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Probiotic *Enterococcus durans* Interference with Oral *Candida albicans* Adhesion: An in vitro Study

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ABSTRACT

Background & Objective: *Candida* species, and most frequently isolated *Candida albicans*, are normal microorganisms of oral cavities; however, *C. albicans* is responsible for oral cavities in children with dental caries. As a new biologic technique, using probiotics has gained popularity in preventing and controlling diseases at present. *Enterococcus durans* has exhibited useful antioxidative properties and antibacterial and probiotic characteristics. This study aims to evaluate the effects of probiotic *Enterococcus durans* on the *in vitro* adhesion of *Candida albicans*.

Materials & Methods: Reference bacteria strain of probiotic *E. durans (*ATCC 6056), *C. albicans* reference strain *(PTCC-5027)*, and 10 clinical samples of *C. albicans* were provided. Adherence inhibition of *Candida albicans* was measured using microtiter plates applying two methods (addition of a mixed suspension of *C. albicans* and *E. durans* simultaneously and addition of *E. durans* 30 minutes before *C. albicans*). Data were analyzed with a repeated measure model. Statistical significance was set at P-value<0.01.

Results: Adhesions of *C. albicans* biofilms decreased in the presence of the probiotic strain *E. durans*. Mean OD620 nm was within the range of 0.45 to 0.49, and 0.33 for OD490 nm.

Conclusion: Using *E. durans* as a probiotic could reduce *Candida albicans* adhesion and, therefore, can be considered as an effective way to decrease its pathogenicity.

Keywords: *Candida albicans, Enterococcus,* Probiotic

Introduction

Candida species are commensal microorganisms and members of normal oral cavity flora; however, these species are opportunistic pathogens which cause candidiasis under specific conditions **(1)**. *Candida albicans* (*C. albicans*) is a microbiologic member of the oral cavity in children with dental caries **(2)**. As reported by Odds, there is a relationship between adhesion rate of *C. albicans* to surfaces and its ability to form colonies and induce diseases **(3)**.

As a new biologic technique, using probiotics has gained popularity in preventing and controlling diseases at present. It is considered as an alternative method to change the microbial ecology of the oral cavity. Competition among probiotics to replace pathogenic microorganisms makes it possible to simultaneously use systemic and local interventions **(4, 5)**. The World Health Organization defines probiotic bacteria as viable microorganisms that are useful for the host's health when used appropriately **(1)**. Different microorganisms have been used as probiotics. Lactic acid bacteria (LAB) is one of the most utilized and effective probiotics. *Enterococci*, as LAB family members, are ubiquitous due to their durability and resistance to growth inhibition factors, including their resistance to high acidity and biliary salts. They are naturally found in different environments including dairy products, meat, vegetables, and cereal **(6-8)**. Also, they are the most predominant microorganisms found in traditional cheese produced from raw milk **(7, 9)**.

Enterococcus Durans (*E. durans*) is a LAB group bacteria and is on the list of safe materials/agents (GRAS). This bacteria is one of the permanent residents of the gastrointestinal tract (10). Evidence has shown its useful antioxidative, antibacterial, and probiotic characteristics. It has a variety of characteristics, including potential adhesion and colonization of the mucosa, the ability to form biofilms, absence of aggressive potential, few virulence factors, specific antimicrobial properties against pathogens, and inducing immune responses (9, 11, 12). In addition, growth of this bacterial species in a selenium-rich environment results in selenium concentration in the bacterial cell mass. As a result, this bacterial species might be an alternative source for organic selenium in foodstuffs (12).

This is the first study on the effect of *E. durans* on *C. albicans*. One of the criteria for the selection of probiotic bacteria is adherence to dental tissue as part of biofilm and competition with the growth of cariogenic bacteria (4). Therefore, this study evaluated the effects of probiotic *E. durans* on the adhesion of *C. albicans in vitro.*

Materials and Methods

Isolation of *Candida albicans*

Reference bacteria strain of *Candida albicans* (*PTCC-5027)* (Pasteur Institute of Iran) and strains isolated from carious teeth of 31 children aged 6-12 years were used in this study. Children's parents received a complete explanation about the aims and methods of the study and then provided written consent for their children to be included in the study. The inclusion criteria consisted of no use of antimicrobial mouthwashes during the previous month, no use of antifungal agents, antihistamines, corticosteroids, and a caries index of >10 as previously reported (13).

Dental plaques were transferred from the carious teeth into Falcon tubes containing 5 mL of BHI (Brain heart infusion) culture medium *(Ibresco Made in Italy)* using a sterile microbrush. The tubes were incubated at 37°C for 24 hours. The samples were cultured on Mycosel Agar (*Quelab Made in UK*) at room temperature for 24 hours. A colony of specimens grown in a Mycosel Agar was sub-cultured in chrome agar Candida (*Ibresco)* medium to separate *C. albicans* which is observed as green colonies on the culture medium (14).

Positive control samples of *C. albicans* (*PTCC-5027)* and *E. durans* (*ATCC 6056*) (Collins *et al.* Rayen Biotechnology co.) were concomitantly cultured with *C. albicans* sample isolations.

Formation of Biofilms using the Microtiter Plate Technique

At this stage, the potential of biofilm formation of the clinical samples was determined using the microtiter plate technique. First, 0.5 McFarland concentrations of the clinical and standard samples were prepared in a BHI broth culture medium (based on standard CLSI) (15). Then, 200 µL of each suspension were transferred into 96 well plates in a microtiter plate (each sample in one raw with eight repetitions). Negative control well only contained BHI broth culture medium. After 24 hours, the contents of the wells were retrieved, and each well was washed with 200 µL of sterile physiologic serum three times. A total of 200 µL of ethanol was used in order to adhere bacteria to the wells for 15 minutes. The quantification of clinical isolates biofilm formation was performed using crystal violet (CV) (Merck, Germany) staining method, according to the protocol described by Silva *et al* (16). In a nutshell, following biofilm formation, the wells were washed with PBS, and CV (1% v/v) was added to the wells, followed by acetic acid (33% v/v) (17). An ELISA reader was used to determine the optical density of the CV stain in the stain solvent at a wavelength of 492 nm. Highly adhering strains with greater capacity to form biofilms were selected for the next stage (9, 18).

The Effects of *E. durans* on Inhibiting *C. albicans* Adhesion

The effects of *E. durans (ATCC 6056)* on inhibiting the adherence of clinical isolated *C. albicans* were evaluated using two techniques: by adding *C. albicans* and *E. durans* to the wells simultaneously (technique 1), and by adding *E. durans* 30 min before adding *C. albicans* (technique 2). For each technique, 100 µL of a *C. albicans* suspension and 100 µL of an *E. durans* suspension were added to each well (each sample in one raw with eight repetitions). Then, the differences between the optical densities of the control wells (standard *C. albicans* well in association with *E. durans* and *E. durans* well alone) and the wells containing clinical samples were evaluated to assess the effects of *E. durans* on adherence (9, 18).

The isolates were categorized as follows based on their optical density using Stepanovic *et al.*'s technique (9, 19).

Statistical Analysis

Data were analyzed with SPSS 20 (SPSS Inc., Chicago, IL., USA) using a repeated measure model. Statistical significance was set at P-value<0.01.

Results

Isolation of the Samples and Determination of Adhesion Capacity

In this *in vitro* study, 10 of 31 clinical isolated samples were *C. albicans* and 21 samples were other microorganisms. All the isolates exhibited moderate adhesion capacity which was similar to the standard samples. The OD620 nm range was 0.45 to 0.49 and 0.33 for OD490 nm (Table 1).

The effects of *E. durans* on *C. albicans* Adhesion using Microtiter Plate Technique

The results were indicative of a significant difference in the potential of adhesion, using both methods (Table).

Table 1. Comparison of adherence reduction of *C. albicans* clinical isolates in the presence of probiotic *E. durans*

	Wavelength (nm)	Mean± SD (before probiotic)	Mean± SD (after probiotic)	*P*- value
Method1	490	1.071±0.416	0.331±0.038	0.000
	620	0.774±0.078	0.499±0.073	0.003
Method2	490	1.062±0.094	0.332±0.066	0.000
	620	0.775±0.095	0.452±0.058	0.001

Mean ± SD of clinical isolates, P-value<0.01 is statistically significant, Method 1: Simultaneous Adding *C. albicans* & probiotic, Method 2: Adding *C. albicans* 30 min after probiotic.

OD = mean OD of the bacteria

ODC= mean OD of the negative control

OD ≤ ODc = no biofilm producer

ODc< OD ≤ 2 ×ODc = weak biofilm producer

2 ×ODc < OD ≤4 ×ODc = moderate biofilm producer

4 ×ODc < OD= strong biofilm producer

Discussion

Biofilm formation is the first step in bacterial infections. They are able to attach to the dental lamina and produce destructive material. We aimed to introduce a methods to reduce the biofilm formation in dental caries. *C. albicans* adhesion to teeth surfaces has a major role in its pathogenicity **(20)**. Therefore adhesion reduction can be an effective way to decrease its detrimental effects **(21)**. present in food and dairy products, *Enterococcus* strains have been known for a long time as effective probiotics **(9)**. *Enterococci* are a type of LAB species and are members of the normal flora of the gastrointestinal tract and fermented food **(12)**. A study by Carasi *et al.* showed that *E. durans* isolated from kefir were able to inhibit different Gram-positive and Gram-negative pathogens **(7)**. Natanzi *et al.* reported the anti-adherence activity of *Lactobacillus acidophilus* on *C. albicans* **(21)**.

Jorgensen *et al.* (2017) evaluated the antifungal potential of *Lactobacillus reuteri* (*L. reuteri*) probiotic bacterial species against six oral *candidal* species. Spectrophotometry and agar overlay inhibition techniques were used to evaluate the potential of *L. reuteri* to inhibit the growth of yeasts. In addition, pH values were evaluated using microsensors. The results indicated that *L. reuteri* produced lactic acid and organic acid decreased the pH of agar culture media and inhibited hyphal morphogenesis induction in *Candida* species **(1)**. Jiang *et al.* (2015) evaluated the growth inhibitory effect of probiotic *Lactobacilli* on oral *Candida* species. The results indicated that these probiotics had positive effects on oral health **(22)**.

Matsubara *et al.* (2010) evaluated the inhibitory effects of probiotic *Lactobacilli* on the early stages of *C. albicans* biofilm formation. The quality of the biofilm formation was evaluated, and the ultrastructural analyses of biofilms of *C. albicans* treated with *Lactobacillus rhamnosus*, *Lactobacillus casei*, and *Lactobacillus acidophilus* and their supernatants were carried out. *Lactobacilli*, depending on the probiotic type and biofilm formation stage, resulted in a significant decrease in the number of cells in biofilms ($P<0.05$). *L. rhamnosus* had no significant effect on the maturation of biofilms ($P>0.05$); however, it resulted in a significant decrease in the early stages of *C. albicans* biofilms **(23)**.

Hasslof *et al.* showed the inhibitory effects of commercial samples of probiotic *Lactobacilli* on oral *S. mutans* and *Candida in vitro*. In the mentioned study, all the *Lactobacillus* species inhibited the growth of *Candida* and *S. mutans* samples; however, the inhibitory effects on *C. albicans* were weaker than those of *S. mutans*, which might be attributed to the resistance of pathogenic bacteria **(24)**.

In the present study, inhibitory effects of *E. durans* on *C. albicans* adhesion were evaluated. Adhesion was evaluated using the microtiter plate technique and the complete culturing of the probiotic agent. In the first technique, adhesion was evaluated by simultaneous addition of the bacteria and *C. albicans* to the culture medium; in the second technique, the probiotic bacteria culture was carried out 30 min before adding *C. albicans*. Based on the results of the present study, the presence of *E. durans* as a probiotic bacterial species resulted in a decrease in adhesion of *C. albicans* to the test surfaces for both techniques; However, in both techniques, there was a significant decrease in binding, which might be attributed to the inter-surface interactions (cell-to-cell and cell-to-surface) and the production of external metabolites that made the organization and structure of the biofilm unstable **(1, 25).**

Considering the useful systemic effects of *E. durans* on increasing the secretion of IgA, regulating the

immune system, and increasing the production of IL-10, further clinical studies are warranted **(7, 11)**. The results of the present study indicate the positive effects of *E. durans* on decreasing *C. albicans* adhesion.

Conclusion

Using *E. durans* as a probiotic could reduce *Candida albicans* adhesion, which might be attributed to the inter-surface interactions and the release of bioactive substances which make the organization and structure of the biofilm unstable and, therefore, can be an effective way to decrease its pathogenicity.

Ethical considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

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Conflict of Interest

Authors declared no conflict of interest.

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