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CULTIVABLE BACTERIAL DIVERSITY ASSOCIATED WITH DENTAL UNITS: MALDI-TOF MS IDENTIFICATION AND ANTIMICROBIAL SUSCEPTIBILITY

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Abstract

Stored water and dental unit components may harbor environmental and potentially opportunistic microorganisms, making microbiological monitoring relevant to biosafety. This study aimed to characterize cultivable bacteria isolated from water stored in dental unit supply bottles and from three-way syringe swabs collected at a teaching dental clinic, as well as to evaluate the inhibitory activity of antimicrobial agents against the recovered isolates. An observational, descriptive, laboratory-based study was conducted between September 2024 and April 2025. Thirty-two samples associated with 16 dental units were analyzed, comprising 16 water samples and 16 three-way syringe swabs. After culture on blood agar and in *Brain Heart Infusion* broth, the isolates were identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). Antimicrobial activity was evaluated using the disk diffusion method according to the recommendations of the *Clinical and Laboratory Standards Institute*. All samples yielded cultivable bacterial growth, and 22 isolates belonging to eight taxonomic categories were recovered. Gram-negative bacteria predominated, accounting for 77.3% of the isolates. The *Burkholderia cepacia* complex was the most frequent group, representing 31.8%, followed by *Elizabethkingia miricola* and *Pseudomonas stutzeri*, each accounting for 13.6%. The *log(score)* values ranged from 2.06 to 2.37. Of the 22 isolates, 17 were subjected to disk diffusion testing and exhibited inhibition zones against the respective antimicrobial agents evaluated. The *Staphylococcus epidermidis* isolates were classified as susceptible for combinations with applicable breakpoints. For the remaining microorganisms without specific validated interpretive criteria, inhibitory activity was reported descriptively, without classification as susceptible, intermediate, or resistant. The findings demonstrated a diverse cultivable microbiota in the evaluated components and reinforce the need for cleaning and disinfection protocols, preventive maintenance, proper water storage, and periodic microbiological monitoring. The absence of quantitative bacterial counts and microbiological indicators of potability precluded classification of the water as suitable or unsuitable for dental use.

Keywords: Bacteria; Dental Equipment; Microbial Sensitivity Tests; Water Microbiology; Water Supply.

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1. Introduction

Water used in dental units is directly involved in different clinical procedures through handpieces, ultrasonic devices, and three-way syringes, in addition to contributing to aerosol generation. Therefore, its microbiological quality is relevant to the biosafety of patients, students, and professionals. Even when the source water meets appropriate quality standards, storage in independent reservoirs and circulation through the dental unit system may favor the recovery of environmental and potentially opportunistic microorganisms (Spagnolo; Sartini; Cristina, 2020).

Dental unit waterlines consist of narrow tubing with a high surface-to-volume ratio and are characterized by intermittent flow and periods of stagnation. These conditions may promote microbial adhesion and biofilm development, from which bacterial cells can subsequently be released into the water used during dental care. Previous studies have demonstrated microbial diversity in the water, reservoirs, and internal tubing of dental units, including environmental bacteria and potentially opportunistic species (Błaszczyk et al., 2022; Dang et al., 2022; Hussain Akbar; Behbehani; Karched, 2023).

Water stored in bottles or reservoirs is not microbiologically equivalent to the output water released by dental instruments because the latter passes through the internal waterlines before reaching the patient. Therefore, the use of distilled water or independent reservoirs alone does not ensure the microbiological quality of dental treatment water unless accompanied by appropriate cleaning, disinfection, maintenance, and monitoring protocols.

As an international reference, the Centers for Disease Control and Prevention recommends that output water used in nonsurgical dental procedures contain no more than 500 colony-forming units per milliliter of heterotrophic bacteria. For surgical procedures, sterile solutions delivered through independent systems should be used (Centers for Disease Control and Prevention, 2024).

In addition to microbial quantification, the identification of bacterial isolates can provide information on the cultivable fraction of the microbiota associated with dental unit components. Methods such as matrix-assisted laser

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desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) can improve the taxonomic resolution of recovered microorganisms. Complementarily, disk diffusion assays can record the inhibitory activity of antimicrobial agents against bacterial isolates. However, clinical classification as susceptible, intermediate, or resistant depends on the availability of specific and validated interpretive breakpoints for each microorganism–antimicrobial combination.

In this context, the present study aimed to characterize cultivable bacteria isolated from three-way syringe swabs and water stored in dental unit supply bottles at a teaching dental clinic located in the interior of Bahia, Brazil, using MALDI-TOF MS. In addition, the study exploratorily evaluated the inhibitory activity of antimicrobial agents against the recovered isolates.

2. Literature Review

Dental unit waterlines have structural characteristics that favor microbial colonization, particularly because their polymeric tubing has a small diameter, a high surface-to-volume ratio, intermittent flow, and periods of water stagnation. These conditions reduce the shear forces exerted by water and facilitate the initial adhesion of microorganisms to internal surfaces. Following adhesion, microbial cells can produce an extracellular matrix and form organized microbial communities known as biofilms (Spagnolo; Sartini; Cristina, 2020; Hussain Akbar; Behbehani; Karched, 2023).

Biofilms act as reservoirs of microorganisms and can continuously release planktonic cells into the water circulating through dental units. Contamination may originate from the water supply, independent reservoirs, handling of dental unit components, or the backflow of fluids from the oral cavity when antiretraction devices fail to operate properly. The extent of contamination may also be influenced by the frequency of equipment use, periods of inactivity, and the effectiveness of cleaning and disinfection procedures (Spagnolo; Sartini; Cristina, 2020; Błaszczyk et al., 2022).

In this context, heterotrophic bacterial counts provide an estimate of the

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cultivable microbial load but do not, by themselves, identify the species present or indicate their potential clinical relevance. Therefore, microbiological assessment can be expanded through the identification of bacterial isolates, investigation of drinking-water quality indicators, and analysis of different points within the system, including the source water, independent reservoirs, water released by dental instruments, and the internal surfaces of the waterlines (Błaszczyk et al., 2022; Dang et al., 2022).

3. Methodology

Water samples were collected directly from the bottles or reservoirs used to supply the dental units. The water stored in these containers was obtained through a distillation process performed at the institution. A total of 100 mL of water was collected aseptically from each bottle and transferred to a sterile glass container containing 0.1 mL of 10% sodium thiosulfate solution to neutralize any residual chlorine.

Each container was labeled with the number of the dental unit to which the corresponding bottle was connected. After collection, the tubes containing the swabs and the containers holding the water samples were placed in thermal boxes and immediately transported to the Microbiology Laboratory of São Francisco River University Center (UniRios).

Aliquots of 100 µL from the swab suspensions and water samples were inoculated onto blood agar by surface plating under aseptic conditions in a laminar flow cabinet. Additional aliquots were inoculated into *Brain Heart Infusion* (BHI) broth as an enrichment step. The plates and tubes were incubated in a bacteriological incubator at 36.5 °C for 24–48 hours.

Cultures showing growth in BHI broth were subsequently inoculated onto solid BHI medium. After incubation, colonies with distinct macroscopic characteristics were selected and subjected to successive subcultures on solid medium until pure cultures were obtained. The use of BHI broth as an enrichment medium was considered when interpreting the results because this step may favor the recovery of certain microorganisms. Consequently, the recovered isolates do not necessarily represent the entire bacterial community present in the samples.

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The bacterial isolates were sent to the Department of Microbiology at the Federal University of Pernambuco (UFPE), where they were identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) using a Bruker Autoflex III Smartbeam instrument with the integrated Bruker Biotyper software and database.

The mass spectra obtained were compared with profiles available in the system reference library, and the results were expressed as *logs(score)* values. Identifications with *log(score)* values greater than 2.0 were accepted at the species level according to the criteria established for the platform.

Of the 22 isolates recovered, 17 were subjected to disk diffusion testing. The isolates of *Cupriavidus metallidurans*, *Microbacterium ginsengisoli*, and *Streptomyces rochei* were not included in the antimicrobial assay because the cultures were no longer viable. Antimicrobial activity was evaluated using the agar disk diffusion method, also known as the Kirby–Bauer method, according to the recommendations of the *Clinical and Laboratory Standards Institute* (CLSI).

Pure bacterial cultures were transferred with a sterile inoculating loop to plates containing Mueller–Hinton agar and incubated at 36 °C for 48 hours. After incubation, isolated colonies were suspended in tubes containing 4 mL of sterile 0.85% saline solution. The suspensions were homogenized, and the inoculum density was adjusted to the 0.5 McFarland standard. A sterile swab was then immersed in each suspension and used to spread the inoculum uniformly over the entire surface of fresh Mueller–Hinton agar plates.

The antimicrobial panels were defined according to the bacterial isolate evaluated. For *Sphingomonas melonis*, ampicillin (10 µg), tetracycline (30 µg), and gentamicin (10 µg) disks were used. Isolates identified as *Burkholderia cepacia* were tested against cefotaxime (30 µg), tetracycline (30 µg), and streptomycin (10 µg). For *Staphylococcus epidermidis*, oxacillin (1 µg), gentamicin (10 µg), and clindamycin (2 µg) were used. The *Burkholderia pyrrocinia* isolate was tested against cefotaxime (30 µg), rifampin (5 µg), and tetracycline (30 µg), whereas the *Burkholderia cenocepacia* isolate was tested against cefotaxime (30 µg), streptomycin (10 µg), and tetracycline (30 µg). For *Elizabethkingia miricola*, imipenem (10 µg), rifampin (5 µg), and

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clindamycin (2 µg) were used. The *Pseudomonas stutzeri* isolates were tested against imipenem (10 µg), gentamicin (10 µg), and cefotaxime (30 µg).

After disk application, the plates were incubated at 35 °C for 24 hours. The diameters of the inhibition zones were measured in millimeters using a precision caliper and recorded individually for each isolate–antimicrobial combination. The general disk diffusion procedure was based on CLSI M02, 14th edition, whereas the applicability of categorical interpretation was assessed according to CLSI M100, 35th edition. When applicable, CLSI M45, 3rd edition, was consulted for infrequently isolated bacteria.

The isolates were classified as susceptible (S), intermediate (I), or resistant (R) only when specific and validated breakpoints were available for the microorganism–antimicrobial combination and the method employed. For *S. epidermidis*, the result obtained with the 1 µg oxacillin disk was interpreted as susceptible when the inhibition zone diameter was ≥18 mm and resistant when it was ≤17 mm, with no intermediate category. For members of the *B. cepacia* complex, including *B. cepacia*, *B. pyrrocinia*, and *B. cenocepacia*, no S/I/R classification was assigned because valid disk diffusion breakpoints were unavailable in the contemporary CLSI editions consulted. Similarly, results for the other species without specific interpretive criteria were reported exclusively as inhibition zone diameters and *in vitro* inhibitory activity, without extrapolating breakpoints established for other microorganisms.

The data were analyzed using descriptive statistics. Absolute and relative frequencies of the taxa were calculated based on the total number of recovered isolates; therefore, these proportions should not be interpreted as prevalence among the samples or dental units. Individual *log(score)* values were recorded for the MALDI-TOF MS results. Antimicrobial assay data were presented for each isolate, including its identification code, sample origin, corresponding dental unit, antimicrobial agent, observed inhibition zone diameter, availability of an applicable breakpoint, and, when scientifically appropriate, categorical interpretation.

The study analyzed exclusively environmental samples obtained from dental unit components. It did not involve human participants, human biological material,

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access to identifiable personal data, or animal experimentation. Therefore, obtaining written informed consent was not applicable.

4. Results and Discussion

All 32 samples analyzed, comprising 16 water samples collected from dental unit supply bottles and 16 samples obtained using three-way syringe swabs, yielded cultivable bacterial growth. A total of 22 bacterial isolates were recovered from these samples.

The 22 isolates were purified through successive subcultures and identified by MALDI-TOF MS. For presentation of the results, the identifications were organized into eight taxonomic categories, one of which corresponded to the *Burkholderia cepacia* complex. Gram-negative bacteria predominated, accounting for 17 of the 22 isolates (77.3%), whereas Gram-positive bacteria represented five isolates (22.7%).

Members of the *B. cepacia* complex were the most frequently recovered microorganisms, totaling seven isolates (31.8%). This group comprised isolates identified as *B. cepacia*, *B. pyrrocinia*, and *B. cenocepacia*. *Elizabethkingia miricola* and *Pseudomonas stutzeri* were represented by three isolates each, individually accounting for 13.6% of the recovered isolates. *Cupriavidus metallidurans*, *Microbacterium ginsengisoli*, *Sphingomonas melonis*, and *Staphylococcus epidermidis* were represented by two isolates each, with an individual relative frequency of 9.1%. *Streptomyces rochei* was identified in one isolate (4.5%). The distribution of the taxa, their frequencies among the recovered isolates, and the corresponding *log(score)* results are presented in Table 1.

Table 1. Distribution and mean identification scores of bacterial isolates recovered from dental unit supply bottle water samples and three-way syringe swabs.

Identified taxon	Number of isolates	Number of isolates	Number of isolates
Complexo *Burkholderia cepacia*[1]	7	31,8	2,11
Elizabethkingia miricola	3	13,6	2,16

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Pseudomonas stutzeri	3	13,6	2,13
Cupriavidus metallidurans	2	9,1	2,37
Microbacterium ginsengisoli	2	9,1	2,36
Sphingomonas melonis	2	9,1	2,12
Staphylococcus epidermidis	2	9,1	2,32
Streptomyces rochei	1	4,5	2,06
Total	**22**	**100,0**	**–**

[1]The *B. cepacia* complex comprised isolates identified as *B. cepacia*, *B. pyrrocinia*, and *B. cenocepacia*. Relative frequencies were calculated based on the total of 22 bacterial isolates.

Source: Prepared by the authors (2026).

The individual *log(score)* values were greater than 2.0 and ranged from 2.06 to 2.37. The highest mean score was observed for *C. metallidurans* (2.37), followed by *M. ginsengisoli* (2.36) and *S. epidermidis* (2.32). The lowest value was recorded for *S. rochei* (2.06). The identifications corresponded to the best-matching profiles available in the reference library used by the MALDI-TOF MS system. For uncommon and closely related taxa, particularly members of the *B. cepacia* complex and the genus *Elizabethkingia*, the results were interpreted in light of the library coverage and the absence of confirmation by molecular sequencing.

The predominance of Gram-negative bacteria and the diversity of the identified taxa are consistent with studies reporting heterogeneous microbial communities in dental unit water systems. Dang et al. (2022), when evaluating water samples from dental units, observed variations in bacterial composition and identified potentially opportunistic genera, including *Pseudomonas* and *Sphingomonas*. Other studies based on culture-dependent methods, MALDI-TOF MS, and 16S rRNA gene sequencing have also identified diverse bacterial communities in dental unit water, reservoirs, and internal tubing (Spagnolo; Sartini; Cristina, 2020; Błaszczyk et al., 2022; Hussain Akbar; Behbehani; Karched, 2023).

Comparisons with these studies should be made cautiously because many investigations have primarily evaluated water discharged from dental instruments and

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samples collected from the internal tubing, whereas the present study examined water stored in supply bottles and samples obtained by swabbing three-way syringes. Structural characteristics of dental unit waterlines, including the small diameter of the tubing, high surface-to-volume ratio, intermittent flow, and periods of stagnation, may promote microbial adhesion and biofilm formation. However, because the internal tubing was not sampled directly and no specific methods were used to demonstrate or quantify biofilms, the findings indicate the recovery of bacteria from the evaluated components but do not confirm the presence of established biofilms within the internal waterlines.

The predominance of isolates belonging to the *Burkholderia cepacia* complex deserves attention because these microorganisms can survive in aqueous environments with limited nutrient availability, adhere to plastic surfaces, and persist in certain water-based products. Some species within this complex may cause opportunistic infections, particularly in patients with cystic fibrosis, chronic respiratory diseases, or immunological impairment, and may exhibit intrinsic and acquired mechanisms of antimicrobial resistance (Tavares et al., 2020; Rhodes; Schweizer, 2016).

The isolation of these bacteria from environmental samples does not demonstrate that patients were exposed or that an infection associated with dental treatment occurred. Instead, this finding should be interpreted as an indication of the need to investigate the source of contamination and review procedures related to water storage, cleaning, disinfection, and dental unit maintenance.

Elizabethkingia miricola and *Pseudomonas stutzeri* each accounted for 13.6% of the isolates. *E. miricola* is an environmental bacterium found in aquatic systems and, although it is rarely associated with disease, it has been reported as a cause of opportunistic infections in vulnerable hospitalized patients (Soler-Iborte; Rivera-Izquierdo; Valero-Ubierna, 2024). *P. stutzeri*, in turn, is widely distributed in the environment and exhibits considerable metabolic diversity, being occasionally recognized as an opportunistic human pathogen (Lalucat et al., 2006).

The identification of predominantly environmental species, including *Sphingomonas melonis*, *Cupriavidus metallidurans*, *Microbacterium ginsengisoli*, and

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Streptomyces rochei, further demonstrates the microbial diversity of the evaluated components. Nevertheless, their recovery should not automatically be interpreted as indicating an equivalent pathogenic potential among all identified species.

The presence of *Staphylococcus epidermidis* among 9.1% of the isolates should also be interpreted cautiously. This species is part of the normal microbiota of human skin and mucous membranes but may act as an opportunistic pathogen, particularly in infections associated with medical devices, because of its ability to adhere to surfaces and form biofilms (Otto, 2009). In the context of this study, its recovery may have been associated with equipment handling, contact-mediated transfer, or environmental contamination.

A total of 17 isolates were subjected to disk diffusion testing and showed inhibition zones in response to the respective antimicrobial agents evaluated. For microorganism–antimicrobial combinations for which applicable interpretive criteria were available, inhibition-zone diameters were equal to or greater than the adopted susceptibility breakpoints, and no intermediate or resistant profiles were identified. The isolates of *C. metallidurans*, *M. ginsengisoli*, and *S. rochei* were not tested because of loss of culture viability.

For infrequently isolated species lacking specific interpretive breakpoints, inhibitory activity against the tested antimicrobial agents was recorded. In these cases, the findings were reported descriptively and were not categorized as susceptible, intermediate, or resistant. The antimicrobial agents tested, the inhibition-zone diameters obtained, and the applicable interpretation of the results are presented in Table 2.

Table 2. Antimicrobial agents evaluated, consulted reference values, and interpretation of the bacterial isolates.

Microorganism (No. of isolates)	Antimicrobial agents evaluated and consulted reference values	Observed result	Classification
Sphingomonas melonis (2)	Ampicillin ≥17 mm; tetracycline ≥19 mm;	All isolates exhibited inhibition zones	No specific breakpoint

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	gentamicin ≥15 mm	against the antimicrobial agents tested.	
Burkholderia cepacia (5)	Cefotaxime ≥18 mm; tetracycline ≥19 mm; streptomycin ≥15 mm		No specific breakpoint
Staphylococcus epidermidis (2)	Oxacillin ≥18 mm; gentamicin ≥15 mm; clindamycin ≥21 mm		Susceptible in the interpretable combinations
Burkholderia pyrrocinia (1)	Cefotaxime ≥18 mm; rifampicin ≥20 mm; tetracycline ≥19 mm		No specific breakpoint
Elizabethkingia miricola (3)	Imipenem ≥23 mm; rifampicin ≥20 mm; clindamycin ≥21 mm		No specific breakpoint
Pseudomonas stutzeri (3)	Imipenem ≥23 mm; gentamicin ≥15 mm; cefotaxime ≥18 mm		No specific breakpoint
Burkholderia cenocepacia (1)	Cefotaxime ≥18 mm; streptomycin ≥15 mm; tetracycline ≥19 mm		No specific breakpoint

Source: Prepared by the authors (2026).

Note: The values shown are consulted reference values and do not represent the inhibition-zone diameters individually measured for each isolate. In the absence of specific CLSI-validated breakpoints for a given microorganism–antimicrobial combination, the results were reported descriptively and were not categorized as susceptible, intermediate, or resistant.

The absence of phenotypic resistance under the conditions evaluated represents a favorable finding; however, it does not mean that all species were susceptible to all antimicrobial agents mentioned in the study. Furthermore, the

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absence of resistance in the disk diffusion test does not exclude the presence of antimicrobial resistance genes, mechanisms not expressed under the experimental conditions, or the possibility of the future selection of resistant isolates. This limitation is particularly relevant to bacteria belonging to the *B. cepacia* complex, whose clinical isolates may exhibit complex antimicrobial resistance profiles (Rhodes; Schweizer, 2016).

The disk diffusion test predominantly evaluates bacterial growth under planktonic conditions and does not fully reproduce the increased tolerance that may occur when microorganisms are organized into biofilms. Therefore, classification as susceptible, intermediate, or resistant should only be applied to microorganism–antimicrobial combinations for which validated interpretive breakpoints are available (Clinical and Laboratory Standards Institute, 2016, 2024, 2025).

Although the presence of culturable bacteria in the evaluated components warrants surveillance measures, the results do not allow the water to be classified as suitable or unsuitable for use, nor do they demonstrate noncompliance with a microbiological limit. Recommendations for water used in dental procedures consider, among other parameters, the heterotrophic bacterial count expressed as colony-forming units per milliliter (Centers for Disease Control and Prevention, 2024). In the present study, quantitative counts in CFU/mL and analyses of drinking-water quality indicators, such as total coliforms and *Escherichia coli*, were not performed. Therefore, the findings represent qualitative evidence of bacterial recovery and do not demonstrate that the water failed to meet microbiological quality standards.

The main limitations of this study include its restriction to a single institution, the limited number of dental units evaluated, the absence of quantitative assessment of the bacterial load, the lack of information on the distribution of isolates according to the source of each sample, the absence of direct evaluation of the internal waterlines, and the lack of molecular investigation of antimicrobial resistance determinants. In addition, culture-dependent methods may fail to detect viable but non-culturable microorganisms, thereby underestimating the existing microbial diversity (Dang et al., 2022).

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5. Conclusion

The identification of 22 bacterial isolates from samples collected from water supply bottles and three-way syringes demonstrated the presence of a diverse culturable microbiota in the evaluated dental unit components. Gram-negative bacteria predominated, particularly isolates related to the *Burkholderia cepacia* complex, and both environmental and potentially opportunistic species were detected.

Among the isolates subjected to antimicrobial susceptibility testing, no phenotypic resistance was detected in the microorganism–antimicrobial combinations for which applicable interpretive criteria were available. For taxa without validated breakpoints, inhibitory activity was reported descriptively, without classification as susceptible, intermediate, or resistant.

The results demonstrate the usefulness of species-level identification for characterizing the culturable microbiota associated with dental unit components and guiding monitoring measures. The findings reinforce the need for documented cleaning and disinfection protocols, preventive maintenance, appropriate water storage, and periodic microbiological monitoring, particularly in dental teaching and healthcare settings.

Future investigations should perform paired sampling of the source water, supply bottles, water discharged from three-way syringes, and the internal waterlines. They should also quantify heterotrophic bacteria and investigate microbiological indicators of drinking-water quality. Sampling at different time points, direct assessment of biofilm formation, and the use of complementary molecular methods are also recommended to identify potential sources and routes of contamination within dental unit water systems.

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