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Laboratory Investigation of Antibacterial and Anti-Biofilm Effects of Curcumin Nanoparticles on *Lacticaseibacillus casei* and *Lactobacillus acidophilus*

Amirhossein Rajabnia[1], Ezzat Allah Ghaemi[2], Ezatolah Kazeminejad[3*]

1. *Dental Research Center, School of Dentistry, Hamadan University of Medical Sciences, Hamadan, Iran*
2. *Laboratory Science Research Center, Golestan University of Medical Sciences, Gorgan, Iran*
3. *Dental Research Center, Golestan University of Medical Sciences, Gorgan, Iran*

ABSTRACT

Background and Aim: Prolonged use of chemical agents to control cariogenic bacteria is frequently associated with adverse side effects. Consequently, increasing attention has been directed toward replacing conventional chemical antimicrobials with herbal alternatives. This study aimed to evaluate the *in vitro* antibacterial and anti-biofilm activities of curcumin nanoparticles against standard strains of *Lacticaseibacillus (L.) casei* and *Lactobacillus (L.) acidophilus*.

Materials and Methods: In this descriptive cross-sectional study, the minimum inhibitory concentration of nano-curcumin was determined and compared with that of chlorhexidine using broth microdilution method. Chlorhexidine mouthwash served as the positive control, whereas physiological saline was used as the negative control. MICs were determined in 96-well microplates according to the Clinical and Laboratory Standards Institute guidelines. Anti-biofilm activity was assessed by a microtiter plate assay employing crystal violet staining. Data were analyzed using SPSS version 22, with statistical significance defined as $P<0.05$.

Results & Conclusion: Chlorhexidine inhibited biofilm formation at concentrations of 6.25 µg/mL for *L. acidophilus* and 12.5 µg/mL for *L. casei*. Notably, nan-ocurcumin reduced biofilm formation significantly in both species ($P<0.05$) at a sub-MIC concentration of 3.125 µg/mL. Overall, curcumin nanoparticles exhibited distinct antibacterial and anti-biofilm effects against *L. casei* and *L. acidophilus*. Given their natural origin, favorable safety profile, and potential to overcome the limitations of synthetic antimicrobials, nan-ocurcumin formulations may represent a promising and biocompatible alternative for the prevention of dental caries.

Keywords: Antimicrobial, Biofilm, Curcumin Nanoparticle, *Lactobacillus acidophilus*, *Lacticaseibacillus casei*

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Corresponding Information: **Ezatolah Kazeminejad**, *Dental Research Center, Golestan University of Medical Sciences, Gorgan, Iran* & Email: Ezztk.edu@gmail.com

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1. Introduction

Tooth decay remains one of the most prevalent infectious diseases of the oral cavity, despite remarkable advancements in dental care **(1)**. Its pathogenesis is closely associated with the activity of cariogenic bacteria such as *Streptococcus (S.) mutans*, *S. sobrinus*, and various Lactobacillus species, which proliferate within biofilm structures on tooth surface **(2, 3)**. These microorganisms metabolize dietary carbohydrates to produce organic acids, resulting in pH reduction,

demineralization of dental tissues, and initiation of caries development **(4)**. The persistence of biofilms-aggregates of microorganisms embedded within an extracellular polymeric substance (EPS)-further complicates prevention and management strategies for dental caries **(5)**.

According to the ecological plaque hypothesis, the microbial composition of dental biofilms changes as caries progresses **(3, 7)**. Early enamel lesions are predominantly colonized by *S. mutans* and other acidogenic Streptococci, whereas Lactobacillus species become increasingly dominant in advanced dentinal caries **(6, 7)**. Members of the *Lactobacillus (L.) casei* group, particularly *L. casei* and *L. acidophilus*, are both highly acidogenic and aciduric, allowing them to survive and proliferate in the low-pH environment characteristic of active dentinal lesions **(6, 7)**. Their elevated prevalence in progressing caries, along with their frequent detection in saliva as biomarkers of a carbohydrate-rich and acidic oral milieu, underscores their clinical significance in dental caries research **(7)**.

The management of dental caries has traditionally relied on chemical antimicrobial agents, most notably chlorhexidine-based mouthwashes **(8)**. Although effective, these agents are frequently associated with adverse effects such as tooth discoloration, disruption of the oral microbiome, and the potential development of microbial resistance **(9)**. Consequently, increasing efforts have focused on identifying safer and more sustainable alternatives derived from natural products **(10)**.

In recent years, herbal bioactive compounds have attracted considerable attention as potential substitutes for the synthetic antimicrobials **(11)**. Among these, curcumin—a natural polyphenolic compound extracted from the rhizome of *Curcuma longa*—has demonstrated a wide spectrum of pharmacological properties, including anti-inflammatory, antioxidant, anticancer, and antimicrobial activities **(12)**. However, its clinical application has been hindered by poor aqueous solubility, low bioavailability, and rapid degradation under physiological conditions **(13, 14)**. Advances in nanotechnology have addressed these limitations through the development of nano-curcumin, a water-dispersible formulation that enhances curcumin stability, solubility, and antimicrobial potency **(15)**.

Previous studies have demonstrated that nano-curcumin possesses significant antimicrobial activity against a wide range of pathogens, including oral bacteria **(16, 17)**. Its ability to disrupt biofilm formation, combined with minimal cytotoxicity **(16)**, underscores its potential for incorporation into dental care products **(18)**.

Although curcumin has been extensively investigated for its antibacterial effects against *S. mutans*, data regarding its efficacy—particularly in nanoform—against *L. casei* and *L. acidophilus* is rare. These species are highly aciduric and play a pivotal role in the progression of dentinal caries. This study aimed to fill this research gap by evaluating the antibacterial and anti-biofilm capacities of nano-curcumin compared to chlorhexidine under controlled laboratory conditions. We hypothesized that nano-curcumin, owing to its enhanced solubility and bioavailability, would exert significant anti-biofilm activity even at sub-inhibitory concentrations.

2. Materials and Methods

2.1 Microorganisms and Chemicals

This descriptive cross-sectional study was conducted at Babol University of Medical Sciences. Standard strains of *L. casei* (ATCC 39392) and *L. acidophilus* (ATCC 435) were obtained in lyophilized form from the Iran Scientific and Industrial Research Center. Curcumin ($C_{21}H_{20}O_6$; purity > 65%; molecular weight 368.38 g/mol) was purchased from Sigma-Aldrich (USA).

2.2 Preparation of Nanoparticles

Curcumin nanoparticles were commercially synthesized by Behnogen Company (Tehran, Iran) using a routine solvent–antisolvent precipitation method **(19)**. In this process, curcumin powder was first dissolved in ethanol as water-miscible organic solvent, and then added dropwise to distilled water under vigorous stirring. The rapid mixing of the solvent and water induced supersaturation and nucleation, resulting in the formation of fine, unencapsulated curcumin nanoparticles. To enhance stability and prevent aggregation, a small amount of stabilizer was added to the aqueous phase before mixing. The resulting nanosuspension was stirred until the solvent evaporated, yielding a stable colloidal dispersion of curcumin nanoparticles.

The particle size and size distribution were determined by dynamic light scattering (DLS) (Malvern Instruments, UK) at 25 °C.

2.3 Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing was performed using the broth microdilution method in sterile 96-well microplates, according to the Clinical and Laboratory Standards Institute (CLSI M07-A11) guidelines **(20)**. De Man, Rogosa, and Sharpe (MRS) broth (Merck, Germany) was used as the culture medium to support the optimal growth of Lactobacillus species. Standard bacterial suspensions

were prepared by adjusting the turbidity of each culture to 0.5 McFarland standard (approximately 1 × 10^8 CFU/mL) and further diluted 1:200 in MRS broth to obtain a final inoculum concentration of approximately 5 × 10^5 CFU/mL/ well. Serial two-fold dilutions of nano-curcumin and chlorhexidine mouthwash (0.2%, equivalent to 2000 µg/mL) were prepared, and 100 µL of bacterial suspension was added to each well, with physiological saline included as the negative control. Plates were incubated at 37°C for 24 hr under microaerophilic conditions, and the minimum inhibitory concentration (MIC) was defined as the lowest concentration showing no visible bacterial growth. All tests were conducted in triplicate.

2.4 Anti-Biofilm Effect Assessment

The anti-biofilm activity of nano-curcumin and chlorhexidine was evaluated using the standard microtiter plate assay described by Stepanović et al **(21)** with minor modifications. Overnight cultures of *L. casei* and *L. acidophilus* grown in MRS broth were diluted 1:100 in fresh MRS medium. A total of 200 µL of each bacterial suspension was added to the wells of sterile flat-bottom 96-well microplates, along with varying concentrations of nano-curcumin or chlorhexidine, ranging from 50 µg/mL to 3.125 µg/mL. Control wells containing bacterial suspensions without any antimicrobial agents served as the negative control (biofilm control), while wells containing sterile broth only served as the blank. Plates were incubated at 37°C for 24 hr under microaerophilic conditions to allow biofilm formation. After incubation, the wells were gently washed three times with phosphate-buffered saline (PBS, pH 7.2) to remove non-adherent cells, air-dried, and then fixed with methanol for 15 min. The adherent biofilms were stained with 0.1% crystal violet (CV) for 15 min and washed again with distilled water. The dye bound to biofilm cells was solubilized with 95% ethanol, and absorbance was measured at 570 nm using an ELISA microplate reader. The percentage of biofilm inhibition was calculated using the formula:

Biofilm inhibition (%) = [(A_control − A_sample) / A_control] × 100

Where A_control is the mean absorbance of control wells (bacteria without agents) and A_sample is the mean absorbance of treated wells.

Prior to treatment, the biofilm-forming capacity of each strain was confirmed according to the Stepanović classification criteria, based on the optical density (OD) measurements of untreated controls **(21)**:

OD≤ODC: No biofilm formation

ODC<OD≤ (2×ODC): Weak biofilm formation power

(2×ODC)<OD≤(4×ODC): Moderate biofilm formation power

(4×ODC)<OD: Strong biofilm formation power **(21)**.

2.5 Statistical Analysis

Data were analyzed using SPSS software version 24. Paired-sample *t*-tests were performed for each group, with statistical significance set at $P \leq 0.05$.

3. Results & Discussion

DLS analysis showed that the curcumin nanoparticles had a mean particle size of 333.8 ± 9.2 nm and a polydispersity index (PDI) of 0.236, indicating a relatively uniform size distribution (Figure 1).

The MIC values for nano-curcumin and chlorhexidine against *L. acidophilus* and *L. casei* are presented in Table 1. Nano-curcumin inhibited the growth of *L. acidophilus* at 6.25 µg/mL and *L. casei* at 12.5 µg/mL. In comparison, chlorhexidine inhibited *L. acidophilus* and *L. casei* at 3.12 and at 6.25 µg/mL, respectively. These differences were statistically significant for both bacterial strains ($P<0.05$).

The anti-biofilm effects of the test agents are summarized in Table 2. Chlorhexidine inhibited biofilm formation in *L. acidophilus* at concentrations ≥ 6.25 µg/mL and in *L. casei* at concentrations ≥ 12.5 µg/mL. Nano-curcumin demonstrated biofilm inhibition in both bacterial strains at all tested concentrations down to 3.125 µg/mL. Statistical analysis confirmed that the inhibitory effect of nano-curcumin at 3.125 µg/mL was significant compared to the control ($P=0.04$).

Table 1. The result of antimicrobial susceptibility testing.

Type of antimicrobial agent	Chlorhexidine (µg/mL)	Nano-curcumin (µg/mL)	*P*-value
L. acidophilus	3.12	6.25	0.04
L. casei	6.25	12.5	0.02

Table 2. Anti-biofilm effects of chlorhexidine and nano-curcumin at different concentrations.

Type of antimicrobial agent		Concentrations (µg/mL) used for anti-biofilm properties investigation				
		50	25	12.5	6.25	3.125
CHL	*L. acidophilus*	+	+	+	+	-
	L. casei	+	+	+	-	-
NPCru	*L. acidophilus*	+	+	+	+	+
	L. casei	+	+	+	+	+

CHL: chlorhexidine; NPCru: nano-curcumin; "+" indicates biofilm inhibition; "–" indicates no inhibition.

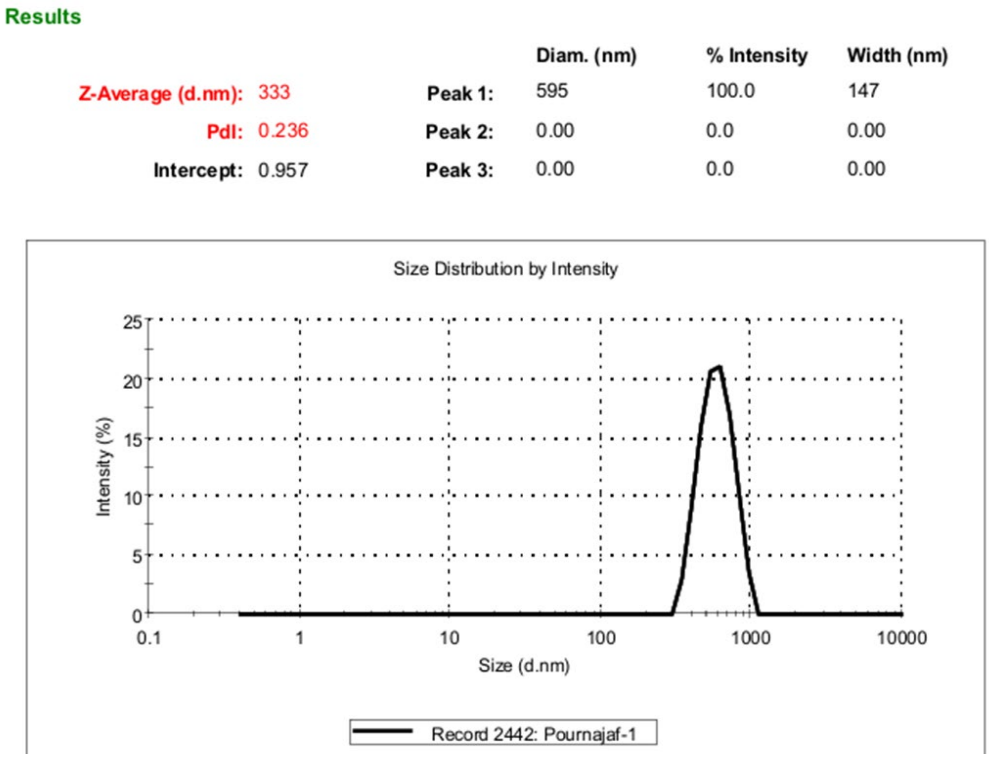


Figure 1. Size distribution of synthesized curcumin nanoparticles. The analysis indicated a relatively uniform size distribution **(Prepared by Authors, 2025)**.

This study demonstrated that nano-curcumin possesses significant antibacterial and anti-biofilm activity against *L. acidophilus* and *L. casei*. The MIC values for nano-curcumin were 6.25 µg/mL for *L. acidophilus* and 12.5 µg/mL for *L. casei*. In contrast, chlorhexidine, inhibited *L. acidophilus* at 3.12 µg/mL and *L. casei* at 6.25 µg/mL. These results suggest that nano-curcumin may possess strong antimicrobial efficacy comparable to chlorhexidine. Such findings support its potential as a novel natural antimicrobial compound for the caries prevention.

Importantly, nano-curcumin was able to inhibit biofilm formation at concentrations below its MIC, demonstrating a pronounced sub-MIC anti-biofilm effect. This property is clinically valuable because it allows disruption of early biofilm establishment without imposing selective pressure that promotes antimicrobial resistance. The improved solubility, stability, and bioavailability of nano-curcumin nanoparticles enhance their ability to interact with bacterial cell walls and biofilm matrices, leading to effective suppression of biofilm development **(22, 23)**.

Our results were consistent with previous research, although some variations exist. Azizi et al **(24)** demonstrated that curcumin-loaded nanoparticles improved curcumin bioavailability and adherence to dental enamel, resulting in caries prevention by *S. mutans*. Their lower MIC values compared to ours may be attributed to species-specific differences and variations in nanoparticle formulation **(24)**. Conversely, Helalat et al **(25)** reported no significant biofilm inhibition, likely due to differences in experimental conditions, bacterial strain sensitivity, or curcumin preparation method. The enhanced activity

observed in our study may result from optimized nanoparticle penetration and controlled release characteristics.

In agreement with previous reports, curcumin and its nano-formulations have exhibited broad antimicrobial activity beyond Lactobacillus species. Vieira et al **(28)** demonstrated inhibitory effects against *S. mutans* and *S. mitis* **(26)** and Trigo-Gutierrez et al **(29)** highlighted curcumin versatility, biocompatibility, and low cytotoxicity **(27)**.

Mechanistically, nano-curcumin exerts antibacterial and anti-biofilm actions by disrupting bacterial membrane integrity, interfering with quorum-sensing communication, and suppressing biofilm matrix synthesis **(28, 29)**. The addition of a polystyrene coating enhances nanoparticle stability and provides sustained release of curcumin, thereby prolonging antimicrobial activity **(30)**. Compared with conventional mouthwashes such as chlorhexidine, which may cause tooth discoloration and oral microbiome disruption **(31, 32)**, nano-curcumin represents a safer, biocompatible, and effective alternative for incorporation into the oral hygiene products.

Despite its promising results, this study has certain limitations. Physicochemical characterization was limited to DLS, without complementary transmission electron microscopy (TEM) or Fourier-transform infrared spectroscopy (FTIR) analyses. The lack of cytotoxicity and biocompatibility testing restricts conclusions about safety. Moreover, the *in vitro* conditions cannot fully mimic the complex oral environment, where saliva flow, temperature, and multispecies interactions may influence antimicrobial efficacy. Additionally, short contact times of oral products *in vivo* may reduce the clinical impact observed under prolonged laboratory exposure.

The present study provides a valuable foundation for the potential development of nano-curcumin-based mouthwashes and other oral care products. Owing to its combined antibacterial, anti-biofilm, and favorable safety profiles, nano-curcumin emerges as a promising candidate for caries prevention and management. Nonetheless, well-designed *in vivo* studies and randomized clinical trials, incorporating comprehensive nanoparticle characterization, standardized cytotoxicity testing, and advanced statistical analyses, are required to validate its efficacy and establish optimal dosing protocols.

4. Conclusion

In summary, nano-curcumin demonstrated substantial antibacterial and anti-biofilm activity against *L. casei* and *L. acidophilus*. Although chlorhexidine achieved growth inhibition at lower MICs, nano-curcumin exhibited superior biofilm suppression while offering the advantages of natural origin, minimal side effects, and potential cost-effectiveness. Considering the growing demand for plant-derived oral care products, nano-curcumin warrants further investigation as a viable and biocompatible alternative to synthetic antimicrobials, with particular emphasis on formulation optimization and clinical validation.

5. Declarations

5.1 Acknowledgment

We would like to grateful to the Dental Research Center, Golestan University of Medical Sciences, Gorgan, Iran for their contribution to this study.

5.2 Ethical Considerations

This study was approved by the Ethics Committee of Golestan University of Medical Sciences under the approval code IR.GOUMS.REC.1399.391. All experimental procedures were conducted in accordance with the ethical guidelines and regulations of the university.

5.3 Authors' Contributions

All the study design, data collection, and statistical analysis were conducted collaboratively. The manuscript was written under supervision, and all contributors reviewed and approved the final version.

5.4 Conflict of Interests

The The authors declare no competing interests.

5.5 Financial Support and Sponsorship

This This research did not receive any specific grant.

5.6 Using Artificial Intelligence Tools (AI Tools)

All authors declare that AI-assisted tools were used only for language refinement. No AI-generated content, data fabrication, or result manipulation occurred. The authors take full responsibility for the accuracy and integrity of the manuscript.

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