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Isolation and Characterization of Probiotic Lactic Acid Bacteria from Human Saliva

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Abstract

Probiotics, which are non-pathogenic microorganisms, interact with the gastrointestinal microbiota and offer various health benefits. These include boosting the host's immune response, acting as antiallergic agents, and exhibiting antimicrobial, anti-cancer, and anti-inflammatory properties. Probiotics are also capable of restoring the disrupted microbiome in a dysbiotic gut. While they can be isolated from different environments, it is often recommended that probiotics intended for human use should be sourced from human origins. The present study shows the successful isolation and identification of lactic acid bacteria from saliva. The lactic acid bacteria were isolated from the collected saliva samples using MRS medium. The isolated bacterial strains were tested for hemolytic activity to verify their non-pathogenic nature. Further, the strains were partially identified by biochemical and microscopic observations; afterwards, the bacterial isolates, which showed non-hemolytic, were tested for their resistance potential against the standard antibiotics. The observed result shows that among the 94 individual isolates, only 12 showed non-hemolytic activity on the blood agar medium. Moreover, the isolated lactic acid bacteria belong to the *Lactobacilli* genus. The tested lactic acid bacterial strains almost showed resistant patterns against many tested antibiotics. The study's findings demonstrate the variety of microbial species in human saliva. Given that these strains are derived from humans, they are likely to exhibit peak efficiency in applications related to food and pharmaceuticals designed for human consumption.

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Introduction

Probiotic lactic acid bacteria, commonly referred to as LAB, are a group of microorganisms known for their potential health benefits. LABs are frequently found in fermented foods and are considered promising probiotic candidates due to their ability to produce lactic acid and antimicrobial components (Sandi *et al.,* 2019). These bacteria are crucial in regulating intestinal microbial homeostasis, influencing nutrient availability, and modulating local and systemic immune responses (Hossain *et al.,* 2016). The probiotic effects of LAB are attributed to various factors, including their ability to adhere to human cells, exclude pathogenic microbes, and exhibit immunomodulatory and anticarcinogenic activities (Moroeanu *et al.,* 2015). Studies have highlighted the significance of LAB, particularly *Lactobacillus* and *Bifidobacterium* species, in conferring probiotic benefits (Sjofjan *et al.,* 2021). LAB, such as *Lactobacillus casei, Lactobacillus plantarum*, *Lactobacillus fermentum* and *Lactobacillus salivarius*, are commonly associated with probiotic properties (Tamang and Lama, 2022). These bacteria are known for enhancing antimicrobial immune protection, thereby aiding in protection against microbial pathogens (Cross, 2002).

Consequently, the search for new bacterial strains with various inherent attributes has emerged to explore their potential utility in treating a wide range of disorders. For example, The application of *Lactobacillus plantarum* ATCC 10241 probiotic strain in a burn model has revealed its potential to hinder Pseudomonas aeruginosa's growth by stimulating phagocytosis of this pathogen by tissue phagocytes, reducing apoptosis, and ultimately promoting tissue repair (Valdéz *et al.,* 2005). In recent years, the issue of antibiotic resistance has gained significant attention, leading to increased research on probiotics and their products as possible alternatives.

The use of probiotics can help in the fight against pathogens through various mechanisms, including competitive exclusion, boosting the function of the intestinal barrier, and producing antimicrobial compounds such as peptides (Fijan, 2016; Besser *et al.,* 2019).Several Lactobacillus strains have demonstrated the ability to inhibit the growth of various multi-drug resistant bacterial pathogens, including MRSA. (methicillin-resistant *Staphylococcus aureus*), *Streptococcus mutans*, *Escherichia coli*, *P. aeruginosa*, *Klebsiella pneumoniae*, *Shigella* spp. and *Clostridium difficile* (Chen *et al.,* 2019; Nami *et al.,* 2019b). Probiotics are present in various environments, including dairy products, fermented foods, and the human body.

However, probiotics derived from humans are commonly recommended for human use (Sanders, 2008; Kumar *et al.,* 2020). This study aimed to isolate probiotic LAB strains from saliva of healthy individuals and evaluate their probiotic potential.

Materials and methods

Materials, reagents, and strains

Culture media and all antibiogram discs, including gentamycin, cefixime, penicillin, chloramphenicol, streptomycin, erythromycin, ampicillin, ciprofloxacin, kanamycin, vancomycin, tobramycin, and clindamycin, were purchased from Himedia, India.

Collection of saliva samples

Samples were obtained from twelve healthy children between the ages of 3 to 11 years. People were informed regarding the study, and written consent forms were provided. The ethics committee of the Cuddalore District Medical College and Hospital approved this study for the collection of saliva samples. Samples were transported to the laboratory on ice and were immediately diluted with peptone water, spread onto de Man-Rogosa-Sharpe (MRS) agar medium and Brain Heart Infusion (BHI) agar, then incubated for 48–72 h at 37°C under aerobic and microaerophilic (by using an anaerobic jar) conditions.

Isolation of lactic acid bacteria from saliva

The organisms were isolated using the pour plate technique. 1 ml aliquots of the samples were plated into MRS (Man, Rogosa, and Sharpe) agar (pH 6.2).

The plates were incubated at 37 °C for 2-3 days under anaerobic conditions. After incubation, individual colonies were selected and transferred into sterile broth mediums. The isolates were purified by selecting colonies using the streak plate technique.

Heamolytic activity

Fresh bacterial cultures were streaked onto blood agar media containing 5–10% sheep blood and incubated for 24 h at 37 °C. The isolates were then examined for the presence of clear zones surrounding the colonies. Clear zones are considered β-hemolysis, greenish zones as α-hemolysis, and the absence of zones indicating no hemolysis is known as gamma hemolysis. Colonies showing beta or alpha hemolysis were excluded, and only those with gamma hemolysis were selected (Halder *et al.,* 2017).

Biochemical and morphological characterization

Morphological characterization was carried out using the Gram staining technique, and biochemical characterization was performed using the catalase test and analysis of carbohydrate fermentation profiles. Physiological tests included the ability to grow in the presence of NaCl [3% and 4.5% (w/v)] and at temperatures of 15 °C and 45 °C. All catalase-negative and Gram-positive bacilli or cocci, the morphology of which was similar to LAB bacteria, were classified as potential probiotic strains.

Antibiotic susceptibility test

The antibiotic susceptibility test was conducted using the disc diffusion assay method. Fresh overnight cultures of bacterial isolates were spread onto MRS or BHI agar plates, and 13 antibiogram disks were then carefully placed on the agar plates, which were subsequently incubated at 37 °C for 24 h.

The antibiotic disks consisted of gentamycin (10 µg), cefixime (5 µg), penicillin (10 µg), chloramphenicol (30 µg), streptomycin (10 µg), erythromycin (15 µg), ampicillin (10 µg), kanamycin (30 µg), vancomycin (30 µg), ciprofloxacin (5 µg), Tobramycin (10 µg), tetracycline and clindamycin (2 µg). Finally, results were reported according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (Kook *et al.,* 2019).

Antibacterial activity of isolated strains against bacterial pathogens

To detect the LAB inhibitory properties against chosen pathogens, the well diffusion assay method was used (Chen *et al.*, 2019). Briefly, bacterial isolates cultured at 37 °C for 24–48 h were centrifuged for 10 min at 10,000 rpm, and the resulting supernatants were then separated and used against eight pathogenic bacterial strains including, *Haemophilus influenzae* (ATCC- 49247), Methicillin-resistant *Staphylococcus aureus* (MRSA) (ATCC 33591), *Escherichia coli* (ATCC 25922), *Streptococcus pneumoniae* (ATCC - 19615), *Klebsiella pneumoniae* (ATCC - 13883), *Listeria monocytogenes* (ATCC 19115), *Pseudomonas auroginosa* (ATCC 9027), *Streptococcus pyogenes* (ATCC – 49619)were obtained from the American Type Culture Collection (ATCC). After 24 h of incubation, the inhibition zones around the wells were measured. Each test was conducted in triplicate.

Results

Isolation of probiotic lactic acid bacteria

The collected saliva samples were subjected to the isolation of probiotic *lactobacilli* using an MRS medium. We found 94 colony-forming units in the collected saliva sample. Different streaking methods were employed to purify the isolates, and the morphologically distinct isolates were removed from the plates (Fig. 1 &Table 1). Further, the purified strains were designated SA1, SA2, SA3, etc.

Biochemical and morphological test

Results were shown in Table 1, all the strains were Gram-positive and catalase-negative and could grow in the presence of 3% (w/v), 4.5% NaCl (w/v), and at the high temperature of 45 °C. All the strains are rod-in shape. Sugar fermentation patterns confirmed that all the strains were fermented all the sugars that we tested except Mannose and Rhamnose. The rod-shaped isolates were likely *Lactobacillus* strains (Fig. 2 & Table 2).

Table 1. Isolation of probiotic bacteria from saliva.

S. No	Samples	Total colony forming units (CFU/mL × 10^6)	Total number of isolates
1	S1	21.02±0.24	6
2	S2	19.22±0.18	5
3	S3	20. 14± 0.87	7
4	S4	26. 06 ±0.55	9
5	S5	19.04 ±0.78	8
6	S6	21.18 ±0.66	5
7	S7	25.08±0.98	9
8	S8	23. 13± 0.35	7
9	S9	27.45± 1.2	9
10	S10	29.71 ±0.71	10
11	S11	19.33 ±0.42	8
12	S12	30.19 ±1.7	11
		Total	94

Hemolytic activity of isolated labs

Initially, we checked the hemolytic activity of isolated strains. Among the isolated 94 strains, SA1, SA-4, SA-7, SA-8, SA-19, SA-21, SA-37, SA 41, SA 42, SA48 and SA 49. No hemolytic activity was observed on the blood agar plates. The selected non-hemolytic bacteria were subjected to further study (Fig. 3).

Table 2. Biochemical examination of isolated probiotic strains.

Property	SA1	SA-4	SA-7	SA-8	SA-19	SA-21	SA-37	SA- 39	SA 41	SA 42	SA48	SA 49
	Morphological and Physiological characteristics											
Shape	Rod	Rod	Rod	Rod	Rod	Rod	Rod		Rod	Rod	Rod	Rod
Gram staining	+	+	+	+	+	+	+	+	+	+	+	+
Catalase	-	-	-	-	-	-	-	-	-	-	-	-
	Carbohydrate fermentation											
Glucose	+	+	+	+	+	+	+	+	+	+	+	+
Galactose	+	+	+	+	+	+	+	+	+	+	+	+
Maltose	+	+	+	+	+	+	+	+	+	+	+	+
Mannose	-	-	-	-	-	-	-	-	-	-	-	-
Manitol	+	+	+	+	+	+	+	+	+	+	+	+
Cellobiose	+	+	+	+	+	+	+	+	+	+	+	+
Rhamnose	+	+	+	+	+	+	+	+	+	+	+	+
L–Arabinose	-	-	-	-	-	-	-	-	-	-	-	-
Fructose	+	+	+	+	+	+	+	+	+	+	+	+
L–xylose	+	+	+	+	+	+	+	+	+	+	+	+
Sorbitol	+	+	+	+	+	+	+	+	+	+	+	+
Sucrose	+	+	+	+	+	+	+	+	+	+	+	+
Lactose	+	+	+	+	+	+	+	+	+	+	+	+
Inositol	+	+	+	+	+	+	+	+	+	+	+	+

+: Positive; -: Negative.

Antibiogram of isolated strains

All the strains were tested for their resistance activity against the standard antibiotics. All the strains expressed their different antibiotic resistance pattern. The antibiogram pattern showed that all the strains were sensitive to the antibiotic ciprofloxacin. However, it showed an effective resistant pattern against all the tested antibiotics (Table 3).

Antibacterial activity of cell-free supernatants of isolate bacteria

The antibacterial activity of the cell-free supernatants from 12 lactic acid bacterial strains was tested against selected bacterial pathogens on solid MHA plates. The cell-free supernatant from strains SA 1, SA 37, SA 21, and SA 41 exhibited the highest growth inhibitory activity against all the test pathogens.

Table. 3. Antibiotic susceptibility of isolated probiotic strains.

Antibiotic	SA 1	SA4	SA 7	SA8	SA 19	SA 21	SA 37	SA 39	SA 41	SA 42	SA48	SA 49
Gentamycin	S	S	S	S	S	S	S	S	S	S	S	S
Cefixime	R	MS	S	S	S	S	S	R	S	S	S	S
Penicillin	R	R	R	S	S	S	R	R	R	R	R	R
Chloramphenicol	S	S	S	S	S	S	S	S	S	S	S	S
Streptomycin	R	S	S	S	S	MS	S	S	S	S	S	MS
Erythromycin	S	S	S	S	S	S	S	S	S	S	S	S
Ampicillin	s	S	S	S	S	S	S	S	S	S	S	S
Ciprofloxacin	S	S	S	S	S	S	S	S	R	S	S	R
Kanamycin	s	S	S	MS	MS	MS	R	S	R	R	R	R
Vancomycin	R	S	S	S	S	S	S	S	R	R	R	R
Tobramycin	MS	S	S	S	S	S	S	S	R	S	S	S
Clindamycin	S	S	S	R	S	S	S	S	S	S	S	S

R: Resistant; S: Sensitive; MS: moderate sensitivity.

This was followed by the cell-free supernatant from strain SA 48, which showed growth-inhibitory activity against five test pathogens. The standard antibiotic, Ciprofloxacin demonstrated a zone of inhibition ranging from 20 to 28 mm (Table 4).

Table 4. Antibacterial activity of LAB from saliva.

	Zone of inhibition (mm)												
Test bacterial pathogens	SA 1	SA 4	SA 7	SA 8	SA 19	SA 21	SA 37	SA 39	SA 41	SA 42	SA 48	SA 49	Gentamicin (5 µg)
S. pyogenes	16.34±0.74	13.10±0.05	-	17.10±0.28	-	12.02±0.02	17.05±0.01	10.02±0.15		-	-	-	25.14±0.04
S. aureus	14.08±0.48	-	-	15.14±0.41	-	13.06±0.08	18.65±0.42	-	18.61±0.21	13.12±0.38	14.54±0.14	-	28.02±0.06
Streptococcus pneumoniae	18.74±0.21	-	-	-	11.12±0.72	12.08±0.18	16.36±0.76	-	16.55±0.05	13.12±0.48	-	-	27.28±0.72
Escherichia coli	15.08±0.34	13.42±0.54	11±0.06	-	-	13.54±0.04	17.28±0.44	-	15.04±0.64	-	13.08±0.38	11.34±0.58	25.25±0.44
Klebsiella pneumoniae	17.15±0.49	-	-	-	15.08±0.71	14.24±0.54	18.58±0.28	-	16.25±0.64	-	12.95±0.61	-	29.04±0.84
Pseudomonas auroginosa	15.34±0.74	13.02±0.06	11.12±0.13	16.05±0.01	11.14±0.44	13.14±0.41	15.04±0.84	12.12±0.72	13.14±0.04	15.08±0.18	16.65±0.42	11.02±0.05	23.01±0.69
Haemophilus influenzae	18.61±0.21	-	-	11.01±0.04	13.24±0.21	16.04±0.82	17.12±0.76	12.25±0.64	15.10±0.28	11.13±0.64	13.10±0.28	-	28.01±0.04
Listeria monocytogens	19±0.07	13.32±0.08	10.14±0.04	13.10±0.28	10±0.06	15.25±0.44	19.14±0.74	13.34±0.18	19.08±0.48	11.10±0.05	13.14±0.41	14.06±0.08	27.01±0.69

-: no zone of inhibition; SA: saliva. The values are expressed in the mean ± standard deviation of three replicates.

Discussion

In recent decades, probiotic research has surged due to their numerous health benefits and market demand. Researchers have actively sought out new and promising probiotic species from the human gut and salivary microbiota (Kiliç and Karahan, 2010; Vijayabharathi *et al.*, 2012; Terai *et al.*, 2015), Human-used probiotics are typically sourced from various environments, including both dairy and non-dairy sources. However, probiotics isolated from human or animal intestines exhibit distinct characteristics compared to those from dairy products. For instance, gut-isolated probiotics tend to be more resistant to high bile salt concentrations and low pH levels. Additionally, they demonstrate stronger adherence abilities than dairy-isolated probiotics. Consequently, non-dairy probiotics are promising for individuals with lactose intolerance (Sornplang and Piyadeatsoontorn, 2016; Sardana *et al.*, 2018). Traditional probiotics have a rich history of global use.

Oral probiotics provide an indirect yet holistic approach to restoring microbial balance, promoting

oral health while minimizing adverse effects (Nguyen *et al.*, 2021). On the other hand, a healthy oral cavity increases the likelihood of discovering beneficial strains specifically adapted to the target site, effectively suppressing the growth of pathogens. This is supported by the isolation of several oral probiotic LAB from the oral cavity (Bosch, *et al.*,2012, Strahinic *et al.*, 2007; Azizian *et al.*, 2019). About 94 LABs were isolated from different saliva samples; 12 showed non-hemolytic activity.

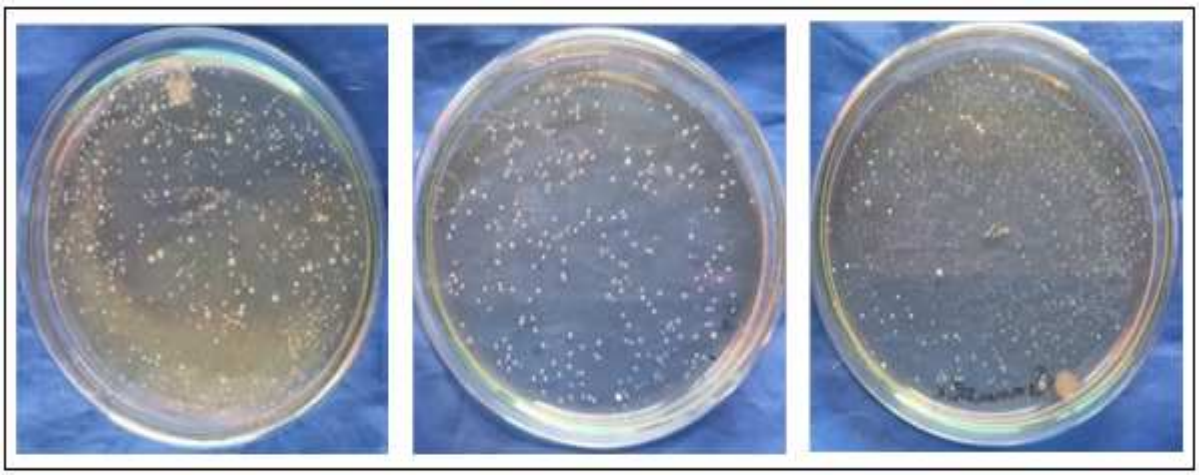

Fig. 1. Isolation of Lactic acid bacteria from collected saliva samples. The individual colonies of saliva-associated probiotics on MRS agar plates.

It is well known that non-haemolysis is an important characteristic of the probiotic application of bacteria. Accordingly, our strains showed non-hemolysis on blood agar plates. A crucial criterion for a suitable probiotic candidate is the absence of antibiotic resistance. In line with existing literature, this study revealed that nearly all isolates exhibited resistance to penicillin, except for SA-8, SA-19, SA-21, which showed sensitivity to penicillin. One possible explanation for these findings is the widespread use of antibiotics. Interestingly, some strains were also resistant to vancomycin and kanamycin.

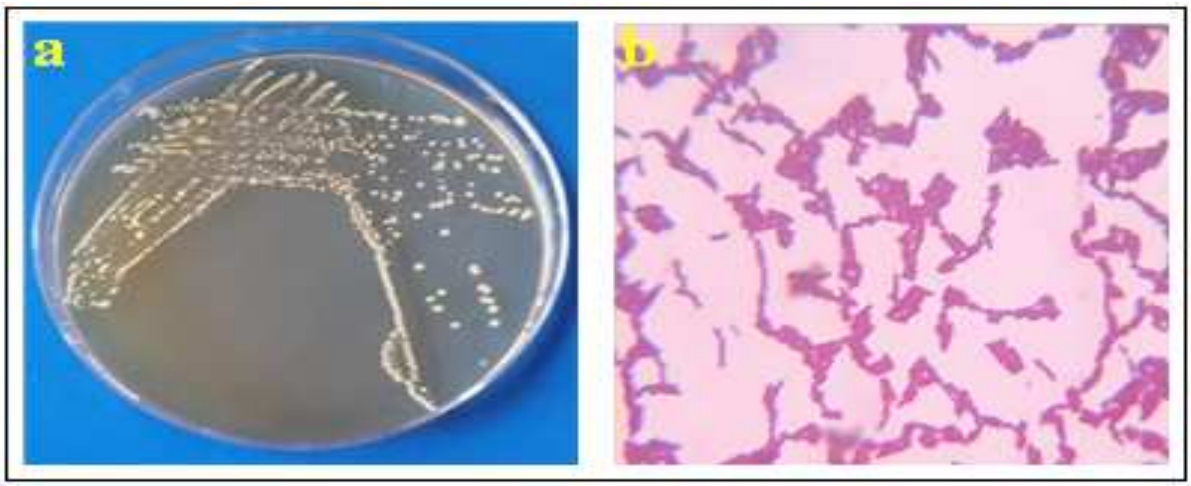

Fig. 2. Morphological characterization of *Lactobacilli* sp. a) Purification of isolated probiotics by quadrant streaking method. b) identification of isolated probiotics by gram staining.

This aligns with previous reports that highlight vancomycin resistance as an intrinsic trait in LAB, including *Lactobacillus*, *Leuconostoc*, and Pediococcus. Notably, many *Lactobacillus* strains, including *L. fermentum*, are commonly employed in the food industry. The vancomycin resistance

observed in this bacterial group is encoded in their chromosomes and is neither transferable nor inducible (Swenson *et al.*, 1990; Tynkkynen *et al.*, 1998; Sharma *et al.*, 2014). In our study, LAB supernatants exhibited inhibitory effects against a range of pathogens, including *H. influenzae*, Methicillin-resistant *S. aureus*, *E. coli*, *S. pneumoniae*, *K pneumoniae*, *L.monocytogenes*, *P. auroginosa*, *S. pyogenes*. These findings emphasize the importance of the selected strains in our studies, as they tend to show broad-spectrum antimicrobial activities *H. influenzae, L.*monocytogenes, *P. auroginosa*. While the extensive antimicrobial effects of LABs primarily stem from organic acid production, we cannot discount the potential activity of antimicrobial peptides and other metabolites produced by these strains (Kivanç *et al.*, 2011; Somashekaraiah *et al.*, 2019).

Fig. 3. Hemolytic activity of isolated probiotics strains on the blood agar plates.

Overall, about 12 potential probiotics with multifaceted attributes were identified as potential probiotics. Following phenotypic characterization, these strains were identified as belonging to the *Lactobacillus* genus. Their unique features make them valuable for the pharmaceutical, cosmetic, and food industries. Additionally, this study highlights human saliva as a promising source of novel probiotics with desirable functional properties.

Conclusion

In this study, we isolated the lactic acid probiotic bacteria from the saliva samples. Based on the microscopic and biochemical analysis, the isolated non-hemolytic strains belong to the *Lactobacillus* genus. Furthermore, the strains were tested for their antibiogram patterns, and the results indicate that all the strains were sensitive to the antibiotics Gentamycin, Chloramphenicol, Erythromycin, Ampicillin and resistant to the other antibiotics. Therefore, they can be considered promising "next-generation" probiotic candidates useful to the pharmaceutical industry.

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Conflict of interest

The authors declare no conflict of interest.

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