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Evaluation of bacterial contamination of miswak toothbrushes in different storage environments: An experimental study

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Abstract

Aim: To evaluate the microorganism levels on miswak toothbrushes and to investigate the effect of storage conditions.

Materials and Methods: 24 Participants kept miswak toothbrushes in the bathroom and room for one month. Thioglycolate was left in the liquids by cutting 1/3 of the miswak hairs, and incubation was provided for microorganisms. Precise identification of microorganisms is achieved with VITEK® 2 Compact. The periodontal health of the participants was evaluated at the beginning and end of the study by evaluating the bleeding on their plaque, gingival indices, sulcus depths, and probing values.

Results: Seventeen (70.80%) participants were female and 7 (29.20%) were male. Half of the participants kept their toothbrushes in the bathroom, and the other half in their room. Lactobacillus spp. was found in 29.2% of toothbrushes, with an average count of $2.43\pm1.71x10^2$ CFU/ml. *Streptococcus mutans* were found in 88.3% of toothbrushes, with an average count of $4.05\pm1.64x10^2$ CFU/ml. Of the toothbrushes tested, 25.0% had *Candida albicans* present with an average count of $3.18\pm1.47x10^2$ CFU/ml. *Lactobacillus spp.* in 29.90% of Miswak toothbrushes, *S. mutans* was found in 83.30% and *C. albicans* in 25%. No significant difference was observed in periodontal index values.

Conclusion: 84% of miswak toothbrushes are contaminated with persistent bacteria in the first month of use, regardless of storage method. Storing miswak toothbrushes in a humid environment results in higher rates of *S. mutans* bacterial contamination and survival. Surprisingly, colonization and survival of *Lactobacillus spp.* and *C. albicans* were higher in miswak toothbrushes stored at room temperature. It should be replaced with miswak toothbrushes every month instead of the previously suggested three months.

Introduction

Dental caries and periodontal disease are primarily caused by microbial dental plaque. Maintaining optimum oral health and maintenance is the primary objective of modern dentistry, which involves the elimination of microbial dental plaque [1]. The microorganisms in dental plaque can produce irritants such as antigens and endotoxins, leading to gingivitis and caries over time [2]. However, effectively removing this plaque from the tooth surface can be challenging. It is recommended to use mechanical and chemical plaque removal methods to maintain good oral health [3]. Tooth brushing is the most essential and effective method to eliminate dental plaque [4].

Using toothbrushes correctly and replacing them regularly is essential to prevent the accumulation of microorganisms [5]. The microorganisms are mainly transferred from the oral cavity to the toothbrush. Contaminated toothbrushes pose health risks, especially for immunosuppressed individuals, organ transplant recipients, cystic fibrosis, and patients with cardiopathies who have endocarditis, increasing the risk of reinfection [3]. They can also be transmitted from contaminated fingers, aerosols from toilet flushes, and bacteria that succeed in the bathroom's humid environment. Previous studies state that toothbrushes are a reservoir for bacteria species such as *Streptococcus spp.*, *Staphylococcus spp.*, *Lactobacillus spp.*, and fungal species such as *Candida spp* [3]. However, storage conditions can effectively prevent this contamination risk [6,7]. Many researches have shown that the toilet's proximity, the humid-

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ity level in the bathroom, the humid conditions of toothbrush storage containers, and shortened brushing sessions can significantly impact the survival of bacteria, leading to increased cross-contamination [8,9].

Recent studies stated that the *Streptococcus* and *Lactobacillus* species play a significant role in the primary microbiota of dental caries. However, these studies have also revealed that fungi, particularly *Candida* species, are frequently encountered in caries lesions [10,11]. Oral hygiene care products made of natural ingredients are gaining popularity worldwide. This global demand for safer, effective, and economical prevention methods has increased due to growing interest. Researchers globally have implemented a multilevel strategy that focuses on natural products as an alternative for prevention. This initiative aims to improve oral health education and reduce the economic burden of oral diseases [12]. Miswak is primarily derived from the Arak Tree (*Salvadora Persica*), commonly found and used in the Middle East [13]. Miswak is commonly used in many countries due to its association with social practices, cultural norms, and religious beliefs [14]. It is used as a popular dental cleaning tool today due to its easy availability and low cost. Miswak sticks are commonly used as a substitute for toothbrushes, typically crafted from plants, shrubs, or trees with potent antimicrobial properties.

A recent systematic review found that Miswak chewing sticks are just as effective as standard toothbrushes in removing plaque buildup mechanically. It has been reported that it possesses better anti-gingivitis properties in comparison to the regular toothbrush [15]. Sodium bicarbonate acts as a mild abrasive to potentially whiten teeth, while silica found in S. persica extracts enhances the mechanical action of plaque removal [16]. Tannins and resins are effective anti-gingivitis agents. They have an astringent effect on the mucous membrane, reducing gingival inflammation, and promoting periodontal health [12]. Clinical studies have shown that users of S. persica chewing sticks exhibit lower plaque accumulation and gingival bleeding scores compared to those who use standard toothbrushes [17]. Combining the mechanical cleaning action of Miswak chewing sticks with their bioactive constituents safeguards against plaque and gingivitis [15].

The contents of miswak fibers, including trimethylamine, salvadorin, vitamin C, flavodin, saponins, sterol, and fluoride, have been shown to positively impact oral health [13]. It can be act an antibacterial agent against cariogenic bacteria. Additionally, its fluoride content promotes remineralization to treat white spot lesions [18]. It is well established that *Salvadora persica*'s essential oils, which are non-polar compounds, exhibit antimicrobial properties against pathogenic bacteria and fungi. Furthermore, these oils are known to help balance saliva pH and eliminate dental plaque [19]. Miswak has a variety of features that make it a crucial tool in preventing the formation of caries. The World Health Organization has recommended using these sticks for oral hygiene maintenance [20].

There have been no studies examining the effect of storage conditions on bacterial contamination of Miswak. This study aimed to compare the effect of storage conditions on Miswak's bacterial load by measuring the microorganisms present on Miswak toothbrushes. The study's null hypothesis is that miswak toothbrushes contain equal bacterial load without being affected by storage conditions.

Materials and Methods

The study has been approved by the Afyonkarahisar Human Research Ethic Committee with the number 2020/13. The study was conducted according to the principles of the Declaration of Helsinki. The study has been presented according to CONSORT guidelines for reporting trials.

Sample size determination

As there were no comparable studies found in the literature, the sample calculation was carried out solely based on the effect size. A sample size of 12 individuals was deemed sufficient for a study on the plaque index of toothbrushes stored in the bathroom. The difference in measurements between the initial and first-month values was taken as 0.9. The Wilcoxon Signed Rank test was used with a power of 0.80 and a significance level of 0.05 for the sample calculations. Since the same procedures would be applied to the toothbrushes stored in the room in the study, a total of 24 people were sufficient for the study [21]. G*Power 3.1 (Heinrich-Heine-Universität Düsseldorf, Düsseldorf, Germany) package program was used for power analysis.

Study population

It was recruited 4th-years 24 dental students who willingly participated in the study. They were divided into two experimental groups, each comprising 12 students.

Inclusion criteria

Individuals included in the study:

- Systemically healthy
- Non-smokers
- Those who brush their teeth twice a day
- Who not used chlorhexidine in the last three months
- Suffers from untreated dental caries
- Having no missing teeth.

Exclusion criteria

- Those who have used miswak before
- Those who are currently undergoing orthodontic treatment
- Those with severely misaligned teeth
- Those who have previously undergone scaling and root planning treatment for periodontitis
- Those who have taken antibiotics in the last month
- Those who have prosthetic crowns.

Randomization and blinding

Participants were asked to choose a color to represent the room or bathroom environment. Before the study started, a blue ball was determined to represent the room, and a red ball was chosen to represent the bathroom environment. An independent researcher (Dr. N.C.K.) who was not involved in the study asked the participants to choose a color to ensure a double-blind randomization procedure. After each participant selected a color, they were informed of the corresponding storage condition, and groups were randomly assigned. Twenty-four participants were thoroughly briefed on the specifics of the study. Additionally, each participant completed a patient consent form to give permission. This study did not include individuals who had active untreated caries or had used chlorhexidine within the past three months. Before the study, participants' plaque was removed professionally without the flour to prevent potential antibacterial effects.

Clinical protocol

The participants' Silness-Löe plaque index (PI), Löe-Silness gingival index (GI), buccal and lingual pocket depths (PD), and bleeding on probing (BOP) values were measured and recorded at the start of the study and again after one month. (Table 1) PI, GI, and PD values were measured from 6 different points of each tooth: mesiobuccal, buccal, distobuccal, distopalatinal, palatal, and mesiopalatinal with a periodontal sond (HUF No:15, Hu-Friedy, Chicago, Illinois, USA). The obtained values were averaged to create a single value [22,23]. The participants' plaque index (PI), gingival index (GI), pocket depths (PD), and bleeding on probing (BOP) values were measured and recorded at the start of the study and again after one month. (Table 1) PI, GI, and PD values were measured from 6 different points of each tooth: mesiobuccal, buccal, distobuccal, distopalatinal, palatal, and mesiopalatinal. The average of PI, GI, and PD values was calculated by dividing the sum of the values obtained from each measured tooth by the product of the number of existing teeth and the number of tooth surfaces measured [24,25]. BOP values were calculated by dividing the number of bleeding areas by the total number of areas probed, then multiplying by 100 to obtain a single value [26]. The obtained values were averaged to create a single value [22,23].

Study design

This study involved 4th-years dental students who were registered in the dentistry faculty. Dental students were randomly divided into two groups. The participants were divided equally into two groups: one in a bathroom storage environment and the other in a room storage environment. Each group consisted of 12 people. (Figure 1) Twenty-four participants were given a miswak-containing "My Miswak Travel" (Fanzoh Private Limited Company, Delhi, India; Klein Dış Ticaret Limited Şirketi, İstanbul Türkiye), toothbrush, categorized by where they stored miswak toothbrush in the bathroom or room (Figure 2).

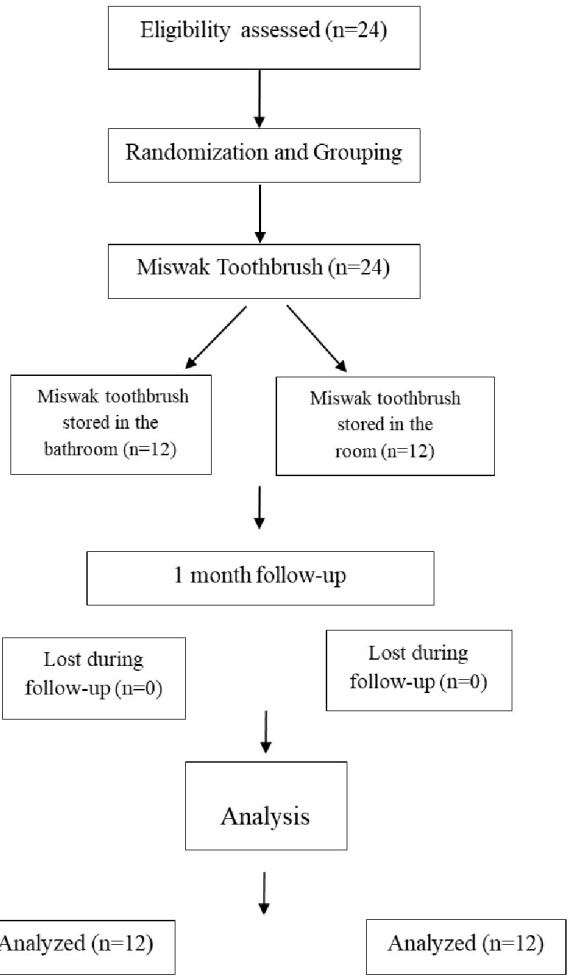

Figure 1. Study flowchart following CONSORT guidelines for clinical trials.

Figure 2. "My Miswak Travel" toothbrush.

Establishing a standardized protocol for oral hygiene techniques

All participants were informed not to store their miswak toothbrushes in plastic covers or environments near toilets [3]. Participants with a bathroom toilet should close the lid before flushing to prevent the spread of aerosols. All participants were given the same toothpaste (Colgate, Maksimum Anti-Çürük, Colgate-Palmolive, Maltepe, İs-

tanbul, Türkiye). Participants who preferred toothpaste were instructed to rinse their miswak toothbrushes thoroughly with tap water. It was recommended to brush the teeth twice a day for two minutes using the modified Stillman technique [27]. It is recommended to clean all tooth surfaces by using the miswak toothbrush with controlled movements. Participants were instructed to refrain from using anti-plaque mouthwash and to clean their teeth with the miswak toothbrush only during the study period. Participants were advised to soak the miswak toothbrush in water before use to soften its stiff fibers. Participants used the miswak toothbrush for a month, stored it correctly. After using their Miswak toothbrushes for 30 nights, participants were instructed to bring them to the researchers in sterile pouches the following morning [28]. All participants were instructed not to store or tamper with the miswak toothbrushes [29,30]. All participants were explicitly informed that they could use dental floss.

Microbiological analysis and colony counting

On return of the toothbrushes, one-third of the miswak bristles were cut using sterile forceps and a scalpel at aseptic conditions. The bristles were left into sterile tubes containing 10 mL Thioglycolate broth (TGB) (Merck Millipore, Darmstadt, Germany) and the samples were immediately transported to the Microbiology Laboratory at Afyon Kocatepe University Faculty of Veterinary Medicine in a cold chain box (+ 4 °C) TGBs were vortexed for 30 s and 1 mL from each TGB with bristles was transferred into sterile tubes consisting of 9 mL Brain Heart Infusion broth (BHIB) (Merck Millipore, Darmstadt, Germany). Broths were incubated at 37 °C for 24 hours. After homogenized by vortexing for 30 s, serial 10-fold dilutions of all samples were prepared.

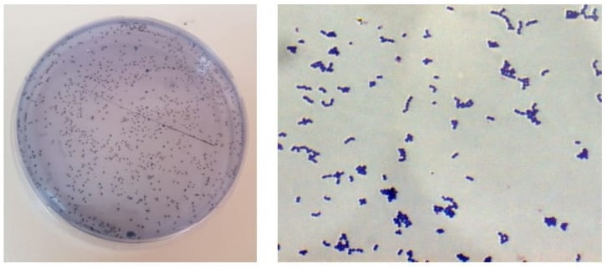

Figure 3. Macroscopic and microscopic views of *S. mutans* isolates.

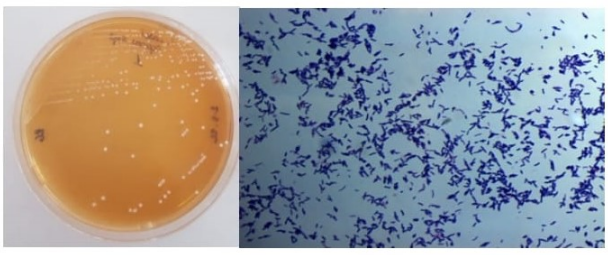

Figure 4. Macroscopic and microscopic views of *Lactobacillus spp.* isolates.

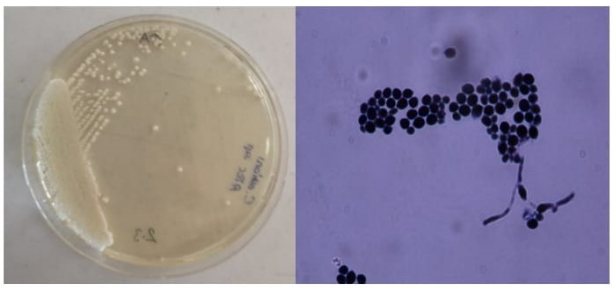

Figure 5. Macroscopic and microscopic views of *C.albicans* isolates.

Aliquots of 0.1 mL was taken from each dilution and inoculated onto bacitracin (200 IU/L) (HiMedia, Mumbai, India) and potassium tellurite (Sigma-Aldrich Inc, St Louis, USA) added Mitis Salivarius agar (MSA) (Merck Millipore, Darmstadt, Germany) for *Streptococcus mutans* (Figure 3), Rogosa agar (RA) (Merck Millipore, Darmstadt, Germany) adjusted the pH to 5.5 with acetic acid 96% for *Lactobacillus spp.* (Figure 4), and Sabouraud Dextrose agar (SDA) (Merck Millipore, Darmstadt, Germany) supplemented with chloramphenicol (Merck Millipore, Darmstadt, Germany) for *Candida albicans* (Figure 5) .

MSA and RA petri dishes were incubated at 35 °C under anaerobic conditions for 24-72 hours, while SDA petri dishes were aerobically incubated at 37 °C for 24-72 hours. Following the incubations, colonies grown on agars were counted twice and calculated as CFU/ml. In addition to, growing colonies on mediums were examined macroscopically (colony morphology) and microscopically (Gram stain). The certain identification of isolates was achieved using VITEK® 2 Compact system (BioMerieux, Marcy l'Etoile-France). In all applications, S. mutans ATCC® 25175, C. albicans ATCC® 10231 and Lactobacillus casei ATCC® 393 were used as quality control strains.

Statistical analysis

Statistical Package for the Social Sciences (SPSS, v.21, IBM Corporation, Armonk, NY, USA) software was used to analyze the data. Descriptive statistics were used to summarize the data, including standard deviation, median (minimum-maximum), interquartile range (IQR) for quantitative variables, and number of patients (percentage) for qualitative variables. The Mann-Whitney U test was used to compare two categories of a qualitative variable based on a quantitative variable, as the data did not meet the normal distribution assumptions. Fisher's exact tests were used to examine the relationship between two categorical variables. The Wilcoxon signed-rank test was used to compare two related quantitative variables due to non-normal distribution. The significance level for statistics was set at 0.05.

Results

Study population and Demographic backround

24 patients were divided into two groups based on storaged condition. The average age was 21.30±1.67 years, with 17 females and 7 males (Table 1). This study was completed with 24 participants initially included. No one was excluded from the study.

Clinical outcomes

Table 2 shows the variance between the initial and one-month measurements of the index outcomes for the miswak toothbrush stored in both the bathroom and the room. The periodontal indexes were utilized to evaluate how the storage conditions of miswak toothbrushes affect periodontal disease. The current study evaluated the effectiveness of miswak toothbrushes under various storage conditions by measuring plaque, gingival index, buccal-lingual pocket depths, and bleeding on probing while regarding time-dependent changes. At the beginning of the study, the IQR values for plaque, gingival index, buccal pocket depth, lingual pocket depth, and bleeding on probing were recorded as 0.69, 1.00, 0.69, 0.67 and 1.92 in the bathroom environment respectively. The measurements made in the room were recorded as 0.42, 1.02, 0.63, 0.69 and 3.50 respectively. During the first month's measurements, the mean values for plaque, gingival indexes, buccal and lingual pocket depth, and bleeding on probing were recorded as 0.71, 0.32, 0.63, 0.74 and 0.03 in the bathroom environment respectively. The measurements made in the room were recorded as 0.39, 0.79, 0.65, 0.66 and 2.56 respectively. No significant difference in miswak toothbrush all periodontal parameter values between baseline and first-month measurements (p>0.05).

Table 1. Descriptive statistics for microbiology results.

Variables		
Storage Condition, n (%)	Bathroom	12 (50.0)
	Room	12 (50.0)
Age	Mean±SD	21.30±1.67
	Median (Min-Max)	21 (20.00-23.00)
Gender, n (%)	Female	17 (70.8)
	Male	7 (29.2)
Lactobacillus spp. (x10^2)	Mean±SD	2.43±1.71
	Median (Min-Max)	2.00 (1.00-6.00)
Lactobacillus spp., n (%)	No	17 (70.8)
	Yes	7 (29.2)
Streptococcus Mutans (x10^2)	Mean±SD	4.05±1.64
	Median (Min-Max)	4.00 (1.30-7.00)
Streptococcus Mutans, n (%)	No	4 (16.7)
	Yes	20 (83.3)
Candida Albicans (x10^2)	Mean±SD	3.18±1.47
	Median (Min-Max)	2.70 (1.70-5.00)
Candida Albicans, n (%)	No	18 (75.0)
	Yes	6 (25.0)

SD:Standard Deviation, Min:Minimum, Max:Maximum.

Table 3 evaluated the effectiveness of miswak toothbrushes under various storage conditions by measuring plaque, gingival index, buccal-lingual pocket depths, and bleeding on probing while regarding storage conditions during the research duration. At the beginning of the study, the IQR values for plaque, gingival index, buccal pocket depth, lingual pocket depth, and bleeding on probing were recorded as 0.01, 0.03, 0.01, 0.01 and -0.01 in the bathroom environment respectively. The measurements made in the room were recorded as 0.02, 0.04, -0.04, 0.02, and 0.74 respectively. No significant difference in miswak toothbrush all periodontal parameter values between baseline and first-month measurements (p>0.05).

Lactobacillus spp. was found in 29.2% of toothbrushes, with an average count of 2.43±1.71x10^2 CFU/ml. *Streptococcus mutans* were found in 88.3% of toothbrushes, with an average count of 4.05±1.64x10^2 CFU/ml. Of the toothbrushes tested, 25.0% had *Candida albicans* present with an average count of 3.18±1.47x10^2 CFU/ml.

It was found that 91.70% of Miswak toothbrushes stored in the bathroom contain *S. mutans*. Additionally, *Lactobacillus* species was present in 33.30% of Miswak toothbrushes. It has been observed that 25% of Miswak toothbrushes stored in the room contain *Lactobacillus* species. Additionally, *S. mutans* were detected in 75% of Miswak toothbrushes, and 25% contained *C. albicans* in the room storage condition. The study investigated the correlation between storage conditions and microbiology results of the miswak toothbrush. However, no significant difference was observed in the data between the toothbrush stored in the bathroom and the one stored in the room (Table 4; p>0.05).

Discussion

It's crucial to maintain good oral hygiene to ensure overall health and a better quality of life. The most fundamental technique to eliminate dental plaque is through tooth brushing [4]. Mechanical or chemical plaque removal methods control dental plaque and related oral bacteria [3]. Toothbrush bristles can get contaminated by oral microflora, storage conditions, or a combination [28]. It was recommended to renew toothbrush every 2-3 months to prevent or reduce contamination of the bristles [6,31].

The aim of the study was to assess the level of microorganisms present on Miswak that were stored in a humid bathroom environment and a dry room environment for individuals aged 20-23 years. This is the first in vivo study assessing a miswak toothbrush's antibacterial properties with storage condition. It was found that varying levels of bacterial contamination in miswak, depending on their storage conditions. The null hypothesis that the antimicrobial potential of toothbrushes containing miswak stored under different conditions is equal has been rejected. The miswak toothbrushes kept in humid conditions displayed significantly higher contamination levels.

After the first use of a toothbrush, the bristles become susceptible to microbial contamination, and the level of contamination increases with each following use [8]. Mehta et al. stated that 70% of toothbrushes were discovered to be highly contaminated with microorganisms after their first

Table 2. Comparisons of periodontal index results for storage conditions of miswak.

Variables	Storage Condition	Time				P value
		Initial		First month		
		Median (IQR)	95% CI for Median	Median (IQR)	95% CI for Median	
Plaque Index	Bathroom	0.69 (0.51)	(0.23-1.10)	0.71 (0.24)	(0.21-0.91)	0.528[a]
	Room	0.42 (0.53)	(0.12-0.88)	0.39 (0.35)	(0.17-0.62)	0.196[a]
Gingival Index	Bathroom	1.00 (0.80)	(0.05-1.20)	0.32 (0.91)	(0.05-1.09)	0.083[a]
	Room	1.02 (0.27)	(0.52-1.17)	0.79 (1.00)	(0.07-1.21)	0.398[a]
Buccal Pocket Depth	Bathroom	0.69 (0.15)	(0.59-0.86)	0.63 (0.18)	(0.58-0.85)	0.952[a]
	Room	0.63 (0.34)	(0.58-0.94)	0.65 (0.35)	(0.55-1.02)	0.055[a]
Lingual Pocket Depth	Bathroom	0.67 (0.10)	(0.61-0.74)	0.74 (0.14)	(0.58-0.79)	0.343[a]
	Room	0.69 (0.22)	(0.60-1.08)	0.66 (0.40)	(0.55-1.03)	0.350[a]
Bleeding on Probing	Bathroom	1.92 (5.29)	(0.01-5.35)	0.03 (2.75)	(0.01-5.55)	0.720[a]
	Room	3.50 (5.92)	(1.78-10.71)	2.56 (10.58)	(0.03-11.10)	0.594[a]

IQR: Interquartile Range, CI: Confidence Interval, [a]: Wilcoxon Signed Rank test.

Table 3. Comparisons of periodontal index difference (Initial-First Month) between storage conditions of miswak toothbrush.

Variables	Storage Condition				P value
	Bathroom		Room		
	Median (IQR)	95% CI for Median	Median (IQR)	95% CI for Median	
Plaque Index	0.01 (0.25)	(0.01-0.40)	0.02 (0.16)	(0.01-0.71)	0.652[a]
Gingival Index	0.03 (0.85)	(0.01-1.06)	0.04 (0.71)	(0.04-1.03)	0.519[a]
Buccal Pocket Depth	0.01 (0.03)	(0.01-0.11)	-0.04 (0.10)	(0.02-0.07)	0.151[a]
Lingual Pocket Depth	0.01 (0.09)	(0.01-0.13)	0.02 (0.10)	(0.01-0.14)	0.151[a]
Bleeding on Probing	-0.01 (1.95)	(0.01-10.67)	0.74 (3.36)	(0.74-3.90)	1.000[a]

SD:Standard Deviation, Min:Minimum, Max:Maximum, [a]: Mann-Whitney U test.

Table 4. Comparisons of microbiology results for toothbrush stored in the bathroom and the room.

Variables		Storage Condition		P value
		Bathroom	Room	
Lactobacillus spp., n(%)	No	8 (66.7)	9 (75.0)	1.000[a]
	Yes	4 (33.3)	3 (25.0)	
Streptococcus Mutans, n (%)	No	1 (8.3)	3 (25.0)	0.590[a]
	Yes	11 (91.7)	9 (75.0)	
Candida Albicans, n (%)	No	9 (75.0)	9 (75.0)	1.000[a]
	Yes	3 (25.0)	3 (25.0)	

SD:Standard Deviation, Min:Minimum, Max:Maximum, [a]: Mann-Whitney U test.

use [32]. Toothbrushes showed significant bacterial contamination after use, except when an oral antiseptic, like mouthwash, was used before brushing [3,33]. The study found that the miswak toothbrush type used for a month was contaminated with *S. mutans*, *Lactobacillus species*, and *C. albicans*.

It is possible for toothbrushes to get infected through contact with other sources. Studies have shown that microorganisms can thrive in closed containers or toothbrushes kept in humid environments for extended periods of time [34]. Microorganisms can survive for more than 24 hours, especially in the presence of humidity [9,33].

Toothbrushes stored in humid conditions and covered with plastic have been found to promote the survival of bacteria [3]. Storing toothbrushes in plastic covers can increase bacterial survival rates, but allowing them to dry for up to 48 hours reduces the biofilm and bacterial survival rate [30,33]. Storing toothbrushes in plastic covers without letting them dry led to higher bacterial survival rates. It has been stated that using plastic covers to store toothbrushes can create an environment for opportunistic bacteria, increasing the risk of reinfection in the oral cavity [32]. After one month of use, 85% of toothbrushes stored in bathrooms with toilets showed heavy bacterial contamination, according to a study on toothbrush storage [35]. Studies show that bacteria from toilets are often found in bathrooms where toothbrushes are stored [36,37].

In a study conducted by Rifaey et al. that lasted four weeks, participants were separated into two groups. The first group was advised to use a standard toothbrush and miswak for the initial two weeks and to only use a toothbrush for the following two weeks. The participants in the second group were given the task of using a regular toothbrush for the initial two weeks and then using both

a regular toothbrush and a miswak for the following two weeks. The researchers then analyzed the colony counts of *S. mutans* between the two groups. The study revealed no significant variation in the number of *S. mutans* colonies between the two groups [38].

A recent study compared the effectiveness of neem, miswak, mango, and banyan chewing sticks in fighting against *S. mutans* and *Lactobacillus species*. These microorganisms are known to play a crucial role in developing and advancing dental caries. The study revealed that higher concentrations of neem, miswak, and mango extracts increased antimicrobial activity. Among these extracts, neem's aqueous extract exhibited the highest antimicrobial activity against *S. mutans* [39].

The miswak extract displayed superior antimicrobial activity against *Lactobacillus acidophilus* [40]. A previous study collected saliva samples from 56 individuals using 30 miswak or 26 standard toothbrushes. The study measured the bacterial species present in their saliva. The study revealed that miswak could have a specific inhibitory impact on the concentration of oral *Streptococcus* species in saliva [41]. It was stated that the effectiveness of the antimicrobial activity of neem and miswak chew sticks and aqueous extracts was compared. Both chewing stick extracts were effective at 50% concentration on *S. mutans* and *Enterococcus faecalis* [39]. Miswak sticks have potential therapeutic effects against *Candida species*, especially *C. albicans* in vitro [42].

In this current study, when compared according to the microorganism, CFU numbers in the toothbrush with miswak, *S. mutans*, *C. albicans,* and *Lactobacillus* species, and numbers were determined in the miswak. It was discovered that 83.3% of the miswak toothbrushes contained *S. mutans*, while 29.2% had *Lactobacillus spp.* and 25% had *C. albicans*. It has been found that even when stored at room temperature with low humidity, miswak toothbrushes have higher bacterial contamination and survival rates. It is possible that the Miswak toothbrush's densely packed bristles prevent proper drying, increasing the likelihood of bacterial growth. It's possible that the low antibacterial effectiveness of miswak toothbrushes contributed to this situation.

This study on the effects of toothbrush types stored under different conditions used these indexes to evaluate the periodontal status. The study examined the effects of various storage conditions on the performance of miswak toothbrushes by evaluating periodontal indexes such as BOP, PI, GI, and PD No significant differences in toothbrushes and storage conditions were found for BOP, PI, GI, and SD similar to previous studies [28].

Based on our research findings, it appears that the patients involved in this study were unable to maintain adequate oral hygiene due to restriction in the effectiveness of the miswak toothbrush in preventing bacterial growth, as well as challenges related to its rigid bristles structure and application techniques.

Limitations

Achieving optimal dental hygiene through brushing requires consideration of multiple factors, such as brushing technique, frequency of brushing, duration of brushing, toothbrush bristle type, toothbrush design, and individual brushing ability. To mitigate this limitation, it was regarded that the participants selected for the study had received professional dental cleaning and did not have untreated cavities before inclusion.

The significance of oral hygiene differs among individuals due to factors like their oral microflora, diet, and socioeconomic status [43]. The success of maintaining oral hygiene through brushing is entirely up to the individual, as it is a personal responsibility that cannot be enforced externally [44]. The possibility exists for effectiveness of individuals' brushing to impact the achieved consequences. However, all study participants were selected as 4th-year dental students to overcome this limitation. They have advanced manual dexterity. Since the researchers were in the same place as all participants, they were advised not to neglect regular tooth brushing by miswak toothbrush. Despite participants frequently reporting complaints about the difficulty in use and pain caused by the hardness of the miswak toothbrush, it continued to be used.

One limitation of this study is that storage conditions for toothbrushes, including room temperature, bathroom humidity, and temperature, were not standardized. Limitations have arisen due to conditions such as the presence of a toilet in the bathroom and the necessity of closing toilet seats before flushing. In current studies, no mention is made of room temperature, bathroom humidity, or temperature levels [3]. In these studies, it is impossible to achieve standardization by equalizing the bath temperature. There is a need for future studies on this subject that will consider bathroom humidity and temperature and room temperature.

This study did not consider the presence or absence of a toilet in the bathroom storage environment. Vitek Compact® can identify *S. mutans*, *Lactobacillus spp.*, and *Candida Albicans*, but is not suitable for identifying intestinal, colonic, or fecal species. It would be impossible to detect contamination through direct or indirect contact with aerosols, regardless of the presence of a bathroom. In future studies, research on the storage conditions of miswak toothbrushes should consider the bathroom environment.

This study evaluated the microbiological potential of miswak toothbrushes and investigated the most suitable storage environment for them. The Vitek® identification device was calibrated for the specific bacterial species under investigation. Bacterial contamination can occur when flushing the toilet with the lid open. Participants were regularly cautioned about this issue. However, investigating possible contamination falls outside the scope of this study. The main research question of this study is where to store the miswak toothbrush. To minimize the impact of the study's limitations, researchers selected participants from dentistry students despite the difficulty of standardization.

Conclusion

Regardless of storage conditions, 83.3% of all miswak toothbrushes became bacterial reservoirs. Additionally, we observed permanent contamination of the toothbrushes

within just one month of use. After analyzing the results of this study, the miswak toothbrushes should be stored in good ventilation, allowing them to air dry without a plastic cover. The miswak toothbrush can act as a bacterial reservoir, even under ideal storage conditions. The results of this research confirmed the situation. Further in-depth studies are necessary to examine their potential as antibacterial agents thoroughly. It has been observed that storing miswak in the bathroom, which is generally considered a humid environment, leads to a higher level of microbial contamination compared to keeping them in a dry room. In other words, miswak should be storaged in an environment where it can dry in order to reduce the risk of recontamination, after independent use, until it is used again. Therefore, additional research is required to validate the findings of this study.

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Ethical approval

The study has been approved by the Afyonkarahisar Human Research Ethic Committee with the number 2020/13.

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