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Bacteriological Assessment of Mini Anchorage Retrieved Following Maxillary Treatments Using Mini Anchorage-Assisted Orthodontic Appliances

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ABSTRACT

In recent years, mini anchorages used in the mouth have been widely used in orthodontics clinics to provide dental movements and to increase skeletal anchorage. In this study, bacteria were isolated from mini anchorages taken from patients who were treated in the Orthodontic Department of Eskişehir Osmangazi University Faculty of Dentistry Clinic and routinely applied mini anchorages to the anterior and posterior regions of the upper palate for jaw widening or support of dental movements. Bacterial isolates were cultured and identified using 16S rRNA gene sequencing. A total of 88 isolates representing 20 different bacterial species were identified. *Streptococcus salivarus* (15 isolates), *Streptococcus anginosus* (12 isolates) and *Enterococcus feacalis* (10 isolates) were the most isolated bacteria in the bacterial composition. *Rothia mucilaginosa (*8 isolates) and *Corynebacteria argentoratense* (6 isolates) were isolated from the Actinobacteria (currently Actinobacteriota) phylum. *Neisseria oralis*, *N. flava,* N*. subflava* and *N. flavescens* species were determined from Proteobacteria (currently Pseudomonadota) phylum.

Keywords: Anchorages, Orthodontics, Bacteria.

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Introduction

In recent years, mini anchorages applied intraorally have been widely used in orthodontic clinics to facilitate tooth movement and enhance skeletal anchorage. Mini anchorages were developed as alternatives to conventional orthodontic anchorage systems, such as extraoral appliances, and have been rapidly adopted in orthodontic practice [1]. They are primarily used in adult patients with missing teeth to assist in tooth movement, to support tooth movement in cases where anchorage is critical, for the intrusion or extrusion of teeth within the jaws, and as replacements for extraoral appliances in children with low compliance to orthodontic treatment [2]. Due to their small size, they are considered safe with respect to the anatomical structures in the area of application. In addition, they offer numerous advantages, such as the ability to apply immediate force following placement, ease of clinical application, adaptability to various orthodontic and orthopedic force systems, low cost, and high patient compliance [2,3].

In some cases, mini anchorages may begin to move within the tissues where they are applied either before or after force application, leading to clinical failure. However, the exact cause of this failure has yet to be clearly identified. Several factors are considered as potential causes of failure, including placement of mini anchorages too close to tooth roots, application of excessive force, the structure of the jawbone where the anchorages are inserted, and pathogenic microbial colonization around the mini anchorage [4,5].

Mini anchorages are applied to the buccal (cheek) and palatal (palate) regions of the oral tissues. To date, nearly all studies on the microbial diversity surrounding orthodontic mini anchorages have focused on those placed in the buccal region [6]. However, mini anchorages are also applied to the anterior and posterior regions of the palate for purposes such as maxillary expansion or intrusion of teeth into the jawbone. It is known that microbial diversity in the palatal region differs and that it is anatomically adjacent to the tongue.

The aim of this study is to identify the bacterial diversity around orthodontic mini anchorages placed in different regions (anterior and posterior) of the upper palate.

This study will also contribute to the identification of local or systemic antimicrobial medications that may be applied to these areas to prevent mini anchorage loss by determining the microflora surrounding palatal mini anchorages.

Material and Methods

Mini Anchorages

A total of 40 mini anchorages, each 8 mm in length and 1.6 mm in diameter, were used—20 obtained from the anterior region and 20 from the posterior region of the

upper palate—from patients undergoing treatment at the Department of Orthodontics, Faculty of Dentistry, Eskişehir Osmangazi University. These patients had mini anchorages routinely applied to the anterior and/or posterior regions of the upper palate for maxillary expansion or to assist in tooth movement (Figure 1). Ethical approval for the study was obtained from the Clinical Research Ethics Committee of Eskişehir Osmangazi University (29.06.2017/17).

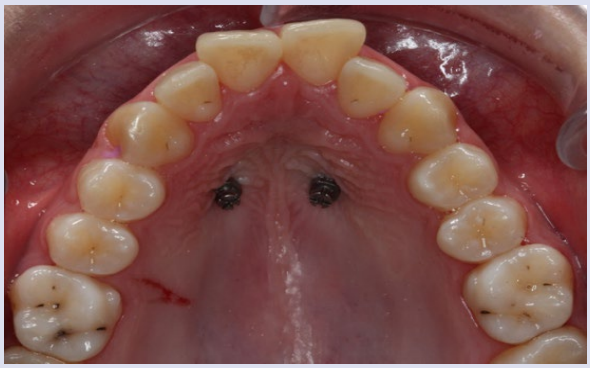

Figure 1. Anchorages placement in the anterior upper palatal (palate) region

Removal of Mini Anchorage and Culturing of the Samples

The removal of the mini anchorages was performed by the same clinician one week after the appliances attached to the anchorages were removed and once the need for support from the anchorages had ended, allowing the natural microflora to develop. Anchorages immediately transferred into Brain Heart Infusion Broth (BHI) medium and transported to the Microbiology Research Laboratory of the Department of Biology at Eskişehir Technical University for microbiological analysis. The collected anchorages were transferred into BHI medium and they were vortexed for 2 minutes. Subsequently, 100 µl of the sample was inoculated onto Blood agar, BHI agar, M17 agar, Mitis Salivarius agar, MRS agar, MacConkey agar, Potato Dextrose Agar (PDA), and Sabouraud Dextrose Agar (SDA) using the spread plate technique. The plates were incubated at 37°C in an incubator containing 5% CO_2 for 24–48 hours.

Isolation and Identification of Microorganisms

After incubation, colonies with different morphologies were selected and purified. Following Gram staining to confirm their purity, they were stored in 20% glycerol at -80°C. Molecular identification of the bacteria was performed based on their 16S rRNA sequences using the primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3'). Amplification of the 16S rRNA gene region was performed using the following PCR mixture: 2.5 µl of 10X Taq Buffer (with KCl and $MgCl_2$), 2.5 µl of 25 mM $MgCl_2$, 2.5 µl of 2.5 mM dNTP mix, 2.5 µl of each of the primers (2.5 mM), 0.25 µl of Taq DNA polymerase (5 U/µl), 11.75 µl of nuclease-free sterile distilled water, and 1 µl of template DNA. PCR conditions: initial denaturation at 94°C for 3 min; followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 60°C for 1 min, and extension at 72°C for 2 min; with a final extension step at 72°C for 10 min. The sequences were compared with other 16S rRNA sequences in the GenBank database on the National Center for Biotechnology Information (NCBI) website using the BLAST program. Species-level identification of the isolates was determined based on the percentage of sequence similarity [7].

The study is limited by the lack of anaerobic culture conditions. Therefore, molecular identification of anaerobic oral microbiota members *Prevotella intermedia*, *Porphyromonas gingivalis*, and *Aggregatibacter actinomycetemcomitans* was planned. For the molecular detection of anaerobic *P. intermedia*, *P.gingivalis*, and *A. actinomycetemcomitans*, total genomic DNA was isolated (Figure 2) and PCR was performed with the given species-specific primers [8].Positive control of total genomic DNA was performed by PCR using the 27F-1492R universal bacterial primers.

Phylogenetic Grouping

The sequences were aligned and edited in MEGA 7.0.26 software with the reference sequences to which they showed high similarity in the BLAST analysis. A phylogenetic tree was then constructed using the Neighbor Joining algorithm within the same program. The validity of the tree topology was verified using the bootstrap method with 1000 replicates [9].

Results

A total of 88 bacterial isolates were purified and molecularly identified based on their 16S rRNA sequences. It was determined that the bacterial isolates belonged to 20 different species (Table 1). Table 2 presents the isolate codes along with the corresponding species names. The phylogenetic groupings of the bacterial isolates were made using trees constructed with the Neighbor Joining algorithm in MEGA 7.0.26 software. As an outgroup, the 16S rRNA sequence of *Flavobacterium* sp. AM179863.1 from the phylum Bacteroidetes was retrieved from GenBank and used (Figure 3).

Table 1. Number of isolates per species

Species	Isolate number
Streptococcus anginosus	12
Streptococcus mitis	4
Neisseria oralis	4
Neisseria flava	2
Neisseria flavescens	2
Streptococcus oralis	3
Corynebacterium argentoratense	6
Rothia mucilaginosa	8
Streptococcus mutans	4
Enterococcus faecalis	10
Streptococcus salivarus	15
Lactobacillus fermentum	3
Lactobacillus rhamnosus	5
Staphylococcus epidermidis	2
Micrococcus luteus	1
Staphylococcus capitis	1
Neisseria subflava	1
Lactobacillus casei	1
Lactobacillus lactis	1
Streptococcus sp.	3

Table 2. Isolate codes and their corresponding species names

Isolate code	Species name	Isolate code	Species name
MCA16.3	*Enterococcus feacalis*	BHIA1.6	*Streptococcus* sp.
MCA16.4	*Enterococcus feacalis*	BHIA1.14	*Streptococcus anginosus*
BHIA16.1	*Enterococcus feacalis*	KAA1.2	*Streptococcus mitis*
MEA17.4	*Enterococcus feacalis*	KAA2.3	*Neisseria oralis*
MRSA17.3	*Streptococcus mutans*	KAA2.7	*Neisseria oralis*
M17A18.5	*Lactobacillus rhamnosus*	KAA3.5	*Neisseria flava*
BHIA18.3.1	*Rothia mucilaginosa*	BHIA3.3.2	*Staphylococcus capitis*
KAA19.1	*Streptococcus salivarus*	BHIA3.2.1	*Streptococcus salivarus*
KAA19.2	*Streptococcus salivarus*	MCA4.8.1	*Streptococcus salivarus*
MCA20.1	*Streptococcus anginosus*	MCA4.3	*Neisseria subflava*
MCA20.3	*Streptococcus anginosus*	KAA5.10	*Neisseria flavescens*
BHIP1.2	*Streptococcus oralis*	KAA5.5	*Rothia mucilaginosa*
KAP1.3	*Streptococcus sp.*	BHIA6.1	*Staphylococcus epidermidis*
MEAP2.1	*Streptococcus anginosus*	KAA6.2	*Micrococcus luteus*
KAP2.3	*Streptococcus oralis*	KAA7.2	*Staphylococcus epidermidis*
KAP2.4	*Streptococcus mitis*	BHIA7.1	*Streptococcus salivarus*
KAP2.10	*Streptococcus anginosus*	MCA8.1.1	*Lactobacillus fermentum*
KAP3.5	*Corynebacterium argentoratense*	MCA8.1.2	*Lactobacillus fermentum*
KAP3.9	*Corynebacterium argentoratense*	KAA9.3	*Rothia mucilaginosa*
KAP3.10	*Corynebacterium argentoratense*	M17A9	*Lactobacillus lactis*
MitP4.2	*Streptococcus salivarus*	KAA10.1	*Enterococcus feacalis*
MRSP4.1	*Lactobacillus rhamnosus*	M17A10.2	*Streptococcus salivarus*
KAP4.4	*Streptococcus sp.*	BHIA11.4	*Streptococcus anginosus*
KAP5.4	*Streptococcus salivarus*	KAA11.2	*Streptococcus anginosus*
M17P7.2	*Streptococcus salivarus*	M17A13.1	*Rothia mucilaginosa*
KAP9.3	*Rothia mucilaginosa*	MCA16.1	*Enterococcus feacalis*
BHIP10.2	*Enterococcus feacalis*	MCP15.1	*Neisseria flava*
BHIP10.1	*Rothia mucilaginosa*	MCP15.2	*Neisseria flavescens*
MCP11.2	*Streptococcus salivarus*	BHIP16.4.1	*Lactobacillus rhamnosus*
KAP11.3	*Streptococcus salivarus*	P16.3	*Streptococcus salivarus*
MCP12.1	*Streptococcus salivarus*	KAP17.1	*Streptococcus oralis*
KAP12.2	*Streptococcus mutans*	BHIP17.2	*Streptococcus mitis*
BHIP13.1	*Lactobacillus casei*	MCP18.4.1	*Lactobacillus rhamnosus*
BHIP13.2	*Enterococcus feacalis*	BHIP18.2	*Streptococcus anginosus*
MCP14.1	*Enterococcus feacalis*	KAP19.1	*Corynebacterium argentoratense*
MCP14.2	*Enterococcus feacalis*	KAP19.2	*Corynebacterium argentoratense*
MCP20.1	*Streptococcus anginosus*	MCA12.4	*Streptococcus anginosus*
KAP20.2	*Corynebacterium argentoratense*	MEAA13.2	*Rothia mucilaginosa*
M17P7.1	*Neisseria oralis*	MCA14.5	*Lactobacillus fermentum*
MCP8.2	*Streptococcus salivarus*	BHIA14.1	*Rothia mucilaginosa*
MCP8.4.2	*Lactobacillus rhamnosus*	BHIA15.3	*Streptococcus mutans*
M17P9.1	*Neisseria oralis*	MCA15.1.2	*Streptococcus mutans*
BHIP5.3	*Streptococcus anginosus*	MCA12.3	*Streptococcus anginosus*
KAP6.3	*Streptococcus salivarus*	KAP6.2	*Streptococcus mitis*

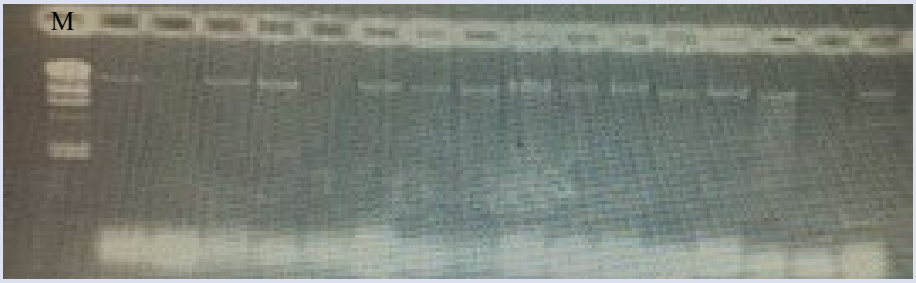

Figure 2. Total genomic DNA. M: 1 kb DNA ladder (New England Biolabs)

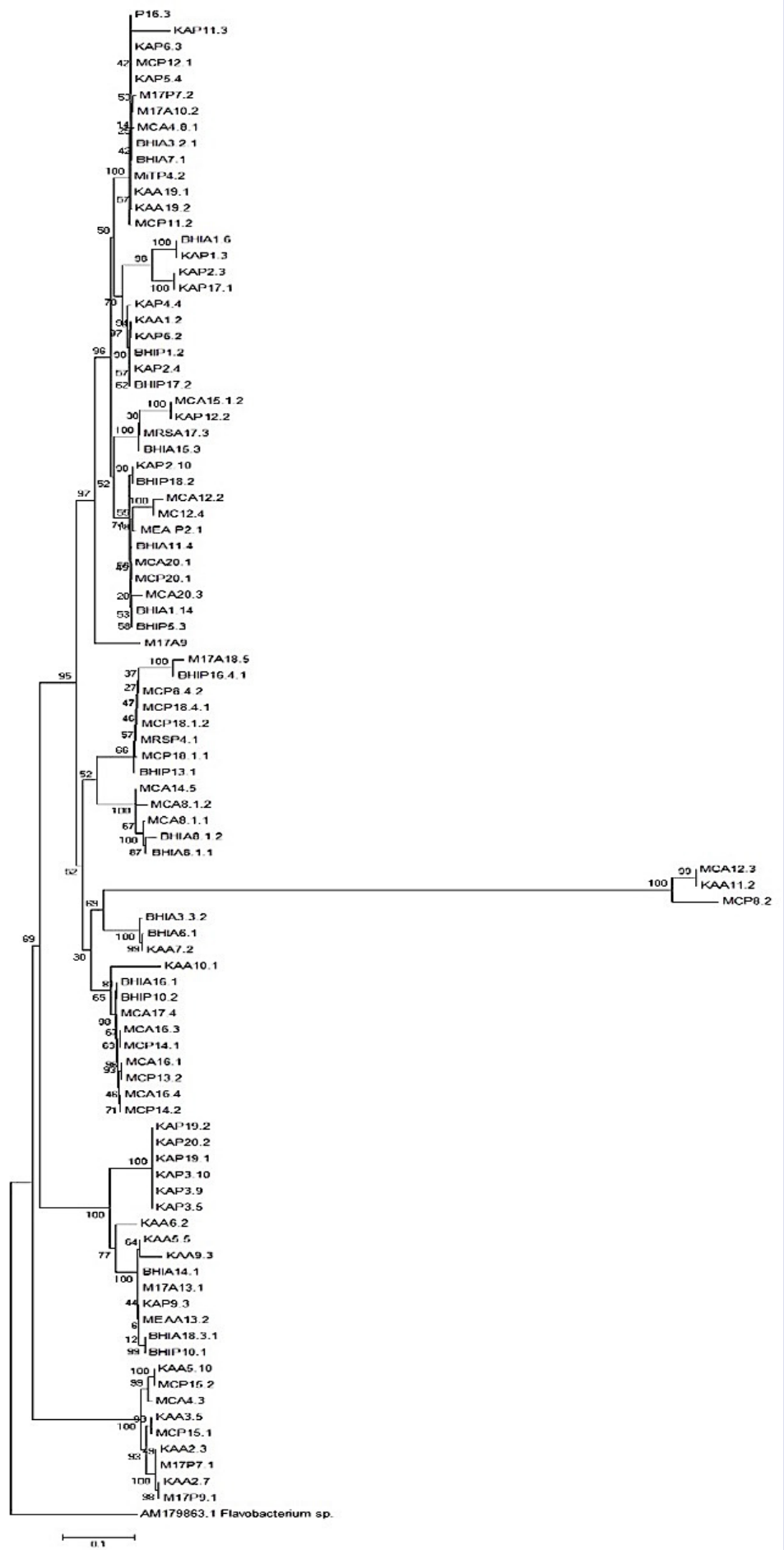

Figure 3. Neighbor Joining tree of the isolates

Discussion

Molecular identification of the bacteria at the species level was performed based on the 16S rRNA gene region. The 16S rRNA gene is the most frequently used gene in bacterial phylogeny and taxonomy studies [9]. Its widespread use is due to several factors: the gene is present in all bacteria; its function has remained unchanged over time, reflecting evolutionary changes in its sequence; its length of approximately 1500 base pairs provides sufficient bioinformatic information; and it contains both conserved and variable regions, which are essential for evolutionary analysis [10,11,12].

Sequence analysis of the 16S rRNA gene region was conducted on 88 bacterial isolates obtained from the mini anchorages used in this study. According to the results, the isolates belonged to the phyla Firmicutes (currently Bacillota) (73.86%), Actinobacteria (15.9%), and Proteobacteria (10.22%). These phyla have also been reported as the dominant groups in both culture-dependent and culture-independent studies of the oral microbiota [13,14,15].

Within the Firmicutes phylum, which represented the majority of the microbial composition, the most frequently isolated species were *Streptococcus salivarius* (15 isolates), *Streptococcus anginosus* (12 isolates), and *Enterococcus faecalis* (10 isolates). From the Actinobacteria phylum, *Rothia mucilaginosa* (8 isolates) and *Corynebacterium argentoratense* (6 isolates) were isolated. From the Proteobacteria phylum, species identified included *Neisseria oralis*, *N. flava*, *N. subflava*, and *N. flavescens*.

When comparing the diversity of bacterial species isolated from mini anchorages placed in the anterior and posterior regions, it was observed that the anterior region contained 17 different bacterial species, while the posterior region contained 14. *Streptococcus oralis* (3 isolates), *Corynebacterium argentoratense* (6 isolates), and *Lactobacillus casei* (1 isolate) were isolated from posterior mini anchorages, whereas *Staphylococcus capitis* (1 isolate), *Neisseria subflava* (1 isolate), *Staphylococcus epidermidis* (2 isolates), *Micrococcus luteus* (1 isolate), *Lactobacillus fermentum* (3 isolates), and *Lactobacillus lactis* (1 isolate) were isolated from anterior mini anchorages.

In a study on microbial colonization of mini implants, biological material was collected from the area surrounding the mini anchorages using paper points. A total of 15 mini anchorages were used in the study. Both cultural and molecular methods were employed for the microbial analysis of the collected samples. BHI medium was selected for non-specific growth, Mitis Salivarius agar for the growth of streptococci, MRS agar for lactic acid bacteria, and Chromagar for *Candida* spp. After 24 hours of incubation under anaerobic conditions, colonies were selected. PCR was performed to detect *Porphyromonas gingivalis* molecularly. The PCR, carried out using primers specific to this species, showed no amplification. In this method, bacterial 16S rRNA primers were used as a control [16].

The bacterial isolates were phylogenetically grouped by constructing a Neighbor Joining phylogenetic tree. As an outgroup, *Flavobacterium* sp. (GenBank accession no: AM179863.1) from the phylum Bacteroidetes (currently Bacteridota) was selected. To test the reliability of the phylogenetic trees, the bootstrap method with 1000 replicates was applied. In the constructed tree, the root group was identified as *Neisseria* sp. In phylogenetic trees, the root represents the sequence that is evolutionarily the most distant from the group.

In another study involving 31 mini anchorages, molecular detection was performed for *Prevotella intermedia* (Pi), *Porphyromonas gingivalis* (Pg), and *Aggregatibacter actinomycetemcomitans* (Aa), which are known as periodontopathogenic microorganisms. As a result, all samples were found to be positive for Pi, 13.3% were positive for Aa, and 33.3% were positive for Pg [8].

Since our study is culture-dependent, the bacterial diversity it reveals is limited. The incubation conditions used were suitable for the growth of facultative anaerobic bacteria capable of growing under aerobic and 5% CO_2 conditions. To overcome this limitation molecularly, total genomic DNA isolation was performed. After visualization of the obtained total DNA on a gel, PCR reactions were set up using primers specific for *Prevotella intermedia*, *Porphyromonas gingivalis*, and *Aggregatibacter actinomycetemcomitans*. The 27F-1492R primers were used as a positive control for the reaction. The 27F-1492R primers are universal 16S rRNA bacterial identification primers. Total genomic DNA isolation yielded DNA as shown in Figure 2. As a result, this total DNA is expected to be the template DNA, and the reaction with these two primers will serve as a positive control.

However, the positive control did not work. It is thought that the cause may be DNA fragmentation either during total genomic DNA isolation or due to prolonged storage at -20°C. Therefore, the molecular detection phase of these bacteria in this study could not be completed.

As a future research recommendation within the scope of the study, it is suggested that total genomic DNA isolation be carried out using commercial kits specifically designed for this purpose, to eliminate DNA damage. In this way, the presence of unculturable bacteria can be revealed molecularly, leading to more accurate information on microbial composition.

Conclusion

This study identified bacterial species colonizing mini anchorages using 16S rRNA gene sequencing, revealing Firmicutes, Actinobacteria, and Proteobacteria as dominant phyla, consistent with the known oral microbiota. The culture-dependent approach limited detection to facultative anaerobes, and unsuccessful positive control PCR prevented molecular confirmation of

specific periodontopathogens. Future studies using optimized DNA isolation methods and culture-independent techniques are recommended to more accurately characterize the microbial diversity of mini anchorages.

Acknowledgments

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Conflicts of interest

There are no conflicts of interest in this work.

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