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Research Thesis

Master's Degree in Dental Medicine

Faculty of Dental Medicine of University of Porto

Bacterial adhesion to ecological brushes – an *in vitro* study

Adesão bacteriana a escovas ecológicas – um estudo *in vitro*

Luiza Teixeira Bittencourt Oliveira

PORTO, 2024

“Live a life you will remember.”

Avicii

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RESUMO

Introdução: Manter a saúde oral é vital. A cavidade oral, inicialmente livre de microrganismos ao nascimento, torna-se rapidamente colonizada. O desequilíbrio da microbiota, que, entretanto, se formou, pode levar a várias doenças orais e sistémicas. Escovar os dentes duas vezes por dia, com pasta de dentes fluoretada, contribui para mitigar estes riscos. No entanto, as escovas de dentes podem ser contaminadas, exigindo práticas de higiene cuidadosas. Existem vários tipos de escovas, inclusive opções ecológicas. Além da remoção de placa bacteriana, a sua segurança e eficácia devem ser averiguadas e garantidas. A Medicina Dentária abraçar a sustentabilidade, mas é crucial garantir a proteção ambiental e a segurança dos produtos, geralmente armazenados em locais propensos a contaminação microbiana.

Objetivo: Analisar e comparar, *in vitro*, a adesão microbiana a escovas de dentes ecológicas e convencionais, quando em contacto com microrganismos relacionados com a saúde oral.

Materiais e Métodos: Foram realizadas experiências *in vitro* para avaliar a adesão bacteriana às cerdas das diferentes escovas de dentes ecológicas: ELGYDIUM Eco, Curaprox CS 5460 Wood, Colgate® Bamboo Charcoal Soft Toothbrush; sendo a escova convencional Colgate® Extra Clean™, utilizada como grupo controlo. Foram utilizados os microrganismos *Staphylococcus aureus*, *Candida albicans, Streptococcus mutans, Escherichia coli* e *Enterococcus faecalis*. A adesão foi avaliada pela quantificação dos microrganismos aderidos - pelo número total de células metabolicamente ativas e cultiváveis, e por microscopia eletrónica de varrimento.

Resultados: Este estudo examina as cerdas das escovas de dentes e as suas propriedades de adesão microbiana. Foram analisados quatro tipos de cerdas com diferentes composições químicas: nylon de alta qualidade (controlo), nylon de base biológica proveniente de óleo de rícino, filamentos de CUREN®, e nylon infundido com carvão. Apesar da adesão semelhante de *Staphylococcus aureus e Candida albicans* entre os grupos, as cerdas ecológicas mostraram uma menor tendência para a adesão de *Candida albicans*. No entanto, os filamentos de CUREN® tiveram uma adesão mais elevada de *Streptococcus mutans*, enquanto o nylon de base biológica e as cerdas infundidas com carvão permitiram uma maior adesão de *Escherichia coli*. Todas as cerdas apresentaram uma tendência para adesão mais elevada de *Enterococcus faecalis* em comparação com o controlo. As imagens da microscopia eletrónica de varrimento revelaram uma adesão dispersa de *Staphylococcus aureus* nas superfícies das cerdas.

Discussão: Este estudo é o primeiro a comparar a adesão microbiana em escovas de dentes ecológicas versus convencionais, utilizando tanto a quantificação de unidades formadoras de colónias quanto a microscopia eletrónica de varrimento para uma visualização detalhada. Incluindo microrganismos como *Staphylococcus aureus, Candida albicans, Escherichia coli, Enterococcus faecalis* e *Streptococcus mutans*, o estudo revelou que diferentes marcas de escovas de dentes e tipos de cerdas influenciam significativamente a adesão microbiana. Estes achados sublinham o papel crítico do design das escovas de dentes e do material das cerdas na prevenção da contaminação microbiana e na melhoria da higiene oral.

Conclusão: Este estudo destaca a importância da seleção de materiais no design das escovas de dentes para uma higiene oral otimizada e considerações ambientais, revelando que a adesão microbiana varia significativamente com base no tipo de cerdas, com opções ecológicas mostrando vantagens e desvantagens distintas em comparação com as convencionais.

Palavras-chave: Escova de dentes ecológica, Escova de dentes tradicional, Adesão bacteriana, Atividade antibacteriana, Sustentabilidade.

ABSTRACT

Introduction: Maintaining oral health is vital. The oral cavity, initially microorganism-free at birth, quickly becomes colonised. An imbalance in the microbiota can lead to various oral and systemic diseases. Brushing teeth twice daily with fluoride toothpaste helps mitigate these risks. However, toothbrushes can become contaminated, necessitating careful hygiene practices. Various types of brushes are available, including eco-friendly options. Besides plaque removal, their safety and efficacy must be ensured. Dentistry is embracing sustainability, but it's crucial to ensure environmental protection and product safety, given their storage in microbe-prone locations.

Objective: To analyse and compare, in vitro, microbial adhesion to eco-friendly and conventional toothbrushes when in contact with microorganisms related to oral health.

Materials and Methods: In vitro experiments assessed bacterial adhesion to bristles of different eco-friendly toothbrushes: ELGYDIUM Eco, Curaprox CS 5460 Wood and Colgate® Bamboo Charcoal Soft Toothbrush; as the conventional toothbrush Colgate® Extra Clean™, used as control group. Microorganisms used were *Staphylococcus aureus, Candida albicans, Streptococcus mutans, Escherichia coli,* and *Enterococcus faecalis*. Adhesion was evaluated by quantifying metabolically active and cultivable adhered microorganisms and by scanning electron microscopy.

Results: Four types of bristles were analysed: high-quality nylon, bio-based nylon from castor oil, ultra-soft CUREN® polyester filaments, and charcoal-infused nylon. Despite similar adhesion of *Staphylococcus aureus* and *Candida albicans* across groups, ecological bristles showed less *Candida albicans* adhesion. However, ultra-soft filaments had higher *Streptococcus mutans* adhesion, while bio-based nylon and charcoal-infused bristles allowed for a greater *Escherichia coli* adhesion. All bristles had higher tendency to *Enterococcus faecalis* adhesion compared to standard nylon. Scanning electron microscopy images revealed scattered *Staphylococcus aureus* attachment on bristle surfaces.

Discussion: This study is the first to compare microbial adhesion on ecological versus conventional toothbrushes using both colony-forming unit quantification and scanning electron microscopy for detailed visualization. By including microorganisms such as *Staphylococcus aureus, Candida albicans, Escherichia coli, Enterococcus faecalis,* and *Streptococcus mutans*, the study revealed that different toothbrush brands and bristle types significantly influence microbial adhesion. These findings underscore the critical role of toothbrush design and bristle material in preventing microbial contamination and enhancing oral hygiene.

Conclusion: This study underscores the importance of material selection in toothbrush design for optimal oral hygiene and environmental considerations, revealing that microbial adhesion varies significantly based on the type of bristles, with eco-friendly options showing distinct advantages and disadvantages compared to conventional ones.

Keywords

Ecological toothbrush, Traditional toothbrush, Bacterial adhesion, Anti-bacterial activity, Sustainability.

INDEX

INDEX OF FIGURES

INDEX OF ABREVIATIONS

ADA - American Dental Association

BHI - Brain Heart Infusion

BA - Blood Agar

CFUs - Colony-forming units

EDS - Energy Dispersive Spectroscopy

SEM - Scanning electron microscopy

CHAPTER I

INTRODUCTION

I. INTRODUCTION

Maintaining good oral health is essential for overall well-being, as it directly and indirectly impacts an individual's health. At birth, the oral cavity is free of microorganisms due to the protected fetal environment. However, shortly after birth, it becomes colonized by various microorganisms. This colonization can be attributed to exposure to environmental agents and changes in the dietary habits as the child grows.[1]

In a healthy adult, the oral cavity hosts over 700 bacterial species and provides ideal conditions for their growth due to its environmental conditions (including temperature and humidity levels). *Streptococcus* predominantly populates most areas, while *Haemophilus* dominates the buccal mucosa, *Actinomyces* thrives in the supragingival plaque, and *Prevotella* is abundant in the low-oxygen subgingival plaque.[2-4]

Although a healthy individual's microbiome is associated with many factors, including age, diet, environmental conditions, and more, their imbalance can contribute to oral and systemic diseases. Dental caries, periodontal and mucosal diseases, oral cancer, peri-implantitis and gastrointestinal-, nervous-, endocrine-, immune- and cardiovascular-related conditions, are some of those which can be influenced by the disruption of the equilibrium within the microbiota.[2,3.]

The American Dental Association (ADA) recommends brushing teeth twice daily, for two minutes, using fluoride toothpaste. This practice has been proven to significantly remove plaque and enhance fluoride levels in both saliva and biofilm fluid, thereby promoting tooth remineralization and reducing the risk of cavities, and other oral and systemic diseases. While various brushing techniques exist, the ADA recommends the use of a technique that relies on placing the toothbrush at a 45-degree angle against the gumline and employing gentle back-and-forth strokes.[5] It is important to clean all tooth surfaces and to use a soft-bristled toothbrush with gentle pressure to minimize the risk of gum injury.[6-11]

Considering that toothbrushing is the primary method for plaque control, a toothbrush should effectively remove adherent biofilms and food debris to reduce tooth decay.[12] Additionally, it must be microbiologically safe, considering toothbrushes are often stored in environments that could harbour bacteria, fungi, or yeast's growth, making them potential reservoirs for pathogenic microorganisms.[1,13]

There are many distinct types of toothbrushes available on the market, including: manual, manual with replaceable head, electric and sonic toothbrushes. These differ in materials, impacting brushing technique.[10-15] Handles can be made from plastic, recycled plastic, bioplastic, beech wood and bamboo, while, bristles may be composed of nylon, charcoal, and infused with castor oil, chlorhexidine, among others.[12,15-17] Despite the differences in design and materials, all toothbrushes share the common goal of removing adherent plaque and food debris from the teeth, promoting oral hygiene.[5]

In the Dental Medicine field, there is a noticeable shift towards more using sustainable healthcare products. This includes the introduction of manual toothbrushes with handles crafted from bamboo, bioplastic, or recycled plastic, often paired with natural bristles.[18] This transformative trend reflects a conscientious effort to harmonize healthcare practices with eco-friendly alternatives. Furthermore, Goal number 12 of the 2030 Agenda for Sustainable Development emphasizes the importance of incorporating environmental considerations into healthcare decisions. It advocates for healthcare professionals to consider the environmental impacts of medical devices when recommending products to patients. This goal serves also as a global call to action, setting ambitious targets to ensure sustainable consumption and production patterns, further highlighting the critical role of sustainability in healthcare.[18,19]

These toothbrushes offer compelling advantages, including reduced carbon footprint and mitigated plastic waste accumulation in landfills and oceans, nevertheless, little is known about their performance in the oral cavity. Despite their commendable environmental credentials, challenges persist, including higher initial costs, limited market visibility, and uncertainties regarding durability and efficacy. [18,22]

While prioritizing eco-friendliness in dental products is crucial, it alone may not be sufficient to ensure optimal human health; these products must also guarantee safety and efficacy. Currently, there is a lack of scientific evidence supporting the effectiveness of eco-friendly toothbrushes in removing plaque and ensuring microbiological safety, unlike their electric toothbrushes or traditional manual counterparts.[16] It's important to address this gap as consumer trust in eco-friendly products relies on scientific validation of their efficacy and safety.

Although the benefits of using a toothbrush are numerous, it is necessary to address the potential contamination of toothbrushes between their uses, which can compromise their microbiological safety. In cariology, researchers have found evidence of intraoral bacterial transmission. Studies have shown that bacteria like *Streptococcus mutans* and periodontopathogens can spread between teeth or via dental instruments and hygiene aids. This transmission poses risks such as the development of dental caries and periodontal disease. Furthermore, the potential transfer of resistant strains could worsen antimicrobial resistance and affect treatment outcomes.[1-3,6] These findings underscore the importance of infection control measures in oral healthcare settings.

According to the literature review conducted for the development of the present study, no studies were found that evaluate ecological toothbrushes regarding their microbial contamination. Contrastingly, several studies have been developed into the assessment of the microbial contamination of traditional toothbrushes, clarifying on crucial aspects of oral hygiene. For instance, Goldsmith RN *et al.* (2007) suggested that different toothbrush characteristics influence *S. mutans* adhesion, emphasizing the need for further research to develop evidence-based guidelines for toothbrush replacement.[20] Subsequently, Karibasappa GN *et al.* (2011) carried out an investigation focusing on the microbial status of manual toothbrushes, focusing on the accumulation of hard deposits between bristle tufts, after 1 and 3 months of use. The identification of various microorganisms, including *S. mutans* and *S. aureus*, raised concerns about the potential for toothbrushes to act as a reservoir for microbial growth, impacting both oral and systemic health.[1] Kim JH *et al.* (2018) extended this exploration, assessing manual toothbrushes contamination by analysing the microbial effects of use duration, frequency, and storage. Their findings

emphasized the importance of proper toothbrush care, recommending disinfection measures such as 1% vinegar for bacterial control and a 1% antimicrobial mouth rinse for optimal reduction of *S. aureus*.[4] Nascimento CD *et al.* (2012) examined bacterial contamination on new toothbrushes before oral use, revealing microbial growth in 47.5% of samples, with the highest prevalence in market-acquired toothbrushes. Notably, chlorhexidine-coated bristles remained uncontaminated, emphasizing the necessity of toothbrush hygiene even before oral exposure.[14] On the other hand, Quirynen M *et al.* (2003) studied toothbrush contamination and the efficacy of toothpastes and coatings in a study involving sixteen volunteers. Results showed that bactericidal coatings were ineffective in reducing bacterial contamination. Notable bacteria included Staphylococci, fungi, Pseudomonas, and others, emphasizing the critical need for rigorous toothbrush hygiene practices.[15] Research also extended to studies with fungi, such as the investigation conducted by Nascimento AP *et al.* (2010), which aimed to evaluate toothbrush contamination by Candida *spp*. in vivo. Further, the efficacy of Periogard® and Neem Sattiva® were assessed as toothbrush disinfection methodologies. The study found contamination of toothbrushes by Candida *spp*. in all tested groups.[13] These collective studies stress the critical role of robust hygiene practices to prevent a wide range of microbial contaminations on toothbrushes, which can have a significant impact for both oral and general health outcomes.[12]

Considering the high rate of microbial contamination of toothbrushes, particularly within the oral environment, where conditions such as dental caries, gingivitis and stomatitis can originate, it is extremely important to extend these investigations to the more recent ecological toothbrushes. Conducting a comparative analysis with conventional toothbrushes regarding microorganisms' adhesion is thus essential to evaluate their biosafety.

Thus, this study aims to analyse and compare, *in vitro*, the microbial adhesion response to both ecological and conventional toothbrushes when exposed to oral-related microorganisms. This research will provide valuable insights into the microbial dynamics on the surfaces of these toothbrushes, evaluating their potential to harbor harmful bacteria. By understanding these dynamics, the study will assess the biosafety of ecological toothbrushes

compared to conventional ones, ultimately presenting translational data to guide consumers and healthcare professionals in making informed decisions about oral hygiene products.

CHAPTER II

MATERIALS AND METHODS

II. MATERIALS AND METHODS

Toothbrush selection

For the selection of ecological toothbrushes, a survey was conducted on the brands that offered these toothbrushes in supermarkets and pharmacies in Portugal. For the control toothbrush, one of the widely used supermarket brand toothbrushes in the country was selected.

Four types of manual toothbrushes were used in this study as follows:

- Group CONT (**Figure 1A**): Colgate® Extra Clean™ (Colgate-Palmolive Company, New York, USA), featuring a plastic handle with a fixed head and plastic bristles.
- Group ELGY (**Figure 1B**): ELGYDIUM Eco (ELGY) (Pierre Fabre Oral Care, France), crafted from European beech wood handle with 100% natural bristles made from a bio-based nylon, which is derived from castor oil.
- Group CURA (**Figure 1C**): Curaprox CS 5460 Wood (CURA) (Curaden AG, Kriens, Switzerland), crafted from Swiss beech wood with ultra-soft bristles composed of CUREN® filaments (polyester filament).
- Group CO.P (**Figure 1D**): Colgate® Bamboo Charcoal Soft Toothbrush (Colgate-Palmolive Company, New York, USA), crafted from sustainable bamboo with nylon-derived charcoal-infused bristles.

Figure 1: Manual toothbrushes used in the study. **A**: Group CONT; **B:** Group ELGY; **C:** Group CURA; **D:** Group CO.P.

Preparation of bristles samples

Tufts were randomly collected from the head of each toothbrush using a sterile haemostat and a No. 11 scalpel blade, under sterile conditions. The haemostat was positioned at the junction between the end of the tuft and its attachment point to the brush head, and the scalpel blade was used to sever the bristles. Each tuft was transferred to an Eppendorf tube.

Morphological Characterization

The bristles of each manual toothbrush were fixed to sample holders using double-sided carbon tape, without any additional manipulation. Subsequently, they were sputter-coated (SPI-Module) with a conductive gold-palladium film and visualized by scanning electron microscopy (SEM), followed by EDS analysis in a FEI Quanta 400 FEG ESEM/EDAX Genesis X4M equipment (Fei Company, Hillsboro, OR, USA).

Microorganisms and growth conditions

The microorganisms used in this study included *Staphylococcus aureus* ATCC 25923*, Streptococcus mutans* DSM 20523*, Escherichia coli* ATCC 25922, *Enterococcus faecalis* ATCC 29212, and *Candida albicans* ATCC 10231. *S. aureus*, *E. coli*, and *E. faecalis* were cultured in Brain Heart Infusion (BHI, Liofilchem) medium, while *S. mutans* was cultured in BHI broth and Blood Agar (Liofilchem), and *C. albicans* was cultured in Sabouraud Dextrose (Liofilchem). All culture media were prepared following manufacturer instructions and sterilized prior to use. Incubation of all microbial cultures was conducted at 37 ºC. A fresh culture suspension was prepared from frozen stock for each experimental trial.

Assessment of Microbial Adhesion

Microbial suspensions were prepared for each strain, in the corresponding growth media, at a concentration of 10^8 colony-forming units (CFUs)/mL. These suspensions were then incubated with individual toothbrush tufts for 2 h at 37 ºC and 180 rpm (Cole-Parmer, Vernon Hills, IL, USA), to allow microbial adhesion. Following incubation, microbial adherence was quantified through CFUs counting and visualized using SEM, as described below.

Colony forming units counting

After the adhesion period, the microbial suspension was removed, and the tufts underwent two rinses with 0.9% NaCl solution (Biochem). Subsequently, the tufts were transferred to new Eppendorf tubes containing 0.5 mL of 0.9% NaCl and subjected to sonication for 10 min in an ultrasonic bath (Bandelin RK 100), to detach adhered microorganisms. Following this process, serial dilutions of the sonicated solutions were prepared and inoculated onto appropriate culture media specific to each microbial strain. After 24 h of incubation at 37 °C, CFUs were enumerated, and the CFUs/mL was calculated.

Scanning electron microscopy

After incubation, the microbial suspension was removed, and the tufts were subjected to two rinses with a 0.9% NaCl solution. Subsequently, the tufts with adhered microorganisms were fixed for 30 min with 3% glutaraldehyde solution (Sigma-Aldrich) and then slowly dehydrated in a gradient ethanol series for 10 min each. The tufts were fixed to sample holders using double-sided carbon tape and sputter-coated with a conductive gold–palladium film. Microorganisms adhered to the tufts were imaged by SEM, as previously described.

Statistical analysis

The data are presented as mean ± standard deviation. For statistical analysis, one-way analysis of variance (ANOVA) with post-hoc Tukey HSD was used, with a significance level set at $p < 0.05$. All analysis were conducted using IBM SPSS Statistics software (Version 29 .0.0.0 (241), 2022).

CHAPTER III

RESULTS

III. RESULTS

Bristles characterization

The bristles of the toothbrushes were composed of different materials as it follows: Group CONT - high-quality nylon (0.178 mm); Group ELGY - 100% natural bristles made from a bio-based nylon, which is derived from castor oil. (0.15 mm); Group CURA - ultra-soft bristles composed of CUREN® filaments (polyester filament) (0.1 mm); Group CO.P - nylon-derived charcoal-infused bristles (0.1 mm) (**Figure 2**). The average diameter of the bristles was approximately 0.1345 mm, determined by each brand's supplier, providing a smooth and comfortable texture during brushing.

SEM visualization revealed that the bristles of all toothbrushes had smooth surface (**Figure 2**). Bristles from Group CO.P displayed some nano-topographical spots, which did not affect the straight pattern of the bristles (**Figure 2D**).

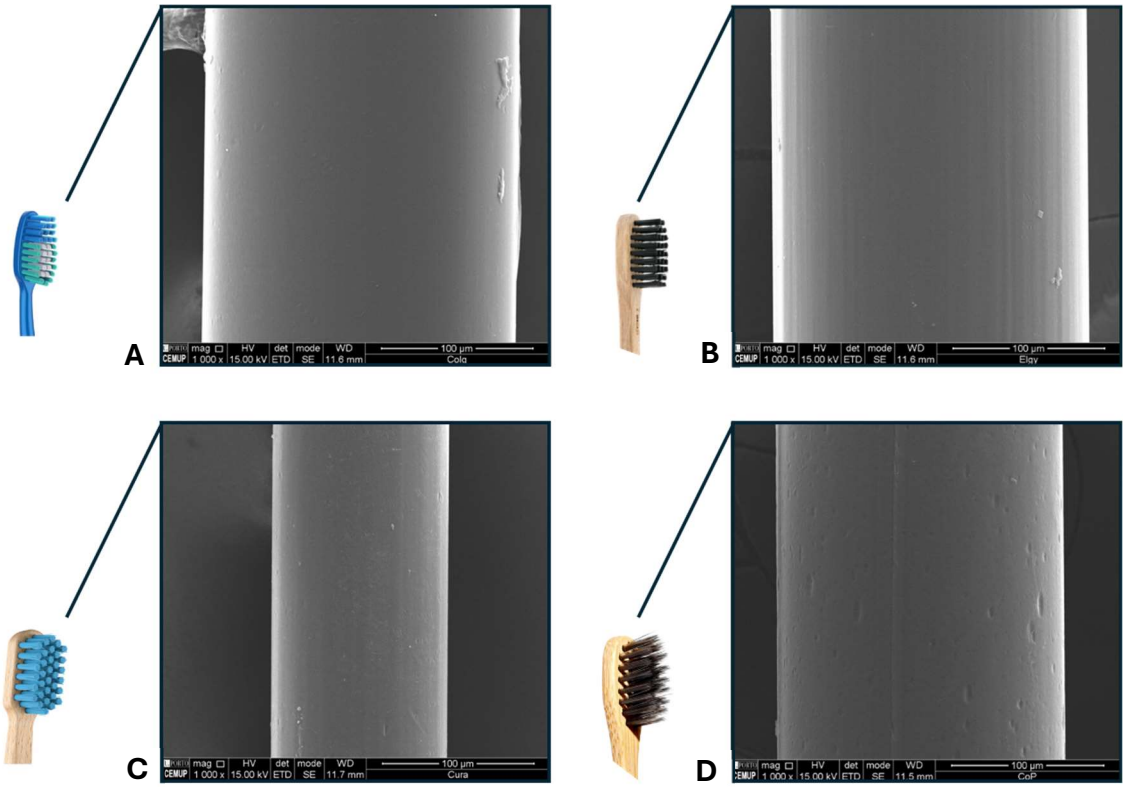

Figure 2: Toothbrush bristles used in the study and bristles imaged by SEM. **A**: Group CONT; **B:** Group ELGY; **C:** Group CURA; **D:** Group CO.P.

In combination with SEM, Energy Dispersive Spectroscopy (EDS) microanalytical technique was used to identify and quantify the elements present in all samples. Carbon (C) and oxygen (O) were identified as the primary elements of the bristles, with variations with their quantities, which is in line with the reported information by the manufacturers, supporting the polymeric nature of the bristles (**Figure 3**).

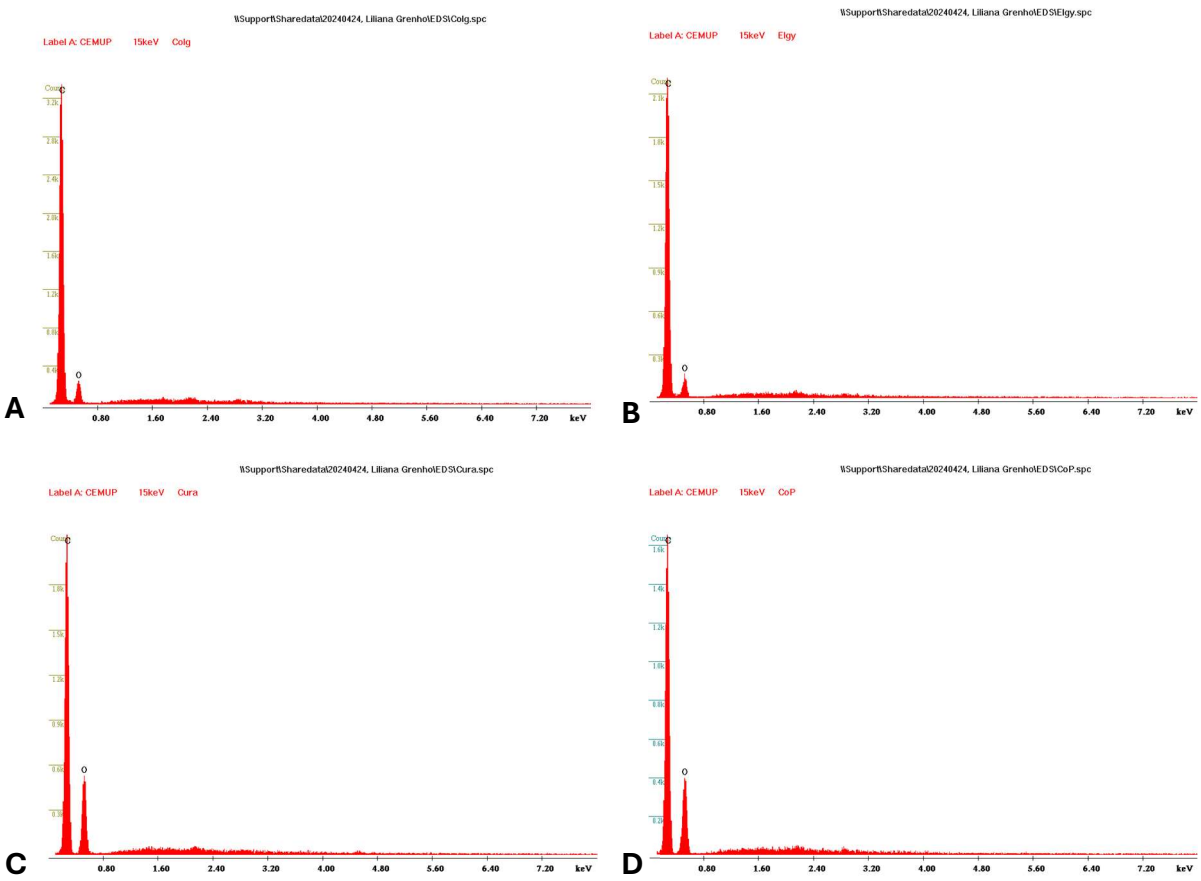

Figure 3: EDS spectra. **A**: Group CONT; **B:** Group ELGY; **C:** Group CURA; **D:** Group CO.P.

Microbial adhesion

In vitro experiments were conducted to evaluate the adhesion of distinct microorganisms to the toothbrush bristles, specifically *S. mutans, S. aureus, C. albicans, E. coli* and *E. faecalis*. The cell adhesion profile was initially determined through quantification of the sessile population by cultivable cells/CFUs method. The results are presented in **Figure 4**.

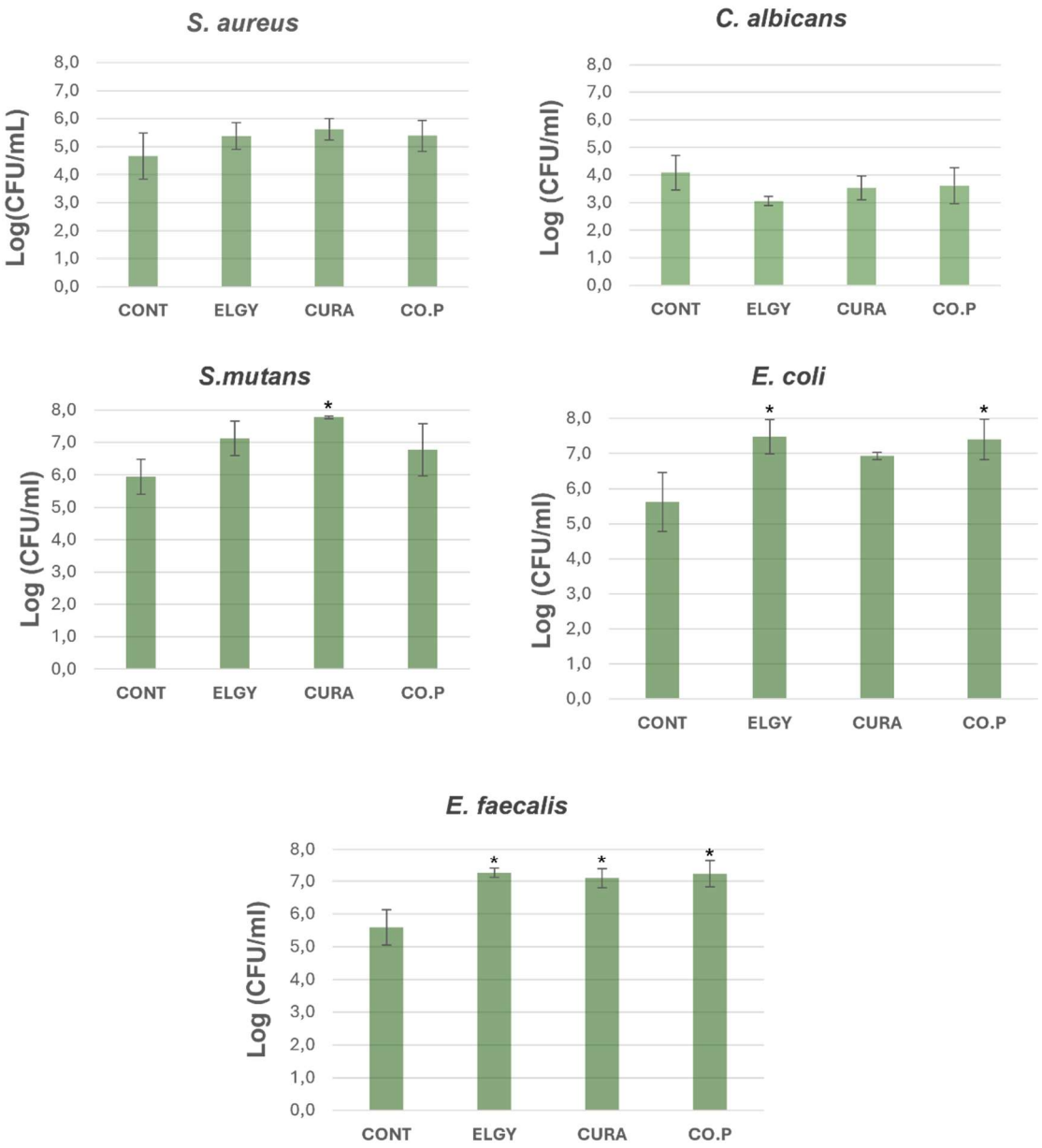

Figure 4: Quantification of adhered microrganisms - *S. aureus*, *C. albicans, S. mutans, E. coli,* and *E. faecalis*, after 2 h of incubation, with the different bristles, using the CFUs method. *Significantly different from Group CONT, $p < 0.05$.

Regarding *S. aureus* (1) and *C. albicans* (2), no statistically significant differences were observed between the groups analyzed: 1 - ($p = 0.286$) and 2 – ($p = 0.133$), indicating that these microorganisms exhibited a similar adhesion profile across all bristles assessed. However, it is noteworthy that *C. albicans* showed a slightly lower tendency of adhesion to the ecological bristles as compared to the Group CONT.

In case of *S. mutans*, the Group CURA exhibited a significantly higher count of adhered bacteria compared to the Group CONT ($p = 0.015$). However, no significant differences were observed among the other groups compared to the Group CONT.

E. coli exhibited significantly higher adhesion to Group ELGY ($p = 0.020$) and Group CO.P ($p = 0.024$) compared to Group CONT, while no differences were denoted between Group CURA and Group CONT.

Lastly, for *E. faecalis*, Group CONT displayed significantly lower counts than all other groups: ELGY ($p = 0.002$), CURA ($p = 0.004$), and CO.P ($p = 0.002$).

The bristles exposed to *S. aureus* were further analysed using SEM. It was observed that the bacteria adhered pointwise along the bristle surface, displaying their typical morphology of Gram-positive cocci arranged in small clusters (**Figure 5**).

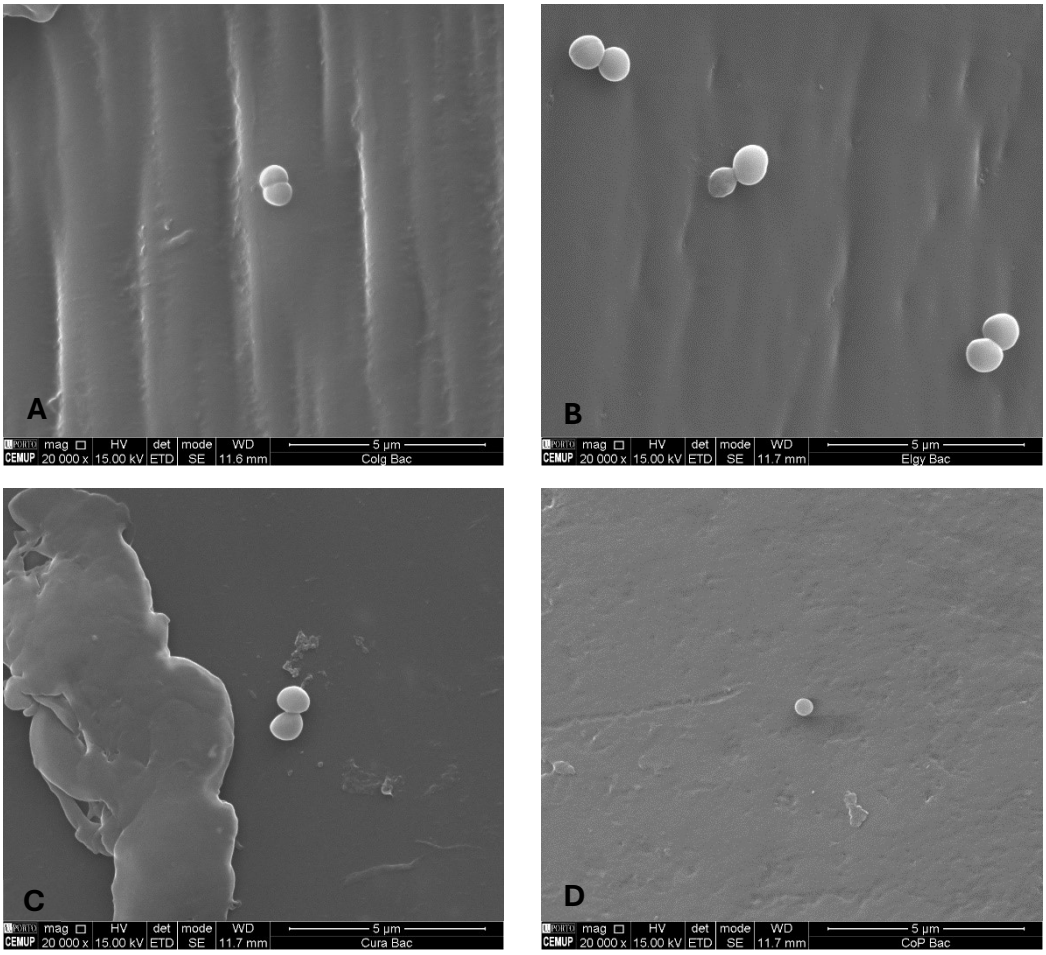

Figure 5: Representative micrographs of *S. aureus* attached to the bristles. **A:** Group CONT; **B:** Group ELGY; **C:** Group CURA; **D:** Group CO.P.

CHAPTER IV

DISCUSSION

IV. DISCUSSION

Research has consistently shown that oral hygiene products harbour a wide range of microorganisms, including bacteria, fungi, and yeast, as they are often stored in environments conducive to microbial growth. The microorganisms can potentially serve as a potential reservoir for pathogenic bacteria.[4,13,14,16] Based on a review of the literature, there has been a lack of studies that examine the adhesion of microorganisms to ecologic toothbrushes compared to conventional ones. Thus, the present study is the first to address this gap.

The present study's methodology was developed based on the techniques implemented by Goldsmith et al. (2007), enhancing and expanding it to include a broader range of microorganisms, such as *S. aureus, C. albicans, E. coli,* and *E. faecalis*, in addition to *S. mutans.* This comprehensive list of microorganisms provides a more detailed analysis of microbial adhesion. Like the previous study, the methodology utilized controlled incubation conditions; therefore, the results from both methods are reproducible and comparable.[20] This methodology combined CFUs and SEM for a detailed and visually illustrative representation of microbial interactions with the bristles substrates. Furthermore, the study also differentiated between adhesion on ecological and regular toothbrushes, addressing environmental and overall public health concerns. This methodological adaptation allowed for a more complete and relevant evaluation of the most common toothbrush brands, available in the supermarkets and pharmacies in Portugal - where the study was conducted; significantly contributing to the knowledge in the field of oral hygiene.

Microorganisms in the oral cavity may be involved in the development of various diseases and complications. For instance, *S. aureus* can cause stomatitis and dental abscesses, and may spread throughout the body and cause toxic shock syndrome.[24] *C. albicans* can cause the development of oral candidiasis in the form of the appearance of white formations in the oral cavity, often accompanied by pain and burning.[25] *E. coli*, typically associated with gastro-intestinal infections, can contribute to the formation of ulcers and inflammation of the gums.[26] *E. faecalis* is known to be the causative agent of odontogenic

infections in the form of a periapical abscesses.[27] *S. mutans*, responsible for cavity formation, can harm the teeth causing cavities and subsequent spread of the bacteria and other infections.[28] These pathogens' ability to cause significant oral and systemic health issues underscores the importance of analysing their adhesion to toothbrush bristles, which can act as reservoirs for these microorganisms.

In the scientific research, the methodologies employed can significantly influence the outcomes and data comparative assessment. As compared to Goldsmith et al. (2007), this study incorporates minor improvements that underscore the importance of precision in research.[20] This study delves deeper by focusing on a wider variety of microorganisms. In addition, the experiment was conducted in a regulated laboratory setting, with brief agitation of the bristles in a warm solution to assess microbial adhesion. State-of-the-art imaging techniques such as scanning electron microscopy (SEM) were used to gain a comprehensive understanding of microbial adhesion. Additionally, the traditional and effective method of quantifying microbial amounts using agar plates was employed.

The study hypothesis proposed that toothbrush groups, distinguished by company brand, bristle type, and shape, would influence the adhesion of *S. aureus, C. albicans, S. mutans, E. coli,* and *E. faecalis* to both eco-friendly and conventional toothbrushes, as measured by the number of recoverable microorganisms. The findings support the hypothesis, indicating that after 2 hours of culture, all the four toothbrush groups harbour varying CFU counts. These differences in microorganism adhesion can be attributed to several factors, including variations in bristle material, bristle tufting, bristle length, and the number of bristles per tuft. Such factors influence the surface area and texture of the toothbrush bristles, which in turn affect the extent of microbial adhesion. Furthermore, the outcomes indicate that the CURA and ELGY bristles were more prone to adhesion by these Gram-negative bacteria. In addition to that, the CURA bristles were found to be more susceptible to adhesion by *S. mutans*, while all ecological bristles were more susceptible to *E. faecalis* adhesion.

The study encountered several practical limitations that could have contributed to variability in the data. One notable issue was the variability in the number of bristles per tuft. These varied between brands, and during the cutting process, which is not always precise, leading to some bristles spreading out and resulting in differing numbers per sample. Additionally, the bristle length was also affected by the same reasons, contributing to inconsistencies in samples. Another limitation involved the selection of times points for microbial adhesion assessment.

While no studies in the literature directly compare data with ecological toothbrushes, some existing research provides potential points of comparison. In the context of toothbrush microbial contamination, distinct yet complementary findings emerge from the comparison of the current study and the research conducted by Karibasappa et al. (2011), which demonstrated that toothbrushes stored in bathrooms with attached toilets exhibited higher contaminations levels, including potentially harmful bacteria such as *S. aureus* and *E. coli*. In that study, as already mentioned, it was emphasized that proper storage and regular replacement of toothbrushes is crucial to reduce health risks.[1] Goldsmith et al. (2007) further revealed the influence of the bristles wear time on the growth of *S. mutans*[19], which is also relevant to understanding how different toothbrush designs and materials impact microbial adherence. These complementary findings underscore the importance of both proper toothbrush maintenance and the selection of effective bristle types. Together, they offer a comprehensive framework for enhancing toothbrush hygiene and reducing microbial contamination.

On another study, Nascimento CD et al. (2012) focused on microbial contamination on toothbrushes, similar to this current study, the research interests and findings are markedly different. Nascimento et al. investigated microbial inoculum levels on toothbrush bristles in vitro after 21 days of purchase, considering various purchasing environments such as industry, drugstore, market, and perfumery. Their results indicated bacterial growth and gram-positive sporulating bacilli in 47.5% of toothbrushes not coated with chlorhexidine.[14]

Microbial contamination on toothbrushes was also the focus of a review by Kim JH et al. (2018). This study examined how the duration of toothbrush use, frequency of use, storage locations, and sterilization methods impact microbial presence. The findings indicated that microbes thrive in environments with increased use duration and higher relative humidity. Furthermore, the study found that sterilization methods such as soaking in vinegar or using a mouthwash effectively reduced microbial contamination.[4] This underscores the importance of not only toothbrush design but also proper maintenance and storage practices to minimize microbial growth and enhance oral hygiene.

According to the present results and the comparison with existing studies, it is evident that as research in this field expands, our scientific understanding deepens. This research explored a wider range of microorganisms and analysed their adhesion to different types of toothbrush bristles. However, further research is necessary to explore the interactions between the materials composing toothbrushes and the oral environment or the cells of the oral cavity, as these materials may release substances that affect these cells, evidencing the need of further compatibility and biocompatibility studies. Additionally, mechanical testing of the bristles is essential to analyse their rigidity and durability. These factors can influence the efficiency of toothbrushes in maintaining oral hygiene and reducing microbial adhesion. Future studies could also examine the impact of different brushing habits and storage conditions on microbial contamination. By doing so, we can develop comprehensive strategies to improve toothbrush hygiene and ultimately enhance oral health. Thus, ongoing research in this field is crucial for uncovering new insights and developing innovative solutions to minimize microbial contamination on toothbrushes.

CHAPTER V

CONCLUSION

V. CONCLUSION

This study aimed to analyse and compare, *in vitro*, the microbial adhesion response to eco-friendly and conventional toothbrushes in contact with microorganisms related to oral health. The findings indicate that there were no statistically significant differences in the adhesion of *S. aureus* and *C. albicans* across all bristle groups, though *C. albicans* showed a slightly lower tendency to adhere to eco-friendly bristles compared to the conventional Group CONT.

Significant differences were observed for *S. mutans*, with the eco-friendly Group CURA exhibiting a higher count of adhered bacteria compared to the conventional Group CONT, indicating greater susceptibility. For *E. coli*, significantly higher adhesion was noted on the eco-friendly Group ELGY and Group CURA bristles compared to Group CONT. In the case of *E. faecalis*, the conventional Group CONT displayed significantly lower adhesion counts than all eco-friendly groups, suggesting that the ecological bristles were more prone to bacterial adherence.

Further SEM analysis of *S. aureus* adhesion revealed pointwise bacterial attachment along the bristle surfaces, characteristic of Gram-positive cocci in small clusters.

These results underscore the varying microbial adhesion properties of different toothbrush bristles. While some eco-friendly bristles exhibited higher microbial adhesion for certain bacteria, the conventional bristles generally showed lower adhesion, particularly against *E. faecalis*. This study highlights the importance of material selection in toothbrush design to optimize oral hygiene and suggests that while eco-friendly materials offer environmental benefits, their microbial adhesion properties require further investigation to ensure they meet health standards.

VI. REFERENCES

1. Karibasappa GN, Nagesh L, Sujatha BK. Assessment of microbial contamination of toothbrush head: an *in vitro* study. Indian J Dent Res. 2011 Jan-Feb;22(1):2-5. doi: 10.4103/0970-9290.79965. PMID: 21623099.

2. Gao L, Xu T, Huang G, Jiang S, Gu Y, Chen F. Oral microbiomes: more and more importance in oral cavity and whole body. Protein Cell. 2018 May;9(5):488-500. doi: 10.1007/s13238-018-0548-1. Epub 2018 May 7. PMID: 29736705; PMCID: PMC5960472.

3. Zhang Y, Wang X, Li H, Ni C, Du Z, Yan F. Human oral microbiota and its modulation for oral health. Biomed Pharmacother. 2018 Mar; 99:883-893. doi: 10.1016/j.biopha.2018.01.146. Epub 2018 Feb 20. PMID: 29710488.

4. Kim JH, Kim DA, Kim HS, Baik JY, Ju SH, Kim SH. Analysis of microbial contamination and antibacterial effect associated with toothbrushes. J Dent Hyg Sci. 2018; 18:296-304.

5. American Dental Association. Toothbrushes [Internet]. Chicago: American Dental Association; c2019 [cited 2024 May 18]. Available from: https://www.ada.org/en/member-center/oral-health topics/toothbrushes

6. Arweiler NB, Netuschil L. The Oral Microbiota. Adv Exp Med Biol. 2016; 902:45-60. doi: 10.1007/978-3-319-31248-4_4. PMID: 27161350

7. Creeth J, Gallagher A, Sowinski J, *et al.* The effect of brushing time and dentifrice on dental plaque removal in vivo. J Dent Hyg. 2009 Summer;83(3):111-16.

8. Newby EE, Martinez-Mier EA, Zero DT, *et al.* A randomised clinical study to evaluate the effect of brushing duration on fluoride levels in dental

biofilm fluid and saliva in children aged 4-5 years. Int Dent J. 2013;63 Suppl 2:39-47.

9. Darby ML, Walsh MM. Dental Hygiene: Theory and Practice. St. Louis: Saunders; 2010.

10. Cifcibasi E, Koyuncuoglu CZ, Baser U, *et al.* Comparison of manual toothbrushes with different bristle designs in terms of cleaning efficacy and potential role on gingival recession. Eur J Dent. 2014;8(3):395-401.

11. Zanatta FB, Bergoli AD, Werle SB, Antoniazzi RP. Biofilm removal and gingival abrasion with medium and soft toothbrushes. Oral Health Prev Dent. 2011;9(2):177-83.

12. Food and Drug Administration. Title 21, Chapter I—Food and Drug Administration, Department of Health and Human Services, 21 CFR Ch. I (4–1–22 Edition).

13. Nascimento AP, Watanabe E, Ito IY. Toothbrush contamination by Candida spp. and efficacy of mouthrinse spray for their disinfection. Mycopathologia. 2010;169(2):133-8.

14. Nascimento CD, Scarabel TT, Miani PK, Watanabe E, Pedrazzi V. *In vitro* evaluation of the microbial contamination on new toothbrushes: a preliminary study. Microsc Res Tech. 2012 Jan;75(1):42-5.

15. Quirynen M, De Soete M, Pauwels M, Gizani S, Van Meerbeek B, van Steenberghe D. Can toothpaste or a toothbrush with antibacterial tufts prevent toothbrush contamination? J Periodontol. 2003 Mar;74(3):312-22.

16. Hope CK, Petrie A, Wilson M. *In vitro* assessment of the plaque-removing ability of hydrodynamic shear forces produced beyond the bristles by 2 electric toothbrushes. J Periodontol. 2003 Jul;74(7):1017-22.

17. Noremylia MB, Aufa AN, Ismail Z, Hassan MZ. An Overview of the Potential Usage of Bamboo Plants in the Medical Field. In: Smith J,

Johnson A, editors. Advances in Medical Botany. New York: Academic Press; 2023. p. 45-60.

18. Duane B, Ashley P, Saget S, Richards D, Pasdeki-Clewer E, Lyne A. Incorporating sustainability into assessment of oral health interventions. Br Dent J. 2020 Sep;229(5):310-314. doi: 10.1038/s41415-020-1993-9. PMID: 32918024.

19. The 2030 Agenda for Sustainable Development. United Nations, 2015. Available from: https://sdgs.un.org/topics/sustainable-consumption-and-production. Access on 20/10/2023.

20. Goldsmith RN, Shey Z, Houpt MI, Fine D, Schreiner H, Greenberg B. Toothbrush bristle wear and adherence of *Streptococcus mutans*. Pediatr Dent. 2007 May-Jun;29(3):243-7. PMID: 17688023.

21. Ikawa T, Mizutani K, Sudo T, Kano C, Ikeda Y, Akizuki T, Kobayashi H, Izumi Y, Iwata T. Clinical comparison of an electric-powered ionic toothbrush and a manual toothbrush in plaque reduction: A randomized clinical trial. Int J Dent Hyg. 2021 Feb;19(1):93-98. doi: 10.1111/idh.12475. Epub 2020 Nov 1. PMID: 33029896.

22. Lyne A, Ashley P, Saget S, *et al.* Combining evidence-based healthcare with environmental sustainability: using the toothbrush as a model. Br Dent J. 2020;229(5):303–309.

23. van der Mei HC, Rustema-Abbing M, Bruinsma GM, Gottenbos B, Busscher HJ. Sequence of oral bacterial co-adhesion and non-contact brushing. J Dent Res. 2007 May;86(5):421-5. doi: 10.1177/154405910708600506. PMID: 17452561.

24. Marsh PD. Dental plaque as a biofilm and a microbial community - implications for health and disease. BMC Oral Health. 2006;6(Suppl 1):S14.

25. Samaranayake LP, MacFarlane TW. Oral candidosis. Clinics in Dermatology. 2000;18(5):553-562.

26. Gendron R, Grenier D, Maheu-Robert L. The oral cavity as a reservoir of bacterial pathogens for focal infections. Microbes and Infection. 2000;2(8):897-906.

27. Hajishengallis E, Parsaei Y, Klein MI, Koo H. Advances in the microbial etiology and pathogenesis of early childhood caries. Molecular Oral Microbiology. 2017;32(1):24-34.

28. Slots J, Rams TE. Antibiotics in periodontal therapy: advantages and disadvantages. Journal of Clinical Periodontology. 1998;25(3):133-138.v

CHAPTER VI

ANNEXES