



# In vitro comparative analysis of the antibacterial and antibiofilm effects of Persica, Matrica, Listerine mouthrinses against *Streptococcus mutans*

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## ABSTRACT

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Dental plaque, a structured microbial biofilm, is a primary etiological factor in dental caries. Mouthrinses are widely used to control plaque and cariogenic bacteria, yet comparative data on their antibacterial and antibiofilm efficacy, particularly for herbal formulations, remain limited. This study aimed to evaluate and compare the antibacterial and antibiofilm activities of Persica (*Salvadora persica*), Matrica (*Matricaria chamomilla*), and Listerine with 0.2% chlorhexidine against *Streptococcus mutans* *in vitro*. The minimum inhibitory concentration (MIC) assay in 96-well microtiter plates was used to determine antibacterial activity, while antibiofilm effects were assessed using the microtiter plate crystal violet staining method. Mouthrinses were tested at serial dilutions, and bacterial growth and biofilm formation were quantified. A p-value < 0.05 considered statistically significant. Chlorhexidine and Listerine demonstrated the strongest antibacterial activity, both exhibiting MIC values of 3.12%, followed by Matrica (6.25%) and Persica (12.5%). In the antibiofilm assay, chlorhexidine completely inhibited biofilm formation at all tested concentrations, whereas Persica and Listerine showed moderate inhibition at higher concentrations. Matrica exhibited the lowest antibiofilm effect, with significant inhibition observed only at the 50% concentration. Statistical analysis confirmed significant differences among the mouthrinses for both antibacterial (p = 0.03) and antibiofilm activities (p = 0.01). Chlorhexidine remains the most potent mouthrinse against both planktonic *S. mutans* and biofilm formation. Herbal formulations, particularly Persica and Listerine, demonstrated concentration-dependent antibacterial and antibiofilm effects. These findings highlight the potential of herbal mouthrinses as adjuncts in oral care, though they are generally less effective than chlorhexidine, especially at lower concentrations.

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## 1. Introduction

Dental plaque is a highly organized microbial biofilm that develops on oral surfaces and represents the primary etiological factor in the onset of dental caries and periodontal diseases [1]. This biofilm is composed of diverse microorganisms embedded within a self-produced extracellular polymeric substance (EPS) matrix consisting of polysaccharides, proteins, lipids, and extracellular DNA. The EPS matrix provides structural integrity and protection, enabling microorganisms to adhere to tooth surfaces, evade host defense mechanisms, and resist antimicrobial agents [2]. If plaque biofilm is not effectively controlled, it leads to sustained acid production, enamel demineralization, gingival inflammation, and progression toward advanced oral and periodontal pathologies.

Dental caries is a multifactorial disease influenced by microbial composition, time, fermentable carbohydrate availability, and the susceptibility of the tooth surface. Additional contributing factors include dietary habits, oral hygiene practices, socioeconomic status, age, and genetic background [3]. Among the oral microbiota, *Streptococcus mutans* plays a central role in the initiation of dental caries due to its strong acidogenic and aciduric properties and its ability to synthesize extracellular glucans that facilitate bacterial adhesion and biofilm maturation. In contrast, Lactobacillus species are more commonly associated with lesion progression and dentinal caries rather than disease initiation [4]. Biofilms represent a distinct microbial lifestyle characterized by altered metabolic activity, enhanced gene regulation, and increased resistance to antimicrobial agents compared with planktonic cells [5]. In the oral cavity, dental plaque biofilm initially appears as a colorless, sticky deposit and gradually develops into a complex, structured microbial community [6]. Importantly, bacteria within biofilms can tolerate antimicrobial concentrations several times higher than those required to inhibit planktonic bacteria, making biofilm control a critical challenge in preventive dentistry [7]. Dental caries remains highly prevalent worldwide and poses a significant public health burden. In Iran, epidemiological data indicate a high prevalence of dental caries across different age groups, particularly among school-aged children [8]. According to World Health Organization (WHO) reports, approximately 60–90% of children globally experience dental caries, underscoring the need for effective preventive strategies [9]. Preventive approaches include dietary modification, mechanical plaque removal through tooth brushing and flossing, and the adjunctive use of antimicrobial mouthrinses and topical fluoride agents [3].

Mouthrinses play an important role in oral hygiene by reducing microbial load, inhibiting plaque accumulation, and suppressing cariogenic bacteria. However, despite extensive research, no ideal mouthrinse has yet been developed that simultaneously exhibits broad antimicrobial activity, antibiofilm

efficacy, low cytotoxicity, absence of adverse effects, and acceptable taste [10]. Consequently, a wide range of chemical and herbal mouthrinses are currently used in clinical practice, including chlorhexidine, essential oil-based rinses such as Listerine, fluoride- or xylitol-containing products, and herbal formulations such as Persica and Matrica [11,12]. Chlorhexidine gluconate is widely regarded as the gold standard chemical plaque control agent due to its broad-spectrum antimicrobial activity and substantivity. In Iran, chlorhexidine is commonly available in a 0.2% formulation and is endorsed by the American Dental Association for clinical use [13]. Nevertheless, its long-term use is associated with adverse effects such as tooth staining, taste alteration, and mucosal irritation, prompting interest in alternative agents [14]. Listerine, one of the earliest commercially available antiseptic mouthrinses, contains essential oils including thymol, eucalyptol, menthol, and methyl salicylate and has demonstrated efficacy in plaque reduction and gingival health improvement, with fewer side effects compared to chlorhexidine [15,16].

Herbal mouthrinses have gained increasing attention due to their natural origin, cultural acceptance, and favorable safety profiles. Persica is an herbal formulation containing *Salvadora persica* (miswak), *Achillea millefolium*, and *Mentha piperita*. The WHO has endorsed *S. persica* as an effective oral hygiene tool, although data on the combined antimicrobial and antibiofilm efficacy of its commercial formulations remain limited [17,18]. Matrica (kamisol), based on *Matricaria chamomilla* (chamomile), is known for its antimicrobial, anti-inflammatory, and wound-healing properties and is commonly used in oral care products [19,20]. Several *in vitro* methods have been employed to evaluate the antibacterial efficacy of mouthrinses against *S. mutans*, including agar diffusion assays and determination of the minimum inhibitory concentration (MIC), which reflects activity against planktonic bacterial cells [21]. However, MIC values alone do not adequately represent the ability of an agent to prevent biofilm formation. Consequently, biofilm-based assays, particularly the microtiter plate crystal violet method, have gained prominence due to their reproducibility and clinical relevance in assessing antibiofilm activity [22].

Although numerous studies have examined the antibacterial effects of mouthrinses, fewer investigations have simultaneously evaluated both antibacterial and antibiofilm activities using standardized and complementary methods. Moreover, comparative data on commonly used commercial mouthrinses, particularly herbal formulations, remain limited in Iran. Given the critical role of biofilm formation in dental caries pathogenesis and the recognized differences between planktonic and biofilm-associated bacterial susceptibility, a comprehensive evaluation incorporating both MIC determination and biofilm inhibition assays is warranted. Therefore, the present study aimed to comparatively assess the

antibacterial and antibiofilm properties of Persica, Matrica, and Listerine mouthrinses in comparison with chlorhexidine gluconate against *S. mutans* under *in vitro* condition.

## 2. Materials and Methods

### 2.1 Study Design

This study was designed as a comparative *in vitro* experimental investigation conducted in 2020 at the Department of Microbiology, Babol University of Medical Sciences, Iran. The test microorganism, *S. mutans* (PTCC 1683), a member of the normal oral flora, was obtained in lyophilized form from the Iranian Research Organization for Science and Technology (IROST). All experimental procedures were carried out under aseptic conditions to prevent contamination. The study protocol was reviewed and approved by the Ethics Committee of Babol University of Medical Sciences (IR.MUBABOL.REC.1399.496).

The mouthrinses evaluated in this study included Persica (Poorsina Pharmaceutical Laboratories, Tehran, Iran), Matrica (Barij Essence Pharmaceutical Co., Kashan, Iran), Listerine (Johnson & Johnson, Italy), and chlorhexidine gluconate 0.2%, (Morva-sept Solution) which served as the positive control. Physiological saline was used as the negative control. All tests were performed in triplicate to ensure reproducibility and reliability of results.

### 2.2 Determination of antibacterial activity

The antibacterial activity of the evaluated mouthrinses against *S. mutans* was assessed by determining the MIC using the broth microdilution technique in 96-well microtiter plates, in accordance with the Clinical and Laboratory Standards Institute (CLSI, 2020) guidelines for streptococci [23].

Briefly, 100  $\mu$ L of cation-adjusted Mueller–Hinton broth (CAMHB) supplemented with 5% lysed horse blood was dispensed into each well. An equal volume (100  $\mu$ L) of the undiluted mouthrinse was then added to the first well, followed by serial two-fold dilutions across ten consecutive wells to achieve final concentrations ranging from 100% to 0.195. Subsequently, 100  $\mu$ L of a *S. mutans* suspension standardized to a 0.5 McFarland turbidity was inoculated into each well, including the control wells. The positive control consisted of CAMHB supplemented with lysed horse blood and bacterial inoculum without mouthrinse, while the negative control contained only the supplemented broth without bacterial inoculation. The microplates were incubated at 37 °C for 24 hours under microaerophilic conditions. The MIC was defined as the lowest concentration of the mouthrinse that completely inhibited visible bacterial growth. All assays were performed in triplicate to ensure the reliability and reproducibility of the results.

### 2.3 Anti-biofilm activity assay

The antibiofilm activity of the tested mouthrinses against *S. mutans* was evaluated using the microtiter plate crystal violet staining assay, as previously described with minor modifications [24]. Briefly, 100  $\mu$ L of Brain Heart Infusion (BHI) broth was dispensed into each well of a sterile 96-well flat-bottom microtiter plate. Subsequently, 80  $\mu$ L of different concentrations of the mouthrinses (prepared in BHI broth to achieve final concentrations of 50%, 25%, 12.5%, 6.25%, and 3.12%) were added to the designated wells. Finally, 20  $\mu$ L of a *S. mutans* suspension adjusted to 0.5 McFarland standard was inoculated into each well, resulting in a total final volume of 200  $\mu$ L per well. Wells containing bacterial suspension without mouthrinse served as the positive control (biofilm formation control), while wells containing only sterile BHI broth without bacterial inoculation were used as the negative control. The plates were incubated at 37 °C for 24 hours under microaerophilic conditions to allow biofilm development. Following incubation, the contents of the wells were gently aspirated, and each well was washed three times with sterile saline to remove non-adherent (planktonic) cells. The plates were then air-dried at room temperature. The remaining adherent biofilms were fixed by adding 200  $\mu$ L of methanol to each well for 15 minutes. After fixation, the methanol was discarded and the plates were allowed to dry completely. The fixed biofilms were stained with 200  $\mu$ L of 0.2% (w/v) crystal violet solution for 5 minutes. Excess stain was removed by gently rinsing the wells with distilled water, followed by air drying. Subsequently, the bound crystal violet was solubilized by adding 200  $\mu$ L of 33% (v/v) acetic acid to each well. Biofilm formation was categorized based on optical density (OD) measurements at 570 nm using the method described by Stepanović et al. The cut-off OD value (OD<sub>c</sub>) was calculated as the mean OD of the negative control plus three standard deviations. Isolates were classified as none, weak, moderate, or strong biofilm producers according to their OD values relative to OD<sub>c</sub> [2]. All experiments were performed in triplicate to ensure reproducibility.

### 2.4 Statistical analysis

Data were collected using a standardized checklist and statistically analyzed using SPSS software version 22 (IBM Corp., Armonk, NY, USA). Differences in mean antibacterial and antibiofilm activities among the four groups were assessed using one-way analysis of variance (ANOVA), and Tukey's post hoc test. In all statistical analyses, a p-value less than 0.05 were considered statistically significant.

## 3. Results

In the present study, the antibacterial and antibiofilm activities of Persica, Matrica, Listerine, and 0.2%

chlorhexidine gluconate against *S. mutans* were comparatively evaluated. The antibacterial activity of the tested mouthrinses was determined by measuring their minimum inhibitory concentrations (MICs). Chlorhexidine and Listerine exhibited the lowest MIC values (3.12%), indicating the highest antibacterial potency against planktonic *S. mutans* cells. This was followed by Matrica with an MIC of 6.25% and Persica with an MIC of 12.5%. ANOVA revealed a statistically significant difference in antibacterial activity among the tested mouthrinses ( $p = 0.03$ ).

Chlorhexidine demonstrated a strong antibiofilm effect, completely inhibiting biofilm formation at all tested concentrations (3.12–50%). Persica exhibited antibiofilm activity at concentrations  $\geq 6.25\%$ , whereas Listerine showed significant inhibition only at concentrations  $\geq 25\%$ . Matrica displayed the weakest antibiofilm activity and was able to inhibit biofilm formation only at the highest tested concentration (50%). Statistical analysis of OD570 values using one-way ANOVA followed by Tukey's post hoc test showed a significant difference in biofilm inhibition among the mouthrinses ( $p = 0.01$ ) (Table 1). Notably, the antibiofilm inhibitory concentrations were higher than the corresponding MIC values for some mouthrinses. This observation reflects the inherent differences between planktonic bacterial susceptibility and biofilm-associated resistance, as biofilm formation involves complex mechanisms such as enhanced extracellular polymeric substance production and altered gene expression, which may require higher concentrations of antimicrobial agents to achieve inhibition.

**Table 1.** Inhibitory concentrations of mouthrinses against *Streptococcus mutans* biofilm formation

Mouthrinse	Concentrations				
	50%	25%	12.5%	6.25%	3.12%
Chlorhexidine	+	+	+	+	+
Persica	+	+	+	+	–
Listerine	+	+	–	–	–
Matrica	+	–	–	–	–

#### 4. Discussion

In the present study, the antibacterial and antibiofilm activities of Persica, Matrica, and Listerine were comparatively evaluated against *S. mutans* and benchmarked against 0.2% chlorhexidine gluconate using two standardized *in vitro* assays: determination of the MIC and the microtiter plate crystal violet biofilm assay. Our results demonstrated that chlorhexidine and Listerine exhibited the highest antibacterial efficacy against planktonic *S. mutans*. While, Matrica and Persica showed the lower antibacterial potency. In contrast, the antibiofilm assay revealed distinct concentration-dependent effects.

Chlorhexidine completely inhibited biofilm formation across all tested concentrations, whereas Persica and Listerine exhibited moderate antibiofilm activity only at higher concentrations. Matrica demonstrated the

weakest antibiofilm effect and inhibited biofilm formation exclusively at the highest concentration tested (50%). These findings underscore the fundamental differences between antibacterial activity against planktonic cells and inhibition of biofilm formation, which involves more complex mechanisms such as bacterial adhesion, extracellular polymeric substance production, and biofilm maturation.

The superior antimicrobial performance of chlorhexidine observed in this study is consistent with numerous previous reports highlighting its broad-spectrum antibacterial and antibiofilm efficacy. Sajadi et al. reported that methanolic extracts of chamomile and thyme significantly reduced *S. mutans* counts in pediatric populations, with effects approaching those of chlorhexidine [19]. However, the stronger activity of chlorhexidine observed in the present study may be attributed to differences in extraction methods and solvent systems, as a commercially available ethanolic Matrica formulation was used rather than a methanolic extract. Similarly, Khan et al. demonstrated that *S. persica* essential oil reduced *S. mutans* counts and disrupted biofilm architecture, although chlorhexidine remained more potent at lower concentrations [20]. This observation aligns with our findings, in which Persica exhibited moderate antibacterial and antibiofilm activities that increased in a concentration-dependent manner. Consistent results were also reported by Balto et al., who found that ethanolic and hexane extracts of *S. persica* were effective against *S. mutans*, *Streptococcus sanguinis*, and *Streptococcus salivarius*, yet still demonstrated lower inhibitory efficacy than chlorhexidine [21].

Collectively, these studies support a consistent pattern indicating that herbal mouthrinses possess measurable antimicrobial and antibiofilm potential but generally remain less effective than chlorhexidine, particularly at lower concentrations. Nevertheless, several studies have reported that specific herbal formulations, especially those containing chamomile or thyme, can exhibit antibacterial effects comparable to chlorhexidine under certain experimental conditions [22,25,26]. Such discrepancies are likely attributable to variations in extraction procedures, plant chemotypes, solvent composition, and the resulting concentrations of active phytochemical constituents.

The differences observed in the antibacterial and antibiofilm efficacy of the tested mouthrinses may also be explained by their distinct chemical compositions. Analytical techniques such as thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC) have confirmed the presence of diverse active compounds in these formulations. Listerine contains eucalyptol (0.092%), menthol (0.042%), methyl salicylate (0.060%), and thymol (0.064%), whereas chlorhexidine contains chlorhexidine gluconate.

Matrica includes chamazulene and  $\alpha$ -bisabolol, and Persica comprises hydroalcoholic extracts of *S. persica*,

*Achillea millefolium*, and *Mentha spicata* [27]. This compositional variability likely contributes to the observed differences in antibacterial and antibiofilm performance among the tested mouthrinses. Numerous studies have investigated the effects of herbal mouthrinses on biofilm formation and the control of cariogenic and periodontal bacteria, including *S. mutans*, *Lactococcus lactis*, and *Porphyromonas gingivalis* [28,29].

However, most previous studies have evaluated antibacterial and antibiofilm activities independently. The novelty of the present study lies in its simultaneous assessment of both antibacterial and antibiofilm properties using two complementary *in vitro* methods. Moreover, to the best of our knowledge, this study represents the first comparative evaluation of Persica, Matrica, and Listerine alongside chlorhexidine in Iran, providing valuable baseline data on the antimicrobial efficacy of commonly used commercial mouthrinses. Despite these strengths, this study has certain limitations. *S. mutans* is a key etiological agent in dental caries, but it is not the sole microorganism involved in oral biofilm development. The absence of additional cariogenic and periodontal species represents a limitation, and future studies should include multispecies biofilm models to better reflect clinical conditions. A major strength of this study is the use of commercially available mouthrinse formulations, which enhances clinical relevance, standardization, and safety compared with laboratory-prepared extracts, which may exhibit greater variability [20].

Among the mouthrinses evaluated, Listerine and chlorhexidine exhibited the strongest antibacterial activity against *S. mutans*, while chlorhexidine demonstrated the highest anti-biofilm efficacy across all tested concentrations. These findings suggest that Listerine may serve as a potential alternative to chlorhexidine for reducing *S. mutans*-related oral infections, particularly in adults without xerostomia or risk of accidental ingestion. However, further *in vivo* and clinical studies are recommended to confirm its long-term effectiveness and safety in routine oral hygiene.

### Declaration of artificial intelligence (AI) in the writing process

The authors declare whether AI or AI-assisted technologies were used during the preparation of this manuscript. If used, AI tools were employed solely to improve language quality, grammar, readability, and organizational structure. The authors carefully reviewed and edited all AI-generated content and take full responsibility for the accuracy, integrity, and originality of the final manuscript. No AI tool was used to generate, analyze, or interpret scientific data or images, or to draw scientific conclusions. The use of AI-assisted technologies complies with current publication ethics recommendations and journal policies.

### Authors' contributions

SM: Methodology, Formal analysis, Data Curation, Writing - Original Draft. AP: Conceptualization, Methodology, Writing - Review & Editing. NB: Project administration, Methodology, Validation, Writing - Review & Editing. All authors read and approved the final version of the manuscript.

### Conflict of interest

No potential conflict of interest was reported by the authors.

### Ethical declarations

This study was reviewed and approved by the Research Ethics Committee of Babol University of Medical Sciences, Babol, Iran (Ethics code: IR.MUBABOL.REC.1399.496). As this research was an *in vitro* laboratory experiment using a standard bacterial strain (*Streptococcus mutans* PTCC 1683) obtained from the Iranian Research Organization for Science and Technology (IROST), no human participants or animal subjects were involved, and informed consent was not applicable. The study was conducted in accordance with the principles of the Declaration of Helsinki.

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