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ORIGINAL RESEARCH

Oral Hygiene Habits and Toothbrush Contamination with *Enterococcus faecalis* and *Staphylococcus aureus* in Dental Students: Epidemiological and Molecular Insights

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Introduction: The contamination of toothbrushes with pathogenic microorganisms poses a risk to both oral and systemic health. This study analyzed the presence of *Enterococcus faecalis* and *Staphylococcus aureus* in toothbrushes used by dentistry students and its relationship with their oral hygiene habits.

Materials and Methods: Microbiological culture and polymerase chain reaction (PCR) were used to detect *Enterococcus faecalis* and *Staphylococcus aureus* in toothbrush samples. Resistance (*blaZ, mecA, vanA*) and virulence genes (*tst, lukS/lukF-PV, hla, hlb, hld, sea, seb, sed, sec*) were screened in the *S. aureus* isolates.

Results: Higher contamination was descriptively observed among toothbrushes stored in bathrooms and those used for ≥1 month. A total of 73 toothbrushes were analyzed; *E. faecalis* was detected in 9/73 (12.3%) and *S. aureus* in 4/73 (5.5%). Among the 4 *S. aureus* isolates, *blaZ* was detected in 4/4 (100%), *mecA* in 1/4 (25%), and *vanA* in 0/4 (0%). These findings reinforce the need to improve toothbrush hygiene and storage practices to reduce the risk of pathogenic transmission. The presence of a MRSA strain calls for the implementation of effective prevention and disinfection strategies, especially in dental and community settings.

Keywords: toothbrushing, *Enterococcus faecalis*, *Staphylococcus aureus*, MRSA

Introduction

Dental care and daily oral hygiene practices are essential for preventing oral diseases and maintaining overall health.[1] Toothbrushing is one of the most widely used methods, with the toothbrush serving as the primary tool for the mechanical removal of dental plaque.[1] However, toothbrushes can become contaminated shortly after their first use and may act as vehicles for microbial dissemination in the oral cavity, potentially increasing the risk of infection, particularly in immunocompromised individuals.[2] Improper storage conditions further promote bacterial survival and cross-contamination, while prolonged use favors colonization by diverse microorganisms, including Gram-positive species of clinical relevance.[3]

Beyond microorganisms originating from the oral cavity, storage conditions and duration of use are key factors that promote the introduction and proliferation of pathogens. Storing toothbrushes in shared containers inside bathrooms has been associated with increased microbiological contamination. The warm and humid bathroom environment facilitates bacterial growth and cross-contamination, particularly through aerosols generated by toilet flushing, contact with contaminated hands,

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and exposure to skin microorganisms. Consequently, toothbrushes may act as reservoirs of pathogenic microorganisms, highlighting the importance of appropriate oral hygiene and toothbrush storage practices to minimize the risk of microbial transmission.[4–7] Regional studies in dental settings remain limited, underscoring the need for further investigation into the persistence and behavior of these microorganisms in locally used oral hygiene tools.

Staphylococcus aureus (*S. aureus*) is a Gram-positive coccus frequently found in the environment and on humans, colonizing areas such as the nasal cavities and skin. It can cause a wide spectrum of infections, ranging from localized lesions to life-threatening systemic conditions such as bacteremia, infectious endocarditis, pneumonia, and meningitis.[8,9] In dentistry, *S. aureus* has been isolated from toothbrushes, suggesting that these items may act as potential sources of exposure due to the bacterium's ability to form biofilms and adhere to surfaces, increasing the risk of transmission and reinfection, especially among vulnerable populations.[5,8–11]

Although *S. aureus* possesses numerous virulence and resistance determinants, key genetic markers such as *mecA, vanA, lukS/lukF-PV* and *tst* are of particular concern because of their association with methicillin and vancomycin resistance and their involvement in severe toxin-mediated diseases.[11,12]

Enterococcus faecalis (*E. faecalis*), another Gram-positive coccus, naturally inhabits the gastrointestinal tract and belongs to enterococci, which are commonly used as indicators of fecal contamination. It is capable of causing urinary tract infections, wound infections, and endocarditis. Many enterococcal strains exhibit intrinsic resistance to several clinically relevant antibiotics, including cephalosporins, penicillinase-resistant penicillins, clindamycin, and aminoglycosides, as well as tolerance to cell-wall–active agents such as ampicillin and vancomycin.[13–15] Microorganisms found on toothbrushes, including *E. faecalis*, may contribute to oral infections such as marginal periodontitis and endodontic complications, compromising the success of root canal treatments.[16,17]

The presence of *S. aureus* and *E. faecalis* on toothbrushes poses a potential risk to oral and systemic health, particularly under humid conditions that favor their survival. Recommendations from the Centers for Disease Control and Prevention (CDC) advise against sharing toothbrushes, recommend rinsing them thoroughly after use, avoiding enclosed storage, and replacing them every three to four months.

The aim of the present study was to evaluate the relationship between oral hygiene habits and toothbrush contamination by *S. aureus* and *E. faecalis*, and to characterize resistance and virulence genes to inform infection-prevention strategies and strengthen oral hygiene practices.

Materials and Methods

Study Design and Period

This was a cross-sectional, laboratory-based study conducted during the 2022–2023 academic period.

Study Population

The study population consisted of toothbrushes used by senior-year dentistry students from the city of Cuenca, Ecuador.

Sample Size and Sampling Procedure

A non-probabilistic convenience sampling method was used. A total of 73 toothbrushes were analyzed, corresponding to the number of eligible and consenting participants available during the study period.

The sample size was determined by the number of eligible and consenting students available during the study period, which is a common limitation in school-based observational studies.

Toothbrushes were included if they belonged to senior-year dentistry students from a university in Cuenca, were used for at least 1 day prior to collection, and the students were of legal age (≥18 years) and had signed informed consent.

Toothbrushes were excluded if the corresponding student refused to participate, provided incomplete survey data, was absent at the time of sample collection, was under 18 years of age, or was enrolled in another academic program.

Short-term toothbrush use (<15 days) was intentionally included to capture early bacterial contamination, as previous studies have shown that microbial colonization may occur shortly after initial use.

Study Procedure

All senior-year dentistry students were invited to an informational meeting, during which the study objectives, inclusion and exclusion criteria, and sample collection procedures were explained. Participants were informed that their used toothbrush would be collected and replaced with a new one.

Each participant signed an informed consent form and completed a structured questionnaire using Google Forms. The survey, adapted from Medina et al, was designed to collect demographic information and oral hygiene habits relevant to potential microbial contamination of toothbrushes.

Participants were instructed to place their toothbrush in an individually labeled sterile bag. Each sample was assigned a unique identification code and transported in a refrigerated container to the Molecular Biology and Genetics Laboratory of the CIITT at the Catholic University of Cuenca for analysis. Given the exploratory nature of this study and the limited number of eligible participants during the academic period, a convenience sampling approach was used.

Laboratory Processing of Toothbrushes

In a biosafety cabinet, the toothbrush handles were removed, and the heads were placed into sterile conical tubes containing 20 mL of tryptic soy broth. The samples were incubated at 37 °C for 24 hours.

Isolation and Identification of *Staphylococcus aureus* and *Enterococcus faecalis*

Staphylococcus aureus

After incubation, aliquots were streaked onto Mannitol Salt Agar and incubated at 37 °C for 24–48 hours. Presumptive identification of *S. aureus* was based on mannitol fermentation, colony morphology, Gram staining, and DNase and coagulase tests.

Molecular confirmation was performed by PCR detection of the *nucA* and *femB* genes, following the protocol described by Hamdan et al.[18]

Automated identification systems such as Vitek were not used due to limited availability of equipment; therefore, identification was performed using conventional microbiological methods and PCR, which are considered reliable for species-level identification.

Detection of Resistance and Virulence Genes (*S. aureus*)

DNA Extraction

For DNA extraction from *S. aureus* strains, we used a lysis solution of 1% sodium dodecyl sulfate (SDS) in 0.24 N Sodium hydroxide (NaOH), and then the samples were boiled. Using a bacteriological loop, a portion of the colonies were suspended in 1mL of sterile distilled water in Eppendorf tubes, followed by centrifugation for 10 minutes at 3000 rpm, discarding the supernatant. Subsequently, 50 µL of the lysis solution was added, mixed using a vortex, and the tubes were placed in a dry block heater at 100 °C for 15 minutes. Lastly, 450 µL of nuclease-free water was added, and the samples were centrifuged for 20 seconds to obtain total DNA. The extracted DNA was stored at −20 °C.[12]

Polymerase Chain Reaction (PCR)

The PCR technique was used to identify resistance and virulence genes present in the isolated *S. aureus* strains. The genes analyzed were *blaZ, mecA, vanA, tst, lukS/lukF-PV, hla, hlb, hld, sea, seb, sed, sec*, following the protocols described by Tenezaca et al, Orellana et al, Laica et al, and Pacheco et al (Table 1).[11,12,19,20]

For each gene, a reaction mixture was prepared containing: 10 µL of Promega Green GoTaq 2X Mastermix, 5 µL of ultrapure water, 1.5 µL of each primer and 2 µL of DNA. The primers, amplification program, and ATCC strains (positive controls) for each gene are shown in Table 1. The reactions were carried out in an Agilent SureCycler 8800 thermal cycler.

The amplicons were separated by horizontal electrophoresis on a 1.5% (w/v) agarose gel (0.75 g of agarose in 50 mL of TAE 1X) with 2 µL of SYBR Safe DNA Gel Stain10,000x of Invitrogen incorporated into the TAE 1X buffer. Electrophoresis was carried out at 100 V for 45 minutes. The amplicon sizes were determined based on their migration in

Table 1 Primers, amplification products, reference strains and PCR amplification program used for the detection of virulence and resistance genes in *S. aureus*

Resistance genes *S. aureus*	Primers (5´-3´)	Amplicon (bp)	Control strains ATCC	PCR Conditions	Reference
blaZ	F: GTTGCGAACTCTTGAATAGG R: GGAGAATAAGCAACTATATCATC	674	11632	Initial denaturation: 94 °C for 5 min 34 cycles of: 94°C for 1 min 54°C for 1 min 72°C for 1 min Final extension: 72°C for 10 min	[19]
mecA	F: GTAGAAATGACTGAACGTCCGATGA R: CCAATTCCACATTGTTTCGGTCTAA	310	43300	Initial denaturation: 94 °C for 5 min 30 cycles of: 94°C for 1 min 62°C for 30s 72°C for 35s Final extension: 72°C for 10 min.	
vanA	F: GGGAAAACGACAATTGC R: GTACAATGCGGCCGTTA	732	70021	Initial denaturation: 94 °C for 2 min 30 cycles of: 94°C for 1 min 54°C for 1 min 72°C for 1 min Final extension: 72°C for 10 min	
Virulence genes *S. aureus*	**Primers (5´-3´)**	**Amplicon (bp)**	**Control strains ATCC**	**PCR Conditions**	**Reference**
tst	F: TTCACTATTTGTAAAAGTGTCAGACCCACT R: TACTAATGAATTTTTTTATCGTAAGCCCTT	180	43300	Initial denaturation: 94°C for 5 min 30 cycles of: 94°C for 30s. 55°C for 1 min 72°C for 1 min Final extension: 72°C for 10 min	[11,12,20]
lukS/lukF-PV	F: ATCATTAGGTAAAATGTCTGGACATGATCCA R: GCATCAASTGTATTGGATAGCAAAAGC	433	25923		
hla	F: CTGATTACTATCCAAGAAATTCGATTG R: CTTTCCAGCCTACTTTTTTATCAGT	209			
hlb	F: GTGCACTTACTGACAATAGTGC R: GTTGATGAGTAGCTACCTTCAGT	309	25923		
hld	F: AAGAATTTTTATCTTAATTAAGGAAGGAGTG R: TTAGTGAATTTGTTCACTGTGTCGA	111			
sea	F: GAAAAAAGTCTGAATTGCAGGGAACA R: CAAATAAATCGTAATTAACCGAAGGTTC	560	33592		
seb	F: ATTCTATTAAGGACACTAAGTTAGGGA R: ATCCCGTTTCATAAGGCGAGT	404	11632		
sec	F: GTAAAGTTACAGGTGGCAAAACTTG R: CATATCATACCAAAAAGTATTGCCGT	297	43300		
sed	F: GAATTAAGTAGTACCGCGCTAAATAATATG R: GCTGTATTTTTCCTCCGAGAGT	492	Laboratory isolate		

the agarose gels, compared with the migration of DNA bands from the molecular weight marker 1 Kb Plus DNA Ladder (TrackIt, Invitrogen) under UV transillumination. Photos were taken with a digital camera.[21,22]

Isolation and Molecular Identification of *E. faecalis*

After incubation in tryptic soy broth, samples were streaked onto CHROMagar Orientation and incubated at 37 °C for 24 hours. Presumptive identification of *E. faecalis* was based on turquoise-blue colony coloration. Molecular identification was confirmed by PCR following DNA extraction using the alkaline lysis method. PCR conditions, primers, and positive control strains are detailed in Table 2. The reactions were carried out in an Agilent SureCycler 8800.

The amplicons were separated by horizontal electrophoresis on a 1.5% (w/v) agarose gel (0.75 g of agarose in 50 mL of 1X TAE) with 2 µL of SYBR Safe DNA Gel Stain 10,000x (Invitrogen) submerged in 1X TAE buffer. Electrophoresis

Table 2 Primers Used for Identification of *Enterococcus faecalis*

Gen *E. faecalis*	Primers	Amplicon (pb)	Control Strains ATCC	PCR Conditions	Reference
sodA	NFL1- F: ACTTATGTGACTAACTTAACC FL2 - R: TAATGGTGAATCTTGGTTTGG	360	19433	Initial denaturation: 95°C for 4 min 30 cycles of: 95°C for 30s 55°C for 1 min 72°C for 1 min Final extension: 72°C for 7 min.	[23]

was carried out at 100 V for 45 minutes. The size of the amplicons was determined according to their migration in the agarose gels, compared with the migration of DNA bands from the molecular weight marker 1 Kb Plus DNA Ladder under UV transillumination. Photos were taken with a digital camera.[23]

Data Collection

Survey responses were collected through Google Forms and exported to Microsoft Excel. Laboratory results were incorporated into the same database for subsequent statistical analysis using SPSS version 26.

Data Analysis

Categorical variables were summarized as frequencies and percentages. Continuous variables were assessed for normality and described as mean ± standard deviation when normally distributed, or as median and interquartile range (IQR) when non-normally distributed. Participants were classified according to the presence of *S. aureus* and *E. faecalis*. Associations between microbial presence and oral hygiene habits were evaluated using Fisher's exact test due to sparse data. Student's *t*-test was used to compare means between two groups, and the Wilcoxon rank-sum test was used to compare medians. The prevalence of resistance genes (*blaZ, mecA, vanA*) and virulence genes (*tst*, *lukS/lukF-PV, hla, hlb, hld, sea, seb, sed, sec*) was calculated.

Potential confounding factors such as prior antibiotic use and individual hygiene practices were not controlled for and are acknowledged as limitations of the study.

Ethical Considerations

The study was reviewed and approved by the Comité de Ética de Investigación en Seres Humanos de la Universidad Católica de Cuenca, under code UCACUE-UASB-O-CEISH-2022-104, dated May 5, 2023. Compliance with the ethical principles established in the Declaration of Helsinki was ensured. All participants were informed about the objectives of the study and signed an informed consent form. Confidentiality and anonymity of the collected data were guaranteed; participation was voluntary, with no coercion or risk to those involved.

Results

Study Population and Data Collection

Toothbrushes were collected from undergraduate students enrolled in the final semester of dentistry who voluntarily agreed to participate in the study and met the established inclusion and exclusion criteria. A total of 73 toothbrushes were obtained and constituted the final sample analyzed. In parallel, a structured survey was distributed to participants using Google Forms to collect sociodemographic data and information related to oral hygiene practices and toothbrush storage habits. Completed survey responses were downloaded in Microsoft Excel format, where each participant was assigned a unique identification code. Microbiological and molecular laboratory results corresponding to each toothbrush sample were subsequently incorporated into the same database. The consolidated dataset was then imported into SPSS statistical software (version 26.0) for descriptive and comparative analyses, allowing the integration of survey-based variables with microbiological outcomes.

Survey Results and Demographic Characteristics

Based on the survey responses, most participants were between 20 and 23 years of age (75.3%), were predominantly female (69.9%), and reported residing in urban areas. These sociodemographic characteristics are summarized in Table 3 and illustrated in Figure 1.

With respect to oral hygiene practices, manual toothbrush use was reported by 98.6% of participants, whereas only a small proportion reported using electric toothbrushes. Toothbrush replacement intervals varied, with the most frequently reported replacement period being two months of use (28.8%), followed by longer periods, indicating prolonged toothbrush use among a considerable proportion of the study population.

Regarding toothbrush storage conditions, 89.0% of participants reported storing their toothbrushes inside the bathroom, most commonly on the sink (58.9%). In addition, 80.8% of participants indicated that they did not use any type of protective cover. Oral hygiene practices and toothbrush storage conditions are detailed in Table 3 and illustrated in Figure 2.

Bacterial Colonization of Toothbrushes

The presence of *E. faecalis* on toothbrushes is presented in Table 4. Overall, a colonization rate of 12.3% (9/73) was observed. Most *E. faecalis*–positive samples were observed in the 20–23-year group (6/9 positives). Although an association with age was detected ($p = 0.027$), this finding should be interpreted cautiously due to sparse counts, including a single participant in the ≥28-year stratum. For the remaining variables evaluated, including sex, area of residence, toothbrush use duration, type of toothbrush, and storage conditions, higher proportions of *E. faecalis* colonization were observed; however, these associations did not reach statistical significance.

Table 3 Sociodemographic Variables and Hygiene Habits

		Frequency	Percentage (%)
Sociodemographic			
Sex	Masculine	22	30.1
	Feminine	51	69.9
Age range	20-23 years	55	75.3
	24-27 years	17	23.3
	≥28 years	1	1.4
Area of residency	Urban	67	91.8
	Rural	6	8.2
Hygiene Habits			
Duration of toothbrush use	<15 days of use	3	4.1
	≥15 days of use	5	6.8
	1 month of use	14	19.2
	2 months of use	21	28.8
	3 months of use	19	26.0
	>3 months of use	11	15.1
Type of toothbrush	Manual toothbrush	72	98.6
	Electric toothbrush	1	1.4
Storage location	Inside restroom	65	89.0
	Outside restroom	8	11.0
Storage place	Drawer	26	35.6
	Sink	43	58.9
	Other	4	5.5
Toothbrush cover	Yes	14	19.2
	No	59	80.8

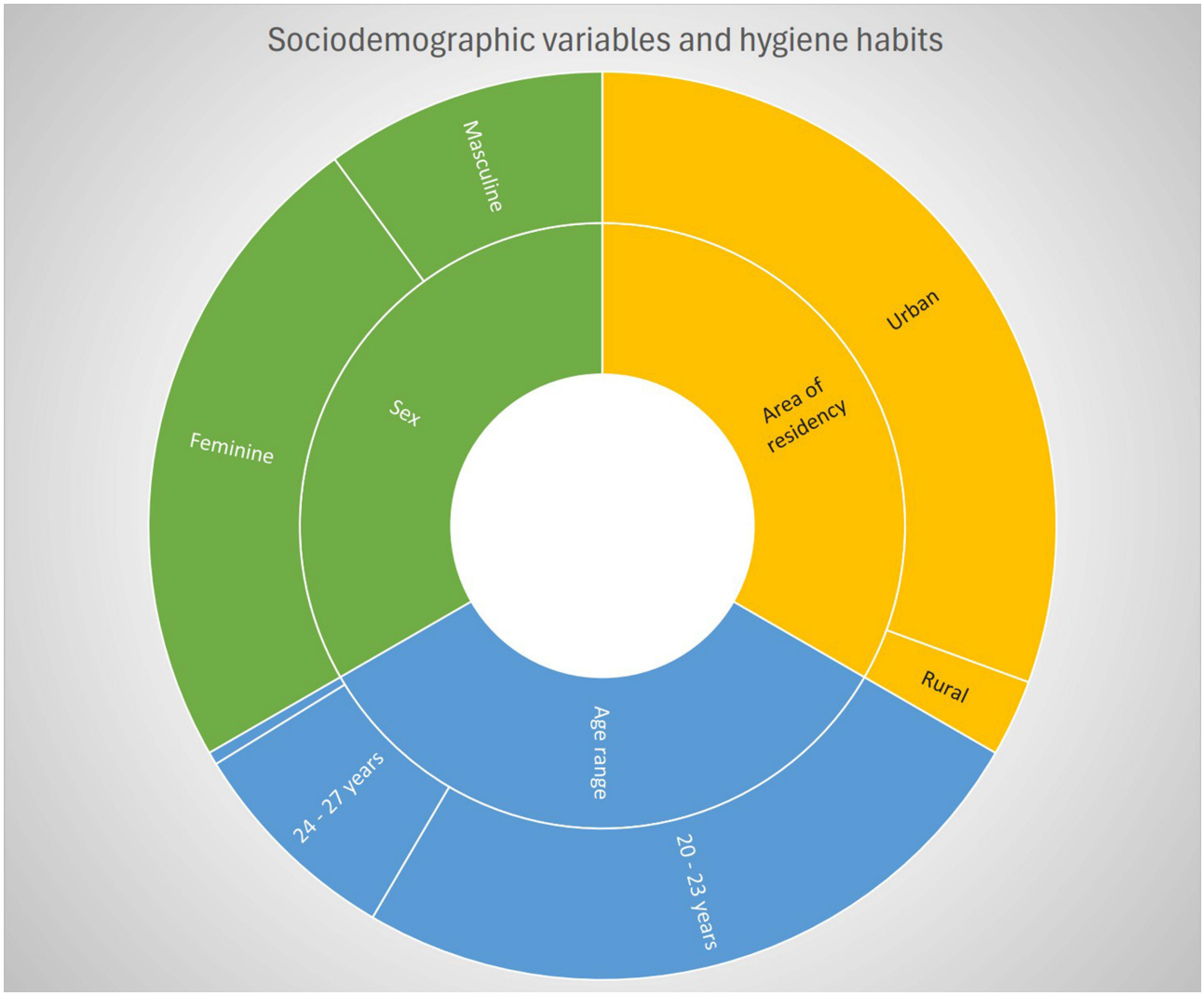

Figure 1 Sociodemographic variables and hygiene habits.

Table 5 summarizes the presence of *S. aureus* on toothbrushes, with an overall colonization rate of 5.5% (4/73). All toothbrushes positive for *S. aureus* belonged to participants residing in urban areas who stored their toothbrushes inside the bathroom. No statistically significant associations were identified between *S. aureus* colonization and the evaluated sociodemographic or oral hygiene variables.

Resistance and Virulence Genes

The distribution of resistance and virulence genes detected in *S. aureus* isolates recovered from toothbrushes is shown in Table 6. All isolates harbored the *blaZ* gene (100%), whereas only one isolate (25%) carried the *mecA* gene. The *vanA* gene was not detected in any of the isolates analyzed.

Regarding virulence-associated genes, *hla* and *hld* were detected in all isolates (100%), *tst* was present in 75% of isolates, and *hlb* was detected in 50%. Enterotoxin genes (*seb, sed*, and *sec*) were detected at lower frequencies, while *sea* and *lukS/lukF-PV* were not identified.

Table 7 Details the resistance and virulence gene profiles of individual *S. aureus* strains. One methicillin-resistant *S. aureus* strain (RAAR61) carrying the *mecA* gene was identified. This isolate harbored multiple virulence-associated genes, including *tst, hla, hld, seb*, and *sec*. The RAAR61 strain corresponded to a female participant aged 22 years, residing in an urban area, who reported using a manual toothbrush for two months and storing it in a bathroom drawer.

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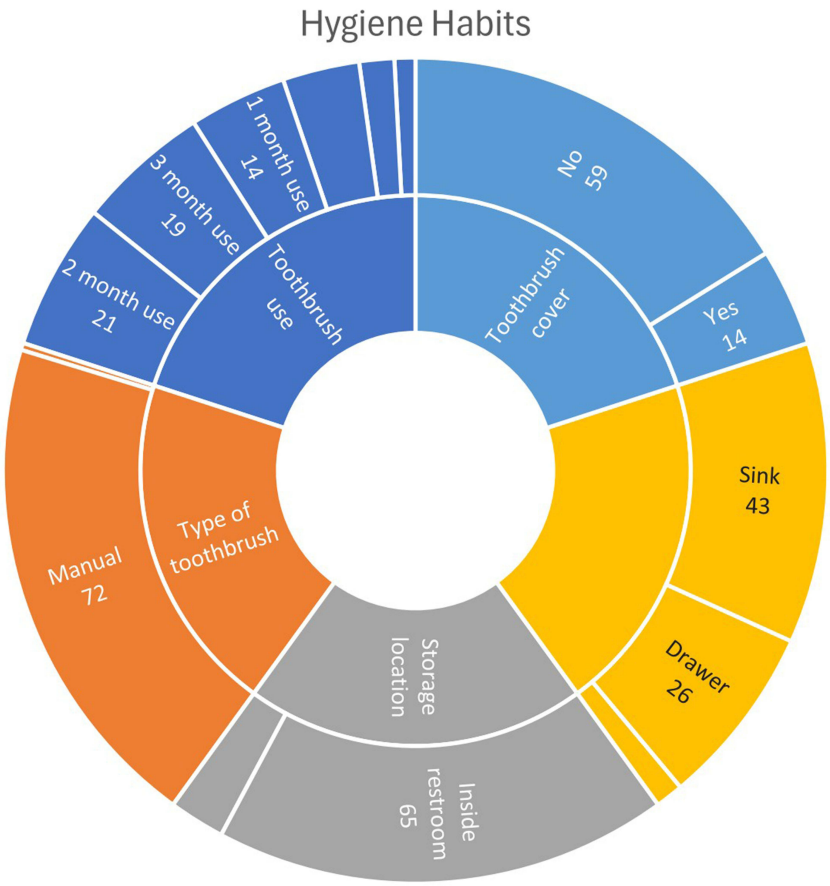

Figure 2 Hygiene Habits.

Figures 3–8 Illustrate the electrophoretic profiles of resistance and virulence genes detected in *S. aureus* isolates, while Figure 9 shows the electrophoretic profiles of amplicons corresponding to *E. faecalis* isolates recovered from toothbrushes.

Novelty of the Results

The novelty of the present study lies in the integrated analysis of sociodemographic characteristics, oral hygiene practices, and microbiological and molecular findings. Unlike previous studies that primarily focused on bacterial

Table 4 Presence of *Enterococcus faecalis* in Toothbrushes According to Participants' Sociodemographic Characteristics and Hygiene Habits

		Negative n (%)	Positive n (%)	Total n (%)	p-value
Sociodemographic characteristics of participants					
Sex	Masculine	19 (26.0%)	3 (4.1%)	22 (30.1%)	
	Feminine	45 (61.6%)	6 (8.2%)	51 (69.9%)	
Total		64 (87.7%)	9 (12.3%)	73 (100.0%)	0.823
Age range	20–23 years	49 (67.1%)	6 (8.2%)	55 (75.3%)	
	24–27 years	15 (20.5%)	2 (2.7%)	17 (23.2%)	
	≥28 years	0 (0.0%)	1 (1.4%)	1 (1.4%)	
Total		64 (87.7%)	9 (12.3%)	73 (100.0%)	0.027

(*Continued*)

Table 4 (Continued).

		Negative n (%)	Positive n (%)	Total n (%)	p-value
Area of residency	Urban	58 (79.5%)	9 (12.3%)	67 (91.8%)	
	Rural	6 (8.2%)	0 (0.0%)	6 (8.2%)	
Total		64 (87.7%)	9 (12.3%)	73 (100.0%)	0.338
Participants' hygiene habits					
Duration of toothbrush use	<15 days of use	3 (4.1%)	0 (0.0%)	3 (4.1%)	
	≥15 days of use	5 (6.8%)	0 (0.0%)	5 (6.8%)	
	1 month use	12 (16.4%)	2 (2.7%)	14 (19.1%)	
	2 months of use	19 (26.0%)	2 (2.7%)	21 (28.7%)	
	3 months of use	16 (21.9%)	3 (4.1%)	19 (26.0%)	
	> 3 months of use	9 (12.3%)	2 (2.7%)	11 (15.1%)	
Total		64 (87.7%)	9 (12.3%)	73 (100.0%)	0.865
Type of toothbrush	Manual toothbrush	63 (86.3%)	9 (12.3%)	72 (98.6%)	
	Electric toothbrush	1 (1.4%)	0 (0.0%)	1 (1.4%)	
Total		64 (87.7%)	9 (12.3%)	73 (100.0%)	0.706
Storage location	Inside restroom	56 (76.7%)	9 (12.3%)	65 (89.0%)	
	Outside restroom	8 (11.0%)	0 (0.0%)	8 (11.0%)	
Total		64 (87.7%)	9 (12.3%)	73 (100.0%)	0.261
Storage place	Drawer	24 (32.9%)	2 (2.7%)	26 (35.6%)	
	Sink	37 (50.7%)	6 (8.2%)	43 (58.9%)	
	Other	3 (4.1%)	1 (1.4%)	4 (5.5%)	
Total		64 (87.7%)	9 (12.3%)	73 (100.0%)	0.544
Toothbrush cover	Yes	13 (17.8%)	1 (1.4%)	14 (19.2%)	
	No	51 (69.9%)	8 (11.0%)	59 (80.9%)	
Total		64 (87.7%)	9 (12.3%)	73 (100.0%)	0.512

Notes: Data are presented as n (%). P-values were calculated using Fisher's exact test. Statistical significance was set at $p < 0.05$.

Table 5 Presence of *Staphylococcus aureus* in Toothbrushes According to Participants' Sociodemographic Characteristics and Hygiene Habits

		Negative n (%)	Positive n (%)	Total n (%)	p-value
Sex	Masculine	20 (27.4%)	2 (2.7%)	22 (30.1%)	
	Feminine	49 (67.1%)	2 (2.7%)	51 (69.9%)	
		69 (94.5%)	4 (5.5%)	73 (100.0%)	0.373
Age Range	20–23 years	53 (72.6%)	2 (2.7%)	55 (75.3%)	
	24–27 years	15 (20.5%)	2 (2.7%)	17 (23.3%)	
	≥28 years	1 (1.4%)	0 (0.0%)	1 (1.4%)	
Total		69 (94.5%)	4 (5.5%)	73 (100.0%)	0.424
Area of residency	Urban	63 (86.3%)	4 (5.5%)	67 (91.7%)	
	Rural	6 (8.2%)	0 (0.0%)	6 (8.2%)	
Total		69 (94.5%)	4 (5.5%)	73 (100.0%)	0.538

(*Continued*)

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Table 5 (Continued).

		Negative n (%)	Positive n (%)	Total n (%)	p-value
Participants' hygiene habits					
Duration of toothbrush use	<15 days of use	3 (4.1%)	0 (0.0%)	3 (4.1%)	
	≥15 days of use	5 (6.8%)	0 (0.0%)	5 (6.8%)	
	1 month of use	14 (19.2%)	0 (0.0%)	14 (19.2%)	
	2 months of use	20 (27.4%)	1 (1.4%)	21 (28.8%)	
	3 months of use	17 (23.3%)	2 (2.7%)	19 (26.0%)	
	> 3 months of use	10 (13.7%)	1 (1.4%)	11 (15.1%)	
Total		69 (94.5%)	4 (5.5%)	73 (100.0%)	0.775
Type of toothbrush	Manual toothbrush	68 (93.1%)	4 (5.5%)	72 (98.6%)	
	Electric toothbrush	1 (1.4%)	0 (0.0%)	1 (1.4%)	
Total		69 (94.5%)	4 (5.5%)	73 (100.0%)	0.808
Storage location	Inside restroom	61 (83.6%)	4 (5.5%)	65 (89.0%)	
	Outside restroom	8 (11.0%)	0 (0.0%)	8 (11.0%)	
Total		69 (94.5%)	4 (5.5%)	73 (100.0%)	0.470
Storage place	Drawer	24 (32.8%)	2 (2.7%)	26 (35.5%)	
	Sink	41 (56.2%)	2 (2.7%)	43 (58.9%)	
	Other	4 (5.5%)	0 (0.0%)	4 (5.5%)	
Total		69 (94.5%)	4 (5.5%)	73 (100.0%)	0.765
Toothbrush cover	Yes	13 (17.8%)	1 (1.4%)	14 (19.1%)	
	No	56 (76.7%)	3 (4.1%)	59 (80.8%)	
Total		69 (94.5%)	4 (5.5%)	73 (100.0%)	0.761

Notes: Data are presented as n (%). P-values were calculated using Fisher's exact test. Statistical significance was set at $p < 0.05$.

Table 6 Frequency of Resistance and Virulence Genes Detected in *Staphylococcus aureus* Isolates Recovered from Toothbrushes

Category	*Gene*	Presence, n (%)
Resistance genes	*blaZ*	4/4 (100%)
	mecA	1/4 (25%)
	vanA	0/4 (0%)
Virulence genes	*tst*	3/4 (75%)
	lukS/lukF-PV	0/4 (0%)
	hla	4/4 (100%)
	hlb	2/4 (50%)
	hld	4/4 (100%)
	sea	0/4 (0%)
	seb	1/4 (25%)
	sed	1/4 (25%)
	sec	1/4 (25%)

Notes: Data are presented as n (%). Percentages were calculated based on the total number of *Staphylococcus aureus*–positive isolates (n = 4) recovered from 73 toothbrush samples analyzed.

Table 7 Resistance and Virulence Genes Detected in *Staphylococcus aureus* Isolates Recovered from Toothbrushes (n = 4)

		Strain RAAR61	Strain RAGC78	Strain EDVM87	Strain SNL987
Resistance genes *S. aureus*	*blaZ*	+	+	+	+
	mecA	+	–	–	–
	vanA	–	–	–	–
Virulence genes *S. aureus*	*tst*	+	+	–	+
	lukS/lukF-PV	–	–	–	–
	hla	+	+	+	+
	hlb	–	+	–	+
	hld	+	+	+	+
	sea	–	–	–	–
	seb	+	–	–	–
	sed	–	–	+	–
	sec	+	–	–	–

Notes: +: presence of the gene; -: absence of the gene.

contamination alone, this research incorporates behavioral factors alongside the detection of antimicrobial resistance and virulence genes in *S. aureus* isolated from toothbrushes of dental students. Notably, the identification of a methicillin-resistant *S. aureus* strain carrying multiple virulence-associated genes underscores the public health relevance of toothbrushes as an often overlooked reservoir of clinically relevant and potentially pathogenic microorganisms.

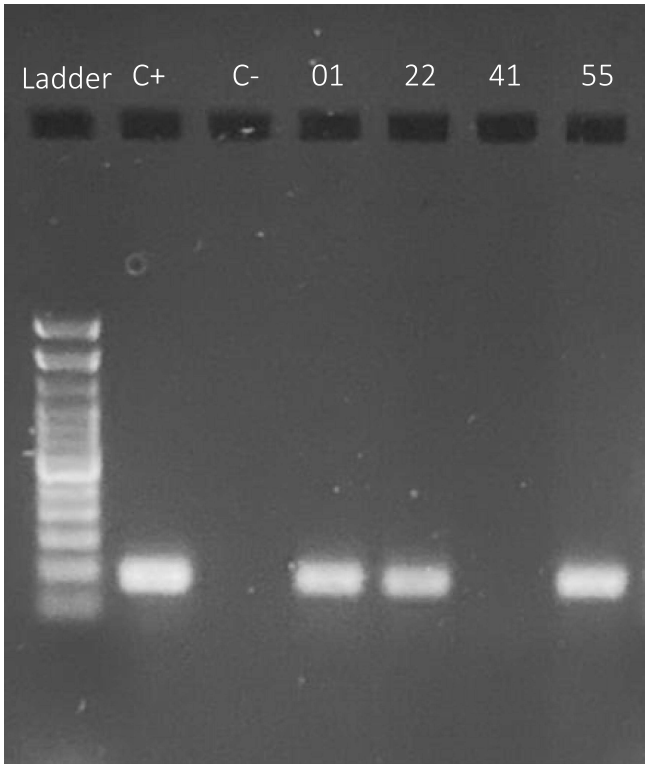

Figure 3 PCR product for the *tst* gene (180 bp) in *Staphylococcus aureus* strains isolated from toothbrushes. Lane 1: Ladder; Lane 2: Positive control (*Staphylococcus aureus* ATCC 43300), Lane 3: Negative control (*Streptococcus pyogenes* ATCC 12344), positive samples: 01, 22 and 55 for the *tst* gene.

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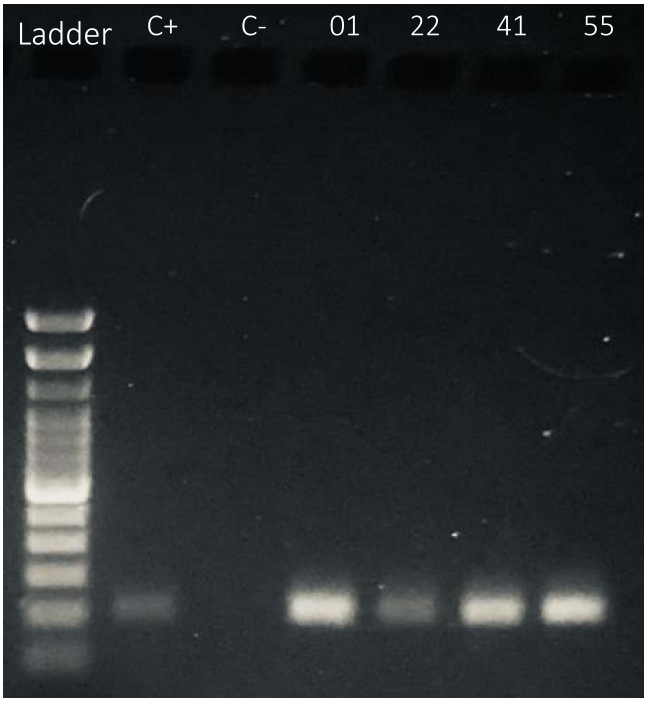

Figure 4 PCR product for the *hla* gene (209 bp) in *Staphylococcus aureus* strains isolated from toothbrushes. Lane 1: Ladder; Lane 2: Positive control (*Staphylococcus aureus* ATCC 25923); Lane 3: negative control (*Streptococcus pyogenes* ATCC 12344); all samples were positive for *hla* gene.

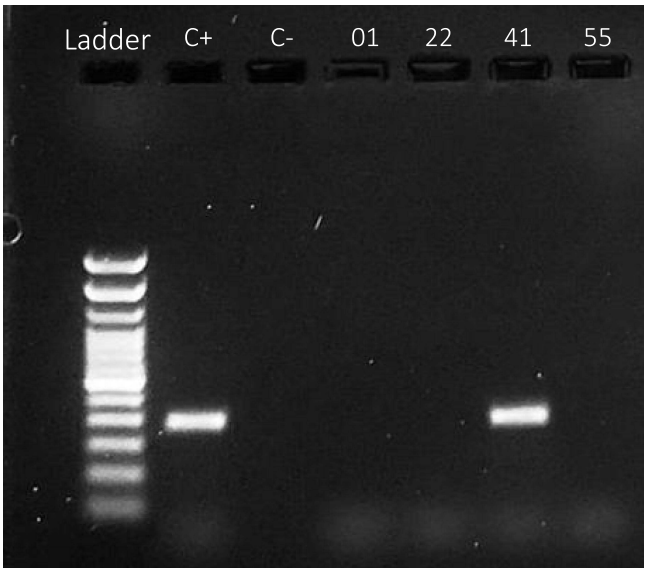

Figure 5 PCR product for the *seb* gene (404 bp) in *Staphylococcus aureus* strains isolated from toothbrushes. Lane 1: Ladder; Lane 2: Positive Control (*Staphylococcus aureus* ATCC 11632); Lane 3: Negative control (*Streptococcus pyogenes* ATCC 12344), Positive sample: 41 for the *seb* gene.

Discussion

Our findings confirm that toothbrushes can become contaminated with *S. aureus* and *E. faecalis*, including strains harboring antimicrobial resistance and virulence-associated genes. In this study, *E. faecalis* was detected in 12.3% of toothbrushes and *S. aureus* in 5.5%, including one methicillin-resistant isolate (1/73; 1.4%). These results support the study objective and highlight toothbrushes as potential reservoirs that may contribute to oral and, potentially, systemic

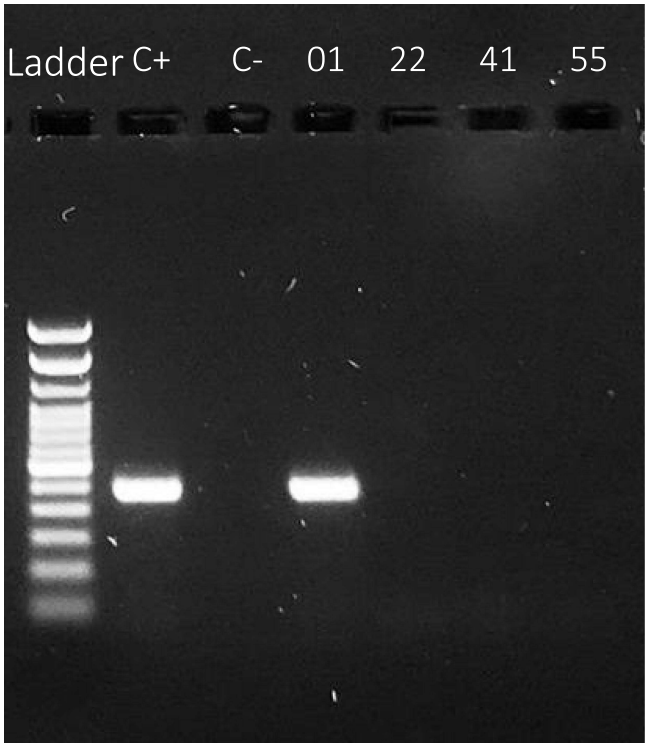

Figure 6 PCR product for the *sed* gene (492 bp) in *Staphylococcus aureus* strains isolated from toothbrushes. Lane 1: Ladder; Lane 2: Positive control (*Staphylococcus aureus isolated in the laboratory*); Lane 3: negative control (*Streptococcus pyogenes* ATCC 12344), positive sample: 01 for the *sed* gene.

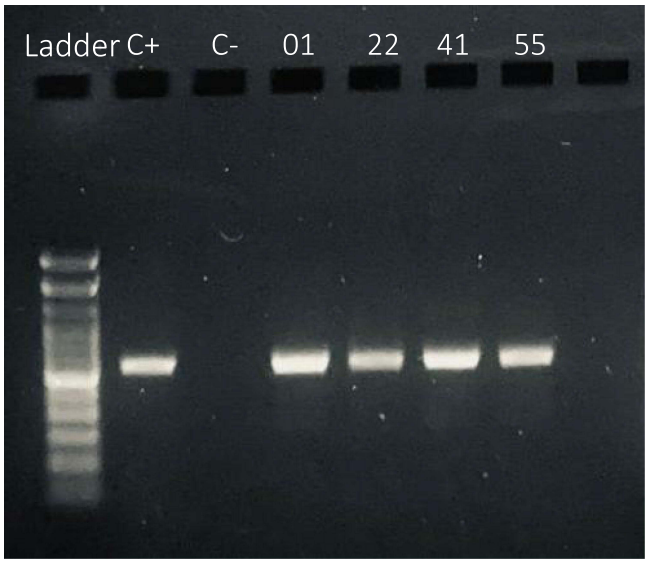

Figure 7 PCR product for the *blaZ* gene (674 bp) in *Staphylococcus aureus* strains isolated from toothbrushes. Lane 1: Ladder; Lane 2: positive control (*Staphylococcus aureus* ATCC 11632); Lane 3: negative control (*Streptococcus pyogenes* ATCC 12344), all samples were positive for *blaZ* gene.

exposure to opportunistic pathogens. Molecular resistance profiling was performed for *S. aureus* isolates; resistance mechanisms in *E. faecalis* were not evaluated and warrant further investigation.

Considering that the toothbrushes analyzed were used by dentistry students with formal knowledge of oral hygiene, the observed contamination is concerning. Most contaminated toothbrushes were manual, lacked a protective cover, were stored inside bathrooms, and had been used for more than one month. Similar factors have been associated with higher

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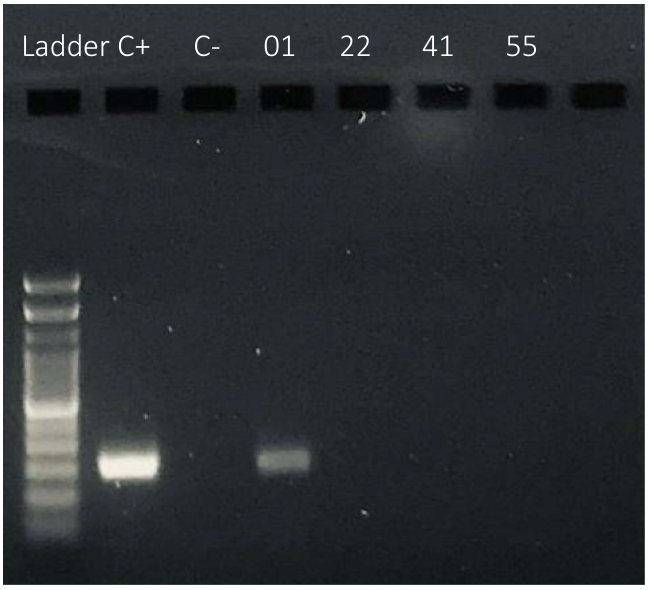

Figure 8 PCR product for the *mecA* gene (310 bp) in *Staphylococcus aureus* strains isolated from toothbrushes. Lane 1: Ladder; Lane 2: positive control (*Staphylococcus aureus* ATCC 43300); Lane 3: negative control (*Streptococcus pyogenes* ATCC 12344), positive sample: 01 for the *mecA* gene.

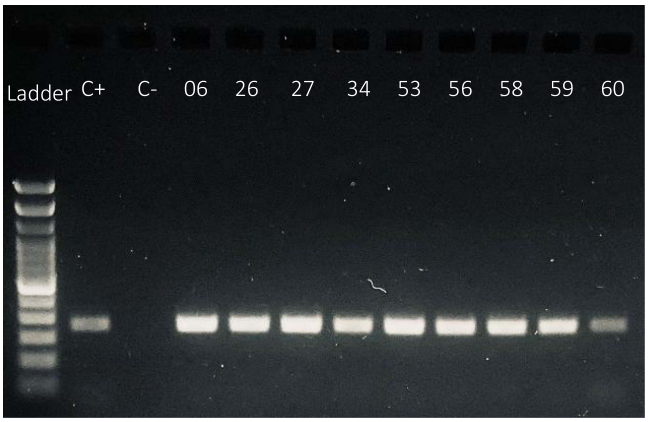

Figure 9 Electrophoretic runs of the amplicons corresponding to *E. faecalis* isolated in toothbrushes.

microbial loads in previous studies, suggesting that storage environment and hygiene-related behaviors play a key role in toothbrush contamination.[3,4,15] These findings reinforce the need to strengthen community recommendations regarding appropriate storage conditions, periodic disinfection, and timely toothbrush replacement.

The prevalence of *S. aureus* in our study (5.5%), including one MRSA-positive toothbrush, supports the view that toothbrushes may serve as vehicles for transmission of opportunistic pathogens. In Ghana, Twumwaa et al reported substantially higher contamination (94% *S. aureus*), with MRSA detected in 12.8% of samples.[6] Such differences may reflect variability in environmental conditions (eg, humidity and temperature), hygiene practices, storage habits, and duration of toothbrush use, all of which may influence bacterial survival and persistence on toothbrush bristles.

In European cohorts, Volgenant et al reported a low MRSA prevalence (1.5%) among dental students and did not observe major differences based on clinical practice exposure.[24] This aligns with our low MRSA frequency, while also emphasizing that low prevalence does not necessarily imply low relevance, particularly when isolates carry clinically meaningful resistance and toxin-associated genes. The consistent observation that bathrooms are common storage locations in different settings suggests that storage environment remains a modifiable risk factor.

A particularly relevant finding was the identification of the RAAR61 strain, resistant to methicillin (*mecA*) and penicillin (*blaZ*), recovered from a toothbrush used for two months and stored in a bathroom drawer. This isolate also carried virulence genes *(tst, hla, hld, seb*, and *sec*), suggesting clinically important pathogenic potential. The detection of *blaZ* and *mecA* in a non-hospital context underscores the circulation of resistance determinants in the community and supports the need to consider everyday items as part of broader antimicrobial resistance surveillance and prevention efforts.

The prevalence of *E. faecalis* (12.3%) is consistent with Romero et al (18%) in toothbrushes stored in bathrooms, which is clinically relevant given the role of *E. faecalis* in persistent endodontic infections and root canal treatment failures.[25]

From a practical and policy perspective, our findings indicate that toothbrushes should not be considered harmless instruments, as they can harbor potentially pathogenic and resistant bacteria. Recommendations should prioritize regular replacement, proper storage away from humid bathroom environments when possible, and periodic disinfection, particularly after the first month of use.[3,4,15]

This study is limited by the modest sample size, the focus on only two bacterial species, the absence of environmental sampling (eg, sinks, bathroom surfaces), and the lack of broader microbiological characterization beyond the targeted species assessed.[17,25,26] In addition, although this study did not evaluate other resistance determinants in *E. faecalis*, efflux pumps represent a relevant mechanism contributing to multidrug resistance; for example, the *lsaE* efflux-associated gene has been linked to macrolide resistance in multidrug-resistant enterococci.[27] Future studies should evaluate disinfection strategies under real-world conditions and expand molecular characterization to include additional resistance mechanisms and a wider microbial spectrum. Moreover, emerging microbiota-based therapies aimed at restoring a balanced oral ecosystem may represent complementary preventive approaches to reduce colonization by opportunistic pathogens.[28] Finally, these results support reinforcing infection control practices in dentistry—through training, adherence to evidence-based guidelines, and risk communication—to minimize cross-contamination and limit the dissemination of virulent and resistant oral microorganisms.[29]

Conclusions

In conclusion, this study demonstrates that toothbrushes can act as reservoirs for *E. faecalis* and *S. aureus*, with detection rates of 12.3% and 5.5%, respectively, including one methicillin-resistant *S. aureus* (MRSA) isolate (1/73; 1.4%). The detection of bacteria carrying resistance and virulence-associated genes underscores the potential of toothbrushes to contribute to the persistence and transmission of opportunistic pathogens, with possible implications for both oral and systemic health.

Toothbrushes stored in bathrooms and those used for prolonged periods showed more frequent contamination, reinforcing the importance of appropriate storage, regular replacement, and periodic disinfection, particularly after the first month of use. These findings support strengthening infection prevention and educational measures in both dental and community settings to minimize cross-contamination. Future research should expand microbial profiling (including additional pathogens and broader microbiome approaches), evaluate disinfection protocols under real-world conditions, and assess the impact of targeted oral hygiene education and infection control interventions on toothbrush contamination and related health risks.

Confidentiality and Privacy

The authors declare that they have followed the established protocols related to information protection and data disclosure.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare that there are no conflicts of interest associated with this study.

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