https://doi.org/10.22491/2357-9730.153709

Original article

Comparative evaluation of alternative therapies against oral opportunistic microorganisms: an *in vitro* study

Regis de Souza Valentim[1], Jullierme de Oliveira Morais[1], Ana Beatriz Costa Almeida[1], Edine Maria de Medeiros Campos Pinheiro[1], Régia Carla Medeiros da Silva[1], Francisco Canindé de Sousa Júnior[2], Maria Raquel Cavalcanti Inácio[2], Ítalo de Macedo Bernardino[1], Ana Rafaela Luz de Aquino Martins[1], Silvana Maria Zucolotto[2], Maria Regina Macedo Costa[1], Ruthineia Diógenes Alves Uchôa Lins[1].

ABSTRACT

Introduction: Opportunistic Gram-negative microorganisms such as *Acinetobacter baumannii*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa* may colonize the oral cavity and contribute to hospital-acquired infections, particularly in critically ill patients. Alternative strategies to chlorhexidine have been investigated for controlling these pathogens. **Methods:** This *in vitro* experimental study was conducted at the Federal University of Rio Grande do Norte. Strains of *A. baumannii*, *K. pneumoniae*, and *P. aeruginosa* were tested against the probiotic *Lacticaseibacillus rhamnosus* (PLR), antimicrobial photodynamic therapy (aPDT; methylene blue + 660 nm laser), and the hydroethanolic extract of *Spondias mombin* L. (SME), using 0.12% chlorhexidine (CHX) as a positive control. Outcomes included bacterial growth inhibition, anti-adherent activity, and antibiofilm effects. Data were analyzed using ANOVA and Tukey's test ($p < 0.05$). **Results:** PLR inhibited all tested microorganisms and showed no significant difference compared with CHX for *A. baumannii* and *K. pneumoniae* ($p > 0.05$). aPDT demonstrated greater anti-adherent activity against *K. pneumoniae* ($p < 0.05$), whereas PLR showed superior performance against *P. aeruginosa* ($p < 0.05$), both comparable to CHX. Significant differences were observed among groups in monospecies biofilms ($p < 0.05$), although PLR did not differ from CHX. In multispecies biofilms, PLR exhibited activity equivalent to the positive control. **Conclusions:** PLR and aPDT effectively controlled microbial growth, with PLR demonstrating antimicrobial, anti-adherent, and antibiofilm effects comparable to chlorhexidine, supporting its potential clinical application.

Keywords: *Microbiology; Complementary Therapies; Probiotics; Photochemotherapy; Lacticaseibacillus rhamnosus.*

Clin Biomed Res. 2026;46:1-10

1 Departamento de Odontologia, Universidade Federal do Rio Grande do Norte (UFRN), Natal, Rio Grande do Norte, Brazil.

2 Departamento de Farmácia, Universidade Federal do Rio Grande do Norte (UFRN), Natal, Rio Grande do Norte, Brazil.

Corresponding author:
Regis de Souza Valentim
rsv1989@hotmail.com
Departamento de Odontologia, Universidade Federal do Rio Grande do Norte
R. Senador Salgado Filho, nº1787
CEP 59078-970, Natal, RN, Brazil

BACKGROUND

The U.S. Centers for Disease Control and Prevention (CDC) estimates that antimicrobial resistance causes at least 2.8 million infections and 35,900 deaths annually in the United States[1]. According to the 2019 global report, deaths caused by pathogenic bacteria are expected to rise to 10 million per year by 2050 if no effective measures are taken[2]. This growing threat is particularly critical for vulnerable populations such as the elderly and immunocompromised individuals, whose immune defenses are suppressed or impaired[1]. Opportunistic microorganisms, and especially superinfecting agents, can exacerbate oral infections and often exhibit resistance to broad-spectrum antimicrobials commonly used in clinical practice[3]. These pathogens are found in both community and healthcare settings, and when infections occur during hospitalization, they are classified as nosocomial infections. The most common forms include surgical site, respiratory tract, bloodstream, and urinary tract infections[4].

In hospitalized patients, the oral microbiota undergoes rapid and significant alterations, with a shift from Gram-positive bacilli to predominantly Gram-negative species within 48 hours of admission[5]. Dental biofilm acts as a natural reservoir for these bacteria, contributing to their persistence and dissemination[6]. This scenario highlights antimicrobial resistance as a major challenge for healthcare professionals, with limited antibiotic options leading to therapeutic failure, prolonged hospital stays, higher treatment costs, and increased morbidity and mortality[5,7-9].

The increasing prevalence and complexity of multidrug-resistant microorganisms have prompted the search for effective alternative therapies to combat emerging drug resistance[10]. Among the promising approaches, antimicrobial photodynamic therapy (aPDT), probiotics, and phytotherapeutic agents have demonstrated efficacy in controlling superinfecting pathogens[11-13].

Antimicrobial photodynamic therapy has emerged as a promising strategy for microbial control. This approach involves the activation of a photosensitizer by a specific wavelength of light in the presence of oxygen, generating reactive oxygen species such as singlet oxygen and free radicals. These reactive molecules promote oxidative damage to microbial DNA, proteins, lipids, and cell membranes, ultimately leading to cell death[10,14,15]. Another alternative strategy involves the use of probiotics, defined as beneficial live microorganisms capable of inhibiting pathogen adhesion, colonization, and biofilm formation through mechanisms such as competition for binding sites, production of antimicrobial substances (e.g., bacteriocins and organic acids), and modulation of host immune responses[16,17]. Phytotherapeutic agents have also attracted attention due to the presence of bioactive compounds such as flavonoids, alkaloids, terpenes, and polyphenols, which may exert antimicrobial activity through mechanisms including disruption of microbial membranes, interference with metabolic pathways, and inhibition of virulence factors[18-20].

Beirão et al.[11] (2014) observed that aPDT promoted the inactivation of bacterial biofilms of *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Experiments concluded that probiotic strains of *Lactobacillus acidophilus* and *Lacticaseibacillus rhamnosus* had a significant inhibitory effect against *Klebsiella pneumoniae in vitro*[13]. Plant extracts of *Solanum paniculatum L.* and *Spondias mombin L.* have also been shown to quantitatively reduce the number of microorganisms associated with infections, as reported by Aromolaran et al.[21] (2014), who found that the extract of *Spondias mombin L.* had antimicrobial effects.

Although several studies have investigated the antimicrobial potential of alternative therapies such as antimicrobial photodynamic therapy, probiotics, and phytotherapeutic agents, most of these reports have focused on their individual effects, and comparative evaluations remain limited. In this context, the present study sought to analyze and compare the antimicrobial effects of three alternative approaches: photodynamic therapy with methylene blue, probiotic *Lacticaseibacillus rhamnosus*, and hydroethanolic extract of *Spondias mombin* L. Specifically, we evaluated their inhibitory effects on microbial growth and adhesion of *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, and *Klebsiella pneumoniae*, in planktonic and biofilm models, using 0.12% chlorhexidine gluconate as the positive control.

METHODS

Study design

This *in vitro* experimental study evaluated the antimicrobial activity, antiadherence effect, and antibiofilm potential of three agents against opportunistic and superinfection-related microorganisms. The experimental groups were defined by the therapeutic type: (1) Antimicrobial Photodynamic Therapy (aPDT), (2) Probiotic *Lacticaseibacillus rhamnosus* (PLR) and (3) *Spondias mombin L.* hydroethanolic extract (SME). Two control groups were included: a positive control treated with 0.12% chlorhexidine digluconate (CHX) and a negative control without any treatment.

Microorganisms and culture conditions

Strains of *Acinetobacter baumannii* (INCQS 3583), *Klebsiella pneumoniae* (INCQS 2371), and *Pseudomonas aeruginosa* (INCQS 2796) isolated from human clinical samples were obtained from the Reference Bacterial Collection for Health Surveillance (CBRVS, FIOCRUZ-INCQS, Rio de Janeiro, Brazil) were used. One strain of each species was used in the experiments. These strains belong to a certified reference collection and have been previously characterized using phenotypic and molecular methodologies, ensuring reliability and traceability. Regarding their antimicrobial susceptibility profiles, no antimicrobial susceptibility data were available for *Acinetobacter baumannii* (INCQS 3583), while *Klebsiella pneumoniae* (INCQS 2371) is as an extended-spectrum beta-lactamase (ESBL)-producing strain, and *Pseudomonas aeruginosa* (INCQS 2796) exhibits resistance to third- and fourth-generation cephalosporins and ciprofloxacin, and intermediate susceptibility to imipenem and piperacillin/tazobactam.

The microorganisms were cultured in Brain Heart Infusion (BHI) broth (HIMEDIA®, India) and subsequently plated on Mueller-Hinton agar (KASVI®, Spain). After incubation at 37 °C for 24 h, the colonies were transferred to BHI and adjusted to the 0.5 McFarland standard (≈10^6 CFU/mL) in a spectrophotometer (Biomate 3, Thermo Spectronic, USA) at 625 nm (absorbance range: 0.08–0.13; ≈10^6 CFU/mL).

Preparation of test solutions

The photosensitizer methylene blue was obtained as a ready-made 0.01% solution (SRB Pharmaceutical Products Trade, RN, Brazil). The probiotic strain *L. rhamnosus* EM1107 was provided by the Brazilian Agricultural Research Corporation (Rio de Janeiro, Brazil), and was grown in de Man, Rogosa, and Sharpe (MRS) broth under anaerobic conditions at 37 °C for 24 h, followed by subculture. After incubation, the culture was centrifuged (4000 rpm/15 min), and the cell-free supernatant (CFS) was collected and stored at −20 °C for subsequent analyses, as described by Oliveira et al.[22] (2024). The hydroethanolic extract of *S. mombin L.* was obtained by macerating the dried leaves of this plant, collected in the region of Dom Marcolino Dantas, RN, Brazil; (coordinates: RN 5°28'14" / W 35°27'37"). These were ground in ethanol:water (70:30, v/v) for seven days, followed by filtration, lyophilization and dilution in distilled water at a concentration of 1:1 (500 µg/mL), according to the methodology of Mendonça[23] (2021). Chlorhexidine 0.12% (Riohex Gard®, Brazil) was undiluted, as it represents the gold standard in oral antimicrobial control, whose efficacy could be compromised by dilution.

Antimicrobial activity against planktonic bacteria

The antimicrobial activity of the tested solutions and of CHX 0.12% (Riohex Gard®, Brazil) was evaluated by microdilution adapted from the Clinical and Laboratory Standards Institute[24] (2012), in sterile 96-well microplates (TPP®, Switzerland). Each well received 100 µL of the test solution, 100 µL of nutrient broth (Biolog®, Brazil), and 10 µL of microbial inoculum, in triplicate for each microorganism. No serial dilutions were performed, as predefined concentrations were used for all tested solutions. In the aPDT group, the wells contained methylene blue and were irradiated with a red diode laser (λ = 660 nm, 100 mW, 100 s, 100 mW/cm², 10 J/cm²). Controls included: positive (chlorhexidine 0.12%), viability (VC - culture medium + microbial inoculum), and sterility (SC - medium only). After 24 h of incubation at 37 °C, optical density was measured at 630 nm in a microplate reader (MR-96A, Mindray). Microbial inhibition percentages were calculated as the proportion of absorbance, with viability control wells set as 100% growth and test wells, including those with CHX, set as X%; higher turbidity indicated greater microbial growth[25]. The experimental layout of the 96-well plate used for the planktonic antimicrobial assay is illustrated in Figure 1.

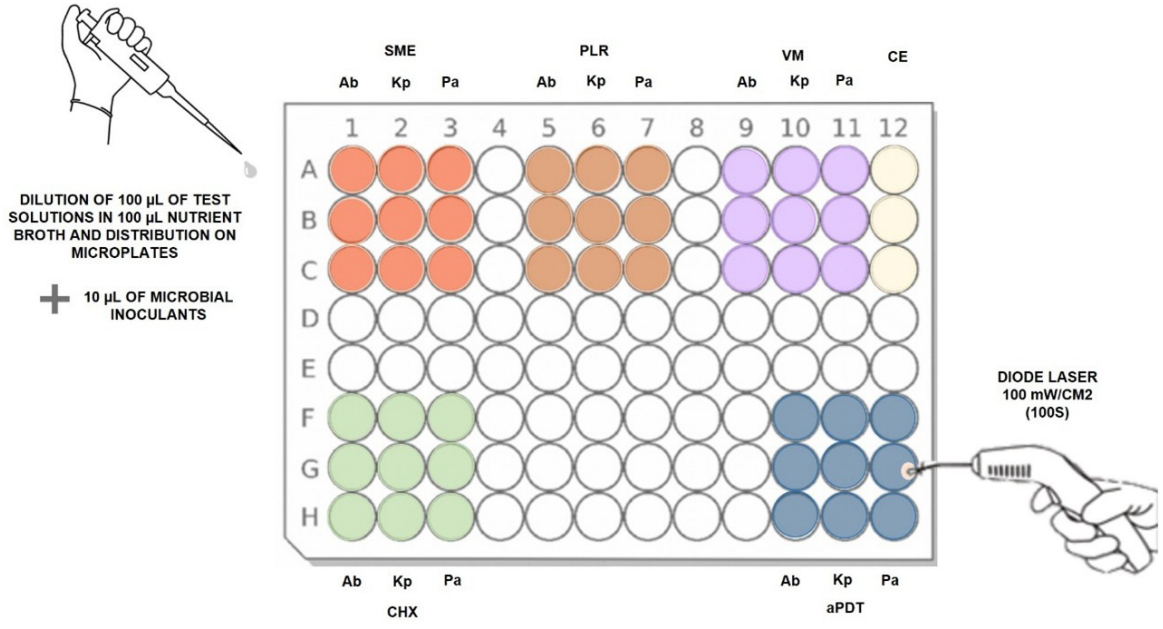

Figure 1: 96-well plate layout for planktonic antimicrobial activity.

Anti-adherence assay

The non-adherence of the test solutions was evaluated using a protocol adapted from Pieri et al.[26] (2010). Glass rods were produced from 75 mm capillaries (Perfecta®, São Paulo, Brazil), sealed, and bent at one end. Each test tube (100 × 15 mm) received 6 mL of tryptic soy broth (TSB; Ion Cult®, India) supplemented with 5% sucrose (Dinâmica®, Brazil), 0.6 mL of the test solution, and 0.1 mL of the bacterial suspension (0.5 McFarland). In the aPDT group, irradiation followed the previously described protocol. The positive control used CHX and the negative control consisted of contaminated broth without any treatment. The preparation of the glass

rods and the experimental setup of the anti-adherence assay are illustrated in Figure 2. After incubation at 37 °C for 24 h, the rods were washed in 0.9% saline solution, transferred to fresh TSB-sucrose, incubated for another 24 h, dried in a laminar flow chamber, and weighed to determine the adhered biomass, subtracting the initial weight. The procedures used for biomass recovery and measurement are illustrated in Figure 3.

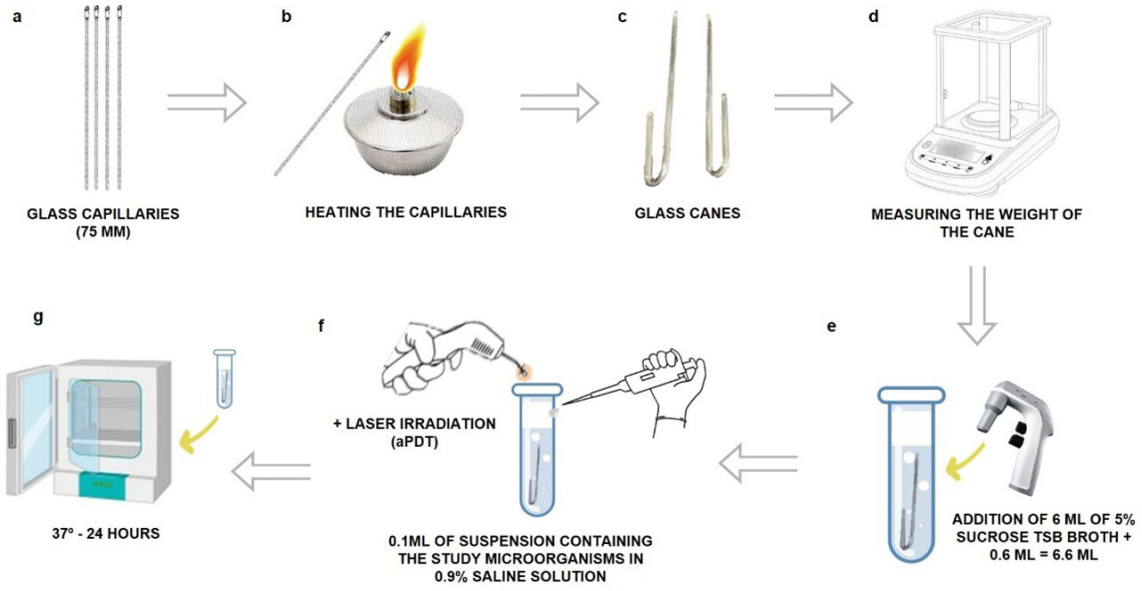

Figure 2: Preparation of glass rods and anti-adherence assay setup. (a) 75-mm capillaries; (b) heating the capillaries to seal and shape their ends; (c) final appearance of the glass rods; (d) weighing the rods on an analytical balance; (e) addition of TSB supplemented with 5% sucrose and the test solution to each tube (final volume 6.6 mL); (f) contamination of the tubes with the microbial suspension. Laser irradiation was applied to the tubes corresponding to the aPDT groups; (g) incubation of the tubes.

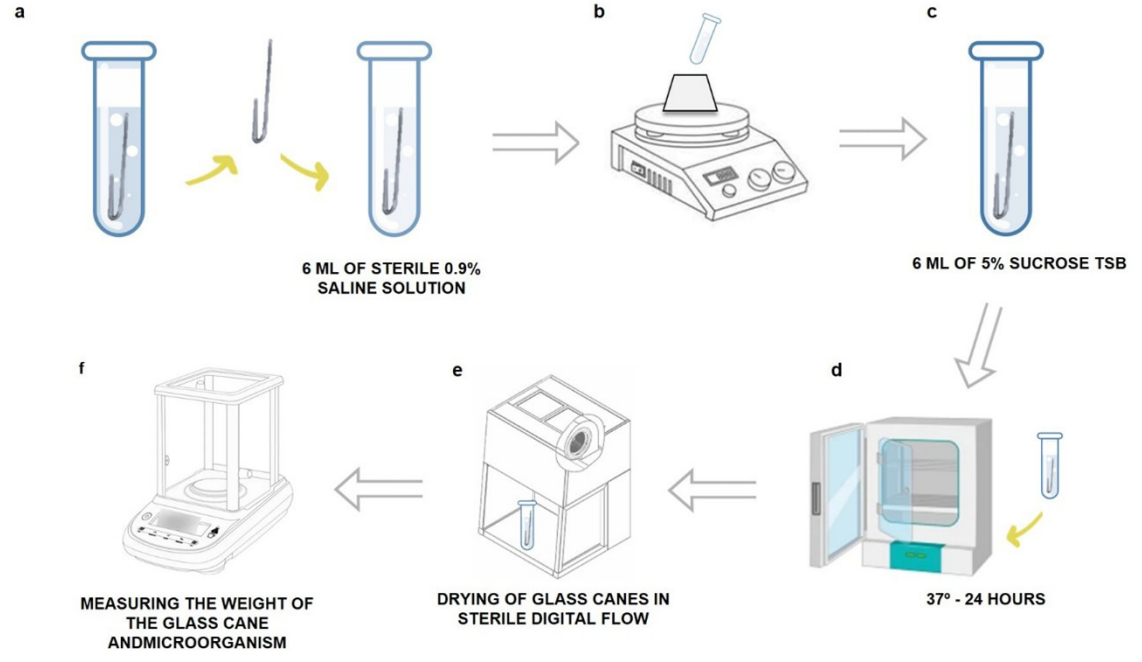

Figure 3: Anti-adherence assay reading procedure. (a) Transfer of the rods to a tube containing 6 mL of sterile saline solution; (b) agitation and removal of the solution; (c) addition of 6 mL of TSB supplemented with 5% sucrose; (d) reincubation at 37 °C for 24 h; (e) drying of the rods in a sterile laminar flow chamber; (f) weighing to determine the biomass adhered to the rods.

Antibiofilm activity on mono- and multispecies biofilms

Biofilms were grown in sterile 96-well polystyrene plates (TPP®, Switzerland) using brain heart infusion broth (BHI; HIMEDIA®, India) supplemented with 5% sucrose (Dinâmica®, Brazil). The antibiofilm activity of the test solutions and the 0.12% chlorhexidine digluconate solution (Riohex Gard®, Brazil; L: 2102840; V: 10/23) was performed against single-species and multiple-species biofilms of the microorganisms in this study, considering the technique proposed by Stepanovic et al.[27] (2000), with adaptations.

For monospecies biofilms, 100 µL of microbial suspension (1 × 10^6 CFU/mL; 0.5 McFarland, absorbance 0.08–0.13) was added to each well (Figure 4). For multispecies biofilms, equal concentrations of the three microorganisms were combined (Figure 5). Plates were incubated for 24 h at 37 °C.

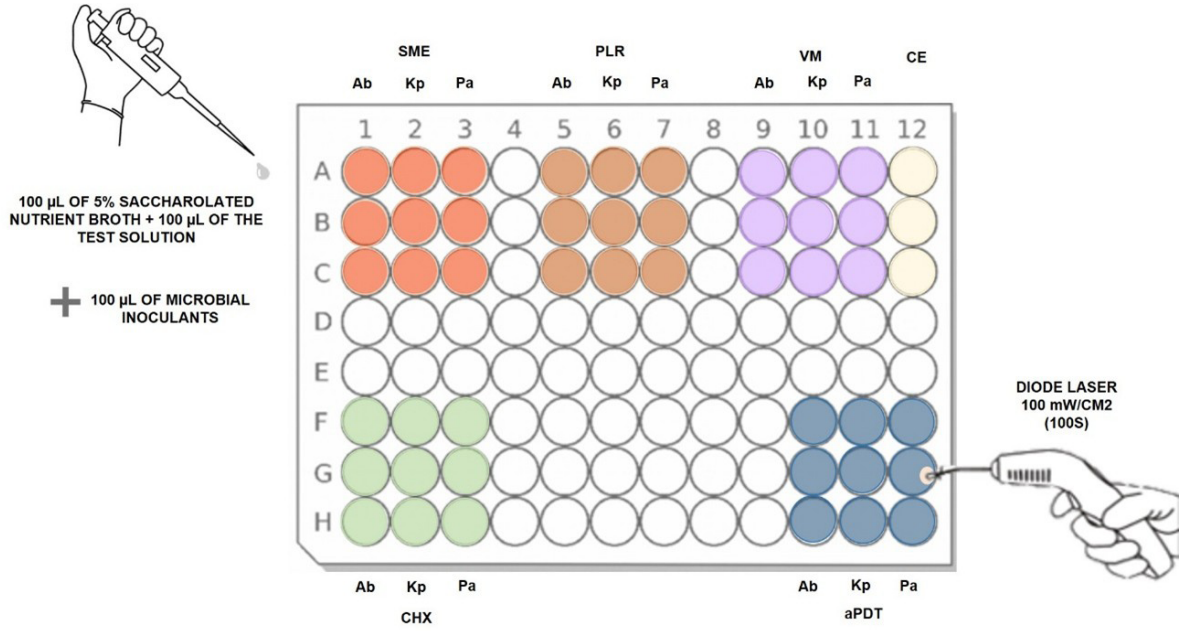

Figure 4: 96-well plate used for monospecies antibiofilm assay.

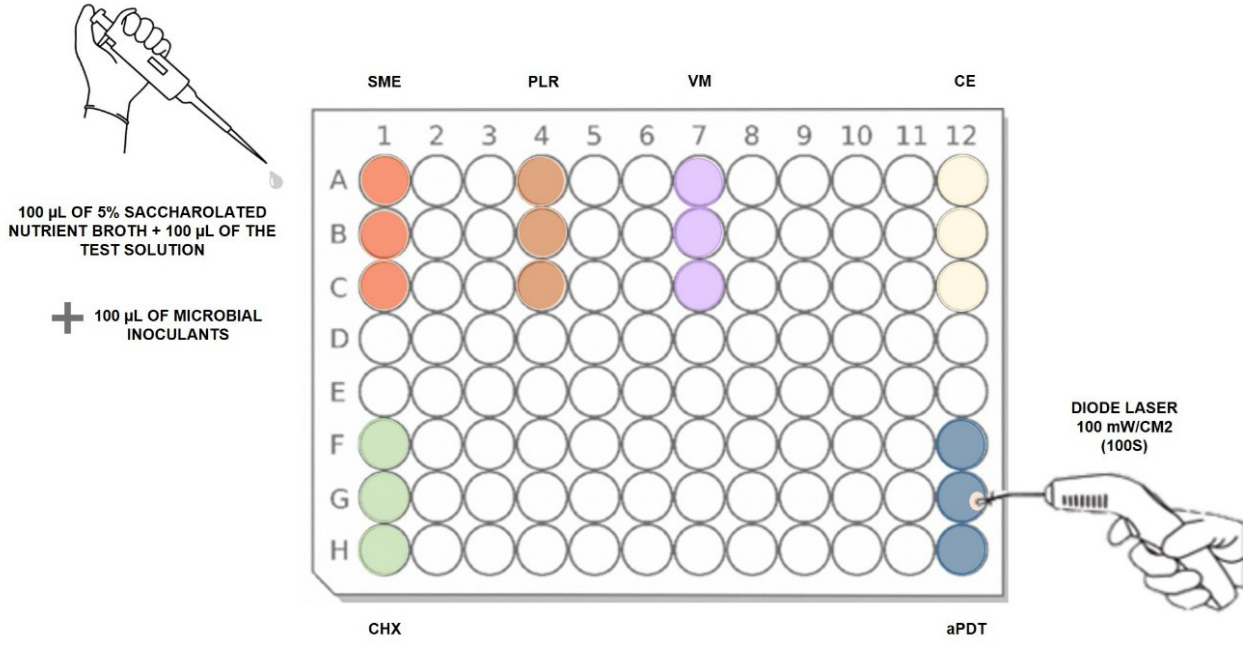

Figure 5: 96-well plate used for multispecies antibiofilm assay.

After incubation, 100 µL of each test solution diluted in 100 µL of 5% sucrose broth was added in triplicate. The aPDT group was irradiated as previously described. Control groups included chlorhexidine (positive control), untreated inoculum (viability control), and sterile medium (sterility control).

Following treatment, the plates were washed twice with 0.9% saline solution, dried at 37 °C for 45 min, and stained with 0.4% crystal violet for 45 min. Excess stain was removed by washing four times with distilled water, and the plates were air-dried. Subsequently, 95% ethanol was added for 45 min to solubilize the bound dye. Finally, 100 µL of the resulting solution was transferred to a new plate and absorbance was measured at 630 nm using a microplate reader (MR-96A, Mindray). Absorbance values were compared with those obtained for chlorhexidine.

Statistical analysis

Descriptive statistics were used to summarize the data through measures of central tendency and dispersion. Normality was assessed, and comparisons were performed using one-way analysis of variance (ANOVA), followed by Tukey's post hoc test. A significance level of $p < 0.05$ was adopted. All analyses were conducted using IBM SPSS Statistics version 25.0, with a 95% confidence interval.

RESULTS

Microbial growth inhibition activity

Data obtained showed statistically significant differences ($p < 0.001$) in the percentages of microbial growth inhibition between the experimental groups for all bacterial species evaluated. Among the three therapies analyzed, PLR demonstrated significant inhibitory effects between species ($p = 0.006$), being more effective against *A.baumannii* and *K. pneumoniae.* CHX also showed a significantly different inhibitory effect between species ($p = 0.043$) (Table 1).

Table 1: Comparative analysis of microbial growth inhibition (%).

Experimental group		*A. baumannii*	*K. pneumoniae*	*P. aeruginosa*	p-value
SME	**Mean**	0.00Aa	0.00Aa	0.00Aa	–
	SD	0.00	0.00	0.00	
PLR	**Mean**	83.62Bb,c	85.58Bc	71.52Ab	0.006*
	SD	4.57	2.21	3.63	
aPDT	**Mean**	51.31Ab	43.14Ab	0.00Aa	0.061
	SD	25.21	28.97	0.00	
CHX	**Mean**	91.97A,Bc	94.39Bc	85.66Ac	0.043*
	SD	1.97	3.83	3.79	
Medium + MO	**Mean**	–	–	–	–
	SD	–	–	–	
p-value		< 0.001*	< 0.001*	< 0.001*	

Anti-adherence effect

Significant differences between the antiadherence effect in the different experimental groups were observed only for the bacterium *K.pneumoniae* ($p = 0.028$). Treatment with PLR was the only one capable of significantly reducing bacterial adhesion, especially against the species *P. aeruginosa* ($p = 0.017$) (Table 2).

Table 2: Comparative analysis of bacterial adherence (mg).

Experimental group		*A. baumannii*	*K. pneumoniae*	*P. aeruginosa*	p-value
SME	**Mean**	2.45Aa	5.45Aa,b	3.25Aa	0.076
	SD	0.64	0.71	1.06	
PLR	**Mean**	3.75Aa	3.95Aa,b	0.75Ba	0.017*
	SD	0.92	0.21	0.07	
aPDT	**Mean**	1.50Aa	2.05Aa	2.65Aa	0.804
	SD	1.70	0.35	2.33	
CHX	**Mean**	0.90Aa	1.50Aa	1.10Aa	0.695
	SD	0.57	0.57	0.85	
Medium+ MO	**Mean**	3.55Aa	7.00Ab	3.65Aa	0.296
	SD	2.33	2.55	0.64	
p-value		0.314	0.028*	< 0.209*	

Antibiofilm potential

The analysis of the antibiofilm potential revealed statistically significant differences between the effects of the treatments for the bacterial species *A. baumannii* ($p < 0.001$), *K. pneumoniae* ($p = 0.003$) and *P. aeruginosa* ($p = 0.005$). The biofilm absorbance in the control group was significantly higher compared to the other groups, indicating a greater biofilm formation capacity. In this group, the biofilm formation capacity varied between species ($p = 0.004$), with *P. aeruginosa* obtaining the densest biofilm (Table 3).

Table 3: Comparative analysis of absorbance (nm) in monospecies biofilms.

Experimental group		*A. baumannii*	*K. pneumoniae*	*P. aeruginosa*	p-value
SME	**Mean**	0.067Ab	0.059Aa,b	0.083Aa,b	0.350
	SD	0.004	0.003	0.033	
PLR	**Mean**	0.040Aa	0.041Aa	0.041Aa	0.593
	SD	0.001	0.001	0.001	
aPDT	**Mean**	0.058Ab	0.064Ab	0.065Aa,b	0.815
	SD	0.007	0.017	0.018	
CHX	**Mean**	0.042Aa	0.041Aa	0.040Aa	0.536
	SD	0.57	0.57	0.85	
Medium+ MO	**Mean**	0.065 Aa	0.067 Ab	0.11Bb	0.004*
	SD	0.005	0.001	0.019	
p-value		< 0.001*	0.003*	0.005*	

In the multispecies analysis, the treatments were able to inhibit biofilm formation ($p < 0.05$). The probiotic and CHX achieved the same level of efficacy, with lower absorbance values (0.045), considered the most effective in reducing multispecies biofilm when compared to the others (Table 4).

Table 4: Comparative analysis of absorbance (nm) in multispecies biofilms.

Experimental group	Mean	SD
SME	0.081b	0.003
PLR	0.045a	0.002
aPDT	0.088b	0.027
CHX	0.045a	0.002
Medium + MO	0.067a,b	0.002

p-value: 0.004*

Note. Mean = average; SD = standard deviation; Medium + MO = culture medium + microorganism. One-way ANOVA followed by Tukey's post hoc test. Lowercase letters compare values vertically. *Different letters indicate statistically significant differences at $p < 0.05$.

DISCUSSION

This study demonstrated that the probiotic *Lacticaseibacillus rhamnosus* was the most effective therapy, showing inhibitory, anti-adherent, and antibiofilm activities comparable to CHX in both monospecies and multispecies biofilms. Antimicrobial photodynamic therapy also exhibited an anti-adherent effect, particularly against *K. pneumoniae*, whereas the hydroethanolic SME did not demonstrate antimicrobial efficacy under the tested conditions. Based on these findings, it is possible to critically analyze the therapeutic potential of these complementary and alternative approaches in comparison with previous evidence.

Complementary, alternative, and integrative therapies are widely used worldwide, either in combination with or as substitutes for conventional methods[28]. Currently, about 88% of the World Health Organization (WHO) member countries recognize these practices and have developed specific legislation on Complementary, Alternative, and Integrative Medicine[29]. The main motivations for their use include symptom relief, improved quality of life, and the potential to increase the effectiveness of conventional therapies, among other factors[30]. Nevertheless, as the safety and efficacy of many of these therapies remain insufficiently supported by scientific evidence, further studies are required to critically investigate their real therapeutic potential[31].

In this study, the aim was to comparatively evaluate the *in vitro* antibacterial, anti-adherent, and antibiofilm effects of different alternative therapies against opportunistic and superinfecting microorganisms from the oral environment. One of the therapies analyzed was phytotherapy, represented by the hydroethanolic SME, a plant native to the northeastern region of

Brazil, where this experiment was conducted. This inclusion was based on previous studies reporting its antimicrobial, anti-adherent, and antibiofilm activities against certain microorganisms, including opportunistic and superinfecting oral pathogens[32,33]. The tested strains included clinically relevant opportunistic pathogens with reported antimicrobial resistance profiles, such as ESBL-producing *Klebsiella pneumoniae* and resistant *Pseudomonas aeruginosa*, reinforcing the clinical relevance of the findings.

In the search for new therapeutic agents capable of controlling opportunistic and superinfecting microorganisms, studies have investigated natural substances with therapeutic potential against infections caused by these pathogens, as well as compounds that may enhance the efficacy of conventional drugs, to which many microorganisms have developed resistance[25]. In this regard, plant-derived products have been highlighted as promising sources of new bioactive molecules for the treatment of infectious diseases[34]. However, In the present study, the hydroethanolic extract of *Spondias mombin L*. did not demonstrate inhibitory activity against the microorganisms tested. This finding corroborates, in part, the data from Silva et al[35] (2012), who observed the absence of activity of the leaf extract against *Klebsiella pneumoniae* and *Pseudomonas aeruginosa,* although they identified efficacy against *Staphylococcus aureus* at concentrations of 0.78 mg/mL.

On the other hand, the results contrast with those of Aromolaran and Badejo[21] (2014), who highlighted the potential of the species in controlling several clinical isolates, including *K. pneumoniae.* However, it should be noted that these authors used significantly higher concentrations (10 to 90 mg/mL) than that used in this research. Furthermore, Freitas et al[33]. (2022) reported activity against a multidrug-resistant strain of *P. aeruginosa* at a concentration of 512 µg/mL, showing that efficacy may be specific to certain isolates. These discrepancies reinforce the possibility that the antimicrobial activity of *Spondias mombin L.* is dose-dependent and influenced by the intrinsic sensitivity of each strain, in addition to factors such as genetic variability of the pathogens and extraction protocols. These data suggest that the concentration of 500 µg/mL used in this work may be below the threshold required for the inhibition of the tested strains, underlining the importance of considering the dose-response curve in phytotherapeutic evaluations.

Regarding antimicrobial photodynamic therapy, results demonstrated a significant anti-adherent effect against *K. pneumoniae* compared to the untreated group, with outcomes similar to those observed for chlorhexidine. These findings are consistent with previous evidence reporting that methylene blue-mediated aPDT reduces bacterial adhesion in multidrug-resistant clinical isolates, including *K. pneumoniae*[36]. The ability of aPDT to interfere with adhesion may be linked to the role of fimbriae and extracellular polysaccharides in mediating bacterial attachment to abiotic surfaces[37,38], which are known to enhance cell surface hydrophobicity.

In contrast, aPDT did not exhibit significant antibiofilm activity against the microorganisms tested, in both single-species and multi-species models. These results differ from those reported by Yang et al.[39] (2018) and Figueiredo-Godoi et al.[40] (2022), who demonstrated effective inhibition of *A. baumannii* and *P. aeruginosa* biofilms. This discrepancy may be attributed to specific methodological variables adopted in this study, such as the initial bacterial density (0.5 McFarland), the concentration of 0.01% methylene blue, and the irradiation protocol (100 s; 10 J/cm²). It is likely that the cell density and the robustness of the extracellular matrix in the 24 hour biofilms limited the diffusion of the photosensitizer and the penetration of the laser. Furthermore, the low availability of oxygen in deeper layers of the biofilm may have restricted the generation of reactive oxygen species, which are essential for the cytotoxicity of aPDT. These factors, known to directly influence the efficiency of photodynamic therapy, suggest that the energy dose or agent concentration used may have reached an efficacy threshold for the adhesion phase, but not for the disruption of the mature biofilm.

Regarding the probiotic *L. rhamnosus*, this therapy demonstrated inhibitory effects against all tested microorganisms, with results comparable to chlorhexidine, showing no statistically significant differences, particularly for *A. baumannii* and *K. pneumoniae*. These findings are consistent with previous studies reporting the inhibitory activity of PLR and other Lactobacillus strains against opportunistic and multidrug-resistant bacteria[4,13]. In the literature, the beneficial effects of Lactobacillus probiotics have been associated with mechanisms such as modulation of host immune responses, competitive exclusion of pathogens, reinforcement of epithelial barrier function, and production of antimicrobial compounds[41], although these mechanisms were not directly evaluated in the present study.

Moreover, in this study, PLR exhibited anti-adherent and antibiofilm activities comparable to CHX, particularly in multispecies biofilms, where its performance was statistically equivalent. This highlights the potential of *L. rhamnosus* as a promising alternative for the control of opportunistic and superinfecting microorganisms in the oral environment, which may be related to its ability to interact within complex microbial communities that resemble clinical conditions.

Among the three therapies tested, PLR and aPDT showed the greatest efficacy in microbial control, with PLR standing out by exhibiting inhibitory, anti-adherent, and antibiofilm effects equivalent to the

gold-standard CHX. These results emphasize the potential of probiotics as complementary therapeutic strategies in the management of infections caused by multidrug-resistant microorganisms.

The limitations of this study should be acknowledged, particularly its solely *in vitro* design, which hinders direct extrapolation to clinical practice. Future studies, including in vivo models and randomized clinical trials, are needed to validate the efficacy and safety of these therapies, as well as to evaluate their performance against clinical isolates and within more complex oral biofilm structures. Nevertheless, these limitations do not detract from the value of our findings, which provide important preliminary evidence to guide further research.

ACKNOWLEDGMENTS

We thank the Coordination for the Improvement of Higher Education Personnel, Brazil, for supporting the postgraduate program, and the Federal University of Rio Grande do Norte for the laboratory infrastructure made available for the development of this study.

REFERÊNCIAS

1. Moussaly M, Zahreddine N, Kazma J, Ahmadieh R, Kan SS, Kanafan ZA. Prevalence of antibiotic-resistant organisms among hospitalized patients at a tertiary care center in Lebanon, 2010–2018. J Infect Public Health. 2021;14:12-6.

2. Interagency Coordination Group on Antimicrobial Resistance. No time to waste: guarding the future against drug-resistant infections [Internet]. Geneva: World Health Organization; 2019 [cited 2025 Jun]. Available from: https://www.who.int/docs/default-source/documents/no-time-to-wait-securing-the-future-from-drug-resistant-infections-en.pdf

3. Passarelli PC, De Leonardis M, Piccirillo GB, Desantis V, Papa R, Rella E, et al. The effectiveness of chlorhexidine and air polishing system in the treatment of *Candida albicans* infected dental implants: an experimental *in vitro* study. Antibiotics (Basel). 2020;9(4):1-12.

4. Dallal MMS, Davoodabadi A, Abdi M, Hajiabdolbaghi M, Sharifi Yazdi MK, Douraghi M, et al. Inhibitory effect of Lactobacillus plantarum and Lb. fermentum isolated from the faeces of healthy infants against nonfermentative bacteria causing nosocomial infections. New Microbes New Infect. 2017;15:9-13.

5. Tuon FF, Gavrilko O, Almeida S, Sumi ER, Alberto T, Rocha JL, et al. Prospective, randomised, controlled study evaluating early modification of oral microbiota following admission to the intensive care unit and oral hygiene with chlorhexidine. J Glob Antimicrob Resist. 2017;8:159-63.

6. Rathbun KP, Sole ML, Yooseph S, Xie R, Bourgault AM, Talbert S. Oral microbiome changes during hospitalization in older adults not receiving mechanical ventilation. Am J Crit Care. 2025;34(3):208-17.

7. Marino PJ, Wise MP, Smith A, Marchesi JR, Riggio MP, Lewis MAO, et al. Community analysis of dental plaque and endotracheal tube biofilms from mechanically ventilated patients. J Crit Care. 2017;39:149-55.

8. Gaber SN, Hemeda EEM, Elsayeh H-AS, Wahed WAY, Khalil MAF, Ibrahim EG. Propolis extract: a possible antiseptic oral care against multidrug-resistant non-fermenting bacteria isolated from non-ventilator hospital-acquired pneumonia. J Pure Appl Microbiol. 2020;14(1):123-31.

9. Silva TO, Ortega LN. A resistência antimicrobiana e custos de cuidado de saúde: uma revisão sistemática. Colloquium Vitae. 2021;13(2):25-39.

10. Wozniak A, Grinholc EM. Combined antimicrobial activity of photodynamic inactivation and antimicrobials–state of the art. Front Microbiol. 2018;9:1-19.

11. Beirão S, Fernandes S, Coelho J, Faustino MAF, Tomé JPC, Neves MGPMS, et al. Photodynamic inactivation of bacterial and yeast biofilms with a cationic porphyrin. Photochem Photobiol. 2014;90:1387-96.

12. Dualibe SAC, Costa MRM, Lima K, Uzeda M. *In vitro* antibacterial activity of two plant extracts against *Enterococcus faecalis*. Afr J Microbiol Res. 2015;9(14):1020-5.

13. Anil A, Raj A, MM A, Kuriakose N, Murali R, Kumar KSH. Avaliação da atividade antimicrobiana de *Lactobacillus acidophilus* e *Lactobacillus rhamnosus* contra *Klebsiella pneumoniae* clinicamente isolada: um estudo *in vitro*. Int J Res Med Sci. 2024;12:1140-6.

14. Zhang QZ, Zhao KQ, Wu Y, Li XH, Yang C, Guo LM, et al. 5-aminolevulinic acid-mediated photodynamic therapy and its strain-dependent combined effect with antibiotics on *Staphylococcus aureus* biofilm. PLoS One. 2017;12(3):1-12.

15. Soria-Lozano P, Gilaberte Y, Paz-Cristobal M, Pérez-Artiaga L, Lampaya-Pérez V, Aporta J, et al. In vitro effect of photodynamic therapy with different photosensitizers on cariogenic microorganisms. BMC Microbiol. 2015;15:187.

16. Elavarasu S, Suthanthiran T, Thangavelu A, Kanagaraj SS, Mohandas L, Sekar S. Evaluation of efficacy of probiotic (BIFILAC) on *Porphyromonas gingivalis*: in vitro study. J Pharm Bioallied Sci. 2016;8(Suppl 1):45-7.

17. Hu L, Zhou M, Young A, Zhao W, Yan Z. In vivo effectiveness and safety of probiotics on prophylaxis and treatment of oral candidiasis: a systematic review and meta-analysis. BMC Oral Health. 2019;19:140.

18. Aleluía CM, Procópio VC, Oliveira MTG, Furtado PGS, Giovannini JFG, Mendonça SMS. Medicines in dentist herbal. Rev Odontol Univ Sao Paulo. 2015;27(2):126-34.

19. Thadhani VM, Choudhary MI, Khan S, Karunaratne V. Antimicrobial and toxicological activities of some depsides and depsidones. J Natl Sci Found. 2012;40(1):43-8.

20. Tartaglia GM, Tadakamadla SK, Connelly SY, Sforza C, Martín C. Adverse events associated with home use of mouthrinses: a systematic review. Ther Adv Drug Saf. 2019;10:1-16.

21. Aromolaran O, Badejo OK. Efficacy of fresh leaf extracts of *Spondias mombin* against some clinical bacterial isolates from typhoid patients. Asian Pac J Trop Dis. 2014;4(6):442-46.

22. Oliveira LFR, Medeiros NS, Assis CF, Sousa Júnior FC. Simultaneous vehiculation of probiotics and yellow mombin (*Spondias mombin L.*) seed extract to develop a new vegan multifunctional ingredient. LWT Food Sci Technol. 2024;198:116018.

23. Mendonça AKP. Potencial antimicrobiano e antibiofilme do extrato hidroetanólico da folha de *Spondias mombin L.* e de suas frações frente a microrganismos superinfectantes do meio ambiente bucal [thesis]. Natal: Universidade Federal do Rio Grande do Norte; 2021.

24. Clinical and Laboratory Standards Institute. Metodologia dos testes a agentes antimicrobianos por diluição para bactéria de crescimento aeróbico: norma aprovada – sexta edição (M7-A6). Wayne: CLSI; 2012.

25. Souza NB, Contessa CR, Almeida L, Manera AP, Moraes CC. Determinação da atividade antimicrobiana do extrato purificado do cogumelo *Agaricus blazei* cultivado por fermentação em estado sólido. Rev CSBEA. 2016;2:1-7.

26. Pieri FA, Mussi MC, Fiorini JE, Scheedorf JM. Efeitos clínicos e microbiológicos do óleo de copaíba (*Copaifera officinalis*) sobre bactérias formadoras de placa dental em cães. Arq Bras Med Vet Zootec. 2010;62(3):578-85.

27. Stepanovic S, Vukovic D, Dakic I, Savic B, Svabic-Vlahovix M. A modified microtiter-plate test for quantification of staphylococcal biofilm formation. J Microbiol Methods. 2000;40(2):175-9.

28. World Health Organization. WHO global report on traditional and complementary medicine 2019 [Internet]. Geneva: WHO; 2019 [cited 2024 Aug]. Available from: https://apps.who.int/iris/handle/10665/312342.

29. World Health Organization. Traditional, complementary and integrative medicine [Internet]. Geneva: WHO; 2021 [cited 2024 Aug]. Available from: https://www.who.int/health-topics/traditional-complementary-and-integrative-medicine#tab=tab_1.

30. Kristoffersen AE, Stub T, Musial F, Fonnebo V, Lillines O, Norheim AJ. Prevalence of and reasons for intentional use of complementary and alternative medicine as an adjunct to future visits to a physician for chronic illness. BMC Complement Altern Med. 2018;18(109).

31. Ng JY. Insight into the characteristics of research published in complementary, alternative, and integrative medicine journals: a bibliometric analysis. BMC Complement Med Ther. 2021;21(185).

32. Lima IPA, Alves RAH, Mayer JSL, Costa MRM, Mendonça AKP, Lima ELF, et al. Antimicrobial activity of *Spondias mombin L.* aqueous and hydroethanolic extracts on *Enterococcus faecalis* and *Pseudomonas aeruginosa*: an *in vitro* study. Research Soc Dev. 2021;10(1):e50710111949.

33. Freitas MA, Cruz RP, Santos ATL, Almeida-Bezerra JW, Machado AJT, Santos JFS, et al. Análise por HPLC–DAD e atividades antimicrobianas de *Spondias mombin L.* (Anacardiaceae). 3 Biotech. 2022;12(61):1-15.

34. Albergaria ETD, Silva MVD, Silva AGD. Levantamento etnobotânico de plantas medicinais em comunidades rurais localizadas na Unidade de Conservação Tatu-Bola, município de Lagoa Grande, PE-Brasil. Rev Fitos. 2019;13(2):137-54.

35. Silva ARA, Morais SM, Marques MM, Oliveira DF, Barros CC, Almeida RR, et al. Chemical composition, antioxidant and antibacterial activities of two *Spondias* species from Northeastern Brazil. Pharm Biol. 2012;50-6:740-6.

36. Khan S, Khan SN, Meena R, Dar AM, Pal R, Khan AU. Photoinactivation of multidrug resistant bacteria by monomeric methylene blue conjugated gold nanoparticles. J Photochem Photobiol B. 2017;174:150-61.

37. Donlan RM. Biofilms: microbial life on surfaces. Emerg Infect Dis. 2002;8:881-90.

38. Di Martino PN, Cafferini B, Joly A, Darfeuille-Michaud A. *Klebsiella pneumoniae* type 3 pili facilitate adherence and biofilm formation on abiotic surfaces. Res Microbiol. 2003;154:9-16.

39. Yang SM, Lee DW, Park HJ, Kwak MH, Park JM, Chol M. Hydrogen peroxide enhances the antibacterial effect of methylene blue–based photodynamic therapy on biofilm-forming bacteria. Photochem Photobiol. 2018;95:833-8.

40. Figueiredo-Godoi LMA, Garcia MT, Pinto JG, Ferreira-Strixino J, Faustino EG, Pedroso LLC, et al. Antimicrobial photodynamic therapy mediated by Fotenticine and methylene blue on planktonic growth, biofilms, and burn infections of *Acinetobacter baumannii*. Antibiotics (Basel). 2022;11:619.

41. Raheem A, Liang L, Zhang G, Cui S. Modulatory effects of probiotics during pathogenic infections with emphasis on immune regulation. Front Immunol. 2021;12:616713.

Received: Feb 19 2026
Accepted: May 15 2026