



Isolation of the Bacteriophages Inhibiting the Expression of the Genes Involved in Biofilm Formation by *Streptococcus mutans*

Zahra Rajabi¹, Rouha Kermanshahi ^{1,*}, Mohammad Mehdi Soltan Dallal², Yousef Erfani³ and Reza Ranjbar ⁴

¹Department of Microbiology, Faculty of Biological Sciences, Alzahra University, Tehran, Iran

²Department of Pathobiology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran

³Department of Medical Laboratory Sciences, School of Allied Medical Science, Tehran University of Medical Sciences, Tehran, Iran

⁴Molecular Biology Research Center, Systems Biology and Poisonings Institute, Baqiyatallah University of Medical Sciences, Tehran, Iran

*Corresponding author: Department of Microbiology, Faculty of Biological Sciences, Alzahra University, Tehran, Iran. Email: rkasra@yahoo.com

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Abstract

Background: The potential of *Streptococcus mutans* for biofilm formation makes it one of the main organisms causing dental caries. Various preventive strategies have been applied to reduce tooth decay.

Objectives: In the current study, we aimed to isolate *S. mutans* bacteriophages from sewage and to investigate their effects on the expression of the genes involved in bacterial biofilm formation in dental caries.

Methods: Eighty-one dental plaque samples were collected. Then to isolate and identify *S. mutans*, bacterial culture media and molecular tests were used. Moreover, the biofilm formation capability of the isolated *S. mutans* was determined. Also, lytic bacteriophages were isolated from raw urban sewage, and phage morphology was determined by transmission electron microscopy (TEM). Real-time PCR was used to assess the effects of the isolated bacteriophages on the expression of the genes involved in biofilm formation.

Results: Overall, 32 (39.5%) samples were positive for the presence of *S. mutans*. All of the isolates contained the *gtfD* gene. The frequencies of other genes were as follows: *gtfB* (17, 53.12%), *gtfC* (19, 53.37%), *SpaP* (13, 40.62%), and *luxS* (23, 17.87%). The isolated *S. mutans* bacteria presented different ranges of biofilm formation ability. Based on TEM results, two sewage-isolated bacteriophages, belonging to Siphoviridae and Tectiviridae families, were able to prevent biofilm formation up to 97%.

Conclusions: Our findings indicate that phage therapy can be an optional way for controlling biofilm development and reducing the colonization of teeth surface by *S. mutans*.

Keywords: Dental Plaque, *Streptococcus mutans*, Bacteriophages, Biofilms

1. Background

Dental caries is one of the most common and among costly and expensive oral diseases for treatment (1). *Streptococcus mutans* is the main etiologic bacterium initiating caries and expressing important virulence factors related to dental caries pathogenesis. *Streptococcus mutans* uses three specific glucosyltransferases (GTFs) to convert sucrose to a sticky extracellular polysaccharide (ECP) that allows them to attach to dental surfaces and form plaque (2). A large number of glucan-binding proteins are known in *S. mutans*, such as SpaP and GbpB and C. The *spaP* gene encodes the P1 protein that has a high affinity for binding to the salivary glycoprotein of *S. mutans* (3). Biofilm maturation is controlled by the Quorum Sensing system (QS). *Streptococcus mutans* also contains two density-dependent

transmission systems. The QS and interspecific transmission systems are encoded by the *luxS* gene (4). Biofilm formation can increase bacterial antibiotic resistance and restrict the ability of the host's inflammatory cells to engulf biofilm-surrounded organisms. Acid production by biofilm forming bacteria destroys tooth enamel, leading to hole creation and tooth decay (5).

A new approach to fight and control biofilm formation by bacteria is to use specific bacteriophages as the viruses invading and killing bacteria (6). They are ubiquitous and approximately 50 times smaller than bacteria (20 - 200 nm), enabling them to effectively penetrate into biofilm layers (7). Phages infect the target bacteria without affecting the normal flora and are naturally eliminated by the eradicating of the bacterium. One advantage of phages is that they can be easily administered either orally, intra-

venously, or nasally (8). There are few reports on the isolation of lytic phages of *S. mutans*, and there is no study on the potential effects of these phages on the expression of the genes contributing to *S. mutans* biofilm formation.

2. Objectives

The aim of this study was to isolate *S. mutans* bacteriophages from wastewater and assess their effects on the expression of the genes involved in biofilm formation by *S. mutans*.

3. Methods

3.1. Isolation and Confirmation of *Streptococcus mutans*

Eighty-one dental plaque samples were taken by a dentist from the patients with dental caries, referred to the dental clinic of Tehran University of Medical Sciences. To isolate *S. mutans*, brain heart infusion (BHI) broth (Merck, Germany) and Mitis-Salivarius agar (QueLab, Canada) were used to incubate bacteria for 24 - 48 h at 37°C with 5% CO₂. After Gram staining, suspected *S. mutans* colonies were confirmed by tests such as catalase, carbohydrate fermentation, and urea hydrolysis to obtain pure cultures (9). *Streptococcus mutans* ATCC35866 was considered as a positive control.

3.2. PCR Identification of *Streptococcus mutans* Isolates

Initially, DNA of the isolated *S. mutans* was extracted using the method described by Hoshino et al. (10). A nanodrop device (Thermo, Lithuania) was used to determine the concentration of the obtained DNA. The oligonucleotide sequences of the primers (Metabion, Germany) used for the PCR amplification of virulence genes have been displayed in Table 1 (10-13). Amplification was carried out by applying a thermocycler (Peqlab, China), and PCR products were visualized by 1% agarose gel electrophoresis.

3.3. Sampling, Isolation, and Purification of Bacteriophages

Raw sewage from urban wastewater of Tehran was supplied, and after overnight, 10 mL of the collected specimens was centrifuged for 10 min at 6000 g and 4°C. The supernatant was passed through a 0.22 μm filter, and 1 ml of the filtered liquid was poured into a tube containing 5 mL of BHI broth with *S. mutans* (10⁶ CFU/mL) to be incubated for 24 h at 37°C while shaking (120 rpm). The tube was centrifuged again for 10 min at 6000 g (4°C), and the supernatant was filtered through a 0.22 μm filter. The filtered liquid was evaluated by the double-layer agar method, which was repeated several times to separate a single plaque. For purification of bacteriophages, the

plate surface was rinsed using 4 mL of the SM buffer and incubated for 5 h while shaking. Finally, the supernatant was passed through a filter and assessed by the double-layer agar method until the isolation of pure plaques. The SM buffer with 20% glycerol was used to preserve bacteriophages. The sensitivity of the isolated phages was measured by the temperature (4°C - 70°C) and pH (3 - 10) tests. To determine bacteriophages' hosts, the agar spot method was used for *Enterococcus faecalis* ATCC29212, *Streptococcus pneumoniae* ATCC 6303, *Streptococcus agalactiae* ATCC12386, *Staphylococcus aureus* ATCC 29213, and the isolated *S. mutans* (14).

3.4. Bacteriophage Morphology

Bacteriophage morphology was studied using transmission electron microscopy (TEM). Drops of the bacteriophage were placed on a copper grid coated with carbon, stained by 2% uranyl acetate, and assessed by a Zeiss-Em10C-100k transmission electron microscope.

3.5. Inhibitory Effects of Isolated Lytic Phages on *Streptococcus mutans* Biofilm Formation

At first, the biofilm formation ability of *S. mutans* isolates was evaluated at 550 nm by the microtiter plate method. Then the inhibitory effects of isolated lytic phages on *S. mutans* biofilm formation were investigated. For this purpose, 100 μL of 0.5 McFarland standard *S. mutans* suspension in the TSB containing 2% sucrose and 50 μL phage was poured into the wells of a microtiter plate and incubated for 24 h at 37°C with CO₂. After washing three times, the plate was left to dry, then stained with 1% crystal violet (Merck, Germany), and incubated at 37°C while shaking (120 rpm) for 15 min. The OD of each well was measured at 550 nm using an ELISA reader. Each test was repeated three times (15). The percentage of biofilm formation was calculated according to this formula:

$$\left[\frac{(C - B) - (T - B)}{(C - B)} \right] \times 100 \quad (1)$$

In this equation, C is the mean OD of control wells, B represents the mean OD of negative controls, and T shows the mean OD of test wells (16).

3.6. Real-Time Quantitative RT-PCR

The biofilms formed in the microtiter plates, with or without bacteriophages (each with 3 replicates) were subjected for RNA extraction using a high-pure RNA isolation kit (Roche, Germany). The quantity and purity of the extracted RNA was measured by a Nano Drop device. Applying a cDNA synthesis kit (Thermo Scientific Revert Aid), the extracted RNA was converted to cDNA based on the manufacturer's instructions. Each reaction tube contained 10

Table 1. Oligonucleotide Sequences of Primers and PCR Amplification Program

Target Gene	Primer Sequence (5' → 3')	PCR Product, bp	PCR Programs	Ref		
<i>gtfD</i>	F: -GGCACCACAACATGGGAAGCTCAGTT-	431	1 cycle: 95°C (5 min); 35 cycles: 94°C (45 s), 59 to 68°C (based on primers, 60 s), 72°C (60 s); 1 cycle: 72°C (5 min)	(10)		
	R: -GGAATGGCCGCTAAGTCAACAGGAT-					
<i>gtfB</i>	F: -AGCAATGCAGCCAATCTACAAAT-	96			(11)	
	R: -ACGAACCTTGCCGTTATTGTCA-					
<i>gtfC</i>	F: -CTCAACCAACCGCCACTGTGTT-	91				(11)
	R: -GGTTTAAACGTCAA AATTAGCTGTATTAG-					
<i>spaP</i>	F: -AACGACCGCTCTTCAGCAGATACC-	192				(12)
	R: -AGAAAGAACATCTCTAATTCTTG-					
<i>luxS</i>	F: -ACTGTCCCCCTTTGGCTGTGTC-	93				(13)
	R: -AACTTGCTTTGATGACTGTGGC-					

μL of the extracted RNA, 1 μL Hexamer primer, 2.5 μL DEPC water, 4 μL 5x standard buffer, 1 μL dNTPs, 0.5 μL RiboLock RNAase, and 1 μL M-MLV, in a total volume of 20 μL . Real-time PCR reaction tubes contained 2 μL of the cDNA, 1 μL of each of forward and reverse primers, 7.5 μL SYBR green PCR master mix, and 5.5 μL DEPC water, applying the Rotor-Gene Q (Qiagene-6000) instrument and SYBR Green PCR Master Mix (Qiagene). As the housekeeping gene, the 16srDNA gene (Metabion, Germany) was used.

3.7. Statistical Analysis

GraphPad Prism 9 software was used for statistical analyses. Due to the normality of the data, one way ANOVA test was utilized to compare means.

4. Results

Out of 81 dental plaque samples, 32 (39.5%) were identified as *S. mutans* strains. Regarding the age of patients, the isolated bacteria were categorized into four different age groups with the highest number (16 cases, 50%) of *S. mutans* strains belonging to the age group of < 7 years. There was a significant relationship between dental caries and the presence of *S. mutans* ($P < 0.05$). Molecular analysis of the *gtfD*, *gtfB*, *gtfC*, *spaP*, and *luxS* genes showed that all *S. mutans* isolates expressed the *gtfD* gene. The frequency of the studied genes was as follows: *gtfB*17 (53.12%), *gtfC*19 (53.37%), *spaP*13 (40.62%), and *luxS*23 (17.87%) (Figure 1).

Regarding the ability of *S. mutans* isolates to form biofilms, it was noticed that 5 (15.6%) isolates produced no biofilms while 3 (9.4%), 21 (65.6%), and 3 (9.4%) isolates formed weak, moderate, and strong biofilms, respectively. Following the purification of phages, the lytic plaques degrading *S. mutans* biofilms were seen on the surface of the plate (Figure 2). Transmission electron microscopy images

revealed the isolation of two lytic bacteriophages. One of them had an icosahedral head and a tail, belonging to the Siphoviridae family. The other one had a non-enveloped icosahedral head and no tail and belonged to the Tectiviridae family (Figure 3). The isolated phages showed a decrease in the number of plaques at 50°C and higher temperatures and revealed high stability at pH = 7 while they tolerated pH = 3 - 6.

Bacteriophage host assessment showed specificity towards the isolated *S. mutans* strains. The addition of the isolated lytic bacteriophages of *S. mutans* prevented the formation of biofilm by these bacteria up to 97%. Real-time PCR findings showed that in the presence of bacteriophages, the expressions of the assessed genes significantly reduced (Figure 4, $P < 0.0001$).

5. Discussion

Dental caries is a damage to a tooth that can happen when decay-causing bacteria in the mouth produce acids and start to decay the tooth's surface (17). When saliva, foods, and oral bacteria are combined together, sticky pale-yellow plaques are formed between teeth and gums. About 4 h after brushing, dental plaques, as structural and functional biofilms, begin to form. Streptococci are known as the main colonizers of oral biofilms, which account for about 63% of the bacteria isolated at the early stages of enamel biofilm formation. *Streptococcus mutans* is able to directly bind to receptors on salivary pellicles (18). In this study, 32 (39.5%) *S. mutans* isolates were identified with the highest number (16, 50%) being related to the patients under the age of seven years. The highest number of referrals was seen in 4-year-old children. There was a significant relationship between dental caries and the presence of *S. mutans* ($P < 0.05$).

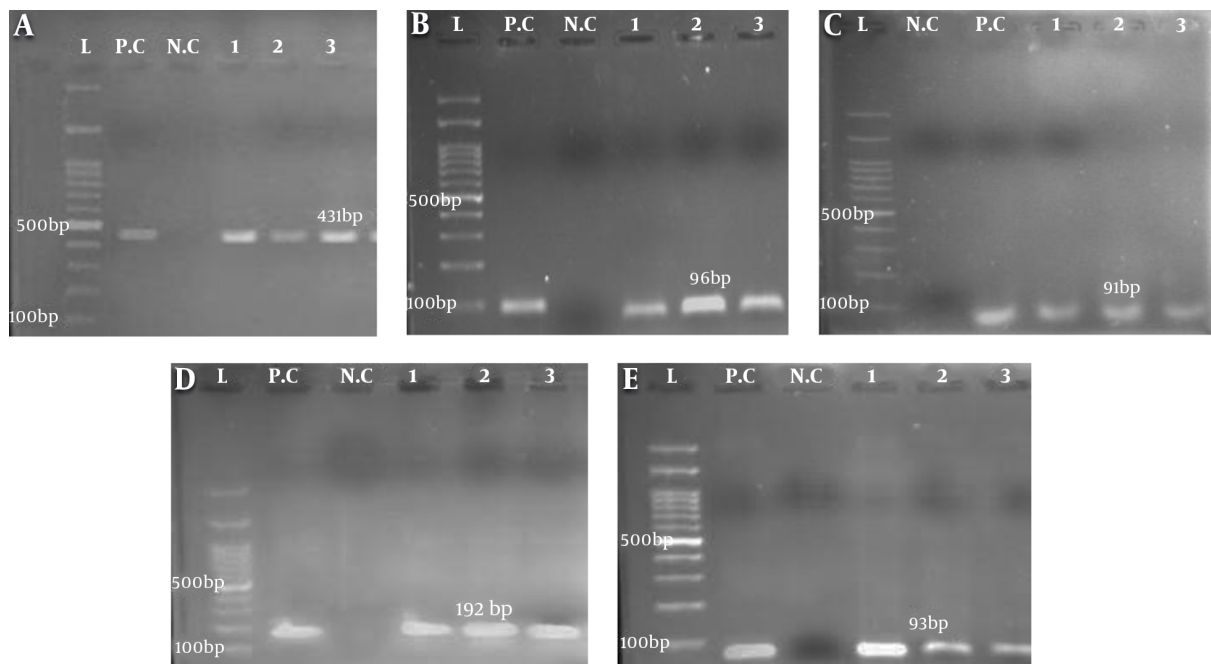


Figure 1. Gel electrophoresis of PCR amplicons of genes from isolated *Streptococcus mutans*, A, *gtfD* (431 bp); B, *gtfB* (96 bp); C, *gtfC* (91 bp); D, *spaP* (192 bp); E, *luxS* (93 bp). L, Ladder (100 bp); P.C, positive control *S. mutans* ATCC35866; N.C, negative control (the tube containing distilled water, gene primers, and Master Mix). The numbers indicate the samples expressing the target genes.

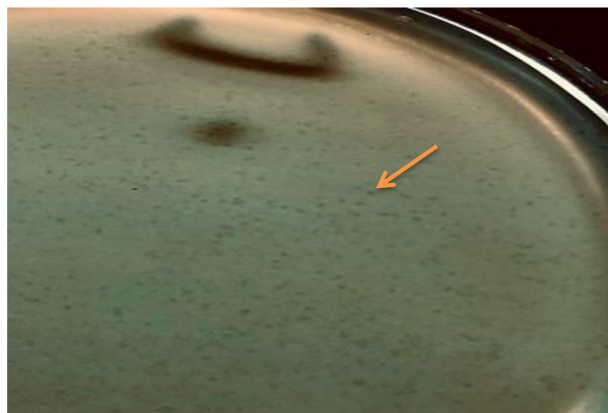


Figure 2. Purified plaques of bacteriophages

Various studies have been performed in Iran and abroad to assess the relationship between *S. mutans* and tooth decay index, especially among preschool children. The results of Batoni et al. (19), Okada et al. (20), Soltan Dalal et al. (21), and Ghasempour et al. (22) indicated that *S. mutans* was the main pathogen linked with dental caries with a remarkably higher frequency than other oral streptococci, especially among children under seven years of

age. Our findings and those of other studies show that the presence of *S. mutans* in the oral cavity exposes individuals to a high risk for dental plaque formation. Differences in the reported frequencies of *S. mutans* probably reflect variations in the genetics, culture, and diets of people in different regions and countries.

Phage therapy is considered a viable alternative for the treatment and control of pathogenic bacteria (23). In this study, we isolated phages from urban wastewater and tested the ability of the bacteriophages in reducing biofilm formation by *S. mutans* and inhibiting the expression of the genes involved in biofilm production. In earlier reports, Beheshti Maal et al. (24) and Dalmaso et al. (25) extracted *S. salivarius* and *S. mutans* phages from the waters of the Caspian Sea and saliva, respectively. However, we here isolated phages from raw urban sewage. According to TEM micrographs, two different lytic bacteriophages were identified, belonging to the Siphoviridae and Tectiviridae virus families. As mentioned, Beheshti Maal et al. (24) isolated two bacteriophages against *S. salivarius* from the Caspian Sea, which belonged to the Tectiviridae and Cystoviridae families. Dalmaso et al. (25) by screening 85 saliva samples, isolated only one phage against *S. mutans*. On the other hand, Bacharach et al. (26) and Hitch et al. (27) could not isolate any phage against *S. mutans* from saliva, which

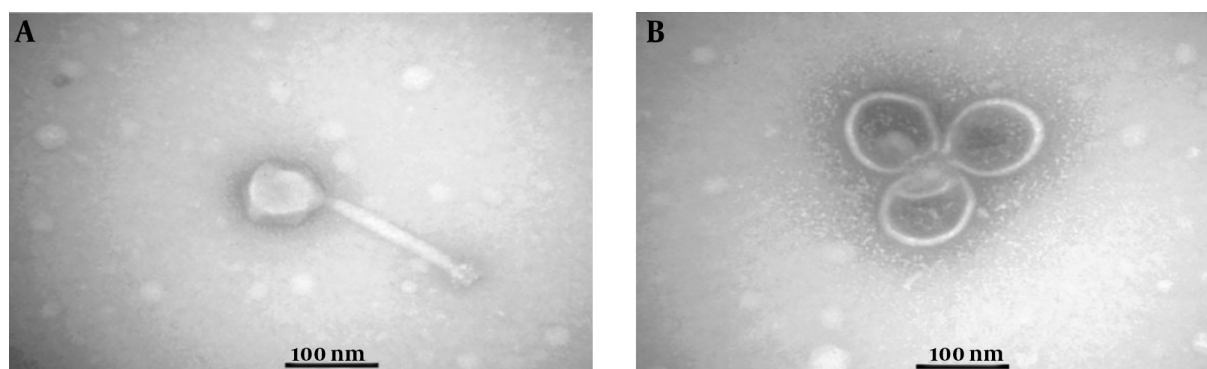


Figure 3. Transmission electron micrographs: A, The morphology of Siphoviridae phage, showing an icosahedral head (88 nm) and a tail (181 nm); B, The morphology of Tectiviridae phage, showing an icosahedral head (108 nm) without a head-tail. The negative control was stained by uranyl acetate 2%. TEM Zeiss -EM10C-100 KV

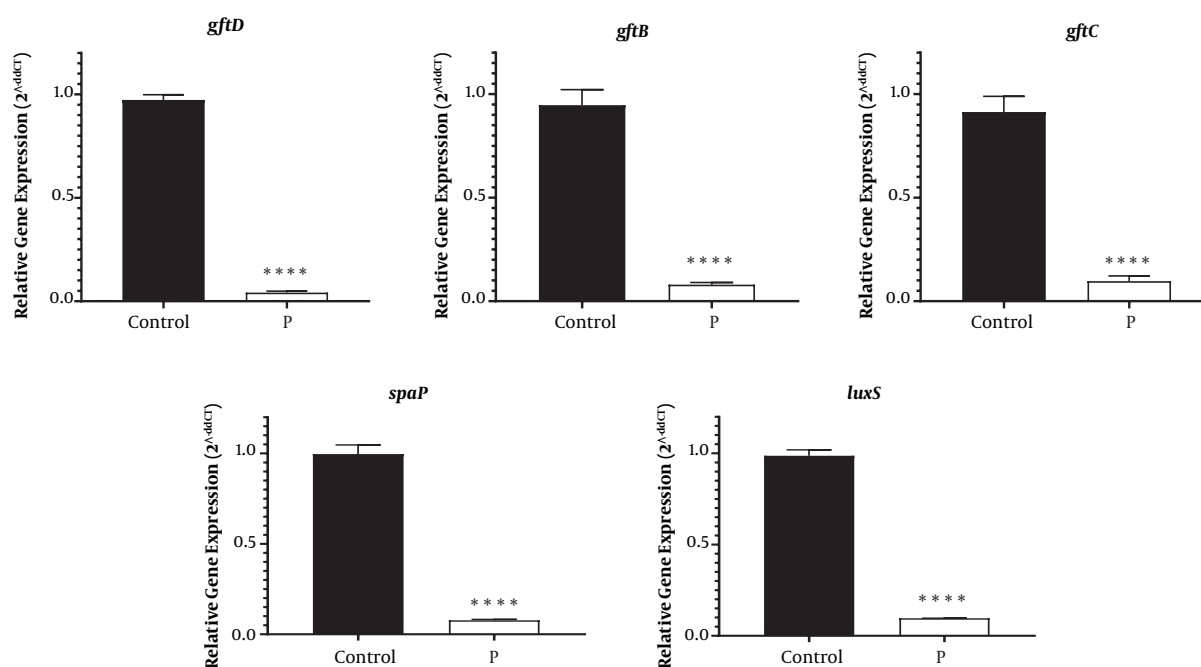


Figure 4. The effects of lytic bacteriophages of *Streptococcus mutans* on the expression of the genes responsible for biofilm formation by the bacterium. Gene expressions before (control) and after treatment by phages (P) have been shown.

is probably due to the difficulty of isolating phages from the oral cavity. So, using a proper source for isolating bacteriophages is important, and according to our study, raw urban sewage can be a better source for phage isolation. The lytic bacteriophages isolated in the present study were specific for *S. mutans*.

In this study, all the isolates contained *S. mutans* specific *gftD*. Besides, *luxS*, *gftB*, and *gftC* genes were identified in 23 (71.87%), 17 (53.12%), and 19 (53.37%) isolates, respectively. The least frequency was related to the *SpaP* gene, ren-

dering positivity in 13 (40.62%) isolates. In another study on 61 dental plaque samples, 19 (41.7%) and 8 (4.1%) isolates expressed the *gftB* and *gftC* genes, respectively (28). However, the relationship between *gftS* genes' expression and the quantity of produced ECP differed among clinical *S. mutans* isolates. Normally, various enzymes can influence the expression and activity of the genes involved in biofilm formation by *S. mutans*. For the first time, we investigated the effects of bacteriophages on the expression of the genes related to biofilm production by a bacterium. Our results

showed that lytic bacteriophages strongly inhibited the expression of the assessed genes. Our molecular results (gene expression) were consistent with the effects of bacteriophages on the biofilm formation ability of *S. mutans*, as evidenced by the microtiter plate method, which its results agreed with those of previous phenotypic reports conducted by Beheshti Maal et al. (24) and Dalmaso et al. (25). Overall, phages and their enzymes can successfully be used to target oral bacteria, in planktonic and biofilm forms, and control the formation of oral biofilms.

5.1. Conclusions

Drug resistance, especially in the biofilm mode, is one of the most challenging issues for treating bacterial infections. To overcome this problem, bacteriophages can be used as new alternatives to limit the growth of pathogenic bacteria and develop new approaches to design effective antibacterial drugs. Based on our results and those of previous studies, phage therapy can provide an efficient way to control biofilm development and help to reduce the colonization of tooth surfaces by *S. mutans*.

Footnotes

Authors' Contribution: Study concept and design: Zahra Rajabi and Rouha Kermanshahi. Data acquisition: Zahra Rajabi, Rouha Kermanshahi, and Mohammad Mehdi Soltan Dalla. Data analysis and interpretation: Zahra Rajabi and Yousef Erfani. Drafting the manuscript: Zahra Rajabi and Reza Ranjbar.

Conflict of Interests: The authors declare no conflict of interest.

Ethical Approval: The study was approved by the ethics committee of Tehran University of Medical Sciences (code: IR-TUMS.SPH.REC.1396.4483).

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