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Original Article

The efficacy of different immersion times in chlorhexidine for reducing the *Lactobacillus acidophilus* contamination of the toothbrush

Fereshteh Fallahi[1], Mina Mohebian[2], Habib Zeighami[3], Neda Gholami[4], Atefeh Yousefi Jordehi[5*]

[1]School of Dentistry, Zanjan University of Medical Sciences, Zanjan, Iran
[2]Department of Oral and Maxillofacial Medicine, School of Dentistry, Zanjan University of Medical Sciences, Zanjan, Iran
[3]Department of Microbiology, School of Medicine, Zanjan University of Medical Sciences, Zanjan, Iran
[4]Oral and Maxillofacial Specialist, Vancouver, British Columbia, Canada
[5]Department of Operative Dentistry, School of Dentistry, Zanjan University of Medical Sciences, Zanjan, Iran

Abstract

Background: *Lactobacillus acidophilus* is involved in plaque formation and progression of caries, and studies show that it can be transmitted through the toothbrush. Thus, decontamination the toothbrush is necessary for oral health. This study aimed to assess the efficacy of different immersion times in chlorhexidine (CHX) for reducing the *L. acidophilus* contamination of the toothbrush.

Methods: This experimental study was conducted on 84 dental students. Primary saliva samples were obtained from the students, and the salivary count of *L. acidophilus* was measured. The students were assigned to four groups, and the groups were standardized in terms of primary salivary *L. acidophilus* count: (I) simple rinse of toothbrush with water, (II) simple rinse of toothbrush with CHX, (III) immersion of toothbrush in CHX for 10 minutes, and (IV) immersion of toothbrush in CHX for 24 hours after use. Saliva samples were collected again from the dental students after 3 weeks of practice. The mean *L. acidophilus* colony counts of primary and secondary saliva samples were calculated. Also, the mean *L. acidophilus* colony counts of toothbrushes were compared among the four groups. Data were analyzed using ANOVA and Tukey's tests. The level of significance was set at 0.05.

Results: The mean *L. acidophilus* colony count of toothbrushes was significantly different between the four groups ($P<0.05$). Groups 3 ($P=0.040$) and 4 ($P=0.012$) showed significantly lower toothbrush microbial count compared with Group 1. No significant difference was noted in the salivary colony count of the four groups after the intervention ($P>0.05$).

Conclusion: Immersion of toothbrush in CHX for 24 hours and 10 minutes was more effective than other methods for the reduction of *L. acidophilus* count of the toothbrush. In order to prevent corrosion of bristles, 10 minutes of immersion is recommended.

Keywords: Chlorhexidine digluconate , Toothbrushing, *Lactobacillus acidophilus*

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Introduction

Microorganisms are in equilibrium in a healthy oral environment.[1] An imbalance in the oral microbial flora results in dental plaque formation, composed of a wide range of bacteria accumulated in a matrix derived from the saliva, forming a thin biofilm on the tooth surface.[2] Dental plaque accumulation is the onset of periodontal disease and enamel demineralization,[3] which can eventually lead to tooth loss and compromise smile aesthetics and masticatory function. Thus, prevention of plaque accumulation on the tooth surface and its removal prior to the onset of inflammatory changes in the gingiva are imperative.[4]

Streptococcus mutans and *Lactobacillus acidophilus* are responsible for dental plaque formation. *L. acidophilus* is involved in the progression of caries due to its aciduric and acidogenic properties.[5]

Plaque control can be performed by mechanical and chemical methods.[6] Daily toothbrushing with toothpaste is the main mechanical method of plaque removal.[7] Use of antiplaque agents such as cetylpyridinium chloride, chlorhexidine (CHX), and essential oils are among the chemical plaque control measures.[8]

Daily use of a toothbrush can lead to its microbial contamination, depending on its storage conditions.[9] Evidence shows that toothbrush bristles can harbor bacteria and cause oral infections[10]. These microorganisms can survive for 24 hours to 7 days.[11] For this reason, the

*Corresponding Author: Atefeh Yousefi Jordehi, Emails: dr.yousefi.j@gmail.com, dryoosefi@zums.ac.ir

decontamination of toothbrushes is recommended by ADA, especially in people with systemic diseases.[12]

Several methods are available for reduction of microbial contamination of the toothbrush, including frequent replacement of the toothbrush, immersion in antimicrobial agents such as CHX, spraying antiseptic agents on the toothbrush, wet or dry heat, and using ozone gas or UV radiation.[13] Among the frequently used mouthwashes, CHX has shown more promising results.[14] CHX is the gold-standard anti-plaque mouthwash with broad-spectrum antimicrobial activity and can prevent smooth-surface caries, disinfect dentures, and inhibit dental plaque formation. It is also used as a root canal irrigant due to its optimal antibacterial activity, relatively long substantivity, and lack of cytotoxicity.[15] Evidence shows that CHX can decrease the bacterial count by 10% to 20% after one time of use.[16]

Immersion of the toothbrush in CHX for an adequately long period of time is an effective method for its decontamination.[17] Immersion of the toothbrush in CHX is simple, but it may have side effects such as microscopic corrosion of bristles, permanent humidity of the toothbrush, and the possibility of growth and proliferation of microorganisms. Thus, this study aimed to assess the efficacy of different immersion times in CHX for reduction of toothbrush *L. acidophilus* contamination.

Materials and Methods

This experimental study was conducted on dental students of the School of Dentistry, in Zanjan University of Medical Sciences.

Since no similar previous study was available for sample size calculation, this study was conducted as pilot on 84 dental students in four groups (n = 21). A total of 14 participants did not show up for the second saliva sampling, and were excluded from the study. Out of the remaining 70 participants of the study, 64.3% (n = 45) were females and 35.7% (n = 25) were males in the age range of 20–25 (Table 1).

The dental students underwent clinical oral examination, and those without active caries were enrolled. The exclusion criteria were periodontal disease, cleft lip or palate, problems affecting the oral microbial flora, problems affecting the toothbrushing ability of participants, use of CHX mouthwash in the past 2 weeks, history of antibiotic therapy in the past 2 weeks, systemic diseases, and medication intake.

Table 1. Frequency of demographic variables

		Group 1	Group 2	Group 3	Group 4	Total
Gender	Male	6	7	5	7	25
	Female	11	9	12	13	45
Age	20	1	0	1	1	3
	21	2	4	2	3	11
	22	6	4	5	4	19
	23	2	1	4	5	12
	24	4	5	2	4	15
	25	2	2	3	3	10

The participants were requested to refrain from toothbrushing in the morning of the sampling day. After obtaining written informed consent from the participants, saliva samples were collected in sterile microtubes. Saline was also added to the microtubes. Next, 50 microliters of this suspension was streak-cultured on MRS culture medium (Merck, Germany), which is specific for *L. acidophilus* cultures.

The culture plates were incubated in a Gas-Pak A (Merck, Germany) at 37°C for 24 hours. After 24 hours, *L. acidophilus* colony count was measured by the naked eye and reported as colony forming units.

The students were then assigned to four groups (n = 21) by block randomization. The complex permuted-block randomization method was used for randomization. This method, in addition to balancing the number of people between the 4 groups, will prevent any predictions in determining the sequence of the intervention. Seven blocks of 12 were considered based on the primary salivary *L. acidophilus* count, the first block was randomly selected, and the random sequence of blocks was obtained via STATA software. Then, subjects within each block were randomly assigned to the treatment groups (Figure 1):

- Group 1: This group included students who were asked to rinse their toothbrush under running water after toothbrushing. This group served as the control group.
- Group 2: The participants were requested to rinse their toothbrush with CHX after toothbrushing.
- Group 3: The participants were asked to immerse their toothbrush in CHX for 10 minutes with no further rinsing with water.
- Group 4: The participants were asked to immerse their toothbrush in CHX until the next time of use (24 hours later).

After the collection of the primary saliva samples, the participants received a toothbrush (Oral B, USA), toothpaste (Pooneh, Iran), and 0.2% CHX (Behsa, Iran) and were provided with instructions for use depending on their group allocation. The used toothbrushes in groups 1, 2, and 3 were capped and kept at room temperature after rinsing or disinfection. After 3 weeks, the secondary saliva samples were collected from the participants as done for the first saliva samples, and they were then cultured. The toothbrushes were also collected and placed in sterile microtubes containing 1 mL of saline. The toothbrushes were then removed from the microtubes in such a way that the toothbrush bristles were in complete contact with the microtube walls (to transfer the bacteria). The solution was then cultured on MRS culture medium, like the saliva

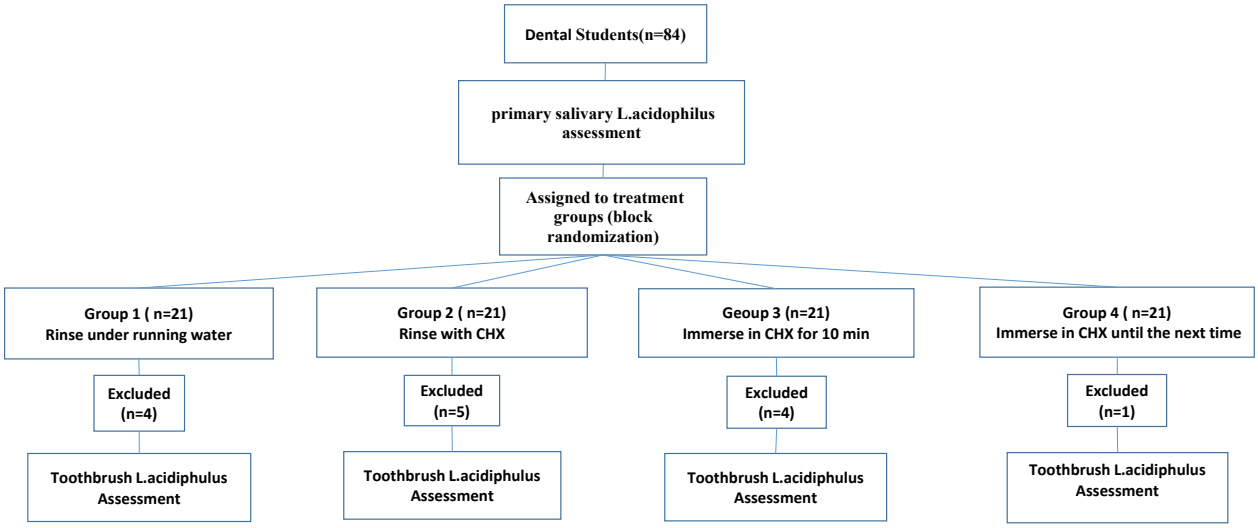

Figure 1. Schematic flowchart of the study protocol

samples. An unused toothbrush was also immersed in saline, and the solution was cultured to serve as control. This study was single blinded and the microbiologists who measured the outcomes (*L. acidophilus* colony count in secondary saliva and toothbrush samples) were blinded to the used toothbrush cleaning process. The coding had been done by a third person who was not involved in outcome assessment.

Data were analyzed using SPSS version 19 (SPSS Inc., Chicago, IL, USA). Normal distribution of data was evaluated using the Kolmogorov-Smirnov test. Since data were normally distributed, ANOVA and Tukey's test were used to analyze the four groups. The level of significance was set at 0.05.

Results

The four groups were not significantly different regarding the mean baseline salivary *L. acidophilus* count ($P=0.429$) or regarding the mean salivary *L. acidophilus* count after the intervention ($P=0.101$). However, the microbial count decreased from group 1 to group 4 (Table 2). A significant difference was noted in the microbial count of toothbrush samples between the groups, and the mean *L. acidophilus* count significantly decreased from group 1 to group 4 ($P=0.014$). Pairwise comparisons by Tukey's test showed significant difference between the groups in such a way that the microbial count of group 3 (immersion in CHX for 10 minutes) was significantly lower than that of control group ($P=0.040$), and there was significant difference between group 4 (immersion in CHX for 24 hours) and the control group ($P=0.012$).

The salivary *L. acidophilus* count after the intervention compared with baseline revealed a significant change in microbial count among the four groups (Table 3). Pairwise comparisons by Tukey's test showed that the reduction of microbial count after the intervention (compared with baseline) in group 4 was significantly greater than in groups 1, 2 and 3 ($P<0.001$, $P=0.025$, $P=0.038$, respectively).

Table 2. Mean *Lactobacillus acidophilus* count in the primary and secondary saliva samples and toothbrush samples

	Group	Mean SD	*P* value	Significant difference
Primary saliva sample	1	363.33±195.91	0.429	
	2	355.88±203.77		
	3	346.50±208.33		
	4	444.44±184.62		
Secondary saliva sample	1	350.00±186.12	0.101	
	2	256.47±164.70		
	3	236.25±140.36		
	4	228.61±117.32		
Toothbrush sample	1	157.33±124.35	0.014	(Group 1 and Group 3: $P=0.40$; Group1 and Group 4: $P=0.012$)
	2	96.47±114.83		
	3	76.00±51.44		
	4	60.28±39.42		

Group 1: control, Group 2: simple rinse of toothbrush with CHX, Group 3: immersion of toothbrush in CHX for 10 minutes, Group 4: immersion of toothbrush in CHX for 24 hours.
SD, standard deviation.

The results after controlling for the effects of gender and age revealed the groups were still significantly different after the intervention regarding the mean microbial count of the toothbrush ($P=0.001$) (Table 4).

The linear regression results regarding the salivary microbial count after the intervention showed that after eliminating the effect of confounders, the difference between the groups was still significant in terms of the mean salivary microbial count (Table 5).

Discussion

This study compared the efficacy of rinsing the

Table 3. Salivary *Lactobacillus acidophilus* count in the four groups after the intervention compared with baseline

	Group	Mean ± SD	*P* value	Significant difference
Change in salivary L. acidophilus count	1	89.57 ± 13.33	<0.001	Group 1 and group 4: $P<0.001$ Group 2 and group 4: $P=0.025$ Group 3 and group 4: $P=0.038$
	2	124.82 ± 99.41		
	3	118.52 ± 110.25		
	4	131.66 ± 215.83		

Group 1: control, Group 2: simple rinse of toothbrush with CHX, Group 3: immersion of toothbrush in CHX for 10 minutes, Group 4: immersion of toothbrush in CHX for 24 hours.
SD, standard deviation.

Table 4. Linear regression results regarding the microbial count of the toothbrush

Variable	Indices			*P* value
	β coefficient	Standardized β coefficient	t	
Gender	12.271	0.067	0.607	0.546
Age	1.166	0.032	0.297	0.768
Intervention group	-30.916	- 0.367	-3.337	0.001

Table 5. Linear regression results regarding the salivary microbial count after the intervention

Variable	Indices			*P* value
	β coefficient	Standardized β coefficient	t	
Before intervention	o.603	0.767	10.395	0.000
Gender	-34.071	-0.105	-1.450	0.152
Age	1.792	0.029	0.408	0.685
Intervention group	-47.432	-0.334	-4.579	0.000

toothbrush with water and CHX and immersion of toothbrush in CHX for 10 minutes and 24 hours to reduce toothbrush *L. acidophilus* contamination. The effect of the above-mentioned methods on the salivary count of *L. acidophilus* after 3 weeks of practice was also evaluated. Our study was conducted on dental students for reliable use of toothbrushes. Similar to many studies, the students who had received antibiotic therapy in the 2 weeks leading to sample collection were excluded to eliminate possibility of false lower *L. acidophilus* load than normal.[18] They were requested not to brush in the morning of the sampling day to ensure the saliva would contain high levels of *L. acidophilus*. To standardize the level of *L. acidophilus*, its baseline salivary count was evaluated; then the participants were assigned to four groups by block randomization based on primary salivary *L. acidophilus* count. Secondary saliva samples were also assessed after 3 weeks. To the best of our knowledge, this is the only study to standardize salivary microbial count among all the groups.

We used the toothpaste to simulate normal conditions, but some researchers believe toothpaste is a disinfectant and did not use it in their studies.[19] MRS culture medium, which is specific for *L. acidophilus* cultures, was used, and the culture plates were incubated in a Gas-Pak at 37°C for 24 hours for anaerobic culture.

The results showed the maximum reduction was recorded in the microbial count of the toothbrush in group 4 (immersion in CHX for 24 hours) followed by group 3 (immersion in CHX for 10 minutes), and the minimum reduction was seen in group 1. The difference in microbial count was significant between groups 3 and 4 and the control group.

As bacterial plaque is responsible for dental caries, chemical plaque control measures in addition to mechanical plaque removal can greatly help in further reduction of microbial load.[20,21] On the other hand, toothbrush contamination is an important topic as the toothbrush can harbor potential pathogens and compromise the health status of individuals, particularly immunocompromised patients.[22,23]

Evidence shows that cariogenic bacteria can be transmitted through dental floss and the toothbrush. There are microbial counts of 10^8 CFU in the toothbrush head, even in healthy individuals.[24] Cobb was the first to suggest the role of contaminated toothbrushes in the recurrence of oral infections.[25] Thus, researchers focused on disinfecting agents for disinfection of toothbrushes. However, no standardized method for storage and decontamination of toothbrushes has been reported yet.[26] Nanjunda Swamy et al reported decreased contamination of toothbrushes following the use of disinfectants.[27] Some studies compared the efficacy of different disinfectants versus CHX for elimination of toothbrush contamination and reported that CHX was more effective for this purpose.[26,28-30] In a review study, Agrawal et al evaluated the efficacy of CHX compared with energy radiation and natural substances and showed its superior efficacy.[31]

CHX is non-toxic, non-irritant, highly effective, rapid in action, and easy to use.[32] Also, it has a prominent role in reduction of bacterial plaque.[33-35] The mechanism of action of CHX is based on the attachment of positively charged CHX digluconate molecules with the negatively charged bacterial cell wall. The CHX molecule mainly binds to the phosphate group, lipopolysaccharide, and carboxyl groups present in proteins. Moreover, CHX prevents bacterial adhesion to surfaces by the removal of calcium and deactivation of glycosyltransferase.[36] Thus,

0.2% CHX was used in the present study for toothbrush decontamination.

The present study showed increased reduction in microbial count with prolongation of immersion time; however, only groups 3 and 4 had a significant difference compared to the control group. Our finding was consistent with Rodrigues et al, who showed that spraying the toothbrush with 0.12% CHX three-times a day caused a significantly greater reduction in microbial count of toothbrush compared with one-time spray or rinsing with water.[13] In our study CHX was used at higher concentration than the CHX evaluated in Rodrigues and colleagues' study, and the results showed that rinsing the toothbrush with CHX daily caused no significant reduction in microbial count compared with rinsing with water. Nevertheless, some researchers reported that CHX reduced the microbial count of the toothbrush within a short span and the effects were much longer lasting compared with the other mouthrinses.[26,32] Talaat et al. sprayed the toothbrush 6 times with 0.12% CHX from a 5 cm distance and found that its bacterial colony counts were significantly lower compared to rinsing with distilled water.[32] Our results were different from their study when we rinsed the toothbrush only 1 time with CHX. However, the findings of our study showed significant reduction in microbial count by increasing the immersion time to 10 minutes and 24 hours.

The immersion of the toothbrush in CHX has drawbacks, such as loss of bristles, their corrosion and discoloration, and reduction of their clinical service life. Also, CHX has inherent side effects such as alteration of the sense of taste, tooth discoloration, and mucosal desquamation.[37] Thus, as the difference in microbial count reduction was not significant between 10 minutes and 24 hours of immersion of the toothbrush in CHX, the immersion of toothbrush in CHX for 10 minutes is recommended.

Future studies on the effects of immersion of the toothbrush in CHX and other antimicrobial agents are recommended on different age groups and also on other outcomes such as dental plaque, gingivitis, and dental caries.

Conclusion

One of the limitations of this study was the non-cooperation of some participants and their exclusion from the study. In spite of the limitations of this study, the results showed that immersion of toothbrush in CHX for 24 hours and 10 minutes was more effective than other methods for reduction of the toothbrush *L. acidophilus* count. In order to prevent corrosion of the bristles, 10 minutes of immersion is recommended.

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Authors' Contribution

Conceptualization: Neda Gholami.
Data curation: Fereshteh Fallahi, Mina Mohebian
Investigation: Fereshteh Fallahi, Mina Mohebian, Habib Zeighami.
Formal analysis: Fereshteh Fallahi, Atefeh Yousefi Jordehi.
Methodology: Neda Gholami, Mina Mohebian, Fereshteh Fallahi, Habib Zeighami.
Project administration: Mina Mohebian.
Supervision: Mina Mohebian, Habib Zeighami
Software: Fereshteh Fallahi, Atefeh Yousefi Jordehi.
Resources: Fereshteh Fallahi, Atefeh Yousefi Jordehi.
Validation: Neda Gholami, Mina Mohebian, Habib Zeighami, Atefeh Yousefi Jordehi .
Visualization: Neda Gholami, Mina Mohebian, Atefeh Yousefi Jordehi.
Writing-original draft preparation: Fereshteh Fallahi, Atefeh Yousefi Jordehi.
Writing-review & editing: Atefeh Yousefi Jordehi, Neda Gholami.

Competing Interests

The Authors of this manuscript declare that they have no conflict of interest, real or perceived financial or nonfinancial in this article.

Ethical Approval

This research was approved by the Ethics Committee of Zanjan University of Medical Sciences. The ethic approval code is IR.ZUMS.REC.1397.250.

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