ORIGINAL ARTICLE

Antimicrobial efficacy of probiotic-containing toothpastes: an *in vitro* evaluation

Ivana Parčina Amižić[1], Livia Cigić[2], Lidia Gavić[2], Marina Radić[3,4], Dolores Biočina Lukenda[2], Marija Tonkić[3,4], Ivana Goić Barišić[3,4]

[1]Dental Practice, Health Center of Split, Dalmatia County, [2]Department of Oral Medicine and Periodontology, School of Medicine, University of Split, [3]Clinical Department of Microbiology and Parasitology, University Hospital Centre Split, [4]School of Medicine, University of Split; Split, Croatia

ABSTRACT

Aim To evaluate, *in vitro* antimicrobial ability of two probiotic toothpastes (one containing *Lactobacillus paracasei*, other containing *Lactobacillus acidophilus*) and one toothpaste without probiotic separately, and in a combination with two different mouthrinses (one containing essential oils and the other containing hexitidine).

Methods Antimicrobial susceptibility was checked by using the ditch method and Clinical Laboratory Standard Institute (CLSI) guidelines. Two different toothpastes with probiotic, toothpaste without probiotic and two different mouthrinses were tested against the following selected microorganisms: *Candida albicans*, *Candida tropicalis*, *Enterococcus faecalis*, *Streptococcus salivarius* and *Staphylococcus aureus*. Kruskal-Wallis test and Mann-Whitney U test were used for the statistical analysis ($p \leq 0.05$).

Results Probiotic toothpastes had better inhibitory effect than toothpaste without probiotic in the case of *Candida albicans* ($p=0.043$) and *Streptococcus salivarius* ($p=0.043$). In all cases, toothpastes had stronger inhibition capacity than mouthrinses ($p \leq 0.05$).

Conclusion Probiotic toothpastes, as a relatively new concept in the prevention of oral infectious diseases such as caries and periodontal disease, can contribute to the prevention of oral infectious diseases.

Key words: *Lactobacillus acidophilus, Lactobacillus paracasei,* mouthrinse, probiotics

Corresponding author:
Lidia Gavić
Department of Oral Medicine and Periodontology, School of Medicine, University of Split,
Šoltanska 2, 21000 Split, Croatia
Phone: +385 21 557 932;
Fax: +385 21 557 895;
E-mail: lgavic@mefst.hr

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INTRODUCTION

The dental plaque, which has been identified as a biofilm, is the primary etiological factor for most frequently occurring oral diseases, dental caries and periodontal diseases (1,2). Microbial biofilms are complex communities of bacteria and are common in the human body and in the environment (1-3). Dental biofilm cannot be eliminated, but it can be reduced and controlled through daily oral care (2). Since teeth comprise only 20% of the mouth's surfaces and the biofilm bacteria contained in oral mucosal reservoirs, the use of topical antimicrobials as an *important* adjunct to *tooth brushing* may also play a role in controlling biofilm (3). Until today, the incorporation of chemical agents with antimicrobial activity into dental products has been proposed as a potential prophylactic method of reducing plaque-mediated disease (4).

According to the World Health Organization (WHO), probiotics are defined as viable microorganisms that confer a health benefit when administered in sufficient doses. The most used genera in various probiotic products are *Lactobacillus* and *Bifidobacteria* (5). Yet, the *Lactobacillus* are the ones that play a significant role in the oral ecosystem and can be linked with oral disease as well as oral health (6). A few studies have revealed that probiotic *Lactobacillus* strains were useful in reducing gingival inflammation and the number of black-pigmented rods in the saliva and subgingival plaque (7-9). Some other clinical trials demonstrated a reduced prevalence of moderate to severe gingival inflammation in adults after regular use of probiotic tablets (10-12). Data of a pilot study suggested a beneficial effect of the probiotic milk drink on gingival inflammation (13). Also, it has been shown that probiotic chewing gums consumed over the period of two weeks caused a reduction in proinflammatory cytokines in patients with gingivitis (14). Possible actions of probiotic bacteria in the oral environment would be a competition of binding sites, production of antimicrobial substances and activation and modulation of the immune response (5,15,16). Unlike probiotic-containing food or supplement product, probiotic-containing toothpaste is a relatively new concept in the prevention of oral infectious diseases such as caries and periodontal disease, and slowly accepted by the common toothpaste user (17). Advantage of this kind of probiotic product is the opportunity for regular, daily, frequent intake of probiotics into the oral environment (18).

Several researches already discovered that *in vitro L. acidophilus* and *L. paracasei* may inhibit growth of mutans streptococci (19-21).

The aim of this research was to evaluate, *in vitro*, antimicrobial ability of two probiotic toothpastes (one containing *Lactobacillus paracasei*, other containing *Lactobacillus acidophilus*) and one toothpaste without probiotic separately, and in combination with two different mouthrinses (one containing essential oils, and other containing hexitidine), against the following selected microorganisms: *Candida albicans*, *Candida tropicalis*, *Enterococcus faecalis*, *Streptococcus salivarius* and *Staphylococcus aureus*.

MATERIALS AND METHODS

Study design

Two probiotic toothpastes (A and B) were obtained from a local drugstore (Table 1). The non-probiotic toothpaste (C) and hexetidine-containing mouthrinses 1 and 2 (Mr1 and Mr2), which were considered standards served as positive controls. The sterile 0.9 saline was used as a negative control in antimicrobial test.

Table 1. Ingredients of toothpastes and mouthrinses used in the study

Agent	Ingredients
Paste A	Aqua, Sorbitol, Hydrated Silica, Glycerin, PEG-32, Sodium Lauryl Sulfate, Aroma, Titanium Dioxide, Cellulose Gum, Sodium Fluoride (0,24%), Saccharin, Sodium Sulfate, Lactobacillus, Sodium Methylparaben, Sodium Hydroxide
Paste B	Aqua,Glycerin, Sorbitol, Hydrated Silica, PEG 8, Sodium Lauryl Sulfate, Celulose Gum, Acorus Calamus Root Powder, Eucalyptus Globulus extract, Origanum Majorana Powder, Rosmarinus Officinalis Powder, Saccharin, Sodium Methylparaben, Lactobacillus acidophilus
Paste C	Aqua, Sorbitol, Hydrated Silica, Glycerin, PEG-32, Sodium Lauryl Sulfate, Aroma, Cellulose Gum, Titanium Dioxide, Sodium Fluoride (0,24%), Saccharin, Triclosan, Sodium Methylparaben, Limonene
Mouthrinse 1 (Mr1)	Aqua, Alcohol, Sorbitol, Poloxamer 407, Benzoic Acid, Sodium Saccharin, Aroma, Eucalyptol, Methyl Salicylate, Thymol, Menthol, Sodium Benzoate, cl 47005, cl 42053.
Mouthrinse 2 (Mr2)	Hexetidine, Ethanol, Propylene glycol, Polysorbate 80, Methyl salycilate, Saccharin Natrium, Anise essential oil, E 124, Citric acid monohydrate, Aqua.

Methods

To prove the probiotic existence in tested toothpastes, the samples of toothpastes were cultured on Columbia agar plate (Bio Rad, France). The plates were incubated for 48 hours at 36 °C in anaerobic conditions and identification of cultivated Gram-positive bacilli confirmed the composition of the paste as stated in declaration.

Suspensions of tested microorganisms were prepared by mixing a pure culture of two control strains, *Enterococcus faecalis* ATCC 29212 and *Staphylococcus aureus* ATCC 29213, and three isolates from routine work, *Streptococcus salivarius*, *Candida albicans* and *Candida tropicalis*. Each isolate was cultivated on solid agar plates and 2-3 colony of each microorganism were mixed in 2 mL of sterile 0.85 saline solutions. The density of 0.5 McFarlands was measured by the densitometer (Densimat, BioMerieux, Marcy l'Etoile, France) for each suspension.

Antimicrobial susceptibility was checked by using the ditch method and Clinical Laboratory Standard Institute (CLSI) guidelines (22). The Muller Hinton agar was used to demonstrate the antibacterial effects on aerobes, while Sabourand dextrose agar was used for *Candida* isolates. Three wells (4 mm in diameter and 3 mm deep) were made using a sterile metallic template. The agar plates were streaked with the suspension of 0.5 Mc Farland turbidity and using a sterile spoon excavator, the toothpastes in their pure form were dispersed into the wells with and without one drop of the ready-to-use mouthwashes. Similarly, ready-to-use mouthwashes were introduced into the wells, while the same amount of 0.9 saline was introduced into the well as a control. The plates were incubated for 24 hours at 36 °C for aerobic bacteria and at 28 °C for *Candida* isolates. The test was repeated twice (n=2) for each microorganism, toothpaste, mouthwash, and the combination of the last two mentioned. After incubation, zones of inhibition (with no growth of bacteria around the wells) were examined around the wells and measured in mm using analog caliper.

Statistical methods

The data were subjected to Kruskal-Wallis test for each pathogen group, and Mann-Whitney U test for pairs of interest, e. g., each toothpaste and mouthrinse were compared with each toothpaste and mouthrinse separately, but also combined. Paste A was compared with paste B, paste C, Mr1, Mr2, paste A+Mr1, paste B+Mr1, paste C+Mr1, paste A+Mr2, paste B+Mr2, paste C+Mr2; paste B with paste C, Mr1, Mr2, paste A+Mr1, paste B+Mr1, paste C+Mr1, paste A+Mr2, paste B+Mr2, paste C+Mr2; paste C with Mr1, Mr2, paste A+Mr1, paste B+Mr1, paste C+Mr1, paste A+Mr2, paste B+Mr2, paste C+Mr2; Mr 1 with Mr 2, paste A+Mr1, paste B+Mr1, paste C+Mr1, paste A+Mr2, paste B+Mr2, paste C+Mr2; Mr 2 with paste A+Mr1, paste B+Mr1, paste C+Mr1, paste A+ Mr2, paste B+Mr2, paste C+Mr2). A $p \leq 0.05$ was considered as statistically significant.

RESULTS

When we observed the effect of the tested toothpastes, mouthrinses or their combinations on the inhibition of *Candida albicans* growth, the inhibition zone was greater after treatment with paste A (Table 2), and that effect was statistically significant in relation to paste B (p=0.046), paste C (p=0.043), Mr1 (p=0.043) and Mr2 (p=0.046). The effect was also statistically significant when the paste A was used alone than in combination with Mr1 (p= 0.046) and Mr2 (p=0.043). Greater zone of inhibition was observed after the use of paste B than Mr1 (p=0.046) and Mr2 (p=0.050). The greater inhibition zone was also observed after the use of paste C than paste B (p=0.046), Mr1 (p=0.043) and Mr2 (p=0.046). It is interesting that inhibition zone was greater after the treatment with paste C alone than in combination with Mr1 (p=0.043).

The effect on *Candida tropicalis* was the greatest after the treatment with the combination of paste C and Mr1 (Table2). When we considered the inhibition zone after the treatment with toothpaste only, the best effect was found after the treatment with paste C (Table 2), and it was statistically significant in relation to paste A (p=0.046) and paste B (p=0.043). Also, the effect after paste A and paste B was statistically different (p=0.046). There was a statistical difference when we compared the effect of Mr1 and toothpastes (paste A: p=0.046; paste B: p=0.043; paste C: p=0.043), but also Mr2 and toothpastes (paste A: p=0.050; paste B: p=o.046; paste C: p=0.046).

Table 2. Antimicrobial activity (mean) against selected microorganisms for tested toothpastes and mouthrinses (Mr)

Paste/ Mouthrinse	Inhibition zone mean in mm (standard deviation)				
	Candida albicans	*Candida tropicalis*	*Enterococcus faecalis*	*Staphylococcus aureus*	*Streptococcus salivarius*
Paste A	29.67* (0.58)	23.00* (1.00)	21.67* (0.58)	23.67* (0.58)	18.33* (0.58)
Paste B	20,00* (2.00)	19.67* (0.58)	19.33* (1.53)	19.00*j (1.00)	18.33*i (0.58)
Paste C	26.67* (1.16)	25.67* (0.58)	27.00* (1.00)	34.33* (1.53)	16.33* (0.58)
Mr1	9.67* (0.58)	9.33* (0.58)	0.00* (0.00)	0.00 (0.00)	0.00* (0.00)
Mr2	11.33* (1.53)	14.00* (1.00)	16.67* (0.8)	28.33* (1.53)	12.67* (0.58)
Paste A + Mr1	27.00 (1.00)	26.00* (1.00)	25.67* (0.58)	25.33* (0.58)	19.00* (1.00)
Paste B + Mr1	22.33 (0.58)	21.33* (0.58)	19.00 (1.00)	18.67 (1.53)	14.67* (0.58)
Paste C + Mr1	25.33 (1.53)	28.00 (1.00)	29.00 (1.00)	45.33* (0.58)	21.67* (0.58)
Paste A + Mr2	25.67* (0.58)	25.67 (1.53)	39.00* (1.00)	41.33* (1.53)	23.67* (0.58)
Paste B + Mr2	19.00 (1.00)	18.67 (0.58)	40.33i (0.58)	36.33j (0.58)	19.33 (0.58)
Paste C + Mr 2	24.50* (0.71)	25.33 (0.58)	40.33* (0.58)	40.33* (0.58)	25.33* (0.58)

*p<0.05

The combination of toothpastes and Mr2 had the greatest effect of *Enterococcus faecalis* (Table 2). Between the toothpaste, the biggest inhibition zone was observed after the treatment with paste C and the results were statistically different in comparison with paste A (p=0.046) and paste B (p=0.050). Considering the effect of toothpaste and mouthrinses applied separately, toothpaste had better effect: paste A had shown better inhibition zone compared with Mr 1 (p=0.043) and Mr2 (p=0.043), paste B compared with Mr 1 (p=0.037) and Mr2 (p=0.046), but also paste C compared with Mr 1 (p=0.037) and Mr2 (p=0.046). Compared the inhibition zone after the treatment with toothpaste alone and in combination with mouthrinses, the effect for paste A was greater after the treatment in combination with Mr1 (p=0.043) and Mr2 (p=0.043). Furthermore, the effects of paste B (p=o.046) and paste C (p=0.046) were better after the combination with Mr2.

Paste C had the best effect on inhibition zone of *Staphylococcus aureus* when it was used alone or in combination with mouthrinses (Table 2). The effect of paste C was statistically significant in relation to paste A (p=0.046) and paste B (p=0.050), when it is used alone. Also, the effect of paste C has significantly increased after its combination with Mr1 (p=0.046) and Mr2 (p=0.046). Considered the effect of toothpaste and mouthrinses applied separately, toothpaste had better effect in case of paste C compared with Mr1 (p=0.037) and Mr2 (p=0.050). Paste A and paste B showed better inhibition zone compared with Mr 1 (p=0.034 for paste A, and p=0.037 for paste B), but Mr2 showed stronger inhibition capacity than paste A (p=0.046), and also than paste B (p=0.050).

As opposite to the inhibition on *S. aureus*, paste C had the weakest influence on *Streptococcus salivarius* of all tested toothpastes (p=0.043). When paste C was combined with mouthrinses, the inhibition zone for *S. salivarius* was significantly better (p=0.043, for both combination). Paste A also showed bigger inhibition zone in combination with Mr2 (p=0.043), while paste B showed weaker effect in combination with Mr1 (p=0.043) (Table 2) than applied alone. Considering the effect of toothpaste and mouthrinses applied separately, toothpaste had better effect, so paste A showed better inhibition zone compared with Mr 1 (p=0.034) and Mr2 (p=0.043), paste B compared with Mr 1 (p=0.033) and Mr2 (p=0.043), but also paste C compared with Mr 1 (p=0.034) and Mr2 (p=0.043).

DISCUSSION

Both tested probiotic toothpastes showed satisfactory antimicrobial activity, although in sum not stronger than the regular toothpaste. *Lactobacillus paracasei*-containing toothpaste had stronger inhibition capacity than *Lactobacillus acidophilus*-containing toothpaste in all cases, apart from *Streptococcus salivarius* group where it had the same inhibition capacity. This coincides with *in vitro* study of Hasslöf et al. (21), but also with *in vivo* study of Glavina et al. (23) investigating the ability of the selection of lactobacilli strains used in commercially available products, to inhibit growth of oral mutans streptococci and *C. albicans in vitro*: with an exception of *L. acidophilus*, the isolated probiotic strains (including *L. paracasei*) displayed strong inhibitory capacities against both microorganisms (23).

Although *L. paracasei*-containing toothpaste had generally stronger antimicrobial activity in comparison with *L. acidophilus* toothpaste, the strongest effect was against *C. albicans.* It was even significantly stronger than hexetidine mouthrinse, which is well known to have consi-

derable antimicrobial activity, but with big flaw in the form of teeth staining (24,25). This is an interesting finding and it should be considered in other oral candidiasis studies, for oral candidiasis accompanied with severe inflammation can significantly degrade the quality of life of immunosuppressed individuals and elderly people (26).

The present study also examined the combined performance of toothpastes and mouthrinses and found that against *C. albicans* all combinations, except *L. acidophilus* paste and essential oil mouthrinse, had weaker inhibition capacity than every single toothpaste, but much stronger than every single mouthrinses. It cannot be suggested that the reason for weaker activity of toothpaste-mouthrinse combinations compared to the one of toothpastes is antiseptic effect of mouthrinse on probiotic culture in toothpastes because this finding also refers to the non-probiotic toothpaste. Further, we found it in testing against *Streptococcus salivarius* where *L. acidophilus*-toothpaste and essential oils-mouthrinse had weaker antimicrobial effect than *L. acidophilus*-toohpaste alone Regular and *L. acidophilus*-toothpaste in combination with hexetidine-mouthrinse had slightly weaker activity than toothpastes against *Candida tropicalis*, but without statistical significance. In all other cases, adding the mouthrinse to the toothpaste increased its antimicrobial activity, especially against *Enterococcus faecalis* and *Staphylococcus aureus*. Such an enhanced antimicrobial effect can be a significant discovery, particular in terms of *Enterococcus faecalis* that has been considered one the most resistant species in the oral cavity and a possible cause of root canal treatment failure (27,28).

The results of this study have shown that *L. paracasei*-containing toothpaste showed the strongest antimicrobial activity against *C. albicans,* even significantly stronger than hexetidine mouthrinse, which should be considered in oral candidiasis studies. Adding the mouthrinse to the toothpaste has increased its antimicrobial activity, especially against *Enterococcus faecalis,* one the most resistant species in the oral cavity. In conclusion, probiotics are defined as viable microorganisms that confer a health benefit when administered in sufficient doses; probiotic-containing toothpaste is a relatively new concept in the prevention of oral infectious diseases. The present study was limited to only a few types of microorganisms and tested agents in their pure form. Therefore, further studies are required to test these types of toothpastes and mouthwashes in different concentrations on different microorganisms.

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Antimikrobna učinkovitost zubnih pasti koje sadrže probiotike: *in vitro* procjena

Ivana Parčina Amižić[1], Livia Cigić[2], Lidia Gavić[2], Marina Radić[3,4], Dolores Biočina Lukenda[2], Marija Tonkić[3,4], Ivana Goić Barišić[3,4]

[1]Ordinacija dentalne medicine, Dom zdravlja Splitsko-dalmatinske županije, [2]Katedra za oralnu medicinu i paradontologiju, Studij dentalne medicine, Medicinski fakultet, Sveučilište u Splitu, [3]Klinički odjel za mikrobiologiju i parazitologiju, Sveučilišna bolnica Split, [4]Medicinski fakultet, Sveučilište u Splitu; Split, Hrvatska

SAŽETAK

Cilj Procijeniti antimikrobne sposobnosti, *in vitro,* dviju zubnih pasti koje sadrže probiotike (jedna sadrži *Lactobacillus paracasei*, a druga *Lactobacillus acidophilus*), jedne zubne paste bez probiotika, te u kombinaciji svake pojedine zubne paste i tekućine za ispiranje usta (jedne koja sadrži eterična ulja i druge koja sadrži heksetidin).

Metode Antimikrobna sposobnost testirana je *ditch*-metodom na agaru, na mikroorganizmima *Candida albicans*, *Candida tropicalis*, *Enterococcus faecalis*, *Streptococcus salivarius* i *Staphylococcus aureus*. Za statističku analizu korišten je Kruskal-Wallisov test i Mann-Whitney U-test ($p \leq 0.05$).

Rezultati Obje testirane zubne paste s probiotikom pokazale su zadovoljavajuće antimikrobno djelovanje, kako pojedinačno, tako i u kombinaciji s tekućinama za ispiranje usta, naročito u slučaju *C. albicans* (p=0.043) i *Streptococcus salivarius* (p=0.043), te jako inhibitorno djelovanje, nego tekućine za ispiranje usta ($p \leq 0.05$).

Zaključak Zubne paste s probioticima mogu doprinijeti u prevenciji oralnih zaraznih bolesti.

Ključne riječi: *Lactobacillus acidophilus, Lactobacillus paracasei,* probiotici, tekućine za ispiranje usta