

Characterization of *Lactobacillus brevis* with Potential Probiotic Properties and Biofilm Inhibition against *Pseudomonas aeruginosa* [†]

Vaishali Singh, Suman Ganger and Shweta Patil *

Department of Microbiology, Vivekanand Education Society's College of Arts, Science and Commerce, Mumbai 400071, Maharashtra, India; singhvaishali268@gmail.com (V.S.); suman.ganger@ves.ac.in (S.G.)

* Correspondence: shweta.patil@ves.ac.in

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Abstract: (1) Background: Probiotics are a live microbial supplement that improve hosts' health by maintaining intestinal microbiota. The evidence suggests that probiotics can be used as a therapeutic strategy to improve overall digestive health. Lactic acid bacteria strains have been extensively used as probiotics. (2) Method: To isolate lactic acid bacteria with probiotic potential from food samples. Probiotic properties such as tolerance to low pH, bile, sodium chloride, lysozyme, antibiotic susceptibility, cell surface hydrophobicity, and antimicrobial activity were determined. (3) Results: Ten different isolates were examined to study their probiotic potential. In this study, *Lactobacillus brevis* was isolated and showed most of the probiotic properties, such as 10% sodium chloride tolerance, 1% bile tolerance, growth in pH 2, and antimicrobial activity against *E. coli*, *S. aureus*, *K. pneumoniae*, and *P. aeruginosa*. Formation of biofilm by *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* was also inhibited by cell free extracts of *L. brevis*, which reveals its therapeutic relevance. In addition, it was found to be stable at low temperature (4°C). (4) Conclusion: The above-mentioned results of *L. brevis* suggest that it has promising potential to be considered "probiotic". Further in vivo assessments could be carried out that would provide its dual role of prevention as well as use in therapy.

Keywords: lactic acid bacteria; probiotics; *Lactobacillus brevis*; probiotic properties; antibiofilm activity; *Pseudomonas aeruginosa*

1. Introduction

The word "probiotic" originated from the Greek *pro-bios*, which means "for life" [1]. Probiotics are microbial cell preparations or components of microbial cells. When administered in adequate amounts, they confer a beneficial effect on the health and well-being of the host. A good probiotic agent needs to be non-pathogenic, non-toxic, and resistant to gastric acid; adhere to gut epithelial tissue; and produce antibacterial substances [2].

Since ancient times lactic acid bacteria (LAB) strains have been used as probiotics because of their health-promoting effects in the host [3]. These organisms have been widely reported to exert many beneficial effects, such as activation of the immune system, prevention of cancer cell growth, maintenance of mucosal integrity, and presentation of an antagonistic environment for pathogens [4]. The inhibitory mechanisms of probiotics include production of antimicrobial compounds such as lactic acid, bacteriocin, cytokines, and butyric acid [5,6]. Lactic acid bacteria (LAB) are characterized as gram-positive cocci or rods, aerotolerant, and able to ferment carbohydrates for energy with the

production of lactic acid [7]. Nowadays, attention is given to obtaining new probiotic bacterial strains from traditional foods and pharmaceutical industries. Globally, interest has developed in the consumption of functional foods or nutraceuticals with potential probiotic microorganisms, and the estimation of the global market is above USD 28.8 billion [8].

Lactobacillus brevis is a heterofermentative gram-positive organism that can be isolated from milk, cheese, sauerkraut, sour dough, silage, and the mouth and intestinal tract of humans [9]. Due to its long-term use in various traditionally fermented food products, *L. brevis* has Generally Recognized As Safe (GRAS) status. *L. brevis* is not typically used in probiotic products, although O'Sullivan et al. (1992) [10] and Collins et al. (1998) [11] have mentioned *L. brevis* in a list of strains currently used in probiotic products. Furthermore, Kishi et al. (1996) [12] reported *L. brevis* strain as a potential probiotic. *Pseudomonas aeruginosa* is a significant cause of infections, especially in immunocompromised hosts. It has numerous virulence factors and shows high resistance to antibacterial agents. It has natural resistance to many drugs, and has an ability to form biofilms, complex adherent structured microbial communities, etc. [13]. Additionally, *Pseudomonas* is also well known food- and milk-spoilage microorganism. Hence, the use of probiotics against antibiotic-resistant and food spoilage microorganisms has been considered recently as an alternative to antibiotics and certain chemical preservatives of food spoilage [13, 14]. Also, *Klebsiella pneumonia* is one of the main causes of urinary tract infections (UTI), pneumonia, intra-abdominal infections, etc. Due to their biofilm formation, they are more recalcitrant to antibiotics. *Lactobacilli* strains have been extensively studied in this regard, as well, because of their remarkable ability to inhibit the growth of pathogenic bacteria by producing bactericidal compounds [15].

The aim of this study is to screen, isolate, and identify lactic acid bacteria from food samples, and to characterize the probiotic potential of the isolates and determine the biofilm inhibition activity formed by *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*.

2. Materials and Methods

2.1. Sample Collection and Isolation of Lactic Acid Bacteria

Samples like milk, yogurt, buttermilk, idli batter, and sauerkraut were collected in a sterile container, stored at 4°C, and plated out within 24 h. Samples were isolated on Rogosa SL agar medium and plates were incubated under low oxygen conditions, at 27 °C for 24–48 h [16]. Single discrete colonies were picked from the Rogosa SL agar plate and preliminary identification was carried out by performing gram staining and physiological and biochemical (catalase, oxidase, indole, methyl red, voges proskauer, citrate utilization, triple iron sugar) tests, including sugar fermentation and gas production and isolation on Homofermentative and Heterofermentative Differential (HHD) medium, referring to *Bergey's Manual of Systematic Bacteriology, Volume 2* [17]. After confirmation of lactic acid bacteria, the cultures were kept in Man, Rogosa and Sharpe (MRS) agar slant and stored at 4°C.

2.2. Determination of Tolerance to Sodium Chloride, Bile Salt, Low pH, and Lysozyme

For determination of sodium chloride tolerance, all the isolates were grown in MRS broth supplemented with different concentrations of sodium chloride ranging from 2% to 10% [18]. The ability of the strains to tolerate bile salts was examined by observing the optimum growth by inoculating various isolates separately into MRS broth tubes supplemented with 0.2–1% bile salts (sodium taurocholate) [19]. For determination of growth at low pH, the isolates were inoculated into MRS broth with varying pH ranging from 2.0 to 6.0 [20]. The broths were inoculated with an overnight culture of the isolates and incubated at low oxygen conditions at 27 °C for 24–48 h, and bacterial growth was monitored by measuring the absorbance at 600 nm. For lysozyme tolerance, cells were suspended in 60 mM phosphate buffer (pH 6.2) supplemented with 100 µg/mL of lysozyme and kept for 24 h. The degree of cell lysis was measured spectrophotometrically by calculating the change in absorbance at 620 nm [21].

$$\% \text{ of survival rate} = [\text{Absorbance after 24 h} / \text{Absorbance at 0 h}] \times 100 \dots \quad (1)$$

2.3. Antibiotic Susceptibility Test

Antibiotic susceptibility testing was performed using the agar disk diffusion method. Antibiotics discs (Hi Media) were placed on MRS agar plates swabbed with 24 h grown cultures. Antibiotic discs such as ampicillin, ciprofloxacin, azithromycin, amikacin, chloramphenicol, gentamicin erythromycin, tetracycline, and vancomycin and were selected with known concentrations based on reviews of literature. The zones of inhibition were measured after incubation at a low oxygen level at 27 °C for 24 h [20].

2.4. Antimicrobial Activity

Antimicrobial activity of the isolates was determined by the scrape and streak method [22] using indicator strains such as *Staphylococcus aureus*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, and *Klebsiella pneumonia* and incubated for 18–24 h at 37°C. Inhibition of the indicator strains was recorded near the streak of the original strain.

2.5. Cell Surface Hydrophobicity

The in vitro cell surface hydrophobicity was determined by the bacterial adherence to a hydrocarbon assay using the method of S. Klayruang et al. [23]. Hydrophobicity was calculated as the percentage decrease in the optical density of the initial aqueous bacterial suspension due to cells partitioning into a hydrocarbon layer. The percentage of cell surface hydrophobicity (% H) of the strain adhering to n-Hexane was calculated using the equation:

$$\% H = [(A_o - A) / A_o] \times 100 \quad (2)$$

2.6. Antibiofilm Activity

Crystal violet (tube method) was carried out for assessing the biofilm inhibition using *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*. The culture tube without cell free supernatant served as control. The results were expressed as the percentage of biofilm inhibition [24].

$$\% I = [(\text{Absorbance control} - \text{Absorbance sample}) / \text{Absorbance Sample}] \times 100 \quad (3)$$

where %I = percentage of biofilm inhibition.

2.7. Stability at Two Different Temperatures

The stability of the selected strain was studied by exposing it to two temperatures (4 °C and 27 °C) and evaluating the viability of the cells by the pour plate method into MRS media.

2.8. Stability in Ice Cream

Ice cream was purchased locally and was used to study the stability of the selected isolate. Ice cream was melted by keeping it at room temperature, into which the isolate was inoculated. After homogenization, the day zero count was determined by the pour plate method into MRS agar. Ice cream was kept in the refrigerator for 7 days at 4 °C, after which the viability of the cells was determined and compared with the initial count.

3. Results

3.1. Screening, Isolation, and Identification of Lactic Acid Bacteria

Ten isolates, namely, MI, MII, BMI, SII, SIV, SIV2, IB1, IB2, IB3, and YK, were selected for their probiotic potential. All isolates were Gram positive and the morphology observed was short rods, cocci in clusters, and rods. In addition, the isolates were catalase, oxidase, and IMViC test negative.

Six isolates, MI, BMI, IB1, IB2, IB3, and YK, were found to be homofermentative whereas four isolates were heterofermentative.

3.2. Tolerance to Sodium Chloride, Bile Salts, Low pH, and Lysozyme

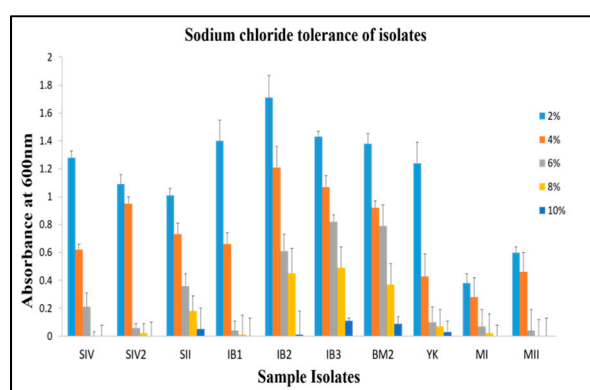
A comparison analysis of all isolates is shown in Figure 1a,b. The survival rates of selective isolates SIV2, SII, and IB2 for 100 µg/mL of lysozyme were 49.50%, 58.00%, and 77.00%, respectively. Out of the 10 isolates that were studied for their probiotic properties, isolate SII matched with probiotic criteria to a promising extent. It was able to tolerate 10% NaCl and grow at pH range 2.0–6.0.

3.3. Antibiotic Susceptibility Test

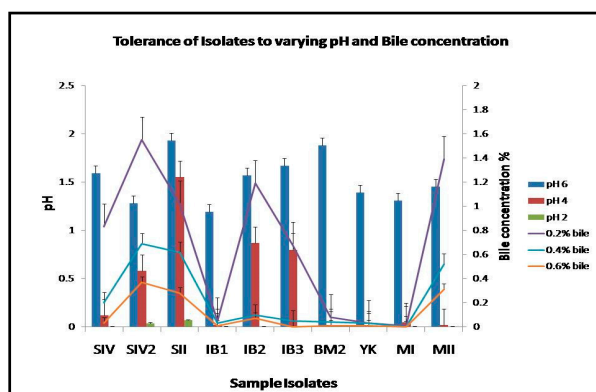
Isolates showed different susceptibility patterns. Isolate SIV, SIV2, SII, IB2, and IB3 were susceptible to ampicillin (10 µg), and all were resistant to vancomycin (30 µg). SII isolate showed resistance towards amikacin (30 µg), ciprofloxacin (5 µg), gentamicin (10 µg), and vancomycin (30 µg) and much less sensitivity towards ampicillin (10 µg) and tetracycline (30 µg) at about 3–5 mm zone of inhibition.

3.4. Antagonistic Activity Against Pathogens

Isolate SII showed inhibitory effects towards all indicator strains such as *E. coli*, *K. pneumoniae*, *S. aureus*, and *P. aeruginosa*, suggesting some antimicrobial compounds were produced that inhibited the growth of indicator strains. This method had an advantage over other methods such as agar well diffusion assay, as some antimicrobial compounds are produced in very small quantities and may require more quantity to diffuse properly into the medium.



(a)



(b)

Figure 1. (a) Sodium chloride tolerance of isolates at different concentrations and (b) bile salt and pH tolerance of isolates at different concentrations of bile and pH values, respectively.

3.5. Cell Surface Hydrophobicity

The hydrophobicity percentage of the isolate SII to n-hexane was found to be 5.03%.

3.6. Antibiofilm Activity of Isolate SII

It was observed that tubes with cell free extracts of SII had the potential to inhibit biofilm formation. A maximum biofilm inhibition of 52.63% of *Pseudomonas aeruginosa* was observed, whereas on *Klebsiella pneumoniae* biofilm the inhibition activity was found to be 22.2%.

3.7. Stability of *L. brevis* at Two Different Temperatures

The initial day count of *L. brevis* kept at 4 °C and 27 °C was found to be 2.89×10^7 cfu/mL. After 7 days, the count of the isolate kept at 27 °C was found to be zero, whereas a 56.13% reduction in the viability of *L. brevis* kept at 4 °C was observed. This suggests that the isolate was more stable at 4 °C than at 27 °C.

3.8. Stability of *L. brevis* in IceCream

The DAY 0 count of *L. brevis* was found to be 4.3×10^5 cfu/mL and DAY 7 cell viability was found to be 3.1×10^5 cfu/mL. This suggests that *L. brevis* is more stable in dairy products, which are stored at lower temperatures, with a 27.90% reduction in viability.

3.9. Molecular and Genetic Analysis of SII

Using the 16S rRNA technique, an identification of isolate SII was carried out at Saffron Lifescience Antalia, Navsari (Gujrat) and was found to be *Lactobacillus brevis* ATCC 14869—Accession no. NR_116238.1

4. Discussion and Conclusions

Ten isolates were evaluated for their probiotic potential, out of which one isolate SII gave encouraging results and was considered for further evaluation of antibiofilm activity, stability testing, etc.

In the present study, *Lactobacillus brevis* ATCC 14869 was isolated from a sauerkraut sample, which was identified by 16S rRNA sequencing. *L. brevis* survival and growth at low pH (pH 2–6) and in the presence of bile (0.2–1%) (*w/v*) suggest that it can survive in extreme conditions of the intestinal tract. Being resistant to low pH is one of the major selection criteria for probiotic strains [25]. Regarding bile salt tolerance of probiotic lactobacilli, their survival in the presence of 0.3% bile salt is physiologically significant, since the bile salts at such a level are present normally in the human intestine [26]. *L. brevis* could tolerate 1–10% (*w/v*) NaCl concentration. NaCl adapted isolates can help to survive in simulated gastric fluid. Probiotic bacteria are exposed to saliva in the oral cavity, which contains lysozyme and electrolytes, and thus lysozyme tolerance is one of the criteria in probiotic selection.

L. brevis showed resistance towards a broad range of antibiotics, suggesting that it can be used in combination with antibiotics for treatment of certain infections, which may aid in rapid recovery of the healthier microbial balance. The results were as expected, as lactobacilli are known to be naturally resistant toward several antibiotics [27]. *L. brevis* showed antimicrobial activity against some of the indicator strains, indicating that they produce antimicrobial compounds [28].

Biofilm production by *P. aeruginosa* is considered an important determinant of its pathogenicity. A maximum biofilm inhibition of 52.63% of *Pseudomonas aeruginosa* was observed. Antimicrobial compounds in the cell free supernatant were believed to halt the growth of the pathogen and even cause death in the cells, rendering the aggregation of cells to form the biofilm unsuccessful. Thus, identification of such LAB strains that have antibiofilm activity would be essential to include as alternatives to the control of biofilms [29]. In a study reported by D Shorkri et al. [13], inhibitory effects of lactobacilli against *P. aeruginosa* and their biofilm formation were investigated.

Cell surface hydrophobicity of *L. brevis* was about 5.03%. Lack of correlation between hydrophobicity and bacterial adhesion has been reported where strains with high hydrophobicity exhibited lower adhesion to a human cell line [30]. Thus, hydrophobicity may be helpful in adhesion, but it is obviously not a prerequisite for a strong adherence capacity. The stability of *L. brevis* was found to be higher in lower temperatures as well as in dairy products such as ice cream, which is stored 4 °C, compared to other temperatures. In a study, it was reported that *L. brevis* ATCC 8287 was a promising strain as a probiotic supplement in dairy products [28]. Thus, from the in vitro methods performed and results discussed it is suggested that *L. brevis* ATCC 14869 has the promising potential to be considered a “probiotic,” and to the best of our knowledge there is no work reported with *L. brevis* ATCC 14869 as a probiotic. Hence, it can be an additional strain that can be considered for probiotics. However, further in vivo trials are needed to determine whether it benefits human health.

A key conclusion from this research is that potential probiotic candidates can be isolated from dairy food, fermented vegetables, etc., and they show a promising potential for further development as a novel probiotic. In future, research efforts can be carried out to exploit other potential probiotic properties of these strains so that they can be used in the medical field and their products can be used in the pharmaceutical industry.

Author Contributions: S.G. and S.P. conceived the presented idea. V.S. carried out the experiment. S.G. and S.P. verified the analytical methods and supervised the findings of this work. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare that there are no conflicts of interest.

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