

Research Article

Comparison of the Effect of Extracted Bacteriocin and Lytic Bacteriophage on the Expression of Biofilm Associated Genes in *Streptococcus mutans*

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Received 11 February 2022; Accepted 26 March 2022; Published 25 April 2022

Academic Editor: Hamid Tebyaniyan

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Today, various biological approaches are used to combat dental plaque biofilms. In the current study, we aimed to compare the effect of extracted bacteriocin and lytic bacteriophage on the expression of biofilm associated genes in *Streptococcus mutans*. *Streptococcus mutans* was isolated from plaques of decayed dental and the existence of their gtf genes was confirmed by PCR. For bacteriocin extraction, at first two probiotic lactobacilli were isolated from traditional foods, then bacteriocin was extracted by partial purification method, and SDS-PAGE was used for estimation of its molecular weight. The previously extracted lytic bacteriophage from raw urban sewage was used against *Streptococcus mutans*. Finally, the effect of isolated bacteriocin and bacteriophage on gtf genes expression level was measured by real-time PCR. Out of 81 dental plaque samples, 32 (39.5%) *Streptococcus mutans* strains were isolated. The frequency of genes was as follows: gtfD32 (100%), gtfB 17 (53.12%), and gtfC 19 (53.37%). 120 traditional food samples (milk, yogurt, pickle, and salty pickle) were evaluated for isolation of lactobacillus. Three strains of *Lactobacillus fermentum* and four *Lactobacillus plantarum* with probiotic potential activity were isolated. Two types of bacteriocins from *Lactobacillus fermentum* and a single type of bacteriocin from *Lactobacillus plantarum* were extracted, and their molecular weights were 60, 58, and 70 kDa, respectively. In our previous study, two bacteriophages belonging to the Siphoviridae and Tectiviridae families were isolated. Real-time PCR results had shown that both bacteriocin and bacteriophage had a decreasing effect on the expression of gtf genes. The different modes in our study for the effects of bacteriocin and bacteriophage showed that both of them had good potential as suitable options to fight dental plaque biofilms, and bacteriophages alone showed a stronger reducing effect.

1. Introduction

Tooth decay is one of the major problems in the world that begins with the formation of dental plaque. Following the formation of dental plaque, tooth decay is caused by turning sugars to acid by oral bacteria and lead to oral disease [1].

Streptococcus mutans is the main etiologic factor that initiates caries and has important virulence factors related to the pathogenesis of dental caries. GTFs are the key enzyme of *Streptococcus mutans* which convert sucrose into a sticky, extracellular polysaccharide which allows them to attach to a dental surface and form plaque. *Streptococcus mutans*

produces three separate Gtfs, which synthesize water-insoluble and soluble glucan [2]. GTFs have an effective role in absorption of other oral bacteria to form a dental biofilm. Biofilm can lead to increasing the antibiotic resistance of bacteria and the inability of host inflammatory cells to phagocytose biofilm cells [3]. Biological approaches are used to combat dental plaque biofilms. The use of microorganisms to promote human health and control of pathogenic bacteria has a very long history [4]. Probiotics are one of beneficial microorganisms which are able to produce bacteriocin. Bacteriocins are protein compounds with antimicrobial properties that prevent the growth of sensitive strains. These proteins have low molecular weight and are resistant to heat and are divided into 4 groups. Some bacteriocins are currently used commercially to inhibit the growth of pathogenic bacteria in food products such as fish, dairy, and meat products [5]. Another new approach used to fight and controlling bacterial biofilm is to use a specific bacteriophage against that bacterium. Bacteriophages are viruses that attack and kill bacteria [6]. These viruses are specific to bacteria and cannot attack eukaryotes. They are approximately 50 times smaller than bacteria (20–200 nm) and are ubiquitous. Due to the small size of bacteriophages, they have a considerable ability to penetrate biofilm layers [7]. Phages infect the target bacterial cell without affecting the normal flora and are naturally eliminated by eradicating the bacterial cell [8]. There are few reports on bacteriocin effect on genes level of *Streptococcus mutans* but there is not any study about the effect of bacteriophages, so in this study we aimed to compare the effect of extracted bacteriocin and lytic bacteriophage on the expression of biofilm associated genes in *Streptococcus mutans*.

2. Material and Methods

2.1. Isolation and Confirmation of *S. mutans*. Eighty-one dental plaque samples were taken by the dentist from patients with dental caries referred to the dental clinic of Tehran University of Medical Sciences. BHI broth (Merck, Germany) and Mitis-Salivarius Agar (QueLab, Canada) were used to isolate *Streptococcus mutans*. Suspected colonies to *Streptococcus mutans* for obtaining pure culture and biotype confirmation were followed by the tests such as catalase, carbohydrate fermentation, and urea hydrolysis [9]. *Streptococcus mutans* ATCC35866 was considered as positive control.

2.2. PCR Identification of Isolated *S. mutans* Genes. At first, DNA extraction of isolated *Streptococcus mutans* was followed by the described method by Hoshino et al. [10]. Nano drop device (Thermo) was used to measure the appropriate concentration of obtained DNA. Oligonucleotide sequences of primers and PCR amplification of virulence genes are displayed in Table 1. Amplification was carried out in a thermo cycler (peQlab). PCR products were introduced to 1% agarose gel electrophoresis.

2.3. Isolation and Molecular Confirmation of Probiotic *Lactobacillus fermentum* and *Lactobacillus plantarum*. One hundred and twenty samples of traditionally prepared milk, yogurt, pickles and salty pickles were collected (30 each of them) from January 2021 until June 2021. Isolation of lactobacillus strains was done by FDA protocol using enrichment in MRS broth and culturing on MRS agar. Appropriate biochemical tests were used for identification of the isolated strains. Probiotic potential activity of isolated lactobacilli was measured by acid and bile resistance. To verify that the phenotypic strains were identified as *Lactobacillus*, we used sequencing of 16srDNA gene of lactobacilli (27F: CTCGTTGCGGGACTTAA and 1522R: GCAGCAGTAGGGAATCTTC) by the PCR method. To perform a molecular reaction, we used Antonsson et al. working protocol with some modifications. The pure culture of *Lactobacillus* isolates was prepared on MRS agar medium and 2 to 3 colonies were dissolved in 50 μ l of STE solution. The suspension was placed in ben-marie (96°C) for 10 minutes (boiling method). The suspension was then centrifuged at RPM 13000 for 5 minutes, and the supernatant was used for PCR [12].

2.4. Extraction of Bacteriocin from Probiotic *Lactobacillus fermentum* and *Lactobacillus plantarum*. In order to extraction of bacteriocin from *Lactobacillus*, these bacteria were concentrated and partially purified by ammonium sulfate precipitation. For this purpose, after culturing of *Lactobacillus fermentum* and *Lactobacillus plantarum*, neutralizing the effect of acid and isolating the microbial mass were performed and then the produced bacteriocin was precipitated and concentrated by precipitation with ammonium sulfate. Concentrated bacteriocin was dialyzed, and its activity was measured. SDS-PAGE technique and Bio Rad device (small gel) were used to evaluate the molecular weight of extracted bacteriocins [13].

2.5. Sampling, Isolation, and Purification of Bacteriophage. We used extracted lytic bacteriophage from our previous study [14], but the brief description is as follows.

Raw sewage from urban wastewater of Tehran was supplied, and after overnight, 10 ml of raw sewage was centrifuged for 10 min in 6000 g in 4°C. The supernatant was passing through a filter 0.22 μ m. 1 ml of filtered liquid was added to a tube containing 5 ml of BHI broth with *Streptococcus mutans* (106 CFU/ml) and incubated for 24 h at 37°C. Tube was centrifuged again for 10 min in 6000 g in 4°C and supernatant filtered through a 0.22 μ m filter. The double layer method (soft agar) was used to confirm the presence of lytic bacteriophage and the time that phage plaques were appeared in order to purify bacteriophage; plate surface was rinsed by SM buffer and transferred to sterile tube. Then, plaque was cut aseptically and added to tubes for repeating the above method until the isolation of pure and separated plaques. SM buffer with 20% glycerol was used to preserve bacteriophages. Host specificity, pH, and temperature tolerance of isolated bacteriophage were also measured. Finally, bacteriophage morphology was studied by using TEM [14].

TABLE 1: Oligonucleotide sequences of primers and PCR amplification program.

Target gene	Primer sequence (5'-3')	PCR product (bp)	PCR programs	Ref
gtfD	F:_GGCACCACAACATTGGGAAGCTCAGTT-R: _GGAATGGCCGCTAAGTCAACAGGAT-	431	1 cycle: 95°C (5 min); 35 cycle: 94°C (45 s), 59°C-68°C (based on primers, 60 s), 72°C (60 s); 1 cycle: 72°C (5 min)	[10]
gtfB	F:-AGCAATGCAGCCAATCTACAAAT-R:_ ACGAACTTTGCCGTTATTGTCA-	96		
gtfC	F:_CTCAACCAACCGCCACTGTT-R: _GGTTTAACGTCAAAAATTAGCTGTATTAG-	91		

TABLE 2: cDNA synthesis kit manufacture.

Component	Amount
Extracted RNA	10 μ l
Hexamer primer	1 μ l
DEPC water	2/5 μ l
5x standard buffer	4 μ l
dNTPs	1 μ l
RiboLock RNAase	0/5 μ l
M-MLV	1 μ l

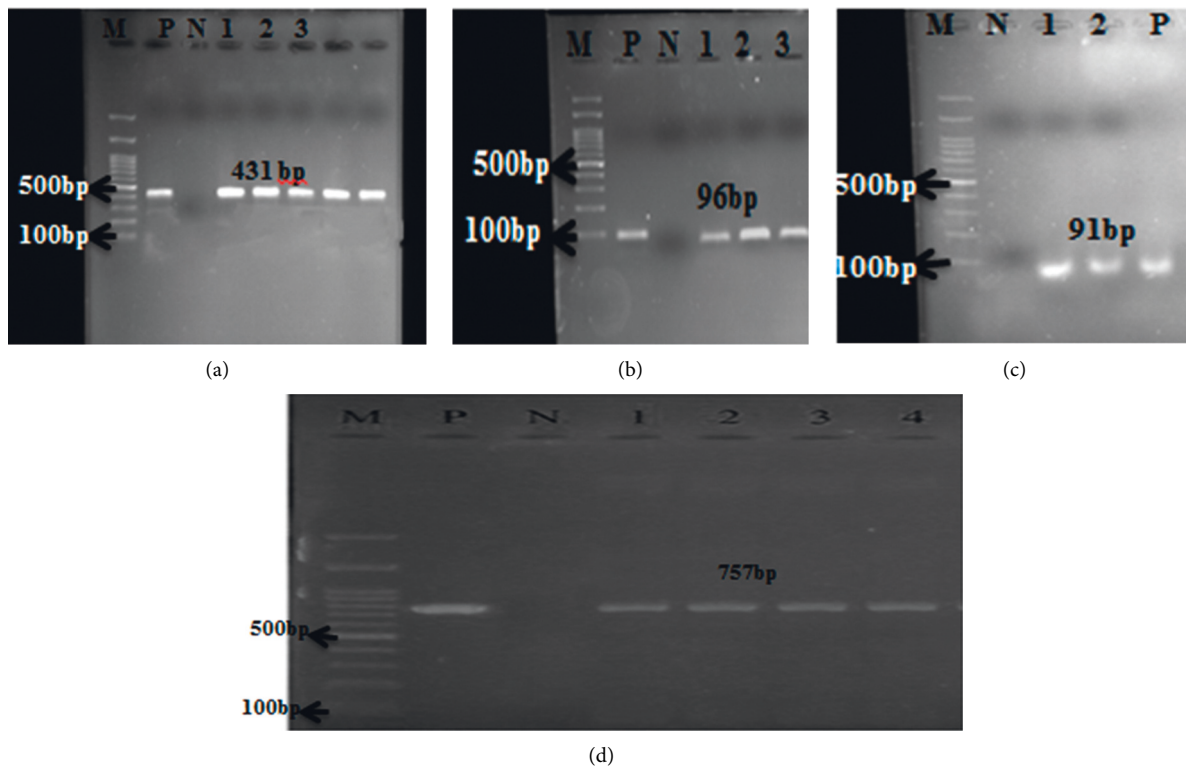


FIGURE 1: Results of gel electrophoresis related to PCR amplification of various genes in *Streptococcus mutans* strains, (a) gtfD (431 bp), (b) gtfB (96 bp), (c) gtfC (91 bp), and (d) 16srDNA gene of *Lactobacillus* (757 bp), M: Marker 100 bp, (P) Positive Control for *Streptococcus mutans* ATCC35866, and Positive Control for *Lactobacillus brevis* ATCC 436. (N) Negative Control (D.W, gene primers, Mastermix), Numbers: The samples contained the gene.

2.6. Real-Time Quantitative RT-PCR. The preparation of biofilm cells on microtiter plates with and without bacteriocin and bacteriophage (in 3 replicates) for RNA extraction was done according to RNA extraction process with high pure RNA isolation kit of Roche (Germany). By the cDNA

synthesis kit (Thermo Scientific Revert Aid), the extracted RNA was converted to cDNA. The real-time PCR reactions (2 μ l cDNA, 1 μ l both forward and reverse primers, 7.5 μ l SYBR green PCR master mix, and 5.5 μ l DEPC water) were performed on the Rotor-Gene Q (Qiagen-6000) instrument

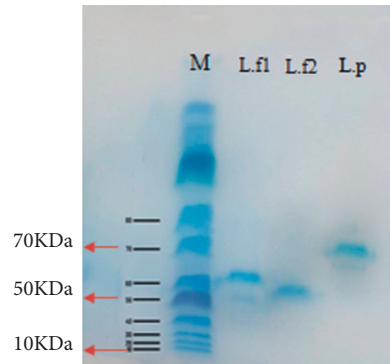


FIGURE 2: Bacteriocins isolated from two strains of *Lactobacillus fermentum* (L.f1 and L.f2) with a molecular weight of 60 and 58 kd, respectively, and bacteriocins isolated from (L.p) *Lactobacillus plantarum* with a molecular weight of 70kd using SDS-PAGE electrophoresis.

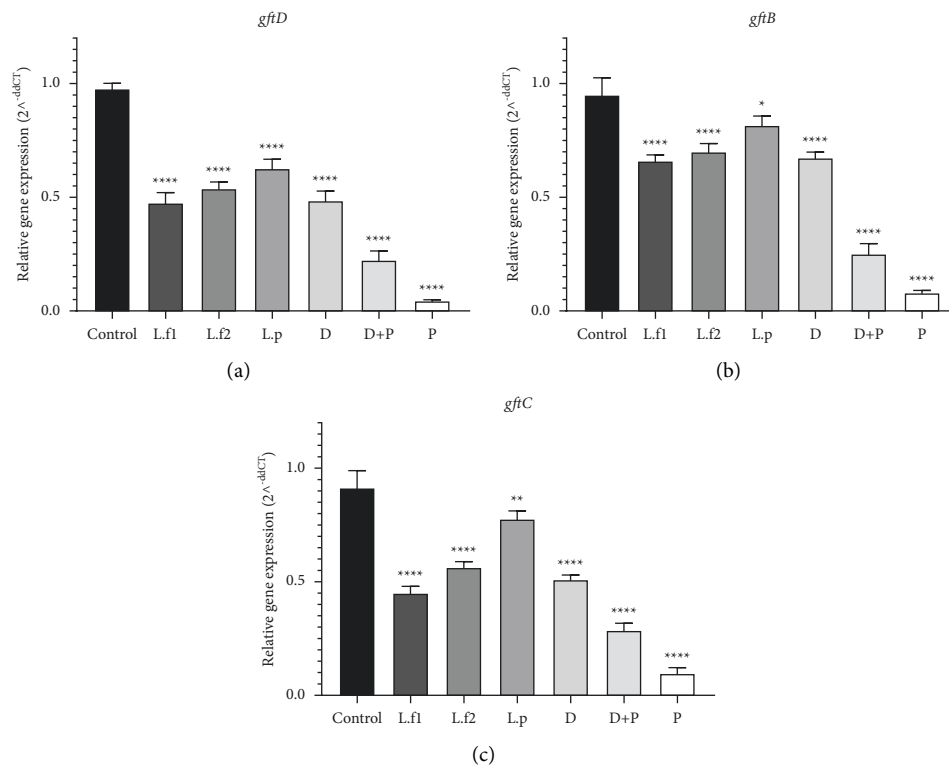


FIGURE 3: Evaluation of the effect of bacteriocins isolated from *Lactobacillus fermentum* 1 (L.f1), *Lactobacillus fermentum* 2 (L.f2), and *Lactobacillus plantarum* (L.p), the sum of all three bacteriocins isolated (D), the sum of all three bacteriocins isolated by lytic bacteriophage (P Specific (D+P), and specific lithic bacteriophage (P) on the expression of native *gtf* gene. (a) *gtfD*, (b) *gtfB*, and (c) *gtfC* of isolated *Streptococcus mutans* from decayed dental plaque. Comparison of gene expression level with control mode shows a significant reduction level.

and SYBR Green PCR Master Mix (Qiagen). 16srDNA gene was used as a housekeeping gene.

3. Results

For isolation of *Streptococcus mutans*, out of 81 dental plaque samples, 32 (39.5%) had positive results. Molecular analysis of the *gtfD*, *gtfB*, and *gtfC* genes in *Streptococcus mutans* showed that all isolates had the *gtfD* gene. The frequency of

the studied genes was as follows: *gtfB*17 (53/12%) and *gtfC*19 (53/37%) (Figures 1(a)–1(c)). Out of 120 samples of milk, yogurt, pickle, and salty pickle from different region in Tehran, 153 isolates of *Lactobacillus* were obtained and confirmed by sequencing of 16srDNA (Figure 1(d)) gene. 18 (11/76%) strains including 4(22/22%) *Lactobacillus plantarum* and 3 (16/66%) *Lactobacillus fermentum* had probiotic potential activity. The most isolated *Lactobacilli* were obtained from milk 67 (43/79%).

The results of extraction and partial purification of bacteriocins for probiotic strains of *Lactobacillus fermentum* and *Lactobacillus plantarum* are shown in Figure 2. Two probiotic isolates of *Lactobacillus fermentum* and one isolate of *Lactobacillus plantarum* had the potential to produce bacteriocin. Bacteriocins isolated from *Lactobacillus fermentum* had a molecular weight of 60 and 58 kDa, respectively, and bacteriocins isolated from *Lactobacillus plantarum* had a 70 kDa molecular weight.

Real-time results showed that in the presence of bacteriophages, the expression of used genes was highly reduced. Graph pad prism9 software was used for statistical analysis of data. Due to the normality of the data, ANOVA-one way test was chosen to compare the means ($p < 0.0001$) (Figure 3).

4. Conclusion

The oral environment is the shelter of a large and diverse number of bacterial microorganisms that are considered as flora. There are more than 700 bacterial species known in the oral cavity, of which 40 species belonged to cause caries. *Streptococcus mutans* is considered as one of the most important etiological factor for tooth decay [15]. In this study, 32 (39.5%) *Streptococcus mutans* was isolated. There was a significant relationship between caries and *Streptococcus mutans* ($p < 0.05$). Various studies have been performed on the relationship between *Streptococcus mutans* and tooth decay index, especially in the preschool children in Iran and abroad. The study results of Neves et al. [16], Bezerra et al. [17], Babaeekhu et al. [18], and Bashirian et al. [19] indicated that *Streptococcus mutans* is the main pathogen to dental caries and frequency of this bacteria is more than other oral Streptococci in many cases, especially in children under 7 years old. In this study, all isolates contained *S. mutans*-specific gtfD (Figure 1(a)) because it was specific for *Streptococcus mutans*. Gharakhani in 2019 by studying 61 dental plaque samples of people with different ages reported 19 isolates (41.7%) with gtfB gene and 8 (4.1%) with gtfC gene [20]. However, the relationship between gtfS genes expression and the quantity of ECP produced differed among clinical isolated of *Streptococcus mutans*. In a normal situation, the existence of various enzymes can influence expression and activity of genes involved in biofilm formation of *Streptococcus mutans*.

Lactobacilli are a heterozygous group of lactic acid bacteria and are especially important because of their importance for improving health, such as preventing colonization of the intestines by pathogenic bacteria and producing metabolite which are able to inhibit the growth of pathogenic bacteria [21]. Lactobacilli are also found in a variety of fermented foods. Bacteriocin is one of the major metabolite of LAB bacteria that it is commercially produced and used in the food industry as a food preservative [22]. To investigate the effects of bacteriocin on the expression level of gtf genes, at first we isolated *Lactobacillus fermentum* and *Lactobacillus plantarum* from 120 traditional foods (milk, yogurt, pickle, and salty pickle) which are usually as common consumed food by the people.

Of the 120 samples, 153 isolates of *Lactobacillus* were obtained, of which three isolates were *Lactobacillus fermentum* and four isolates were detected as *Lactobacillus plantarum* by probiotic potential activity.

SoltanDallal from pickle and salty pickle in Iran [23], Yu in China from traditional Sichuan province pickles [24], Nguyen from traditional fermentation vegetables in Vietnam [25], Parsaeimehr from traditional yogurt in Iran [26], and Yeshambel from cow milk and milk products [27], isolated a large number of probiotic bacteria that most of them belonged to LAB. Due to differences in culture, geography, and vegetation, the frequency of probiotic strains is definitely different but all data refer to beneficial effect of them to inhibit and combat by pathogenic bacteria. In the current study, we isolated two bacteriocins from *Lactobacillus fermentum* which had molecular weight of 60 and 58 kDa, respectively, and bacteriocin isolated from *Lactobacillus plantarum* had a molecular weight of 70 kDa. Adebayo in 2013 extracted bacteriocin with a molecular weight of 70 kDa from *Lactobacillus fermentum* by ammonium sulfate precipitation and dialysis [28]. Sing in 2013 observed 78 kDa extracted bacteriocin from *Lactobacillus fermentum* by the SDS-PAGE method. *Lactobacillus plantarum* in Chun study had 3.5 kDa and 4.7 kDa molecular weight by Tris-Tricine SDS-PAGE methods [29]. Due to the differences in the native of the strains and the differences in the ability to produce bacteriocins, the difference between the molecular weights of the bacteriocins certainly seems to be normal.

Phage therapy is considered a viable alternative for the treatment and control of pathogenic bacteria [30]. In this study, we tested the ability of two extracted phages from our previous study (extracted bacteriophages belonged to the Siphoviridae and the Tectiviridae family of viruses) [14] to reduce expression of the gene involved in biofilm for the first time. Also, we made an effort to investigate the effect of isolated bacteriocin and bacteriophage on gtfD, gtfB, and gtfC genes expression level simultaneously. Our results showed that lytic bacteriophages in comparison with bacteriocins had a strong inhibitory effect on reducing the expression of genes used in this study. The total effect of bacteriocin and bacteriophage together, in comparison with the effect of bacteriocin alone, was in the next order of reduction effect. Among of isolated bacteriocin, the bacteriocin extracted from *Lactobacillus fermentum* 1 had more reduction effect in comparison with two other bacteriocins. Before Tahmourespour in 2011 [31], Raj in 2017 [32], Ahmed in 2014 [15], and Wasfi in 2017 [33] have shown the decreasing effect of isolated probiotic Lactobacilli such as *Lactobacillus fermentum*, *Lactobacillus plantarum*, *Lactobacillus acidophilus*, and *Lactobacillus Casei* on gtfS genes in *Streptococcus mutans* which is in accordance with the results obtained from our current study and showed that bacteriocins as small antimicrobial peptides are one of the most important components of probiotics in the fight against pathogens, especially *Streptococcus mutans*. However, there is no another study about the effect of bacteriophage on reducing the expression of genes associated in *Streptococcus mutans* biofilm but studies on the isolation of *Streptococcus*

mutans lytic bacteriophages and its inhibitory effect on its biofilm formation are very limited, and the results are mentioned in the discussion section of our previous study. However, the results showed a very effective role of bacteriophage on growth inhibition and inhibition of *Streptococcus mutans* biofilm.

Despite many advances in the health and dental industry, tooth decay is still a main problem. Results of our study and other previous studies showed that traditional foods can be considered as good sources of probiotic organisms. Proper selection of probiotic bacterial strains can be one of the most effective selection methods to combat biofilm and reduce the formation of dental plaque. Phage therapy is another way to solve the problem of antibiotic resistance and fight biofilm formation to help treat and kill the desired bacteria and is more effective to decrease the biofilm compared with antimicrobial therapy with synthetic antibiotics. And according to the obtained results, they can be viewed in the future as components in the oral health industry.

Data Availability

The data used to support this study are included within the published paper.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

Authors' Contributions

Zahra Rajabi and Rouha Kermanshahi were responsible for study concept and designing. Zahra Rajabi, Rouha Kermanshahi, and Mohammad Mehdi Soltan Dallal were responsible for data acquisition. Mohammad Reza Afradi and Donya Alinejad prepared the sample. Zahra Rajabi, Yousef Erfani, and Reza Ranjbar analyzed and interpreted the data. Zahra Rajabi and Reza Ranjbar drafted the manuscript. Finally, the paper was revised by Reza Ranjbar.

Acknowledgments

The authors would like to thank the school of Allied Medical Sciences of Tehran University of Medical Sciences, for giving us this opportunity to do our research project in their laboratory. We would also like to thank for the guidance and advice from the Clinical Research Development Unit of Baqiyatallah Hospital, Tehran, Iran.

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