial pathogens such as *Staphylococcus aureus, Pseudomonas fluorescens*, *Streptococcus epidermidis* and *Staphylococcus aureus* Newman. Probiotic isolates *viz.*, PS1, PS17, PS11, PS12 and PS17 strains showed good antagonistic activity when compared to other strains (Table 3). **Lertcanawanichakul and Sawangnop (2008)** evaluated the efficacy of this method to test antibacterial activity and found that the cross streak method is a practical and suitable technique. However, the cross streak method should be supported by well diffusion method.

Table 3 Antagonistic activity of probiotic isolates against microbial pathogens

Isolates	*P. fluorescens*	*S. aureus Newman*	*S. aureus*	*S. epidermidis*
PS1	+	+	+	+
PS3	+	+	+	+
PS6	-	-	-	-
PS11	-	-	-	-
PS12	+	+	+	+
PS13	-	-	-	-
PS14	-	-	-	-
PS15	+	+	+	+
PS16	-	-	-	-
PS17	+	+	+	+

Legend: '+' = Inhibition, '-' = No inhibition

Agar Well Diffusion Method

As a secondary screening technique, the antibacterial activity of the chosen isolates was further evaluated against test pathogens using the agar well diffusion method. The isolates *viz.*, PS1, PS3, PS12, PS15 and PS17 exhibited strong antibacterial activity against *P. fluorescens* and *S. aureus Newman* as observed in Figure 4. One crucial probiotic selection criterion is competition with pathogenic bacteria in the intestinal system, which results in the process of suppressing enteric pathogens. Additionally, this screening may be used as a substitute for treating intestinal infections with antibiotics **(Shokryazdan *et al.*, 2014).** Antimicrobial activity of *W. confusa* has been observed against *Salmonella* serotype *enteritidis, Helicobacter pylori, E.coli, Enterobacter faecalis, L. monocytogenes, S. aureus*, and *Klebsiella pneumonia* **(Nam *et al.*, 2002; Serna-Cock *et al.*, 2013; Reis *et al.*, 2016).**

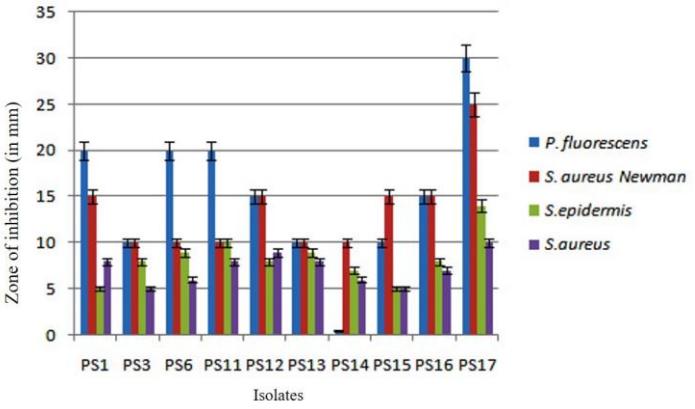
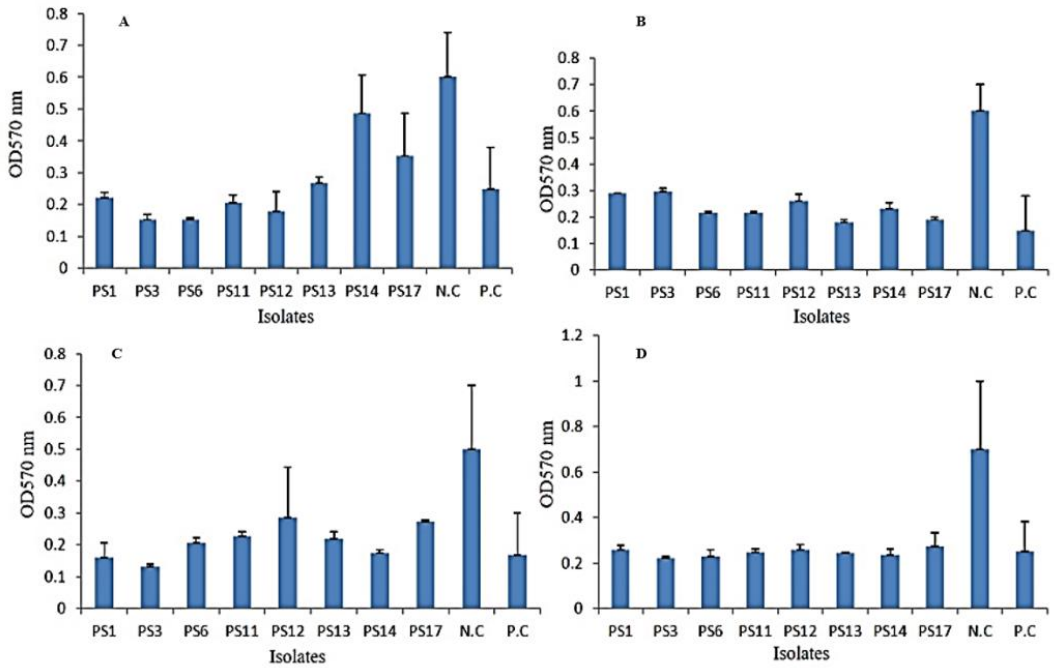


Figure 4 Antibacterial activity by selected probiotic isolates

Antibiofilm activity of probiotic isolates

Inhibitory effect of CFS of probiotic isolates against biofilm formation by *P. fluorescens, S. aureus Newman, S. epidermidis* and *S. aureus* was determined *in vitro* by semi-quantitative method using 96 well polystyrene plate method. All the isolates demonstrated the ability to inhibit biofilm formation of the pathogenic isolates tested, indicating the presence of antimicrobial compounds in the supernatant of the isolates (Figure 5). In addition to its ability to control host serotonin production in the gut, the native microbiota was found to be the most potent antibacterial for the inhabitants when compared to non-native probiotics **(Yano et al., 2015).** According to **Fredua-Agyeman et al. (2020)**, certain native Lactobacillus strains can successfully suppress pathogens in the gut and endure harsh in vivo environments. They recommend combining these strains with commercial probiotics to maximise the native efficacy.



Legend: A) *S. aureus*, B) *P. fluorescens*, C) *S. epidermis* and D) *S. aureus Newman*; N.C- Negative Control; P.C- Positive control
Figure 5 Antibiofilm activity of selected probiotic isolates

Molecular Characterization of selected probiotic strain

The most efficient probiotic isolate as determined during preliminary studies was identified by 16SrRNA sequencing. Using phylogenetic construction, *Weissella confusa* was identified as the isolate. The sequence was submitted to NCBI data base and accession numbers was obtained as MK934416. Gram-positive, catalase-negative, non-endospore-forming *Weissella confusa* cells have a coccid or rod-shaped morphology. This species has been isolated from human skin, human breast milk, human faeces, animal faeces, plant and vegetable vagina, and a variety of fermented foods. *Weissella* has been shown to have substantial cell surface hydrophobicity, auto-aggregation, and moderate biofilm forming capabilities, which may help prevent cancer and enhance immune function **(Fusco, 2015, Fhoula *et al.*, 2018).** Weissella spp. have drawn a lot of interest due to their strong probiotic potential in the management of numerous illnesses. Cabrera-Rubio et al. (2012) revealed that *W. confusa* was one of the primary species in the colostrum of Finnish women who were nursing. Because of its antibacterial properties and intestinal tolerance, Weissella confusa may be a viable probiotic **(Serna-Cock *et al.*, 2010; Lee *et al.*, 2012).**

***In vivo* studies in animal model**

The mice was inoculated with a virulent strain of *S. aureus strain Newman* and effect of probiotic isolate *Weissella confusa* PS17 on growth index, mortality rate and serum uric acid was measured. The response variables noted are given in Table 4. The ability of *W. confusa* PS17 to remain in mice's gastrointestinal tracts following three days of oral dosing was assessed. After the oral administration, the probiotic strain was persistent in fecal sample of Group C mice at 10^4 CFU/g. The mortality rate of mice was reduced when the infected mice was treated with *W. confusa* PS17 compared with the mice treated with pathogenic strain *S. aureus Newman*, indicating that probiotic strain boosted the immune response of the mice. Studies suggested that the probiotic organism can be used as an adjuvants to enhance the immune response **(Lemme-Dumit et al, 2021)**. Serum uric acid levels of treated mice were compared with normal control and infected mice after 2 weeks of administration. The uric level was normalised after the infected mice was treated with *W. confusa* PS17 and this in turn enhanced the survival rate of mice. Studies suggested that the elevated uric acid has effect on cardiovascular and renal disease **(Nakagawa *et al.*, 2006).** Thus, our promising candidate *W. confusa* PS17 has shown the profound effect on improving the condition of health.

Table 4 Effect of Mice on oral administration of *Weissella confusa* PS17

Biological parameters	Group A- Negative Control (Untreated)	Group B – Mice infected with *S. aureus* Newman	Group C – Infected mice treated with Probiotic isolate PS17
Probiotic count in feces (CFU/g)	0	0	$2 \times 10^4 \pm 0.7$
Body weight (g)	20 ± 2	14 ± 2	23 ± 2
Mortality rate (8 animals/group)	6 ± 2	2 ± 1	8 ± 0
Uric acid (mg/dl)	1.0 ± 0.05	3.5 ± 0.06	1.3 ± 0.1

Legend: The values are depicted as Mean ± SE

CONCLUSION

Live microorganisms that are good for their host's health are known as probiotics. Although probiotic strains can be derived from a variety of sources, human origin is the ideal source for applications involving humans. Many studies have demonstrated the positive effects of breast milk on the health of newborns since it contains numerous bioactive chemicals, proteins, carbs, minerals, lipids, vitamins, and good LABs that support the healthy growth, development, and immunity of the infants. The current study aimed to investigate the potential of probiotic isolates to be used for neonates to improve the health benefits and fight against infections. Probiotic isolates were isolated from human milk and were screened for their enzymatic potential to breakdown starch, protein and fat. The isolates were found to be tolerant to low pH of 3.5, 10% salt and 0.3% bile salt. Antagonistic screenings of the probiotic isolates against tests pathogens of humans were significant. *In vivo* study suggests that isolated probiotic bacteria *W. confusa PS17* from human breast milk aids in colonising in the intestinal area and suppresses the population of pathogen *S. aureus* Newman, thereby reduces the mortality rate and develops the host immunity. Studies also reported that the probiotics can be used as an adjuvant in boosting the immune response. Overall, our findings suggest a protective role of the isolated probiotic strain PS17 in an animal model after *S. aureus* Newman challenge. The most efficient probiotic isolates identified as *Weissella confusa* can be further studied in enriching the concentration of intestinal digestive enzyme and improving digestive activity of humans. The present study represents a preliminary basis for *W. confusa* PS17 in probiotic-based therapies or development of functional food with probiotic supplements. Additionally, the isolates can be identified as a possible strain of probiotics and investigated for their possible involvement in the early colonisation of the baby's gut.

Acknowledgement: The authors greatly acknowledge the Department of Life Sciences, Kristu Jayanti College (Autonomous) under the strengthening component of the DBT star college scheme (San. No. 102/IFD/SAN/3995/2019-20) funded by the Department of Biotechnology, Ministry of Science &

Technology and Government of India for the financial support and facilities to carry out this research work and also thank the Department of Biotechnology, Sri Kaliswari College, Sivakasi, India.

REFERENCES

Anteneh, T., & Prapulla, S. (2015). Studies on amylase from probiotic lactic acid bacteria. *Int. J. Mod. Chem. Appl. Sci*, 2(4), 211-214.

Argyri, A.A., Zoumpopoulou, G., Karatzas, K.A., Tsakalidou, E., Nychas, G.J., Panagou, E.Z., Tassou, C.C. (2013). Selection of potential probiotic lactic acid bacteria from fermented olives by *in vitro* tests. *Food Microbiol*., 33(2):282-91. 10.1016/j.fm.2012.10.005

Balouiri, M., Sadiki, M., & Ibnsouda, S. K. (2016). Methods for in vitro evaluating antimicrobial activity: A review. *Journal of pharmaceutical analysis*, 6(2), 71-79. https://doi.org/10.1016/j.jpha.2015.11.005

Bauer, A.W., Kirby, W.M.M., Sherris, J.C & Turck, M. (1966). Antibiotic Susceptibility Testing by a Standardized Single Disk Method. *American Journal of Clinical Pathology*, 45(4), 493–496, https://doi.org/10.1093/ajcp/45.4_ts.493

Beasley, S.S. & Saris, P.E. (2004). Nisin-producing *Lactococcus lactis* strains isolated from human milk. *Applied and environmental microbiology*, 70(8), 5051-5053. https://doi.org/10.1128/AEM.70.8.5051-5053.2004

de Man, J.C.; Rogosa, M.; Sharpe, M.E. (1960). A Medium for the Cultivation of *Lactobacilli*". *Journal of Applied Bacteriology*. 23, 130–135. https://doi.org/10.1111/j.1365-2672.1960.tb00188.x

Cabrera-Rubio, R., Collado, M.C., Laitinen, K., Salminen, S., Isolauri, E. & Mira, A. (2012) The human milk microbiome changes over lactation and is shaped by maternal weight and mode of delivery. *Am J Clin Nutr*, 96, 544–551.

Castillo N.A., Perdigón G. & de Moreno de Leblanc A. (2011). Oral administration of a probiotic *Lactobacillus* modulates cytokine production and TLR expression improving the immune response against *Salmonella enterica* serovar *typhimurium* infection in mice. *BMC Microbiol.* 3:177. https://doi.org/10.1186/1471-2180-11-177

CLSI (2012). Clinical and Laboratory Standards Institute. Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Second Informational Supplement. CLSI Document M 100-S22. Clinical and Laboratory Standards Institute, Wayne.

Conway, P. L., Gorbach, S. L., & Goldin, B. R. (1987). Survival of lactic acid bacteria in the human stomach and adhesion to intestinal cells. *Journal of dairy science*, 70(1), 1-12.

Desai, A. (2008). Strain identification, viability and probiotics properties of *Lactobacillus casei*. *Ph.D Thesis.* School of Biomedical and Health Sciences, Victoria University,Victoria, Australia

Ehrmann, M.A., Kurzak, P., Bauer, J. & Vogel, R.F. (2002). Characterization of lactobacilli towards their use as probiotic adjuncts in poultry. *Journal of Applied Microbiology*, 92(5):966–975.

Fhoula, I., Rehaiem, A., Najjari, A., Usai, D., Boudabous, A., Sechi, L. A., & Hadda-Imene, O. (2018). Functional probiotic assessment and in vivo cholesterol-lowering efficacy of *Weissell*a sp. associated with arid lands living-hosts. *BioMed Research International*, 1654151. https://doi.org/10.1155/2018/1654151.

Fredua-Agyeman, M., & Gaisford, S. (2015). Comparative survival of commercial probiotic formulations: tests in biorelevant gastric fluids and real-time measurements using microcalorimetry. *Beneficial microbes*, 6(1), 141-151.

Fredua-Agyeman, M., Ofosu-Boateng, M., Offei, A. O., Mehta, M., Gaisford, S., & Limayem, A. (2020). Study on the functional properties of potential indigenous probiotics isolated from human samples in West Africa. *LWT*, 133, 109895. https://doi.org/10.1016/j.lwt.2020.109895

Fusco, V., Quero, G.M., Cho, G.S., Kabisch, J., Meske, D. & Neve, H. (2015). The genus *Weissella*: taxonomy, ecology and biotechnological potential. *Front Microbiol.*, 6:155.

González, R., Mandomando, I., Fumadó, V., Sacoor, C., Macete, E., Alonso, P. L., & Menendez, C. (2013). Breast milk and gut microbiota in African mothers and infants from an area of high HIV prevalence. *PloS one*, *8*(11), e80299. https://doi.org/10.1371/journal.pone.0092930

Grajek, W., Olejnik, A., & Sip, A. (2005). Probiotics, prebiotics and antioxidants as functional foods. *Acta Biochimica Polonica*, 52(3), 665-671. https://doi.org/10.18388/abp.2005_3428

Hanson, L. Å., & Korotkova, M. (2002). The role of breastfeeding in prevention of neonatal infection. In *Seminars in neonatology*,7(4), pp. 275-281, WB Saunders. https://doi.org/10.1053/siny.2002.0124

Heikkilä, M. P., & Saris, P. E. J. (2003). Inhibition of *Staphylococcus aureus* by the commensal bacteria of human milk. *Journal of applied microbiology*, 95(3), 471-478. https://doi.org/10.1046/j.1365-2672.2003.02002.x

Herreros, M.A., Sandoval, H., González, L., Castro, J.M., Fresno, J.M. & Tornadijo, M.E. (2005). Antimicrobial activity and antibiotic resistance of lactic acid bacteria isolated from Armada cheese (a Spanish goats' milk cheese). *Food Microbiology*, 22(5):455-459.

Hoque, M. Z., Akter, F., Hossain, K. M., Rahman, M. S. M., Billah, M. M., & Islam, K. M. D. (2010). Isolation, identification and analysis of probiotic properties of *Lactobacillus spp*. from selective regional yoghurts. *World J Dairy Food Sci*, 5(1), 39-46.

Klare, I., Konstabel, C., Werner, G., Huys, G., Vankerckhoven, V., Kahlmeter, G., Hildebrandt,B., Müller-Bertling,S., Witte, W. & Goossens, H. (2007). Antimicrobial susceptibilities of *Lactobacillus*, *Pediococcus* and *Lactococcus* human isolates and cultures intended for probiotic or nutritional use, *Journal of Antimicrobial Chemotherapy*, 59(5), 900–912. https://doi.org/10.1093/jac/dkm035

Jeurink, P.V., Van Bergenhenegouwen, J., Jiménez, E., Knippels, L. M. J., Fernández, L., Garssen, J., & Martin, R. (2013). Human milk: a source of more life than we imagine. *Beneficial microbes*, 4(1), 17-30. https://doi.org/10.3920/BM2012.0040

Klein, G. (2003). Taxonomy, ecology and antibiotic resistance of enterococci from food and the gastro-intestinal tract. *International journal of food microbiology*, 88(2-3), 123-131. https://doi.org/10.1016/S0168-1605(03)00175-2

Kõll, P., Mändar, R., Marcotte, H., Leibur, E., Mikelsaar, M. & Hammarström, L. (2008). Characterization of oral lactobacilli as potential probiotics for oral health. *Oral Microbiology and Immunology*, 23(2), 139–147.

Lee, K.W., Park, J.Y., Jeong, H.R., Heo, H.J., Han, N.S. & Kim, J.H. (2012) Probiotic properties of *Weissella* strains isolated from human faeces. *Anaerobe*, 18, 96–102.

Lemme-Dumit, J. M., Cazorla, S. I., Perdigón, G. D. V., & Maldonado-Galdeano, C. (2021). Probiotic Bacteria and their cell walls induce Th1-type immunity against Salmonella typhimurium challenge. *Frontiers in Immunology*, 12: 1770.

Lertcanawanichakul, M. & Sawangnop, S. (2008). A Comparison of Two Methods Used for Measuring the Antagonistic Activity of *Bacillus* Species. *Walailak J Sci & Tech.*, 5(2): 161-171.

Lu, D., Yahong, Y. & Yuxiang, Z. (2018). Screening and evaluation of probiotics for antimicrobial activity against foodborne pathogenic bacteria. *J. Food Science*, 39(24): 93-101.

Martín, R., Heilig, G. H. J., Zoetendal, E. G., Smidt, H., & Rodríguez, J. M. (2007). Diversity of the *Lactobacillus* group in breast milk and vagina of healthy women and potential role in the colonization of the infant gut. *Journal of applied microbiology*, 103(6), 2638-2644. https://doi.org/10.1111/j.1365-2672.2007.03497.x

Martín, R., Langa, S., Reviriego, C., Jimínez, E., Marín, M. L., Xaus, J. & Rodríguez, J. M. (2003). Human milk is a source of lactic acid bacteria for the infant gut. *The Journal of pediatrics*, 143(6), 754-758. https://doi.org/10.1016/j.jpeds.2003.09.028

Martín, R., Olivares, M., Marín, M. L., Fernández, L., Xaus, J., & Rodríguez, J. M. (2005). Probiotic potential of 3 lactobacilli strains isolated from breast milk. *Journal of Human Lactation*, 21(1), 8-17. https://doi.org/10.1177/0890334404272393

Martín, V., Maldonado-Barragán, A., Moles, L., Rodriguez-Baños, M., Campo, R. D., Fernández, L., & Jiménez, E. (2012). Sharing of bacterial strains between breast milk and infant feces. *Journal of Human Lactation*, 28(1), 36-44. https://doi.org/10.1177/0890334411424729

Matsumiya, Y., Kato, N., Watanabe, K., & Kato, H. (2002). Molecular epidemiological study of vertical transmission of vaginal *Lactobacillus species* from mothers to newborn infants in Japanese, by arbitrarily primed polymerase chain reaction. *Journal of infection and chemotherapy*, 8(1), 43-49. https://doi.org/10.1007/s101560200005

Morrow, A. L., & Rangel, J. M. (2004). Human milk protection against infectious diarrhea: implications for prevention and clinical care. In *Seminars in Pediatric Infectious Diseases* (Vol. 15, No. 4, pp. 221-228). WB Saunders. https://doi.org/10.1053/j.spid.2004.07.002

Nakagawa, T., Mazzali, M., Kang, D. H., Sánchez-Lozada, L. G., Herrera-Acosta, J., & Johnson, R. J. (2006). Uric acid–a uremic toxin?. *Blood purification*, 24(1), 67-70. https://doi.org/10.1159/000089440

Nam, H., Ha, M., Bae, O. and Lee, Y. (2002) Effect of *Weissella confusa* Strain PL9001 on the adherence and growth of *Helicobacter pylori*. *Appl Environ Microbiol*., 68, 4642–4645.

Newburg D.S. (2005). Innate immunity and human milk. *J. Nutr*., 135(5):1308-12. https://doi.org/10.1093/jn/135.5.1308

Olivares, M., Díaz-Ropero, M. P., Martín, R., Rodríguez, J. M., & Xaus, J. (2006). Antimicrobial potential of four *Lactobacillus* strains isolated from breast milk. *Journal of applied microbiology*, *101*(1), 72-79. https://doi.org/10.1111/j.1365-2672.2006.02981.x

Prittesh, P., & Vrutika, L. (2015). Isolation of lactic acid bacteria from different dung samples and In Vitro screening for certain probiotic properties. *International Journal of Scientific Research and Reviews*, 4(1), 41-49.

Reis, N. A., Saraiva, M. A. F., Duarte, E. A. A., de Carvalho, E. A., Vieira, B. B., & Evangelista-Barreto, N. S. (2016). Probiotic properties of lactic acid bacteria isolated from human milk. *Journal of applied microbiology*, 121(3), 811-820. https://doi.org/10.1111/jam.13173

Sahadeva, R.P.K., Leong, S.F., Chua, K,H. (2011). Survival of commercial probiotic strains to pH and bile. *International Food Research Journal*, 18(4):1515–1522.

Sambrook, J., & Russell, D. W. (2006). The condensed protocols from molecular cloning: A laboratory manual. CSHL Press.

Serna-Cock, L., Hern_andez, L.J.V. and Gaona, R.C. (2010). Cin_etica de fermentaci_on y acci_on antimicrobiana de *Weissella confusa* contra

Staphylococcus aureus y *Streptococcus agalactiae*. *Rev Fac Ing Univ Antioquia* 55, 55–65.
Serna-Cock, L., Enr_ıquez, C., Gaona, R.C. and V_asquez, A. (2013) Cellular response of the bovine mammary gland after Weissella confusa infusion to control *Streptococcus agalactiae*. *Rev Colomb Cienc Pec* 26, 280–287.
Sharma, D., Saharan, B. S., Chauhan, N., Procha, S., and Lal, S. (2015). Isolation and functional characterization of novel biosurfactant produced by Enterococcus faecium. Springerplus 4:4. https://doi.org/10.1186/2193-1801-4-4
Shokryazdan, P., Sieo, C.C., Kalavathy, R., Liang, J.B., Alitheen, N.B., Jahromi, M.F. and Ho, Y.W. (2014) Probiotic potential of *Lactobacillus* strains with antimicrobial activity against some human pathogenic strains. *Biomed Res Int.*, 1–16. https://doi.org/10.1155/2014/927268
Verma, S & Sharma, K.P. (2014). Isolation, identification and characterization of lipase producing microorganisms from environment. *Asian J Pharm Clin Res*, 7(4), 2014, 219-222.
Wright, A. L., Bauer, M., Naylor, A., Sutcliffe, E., & Clark, L. (1998). Increasing breastfeeding rates to reduce infant illness at the community level. *Pediatrics*, 101(5), 837-844. https://doi.org/10.1542/peds.101.5.837
Yano, J.M., Yu, K., Donaldson, G.P., Shastri, G.G., Ann, P., Ma, L. (2015). Indigenous bacteria from the gut microbiota regulate host serotonin biosynthesis. *Cell*, 161(2), 264-276.