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Effect of Probiotic *Enterococcus durans* on the Adhesion of Clinically Isolated *Streptococcus mutans*

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Abstract

Background: *Streptococcus mutans* is the most cariogenic microorganism with high adherence ability to the tooth surfaces. The probiotics have attracted attention as a new and bioecological technique to inhibit oral bacterial colonization. *Enterococcal* strains, such as *Enterococcus durans*, are abundant in nature and can be identified as a probiotic.
Objectives: Since the reduction of adhesion can be an effective way to decrease the cariogenic potential of *S. mutans*, the present study aimed to evaluate the inhibitory effect of *E. durans* on the adhesion of *S. mutans*.
Methods: In this in vitro study, the standard strain bacteria of probiotic *E. durans* and *Streptococcus mutans* and 12 clinical samples of *S. mutans* were used. The ability of *S. mutans* biofilm formation was assessed. Then, the effect of *E. durans* on *S. mutans* adhesion was determined via microtiter plate technique by two methods: (1) Adding a mixed suspension of *E. durans* and *S. mutans* simultaneously; (2) adding *E. durans* 30 minutes before the inoculation of *S. mutans* to the system. The data were analyzed with SPSS 20 by using paired *t*-test, and statistical significance was set at $P < 0.05$.
Results: The results showed a decrease in adhesion in the presence of *E. durans*, with the use of both methods ($P < 0.05$).
Conclusions: Based on the results of this in vitro study, the use of probiotic *E. durans* decreased the adhesion of *S. mutans*.

Keywords: *Enterococcus durans*, Probiotics, *Streptococcus mutans*

1. Background

Dental caries is a transmissible infectious disease that affects the hard tissues of the oral cavity and is initiated by the decalcification of the inorganic component of the tooth, followed by the destruction of the organic matrix. Generally, dental caries occurs in the presence of cariogenic bacteria (1). Of all bacterial species, *S. mutans* is the most important and the most cariogenic microorganism. One of the most prominent properties of *S. mutans* is its ability to adhere to tooth surfaces (1). The use of techniques to reduce adhesion and inhibit the colonization of oral bacteria can decrease the dental caries rate. Currently, the use of probiotics has attracted attention as a new and bioecological technique to combat oral diseases because its utilization makes it possible to simultaneously use systemic and local interventions (2, 3).

In recent years, several studies have shown the beneficial effects of probiotics on oral health and on decreasing the counts of cariogenic bacteria; these probiotic agents are presented in the form of dietary supplements, tablets, capsules, and mouthwashes (2, 4-7). Evidence indicates that the mechanism of action of probiotics in the oral cavity is through the completion and production of organic acids and antimicrobial agents, similar to that in other parts of the alimentary tracts and urinary system (8-10). Various microorganisms have been introduced as probiotics, the most important of which belong to the lactic acid bacterial (LAB) family. *Lactobacillus* and *Enterococcus* species belong to the LAB family and are members of the normal flora of the alimentary tract and fermented food. *Enterococcus* species are ubiquitous in nature, which is attributed to their survival and resistance to growth inhibition factors, including resistance to high acidity and biliary salts (11, 12).

Conventional dairy products are one of the essential sources for the isolation of *Enterococcus* bacterial species, and their combination, as an initiator, into industrial dairy products, not only does improve the quality and flavors

of the product but also provides valuable health benefits of probiotics for users (12). *Enterococcus durans* has been introduced on the list of safe materials (GRAS) and identified as one of the permanent residents of the GI tract (13). In humans, *Enterococcus* species are used for the treatment of some diseases, such as diarrhea and inflammatory bowel syndrome, and for regulating the immune system (14, 15). The growth and proliferation of this bacterial species in a culture medium rich in selenium results in its accumulation in the bacterial cell. Therefore, this bacterial species might be an alternative source for selenium in organic foods (15). Besides, evidence has shown the useful probiotic characteristics of *E. durans*, including its potential to adhere to and colonize mucosal surfaces, the absence of aggressive potential, lack of virulence factors, specific antimicrobial activity against pathogens, induction of immune responses, the effect on the expression of proinflammatory cytokines and increasing the induction of secretory IgA (14-16).

Biofilm formation is the first step in bacterial infection, followed by attachment to the dental lamina and the production of destructive materials (17). We aimed to introduce a method to reduce biofilm formation in dental caries. The adhesion of *S. mutans* to tooth surfaces has a significant role in their pathogenicity. Therefore, decreasing the adhesion can be an effective way to decrease the cariogenic potential of *S. mutans* (17).

2. Objectives

The present study evaluated the inhibitory effect of *E. durans* on the adhesion of *S. mutans*.

3. Methods

3.1. Isolation of Streptococcus mutans

The present in vitro study was carried out on carious teeth in 66 children aged 6 - 12 years, who were referred to the Department of Pediatric Dentistry, Faculty of Dentistry, Sanandaj University of Medical Sciences. The inclusion criteria consisted of no use of antimicrobial mouthwashes during the one month before the study (18), no use of antifungal and antihistaminic agents, no use of corticosteroids (19), and a caries index of > 10 (20). The Ethics Committee of Kurdistan University of Medical Sciences approved the protocol of the study under the code IR.MUK.REC.1396/302. Clinical samples were collected after justifying the children's parents and acquiring informed consent forms. The dental plaque was transferred from carious teeth into tubes containing 5 mL of brain-heart infusion culture medium (BHI) with the use of a sterile micro brush. The tubes were incubated at 37°C for 24 hours with the clinical samples through culturing on blood agar medium (Ibresco) using the streak culture technique, gram staining, catalase, growth in bile esculin medium (Ibresco), and growth in Mitis salivarius agar (Ibresco). Then the suspected colonies of *S. mutans* were cultured in brain-heart infusion broth medium and stocked at -20°C. *E. durans* were cultured based on the microbiological method.

3.2. Formation of Micro-Biofilm Using the Microtiter Plate Technique

In this stage, the ability of the isolated colonies of *S. mutans* ATCC35368 (Pasteur Institute of Iran) was determined using the microtiter plate technique. First, the clinical strain of *S. mutans* in the BHI broth culture medium was cultured on the BHI agar medium and incubated for 24 hours in an anaerobic jar (using a candle) at 35°C - 37°C. After 24 hours, 0.5 McFarland concentration of the cellular suspension in the BHI broth culture medium was prepared from each bacterial sample to determine the ability of isolated bacteria to form biofilms. A total of 200 μL of this suspension were transferred into each well in a flat 96-well polystyrene plate (Biosorfa).

The negative control wells contained the BHI broth culture medium. After 24 hours, the contents of the wells were retrieved, and each well was irrigated with 200 μL of sterile physiologic serum three times. A total of 200 μL of absolute ethanol was used for 15 minutes to stabilize bacteria attached to the walls and bottom of the wells. To quantitatively evaluate the production of biofilms after drying of the wells, they were stained with 200 μL of 2% crystal violet (CV) stain for 5 minutes. Then, 200 μL of 33% acetic acid was added to each well, and the optical density of the CV stain in the solvent was determined at a wavelength of 620 nm using an ELISA reader.

Classification of isolates based on optical density was carried, as previously described by Stepanovic et al. (21). Microtiter plate test results were analyzed and compared using the OD values in four categories to show the adherence capabilities of the tested bacteria. Therefore, with the use of the ODs of bacterial biofilms, the strains were categorized into non-adherent (0), and weakly (+), moderately (++), or strongly (+++) adherent, as follows:

OD = mean OD of the bacteria;

ODC = mean OD of the negative control;

$OD \leq ODC$ = no biofilm producer;

$ODC < OD \leq 2 \times ODC$ = weak biofilm producer;

$2 \times ODC < OD \leq 4 \times ODC$ = moderate biofilm producer;

$4 \times ODC < OD$ = strong biofilm producer.

3.3. The Effects of E. durans on the Inhibition of S. mutans Adhesion

The effect of *E. durans* (ATCC6056) (Collins et al., Rayen Biotechnology Co.) on the inhibition of adhesion was evaluated using two methods: (A) By simultaneous adding of *S. mutans* and *E. durans* to the wells; and (B) adding *E. durans* 30 minutes before inoculation of *S. mutans*. Then, the difference in the optical density between the control wells (containing standard *S. mutans* and *E. durans* alone) and the wells containing clinical samples was determined to evaluate the effect of *E. durans* on the adhesion of *S. mutans* (by comparing the size of biofilms in the control and clinical groups) (7, 14).

3.4. Statistical Analysis

Data were analyzed with SPSS 20 using the paired *t*-test. Statistical significance was set at $P < 0.05$.

4. Results

4.1. Isolation of the Samples and Determination of Their Adhesion Ability

In the present in vitro study, out of 66 isolated clinical samples, 12 (18%) were *S. mutans*, and the others were *non-mutans streptococci* (based on laboratory diagnosis in the method). According to the OD value, 5 (41.67%) and 7 (58.33%) of the isolated *S. mutans* exhibited poor adhesion and moderate adhesion, respectively. The standard *S. mutans* sample exhibited moderate adhesion.

4.2. Effect of E. durans on the Adhesion of S. mutans Using the Microplate Technique

The results showed a decrease in adhesion in the presence of *E. durans* with the use of both methods (Table 1).

5. Discussion

Currently, bacteriotherapy is an alternative technique for altering the microbial ecology of the oral cavity, leading to a competition to replace pathogenic microorganisms (22).

The use of probiotic bacteria in competition with the *S. mutans* inhibits the colonization of cariogenic bacteria via reducing the bacterial adhesion to the tissue surface; thereby, reducing their pathogenic potential (6).

In this study, the *enterococcal* strain was selected because it can be found in a wide variety of conventional dairy products with known useful characteristics, including antioxidant, probiotic potential, acid resistance, and bile salt tolerance (12, 14). According to the results of the present, *E. durans*, as a probiotic bacterial species, reduced the adhesion of clinical *S. mutans* to microtiter plates in method 1 ($P < 0.047$) and method 2 ($P < 0.0000$). There is no other report like that of the present study for comparison. The inoculation of *E. durans* before *S. mutans* (method 2) was more effective in decreasing adhesion, which might be attributed to the high potential ability of *E. durans* colonization and competition with *S. mutans* growth (14). Like other probiotic bacteria, *E. durans* can produce biofilms. It is also highly capable of adhesion and colonization with hydrophobicity and autoaggregation properties that should be considered as the first step for competition with adhesion by pathogens (14). Another reason for adhesion reduction might be the secretion of organic acids, hydrogen peroxide, and bacteriocin. This probiotic condition potentially affects the metabolic activity of *S. mutans* (15). Pieniz et al. (14, 15) reported that *E. durans* species, similar to other probiotic microorganisms, has antibacterial properties, which might be attributed to its ability to produce and secrete organic acids, hydrogen peroxide, and bacteriocin. *E. durans* has been demonstrated to inhibit the in vitro growth of *Listeria monocytogenes*, *Escherichia coli*, *Shigella flexneri*, *Staphylococcus aureus*, *Salmonella typhimurium*, *Pseudomonas aeruginosa*, *Aeromonas hydrophila*, and *Corynebacterium phima* (15, 23). Other *enterococcal* strain, *E. faecium*, has a probiotic ability to control the biofilm production of *S. mutans* and *S. sobrinus* (24). Kim et al. (25) showed the positive effect of using *E. durans* against the inhibition of artificial plaque formation and the growth of *S. mutans*.

In vivo studies have also confirmed the beneficial systemic effects of *E. durans* consumption on increasing the secretion of IgA, regulation of the immune system, and increasing the production of IL-10 (16). Probiotic strains can invoke cellular and humoral nonspecific immunity in the oral cavity that might help delay dental caries caused by *S. mutans* (26, 27).

5.1. Conclusions

Based on the results of the present in vitro study, it can be concluded that the use of probiotic *E. durans* decreased the adhesion of *S. mutans*.

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Footnotes

Authors' Contribution: All the authors contributed to drafting and revising the manuscript.

Table 1. The Comparison of Adherence Reduction of *S. mutans* in the Presence of Probiotic *E. durans*[a, b]

	Number	Wavelength, nm	Before Probiotic	After Probiotic	P Value
Method 1	12	620	0.429 ± 0.390	0.390 ± 0.028	0.047
Method 2	12	620	0.396 ± 0.032	0.222 ± 0.188	0.000

[a] Value are expressed as mean ± SD.
[b] Paired *t*-test, P < 0.05, significant; method 1, simultaneous adding of *S. mutans* and probiotic; method 2, adding *S. mutans* 30 min after probiotic.

Conflict of Interests: The authors declare that they have no conflict of interests.

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