Antifungal Effect of Probiotics Versus Miconazole Against *C. albicans* Biofilm on Denture Base Resins. (A Comparative in Vitro Study)

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Abstract

Background: Results of most epidemiological studies found that approximately 65% of removable denture wearers suffer from candida associated denture stomatitis. Adhesion and biofilm formation of C. albicans was regarded as essential for such condition.

Objective: The objective of this study was to evaluate the effect of probiotics on C. albicans metabolic activities and to compare this effect with a different concentration of miconazole nitrate on denture base resins.

Materials and methods: Biofilms were developed on 50 heat-cured PMMA discs (5 mm diameter × 1.5 mm thick) that were polished with waterproof silicon carbide paper (grit p600), submerged in candida cell suspension discs were washed with PBS to remove non adherent cells. Antifungal susceptibility probiotics and a different concentration Miconazole nitrate (0.5, 5, 50, 100 µg/ml) was measured by the XTT reduction assay.

Results: Lactobacillus probiotics possess an inhibitory effect against C. albicans biofilm and this effect is comparable to a different concentration miconazole.

Conclusions: Lactobacillus probiotics can be considered effective alternative antifungal treatment to prevent denture-associated stomatitis.

KeyWords: Probiotics, *candida albicans*, miconazole, acrylic discs.

DOI Number: 10.48047/NQ.2022.20.16.NQ88170 **NeuroQuantology2022;20(16):1712-1717**

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Relevant conflicts of interest/financial disclosures:
The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Introduction:

Candida-associated denture stomatitis (CaDS) is a common oral inflammatory reaction with multifactorial etiology. Usually appears as an erythematous reaction extends to the limits of the maxillary denture bearing area. Severity of the condition has a negative impact on oral tissues and the prosthetic outcome(1).

Although longstanding denture wearing and other predisposing factors such as local trauma, poor hygiene, denture base material, smoking, bad dietary habits and pH of denture plaque have been implicated, C. albicans colonized on the denture surface has been considered a critical factor in such condition (1). These predisposing factors provide a suitable environment for adhesion and biofilm formation of C. albicans which is a highly organized structure with high resistance to antifungal drugs and host defenses (2).

Different approaches have been implemented to treat CaDS as denture base surface modification, antimicrobial agents incorporation into the denture base material and recently the use of several nanoparticles as antifungal agents. However, topical application of antifungal drugs such as nystatin and miconazole still the most common traditional method to treat such condition (3).

Miconazole nitrate belongs to ergosterol biosynthesis inhibitors have been used for more than 2 decades for local fungal infections treatment. However, it posses a several adverse effects and the rate of recurrence remains high (4).

Lactobacillus species are representative probiotics that are widely used in the production of fermented dairy products by playing an important role in the maintenance of human health through stimulating immune system (5). Also, it has antifungal activity against different fungal strains including C. albican. So, they may be used for the prevention and treatment of associated denture stomatitis (6).

The antifungal activity of lactobacillus occurs by two mechanisms. First, Lactobacillus strains produce abundant lactic acid that diffuses through the membrane of target fungus reducing cytoplasmic pH, thereby causing loss of fungus viability. Second, production of various antifungal peptides that may disrupt the fungus membrane or inactivate cytoplasm molecules after internalization (7).

The current study was designed to evaluate the antifungal effect of commercially available lactobacillus probiotics gel against metabolic activities of C. albicans biofilm on both heat-polymerized acrylic and flexible denture base resins and to compare this effect with different miconazole concentrations.

Methods

Candida strain

Candida albican (ATCC 10231) strain obtained from Assiut University Moubasher Mycological Centre subcultured on sabouraud dextrose agar (Difco, Detroit, MI), and stored at 4–6°C. The tested strain was inoculated in Yeast Nitrogen Base medium (YNB) (Difco) supplemented with 100 mM glucose (Sigma-Aldrich, St. Louis), for 24 hours at 37°C. Cells were harvested, washed with PBS, and standardized to 1×107 cells/ml YNB glucose.

Stages of biofilm formation

Biofilms were developed on heat-cured PMMA discs (5 mm diameter × 1.5 mm thick) that were polished with waterproof silicon carbide paper (grit p600).

1. **Adherence phase:** PMMA discs were submerged in Candida cell suspension in 48-well plate then placed on a titer plate shaker at a setting of 1, incubated for 90 min at 37°C. The discs were washed with PBS to remove non-adherent cells.
2. **Biofilm formation phase:** The washed discs were transferred to new 48-well plates, submerged in fresh YNB/100 mM glucose and placed in on the titer-plate shaker for 48 hours at 37°C at the same setting.

Antifungal susceptibility of miconazole

Miconazole nitrate was solubilized in dimethyl sulfoxide and then diluted in YNB-glucose to obtain the desired final concentrations of (0.5, 5, 50, 100 µg/ml). The 48-h Candida biofilms were incubated with the dilution series of antifungals for 24 hours at 37°C. The viability of the Candida cells was measured by the XTT reduction assay.

Testing the antifungal effect of the probiotic

The probiotic supplements used in the study were prolacsan (CMS dental, Denmark), which contains a mixture of lactobacillus brevis and lactobacillus plantarum. Fresh culture of the probiotic bacteria was obtained by culture in Muller Hinton broth for 24 hours.

The turbidity of the bacterial broth culture was adjusted to be equivalent to 0.5 McFarland standard which yields an approximate cell density of 1.5x108 cells/ml. This was then diluted with PBS to obtain bacterial density of 1x107.

Following biofilm formation on denture strips, the strips were transferred to the standardized probiotic bacterial suspensions, incubated for 24 hours at 37°C. The viability of the Candida cells was also measured by the XTT reduction assay.

XTT reduction assay.

The denture strips were then transferred to a new 12-well plate containing 4 ml of PBS in each well. A total of 50 µl of XTT (1 mg/mL) and 4 µl menadione (1 mM) solutions were added to each well. The plate was then incubated at 37°C for 3 to 5 hours. After incubation peroid, the solution from each well was centrifuged and absorbance measured at 492 nm using spectrophotometry. Discs without Candida cells served as negative controls and discs containing Candida cells only without miconazole or probiotic served as positive controls.

Statistical analysis

Numerical data were explored for normality by checking the distribution of data using tests of normality (Kolmogorov-Smirnov and Shapiro-Wilk tests), which revealed a normal (parametric) distribution. Parametric data were presented as mean, standard deviation and confidence intervals and were compared between groups using One-way analysis of variance (ANOVA) test and Tukey's post hoc test. T test was used for comparison between 2 groups. Two ways ANOVA test was used to study the interaction of variables. The significance level was set at P ≤0.05. Statistical analysis was performed with IBM SPSS Statistics for Windows, Version 18; NY: IBM Corporation.

Results

1- Effect of different concentrations of miconazole on acrylic & flexible samples

1-(a) Comparison of each concentration with control

This comparison is presented in **Table (1).**

In acrylic group, comparing each concentration of miconazole with control revealed a statistically significant difference (p=0.00). The difference between control and 0.5mg was (0.88), while the difference between control & 5 mg and 50 mg was (1 and 1.05 respectively), in comparison to a difference (1.13) between control and 100mg.

In flexible group, comparing each concentration of miconazole with control revealed a statistically significant difference (p=0.00). The difference between control and 0.5mg was (0.817), while the difference between control & 5 mg and 50 mg was (1.03), in comparison to a difference (1.18) between control and 100mg.

Table (1): Statistical comparisons (Mean ± SD) of each concentration with control (t test)

	Groups	Mean	Std. Dev	Mean Difference	t value	P value
Acrylic	Control group	1.891	.00	0.884	35.9	0.00*
	0.5 mg	1.007	.06			
	Control group	1.891	.00	1.00	212.5	0.00*
	5 mg	.888	.01			
	Control group	1.05	.00	1.05	240.08	0.00*
	50 mg	.846	.01			
	Control group	1.891	.00	1.13	177.21	0.00*
	100 mg	.760	.01			
Flexible	Control group	1.921	.00	0.817	705.95	0.00*
	0.5 mg	1.104	.00			
	Control group	1.921	.000	1.03	214.92	0.00*
	5 mg	.888	.011			
	Control group	1.921	.000	1.03	318.97	0.00*
	50 mg	.889	.007			
	Control group	1.921	.000	1.18	334.4	0.00*
	100 mg	.741	.008			

1- (b) Comparison of different concentration within the same group

This comparison is presented in **Table (2).**

In acrylic group, comparing different concentrations of miconazole revealed a statistically significant difference (p=0.00). The highest mean value was recorded at 0.5mg was (1.007±0.06), then in 5 mg and 50 mg, with the lowest value recorded in 100mg (0.760±0.01). Post hoc test revealed no significant difference between 5 and 50 mg.

In flexible group, comparing different concentrations of miconazole revealed a statistically significant difference (p=0.00). The highest mean value was recorded at 0.5mg was (1.104±0.00), then in 5 mg and 50 mg, with the lowest value recorded in 100mg (0.741±0.01). Post

hoc test revealed no significant difference between 5 and 50 mg.

Table (2): Statistical comparison of different concentrations within the same group (ANOVA test)

Material		Mean	Std. Dev	95% Confidence Interval for Mean		Min	Max	F	P
				Lower Bound	Upper Bound				
Acrylic	0.5 mg	1.007[a]	.06	.94	1.08	.97	1.10	61.56	0.00*
	5 mg	.888[b]	.01	.88	.90	.87	.90		
	50 mg	.846[b]	.01	.83	.86	.83	.86		
	100 mg	.760[c]	.01	.74	.78	.74	.78		
	Total	.875	.10	.83	.92	.74	1.10		
Flexible	0.5 mg	1.104[x]	.00	1.10	1.11	1.10	1.11	1888.52	0.00*
	5 mg	.888[y]	.01	.87	.90	.87	.90		
	50 mg	.889[y]	.01	.88	.90	.88	.90		
	100 mg	.741[z]	.01	.73	.75	.73	.75		
	Total	.9055	.13	.84	.97	.73	1.11		

Significance level p≤0.05, *significant
Tukey's post hoc test: Within the same material, means sharing the same superscript letter are not significantly different

1-(c) Comparison between both groups (flexible versus acrylic) at each concentration

This comparison is presented in **Table (3).**

At 0.5 mg concentration, flexible recorded a significantly higher value than acrylic (p=0.017).

At 5 mg concentration, there was no significant difference between acrylic and flexible (p=0.954)

At 50 mg concentration, flexible recorded a significantly higher value than acrylic (p=0.00).

At 100 mg concentration, acrylic recorded a significantly higher value than flexible (p=0.039).

Table (3): Descriptive statistics and comparison between both groups (Flexible versus acrylic) at each concentration (t test).

	Groups	Mean	Std. Dev	Mean Difference	t value	P value
0.5 mg	Acrylic	1.007	.055	.097	3.92	0.17*
	Flexible	1.104	.003			
5 mg	Acrylic	.888	.011	0.0004	0.059	0.954 ns
	Flexible	.888	.011			
50 mg	Acrylic	.846	.010	0.044	8.07	0.00*
	Flexible	.889	.007			
100 mg	Acrylic	.760	.014	0.019	2.6	0.039*
	Flexible	.741	.008			

Significance level p≤0.05, *significant, ns=non-significant

1-(d) Effect of miconazole on acrylic and flexible groups (interaction of variables)

Results of two way ANOVA test are presented in **Table (4-a.b.c).**

Regardless of the group material, there was a significant difference between Miconazole concentrations (p=0.00). The highest value was recorded at 0.5 mg conc. (1.055±0.63), followed by 5 mg, then 50 mg, with the least value recorded in 100mg (0.75±0.015). The difference between 5 and 50 mg was not statistically significant.

Regardless of Miconazole concentration, there was a significant difference between group materials (p=0.00). The highest value was recorded in flexible (0.905±0.27), compared to acrylic (0.875±0.015).

The interaction of group material and Miconazole concentration variables had a statistically significant effect (p=0.00).

Table 4a. Descriptive statistics of candida in different concentrations (regardless of group material)

Concentration	Candida (overall in both groups)		P value
	Mean	Std Dev	
0.5 mg	1.055[a]	.063	0.00*
5 mg	.888[b]	.010	
50 mg	.868[b]	.024	
100 mg	.750[c]	.015	

Significance level p≤0.05, *significant
Tukey's post hoc test: Means sharing the same superscript letter are not significantly different

Table 4b. Descriptive statistics of candida in different group material (regardless of concentrations)

Material	Candida (overall in all Miconazole concentrations)		P value
	Mean	Std. Dev	
Acrylic	.875	.015	0.00*
Flexible	.905	.027	

Significance level p≤0.05, *significant

Table 4c. Results of two way ANOVA test for interaction of group material and miconazole concentration variables)

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Material	.009	1	.009	19.963	.000*
Concentration	.474	3	.158	343.831	.000*
Material * Concentration	.020	3	.007	14.455	.000*
Error	.015	32	.000		
Total	32.224	40			
Corrected Total	.518	39			

Significance level p≤0.05, *significant

2-Effect of probiotic on acrylic & flexible samples

2-(a) Comparison of probiotic with control

This comparison is presented in **Table (5).**

In acrylic group, comparing probiotic with control revealed a statistically significant higher value with control (p=0.00). The difference between control and probiotic was (0.80). In flexible group, comparing probiotic with control revealed a statistically significant higher value with control (p=0.00). The difference between control and probiotic was (0.681).

2-(b) Comparison of probiotic in acrylic and flexible group

This comparison is presented in **Table (5).**

A statistically significant higher value was noted in flexible in comparison to acrylic (p=0.00). The difference between acrylic and control was (0.148).

Table (5): Descriptive statistics and comparison of probiotic with control & comparison of probiotic in both groups (t test)

Groups	Mean	Std. Dev	Mean Difference	t value	P value
Control Acrylic	1.891	.000	0.80	469.78	0.00*
Probiotic Acrylic	1.091	.004			
Control Flexible	1.921	.000	0.681	255.16	0.00*
Probiotic Flexible	1.239	.006			
Acrylic	1.091	.004	0.148	46.78	0.00*
Flexible	1.239	.006			

Significance level p≤0.05, *significant

2-(c) Comparison of probiotic with each concentration of miconazole within the same group

This comparison is presented in **Table (6).**

In acrylic group, comparing each concentration of Miconazole with probiotic revealed a statistically significant higher value in probiotic in comparison to 0.5 mg (p=0.027), 5 mg (p=0.00), 50 mg (p=0.00) and 100mg (p=0.00).

In flexible group, comparing each concentration of Miconazole with probiotic revealed a statistically significant higher value in probiotic in comparison to 0.5 mg (p=0.00), 5 mg (p=0.00), 50 mg (p=0.00) and 100mg (p=0.00).

Table (6): Comparison of probiotic with each concentration of miconazole within the same group (t test).

	Groups	Mean	Std. Dev	Mean Difference	t value	P value
Acrylic	Probiotic 0.5 mg	1.091	.004	0.084	3.4	0.027*
		1.007	.055			
	Probiotic 5 mg	1.091	.004	0.203	40.39	0.00*
		.888	.011			
	Probiotic 50 mg	1.091	.004	0.245	52.49	0.00*
		.846	.010			
	Probiotic 100 mg	1.091	.004	0.331	50.16	0.00*
		.760	.014			
Flexible	Probiotic 0.5 mg	1.239	.006	0.135	46.5	0.00*
		1.104	.003			
	Probiotic 5 mg	1.239	.006	0.006	63.87	0.00*
		.888	.011			
	Probiotic 50 mg	1.239	.006	0.35	83.38	0.00*
		.889	.007			
	Probiotic 100 mg	1.239	.006	0.499	112.6	0.00*
		.741	.008			

Significance level p≤0.05, *significant

Discussion

Candida albicans has been regarded as the primary cause of denture-associated stomatitis due to its capability to adhere to oral tissues and denture surfaces. Treatment of oral candidiasis (OCs) mainly based on antifungal drugs such as miconazole however, presence of multi-resistance phenotypes of Candida spp. represent challenge for public health. Consequently, the development of alternative safe therapeutic measures is necessary to prevent the emergence of fungal resistance(1).

Probiotics which defined as "live microorganisms that, when administered in adequate amount, confer health benefit to the host" (8) found to be having a protective role against the Candida spp. However, its role in reducing metabolic activities of candida on denture base remains unclear (9).

The aim of this study was to evaluate the effect of commercially available two lactobacillus probiotics strains Lb. brevis & Lb. plantarum (a total of 6 x 109 CFU) on the metabolic activities of candida albicans on acrylic discs as a standardized model using the XTT reduction assay and to compare this effect with miconazole at a different concentration. Both strains have the qualified presumption of safety (QPS) status from the European Food Safety Authorities (EFSA) and generally recognized as safe (GRAS) from the United States Food and Drug Administration (FDA) (10).

A combination of two strains was used because most researches on probiotics found that a combination of different probiotic species can enhance their effects in a synergetic manner (11). Also, it was stated, "no single probiotic bacterium exhibits all the properties and probiotics strains are often used in combination with each other to increase the beneficial effects (12).

Although results of this study indicated that there was significant reduction in metabolic activities of candida in both acrylic and flexible discs groups when compared with the control (the reduction was higher in acrylic than flexible discs group) regardless the miconazole concentration, this effect increase by increasing miconazole concentration, and the highest inhibition of metabolic activity was recorded in 100mg while 0.5mg recorded the lowest value. These observations are in general agreement with previous reports which found that miconazole possess potent in vitro activity against Candida spp. (13) A similar findings obtained in another study which found that miconazole exhibits high antifungal activity against Candida

biofilms developed on acrylic discs at concentration of 5 g/ mg and the highest inhibition of metabolic activity was observed at 96 mg/ml miconazole. (14)

Although the present study found that the used probiotics strains exhibit a reduction in the metabolic activities of candida on the surface of acrylic resin discs and this inhibitory effect is comparable to miconazole, a more clinical researches are needed and should be conducted to evaluate the effect of probiotics as an alternative treatment.

Conclusions

lactobacillus probiotics strains (Lb. brevis & Lb. plantarum) showed a significant reduction on the metabolic activities of candida albicans developed on acrylic resin discs and considered promising antifungal therapeutic agent for both prevention and treatment of oral candidiasis in denture wearers.

Conflict of interest

The authors confirmed that this article content has no conflict of interest.

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