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CARACTERIZAÇÃO DO MICROBIOMA SALIVAR EM INDIVÍDUOS INSTITUCIONALIZADOS ANTES E APÓS O TRATAMENTO DA DOENÇA CÁRIE

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Brasília, 15 de Julho de 2022.

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Tese apresentada ao Programa de Pós-Graduação em Odontologia da Faculdade de Ciências da Saúde da Universidade de Brasília como requisito parcial à obtenção do título de Doutor em Odontologia.

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Tese aprovada, como requisito parcial para obtenção do grau de Doutor em Odontologia, Programa de Pós-Graduação em Odontologia da Faculdade de Ciências da Saúde da Universidade de Brasília.

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"Entregue suas preocupações ao Senhor, e ele o susterá."

Salmos 55:22

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RESUMO

A doença cárie é resultado de um desequilíbrio do microbioma oral desencadeado pelo consumo de açúcar. Este desequilíbrio microbiológico pode ser confirmado através de estudos de sequenciamento genético bacteriano. O objetivo deste estudo foi avaliar a ocorrência de cárie em dois grupos de pessoas atendidas pelo Instituto Dom Orione em Brasília, correlacionar com o índice de placa e avaliar a resposta ao tratamento realizado, além de avaliar o efeito do tratamento na composição da microbiota salivar dos participantes. Dois grupos não paralelos com dificuldades de higiene bucal, pois compreendem pessoas com deficiências intelectuais e crianças, participaram deste estudo longitudinal. Os participantes foram avaliados clinicamente antes do tratamento, 1 mês e 6 meses após o tratamento, períodos em que foram também coletadas amostras salivares para análise microbiológica. Tratamento restaurador atraumático foi utilizado para tratamento de lesões cavitadas (quando indicado). Todos os pacientes cárie-ativos receberam tratamento não-invasivo (instrução de dieta, de higiene bucal e aplicações tópicas de flúor). 56.25% e 65.7% dos indivíduos do grupo Residente e Visitante apresentaram doença cárie ativa, respectivamente. O sequenciamento do gene 16S rRNA revelou maior abundância dos filos *Firmicutes* (60.91%), *Actinobacteria* (12.07%) e *Proteobacteria* (10.95%) em todas as amostras. Após o tratamento, somente foram observadas alterações relevantes no microbioma após 6 meses em situação de saúde. Foi observada redução dos níveis de lactobacilos, provavelmente refletindo a realização das restaurações de cavidades. Este resultado mostra a importância de alterações na dieta e higiene a longo prazo para que haja reversão da disbiose.

Palavras-chave: Cárie Dentária; Saliva; Microbioma; Disbiose.

ABSTRACT

Dental caries is the result of an imbalance of oral microbiome triggered by sugar intake. This microbiological imbalance can be confirmed by genetic sequencing studies. The aim of this study was to assess the occurrence of dental caries in two groups of people attended by Dom Orione Institute in Brasilia, correlate it with the visual plaque index and assess the response to treatment, in addition to assessing the effect of the treatment in the salivary microbial composition of the participants. Two non-parallel groups with poor oral hygiene, as they include people with intellectual disabilities and children, participated in this longitudinal study. The participants were clinically assessed at baseline, 1 month and 6 months after treatment, when salivary samples were also collected for the microbiological analysis. Atraumatic restorative treatment was used to treat cavities (when needed). All caries-active participants received non-invasive treatment (diet instruction, oral hygiene instruction and topic fluoride applications). 56.25% and 65.7% of individuals from group Residents and Visitors were caries-active, respectively. 16S rRNA sequencing showed a higher relative abundance of *Firmicutes* (60.91%), *Actinobacteria* (12.07%) and *Proteobacteria* (10.95%) in all samples. After treatment, relevant changes in the microbiome were only seen after 6 months in health. There was a reduction in lactobacilli, probably reflecting the restoration of cavities. This result shows the importance of long-term diet and hygiene shift to see the reversion of dysbiosis.

Keywords: Dental Caries; Saliva; Microbiome; Dysbiosis.

SUMÁRIO

1 INTRODUÇÃO, REVISÃO DA LITERATURA E OBJETIVOS

1.1 INTRODUÇÃO

A cárie dentária, doença modulada pelos hábitos alimentares, mediada pelo biofilme dental e considerada um processo dinâmico, resulta na perda de minerais dos tecidos dentários [Machiulskiene et al., 2020], podendo impactar negativamente na qualidade de vida dos indivíduos [Pitts et al., 2017]. Um estudo publicado em 2020 que avaliou a prevalência de doenças bucais de 1990 a 2017, estimou que aproximadamente 2,3 bilhões de pessoas apresentavam dentes permanentes com lesões de cárie não tratadas e 532 milhões de crianças apresentavam lesões de cárie não tratadas em dentes decíduos, sendo a cárie, portanto, considerada ainda hoje uma doença de alta prevalência no mundo [Bernabe et al, 2020].

Pesquisadores ao longo dos anos propuseram diferentes teorias para explicar o processo da doença cárie. Em 1994, Marsh propôs a teoria da placa ecológica [Marsh, 1994], que explicou a relação dinâmica que acontece no biofilme dentário, onde uma mudança no ambiente (pH baixo), causada pelo alto consumo de açúcares fermentáveis gera uma mudança no equilíbrio da microbiota do biofilme, e como consequência acontece a desmineralização da estrutura dental. Sabendo disso, uma medida efetiva para controlar a doença cárie seria a redução do consumo de açúcar ou a utilização de substitutos do açúcar [Marsh, 1994; Marsh and Bradshaw, 1995; Theilade, 1996; Sheiham and James, 2015]. Além disso, a orientação de dieta por um profissional bem-informado sobre a relação dieta-cárie é fator importante no controle da doença. A instrução de dieta acompanhada da mudança de hábitos alimentares já se mostrou efetiva em reduzir a cárie dentária e a sua severidade em crianças [Feldens et al., 2010].

Com base no conhecimento científico que temos sobre a doença cárie e pensando em todas as alternativas para seu tratamento, um consenso de 2018 da European Organization for Caries Research (ORCA) estabeleceu diretrizes para o tratamento da doença em crianças e em adultos [Schwendicke et al., 2020; Splieth et al., 2020]. Nestes consensos fica clara a importância de inativar a doença para o sucesso do tratamento, e não apenas tratar seus sinais clínicos. O tratamento não invasivo que

objetiva inativar a doença e tratar lesões não cavitadas inclui o controle da frequência do consumo de sacarose, hábitos de higiene bucal e uso diário de fluoretos presentes nos dentifrícios. No âmbito do consultório, aplicações tópicas de flúor profissional podem contribuir para a inativação de lesões ativas. Em situações de maior perda mineral do dente, tratamentos invasivos podem ser necessários, como restaurações, tratamento endodôntico ou até mesmo exodontias. Dessa forma, o tratamento ideal deve combinar medidas não-invasivas e invasivas, visando o controle da doença [Schwendicke et al., 2020; Splieth et al., 2020].

No Distrito Federal, há estudos que observaram alta prevalência de cárie em escolares e sua relação com escolaridade; consequência de lesões não tratadas em dentina, que foram moderadas, porém de baixa severidade; entre outros desfechos [de Amorim et al., 2011; Figueiredo et al., 2011; Almeida et al., 2018]. Porém, estudos que avaliam a ocorrência de cárie em grupos com higiene bucal deficiente e o efeito da instrução de dieta associada ao tratamento restaurador na atividade da doença e no microbioma salivar não são conhecidos. Esta avaliação contribui com o conhecimento científico de como estas intervenções combinadas podem influenciar o estado de saúde bucal dos indivíduos, tirando o foco apenas do tratamento restaurador invasivo e dando atenção aos tratamentos não invasivos.

1.2 REVISÃO DA LITERATURA

1.2.1 Dieta e cárie

Antes da revolução industrial que deu início ao consumo de açúcares fermentáveis, observava-se que cárie não era um agravo prevalente. Um exemplo disso foi a observação de uma população isolada na ilha de Tristão da Cunha [Holloway et al., 1962]. Este estudo avaliou a prevalência de cárie na população da ilha e comparou os resultados com um estudo anterior realizado na mesma população antes da segunda guerra mundial, quando a ilha não fazia parte das rotas dos navios e, portanto, sua fonte de alimentos era quase totalmente de produtos cultivados na ilha. Enquanto no estudo anterior a prevalência de cárie era extremamente baixa, não chegando a 15% na faixa etária mais atingida, Holloway et al. encontraram em 1962 uma prevalência de cárie muito maior, atingindo mais de 30% em 4 das 5 faixas etárias avaliadas. Outro exemplo é um estudo que avaliou esqueletos de indivíduos de quatro

sítios arqueológicos no sul da França, em que foi observado menor quantidade de dentes cariados numa população em que o consumo de carboidratos e alimentos cozidos era baixo, e o cultivo de cereais era menos comum comparado às outras populações avaliadas [Grimoud et al., 2011]. De semelhante forma, o estudo de Costa Junior de 1980 concluiu que os baixos índices de dentes cariados observados nos esqueletos de moradores do Alaska foram atribuídos à dieta totalmente desprovida de açúcar refinado e amidos [Costa Junior, 1980]. Já o estudo de Hopewood House mostrou que o tipo de alimentação está diretamente ligado ao desenvolvimento de lesões de cárie, mesmo na ausência de cuidado odontológico ou instrução de higiene bucal [Harris, 1963]. Ainda em 1954, Gustafsson et al. observaram uma relação positiva in vivo de consumo de açúcar e cárie no famoso estudo de Vipeholm [Gustafsson et al., 1954]. Neste estudo, concluiu-se que o consumo de açúcar em maior frequência durante o dia tem relação direta com maiores índices de cárie.

Mais recentemente, revisões como a de Sheiham & James [Sheiham and James, 2015] e Hujoel & Lingström [Hujoel and Lingström, 2017] retomaram essa relação. A exposição ao açúcar pode ser considerada um fator determinante para o desenvolvimento da doença cárie, e sendo esse açúcar a sacarose a cariogenicidade é ainda maior, pois ao metabolizar a sacarose, a bactéria, além de produzir ácido, produz polissacarídeos extracelulares que aumentam a cariogenicidade do biofilme [Dibdin and Shellis, 1988; dos Santos et al., 2002]. Reforçando ainda mais essa relação da sacarose com a cárie, um estudo de 2021 relatou que havia uma maior frequência de consumo de alimentos açucarados em pacientes com experiência de cárie, em comparação a pacientes sem experiência de cárie [Cantoral et al., 2021].

1.2.2 O microbioma bucal na doença cárie

Estudos que avaliaram amostras de biofilme de pacientes com cárie e sem cárie mostraram que em pacientes com cárie, o pH do biofilme permanecia mais baixo por mais tempo após o consumo de carboidratos fermentáveis, enquanto no biofilme de pacientes sem cárie o pH se recuperava mais rapidamente [Edlund et al., 2017]. Este ambiente mais ácido favorece a predominância de microrganismos acidogênicos e acidúricos [Marsh, 1994; Edlund et al., 2017], levando a uma alteração no microbioma bucal, do estado de saúde em equilíbrio (homeostase/eubiose) para uma situação de

desequilíbrio (disbiose) [Radaic and Kapila, 2021]. Apesar de haver uma variação natural entre o microbioma bucal de indivíduos saudáveis [Edlund et al., 2017], quando há uma disbiose normalmente há redução da diversidade microbiana, com redução de microrganismos considerados benéficos à saúde e enriquecimento de microrganismos patobiontes (que podem causar dano ao seu hospedeiro em determinadas circunstâncias) [Petersen and Round, 2014; Jochum and Stecher, 2020].

Quando analisamos estudos que avaliaram a composição da microbiologia salivar ou do biofilme em indivíduos com cárie, há uma diversidade nos resultados encontrados. Normalmente estão mais abundantes bactérias de algumas espécies dos gêneros *Streptococcus*, *Lactobacillus* e Actinomyces [Radaic and Kapila, 2021]. A exemplo destas, a espécie *Streptococcus mutans* já foi amplamente estudada e muitas vezes relacionada à cárie, mas mesmo esta parece não ser necessária para o desenvolvimento da doença, já tendo sido reportado cárie sem a presença desta bactéria [Erickson et al., 2017]. Outros pesquisadores também já estudaram a cariogenicidade do biofilme considerando o potencial acidogênico de outras bactérias além de *S. mutans* [van Houte et al., 1996]. Devemos também considerar a variação microbiológica que pode acontecer nos diferentes estágios da doença. Nos estágios iniciais, durante a evolução das lesões e em estágios mais severos da doença as bactérias mais abundantes podem não ser as mesmas, assim como pode haver variações entre adultos e crianças, o que representa a complexidade do microbioma bucal nas diferentes situações clínicas [Anderson et al., 2018].

Comunidades microbianas são extremamente complexas e sofrem constantes alterações de acordo com variações do meio. Estudos tentam desvendar a forma como o microbioma reage nas diferentes situações clínicas e entender como isso influencia nas doenças bucais. Levando em consideração que até mesmo bactérias presentes em baixa abundância, e também domínios além das bactérias podem ter um papel importante no desenvolvimento das doenças bucais, e dado as limitações que ainda temos nestes estudos [Cena et al., 2021], pesquisas que avaliem a microbiota bucal ainda se fazem necessárias.

1.2.3 Higiene bucal, flúor e a doença cárie

Sabendo que lesões de cárie se desenvolvem nas regiões de acúmulo de biofilme na presença de uma alimentação cariogênica, um dos pilares do tratamento da doença é a instrução de higiene bucal. Um dos objetivos dos procedimentos restauradores em dentes cavitados é, portanto, impedir o acúmulo de biofilme, para que haja inativação e controle da doença [Hilgert et al., 2017]. Em 2017, Hilgert et al. mostraram que a escovação supervisionada foi capaz de reduzir o índice de placa em crianças [Hilgert et al., 2017], porém esse hábito pode não ser mantido (como já foi concluído por outros estudos) [Lindhe and Koch, 1967; Stein et al., 2018] e, portanto, a escovação supervisionada não teria um benefício a longo prazo. A instrução de higiene bucal é uma recomendação que tem potenciais benefícios para a saúde bucal se acompanhada da mudança de hábito do paciente, no entanto, uma meta-análise de ensaios clínicos controlados mostrou que não há evidência de que a higiene oral por si só reduza o risco de cárie, na ausência de fluoretos [Hujoel et al., 2018].

O uso de fluoretos na odontologia surgiu no século 20 com o aumento da prevalência de cárie [Whelton et al., 2019]. Atualmente, tem sido observado que a relação direta entre açúcar e cárie é diminuída com o acesso aos fluoretos [Duggal et al., 2001], devido à sua capacidade de reduzir a perda mineral dos tecidos dentários. Usado como agente coadjuvante no controle da doença cárie, quando o fluoreto está presente na saliva ou no biofilme na sua forma solúvel, ele induz a precipitação de minerais em forma de fluorapatita na estrutura dentária durante a remineralização, o que não reduz a produção de ácidos pelas bactérias ou o acúmulo de biofilme, mas é capaz de reduzir a perda mineral do dente, fazendo com que o tempo necessário para observamos lesões clinicamente seja maior [Tenuta e Cury, 2010]. Diversos estudos publicados na literatura apresentam evidência científica segura dos benefícios do uso de fluoretos no controle da doença cárie [Wong et al., 2011; Bansal et al., 2015; Marinho et al., 2015; Zampetti e Scribante, 2020; Cantoral et al., 2021]. Seja de forma coletiva como na água fluoretada, de uso individual em dentifrícios ou bochechos fluoretados, de uso profissional em forma de gel ou verniz de flúor, ou ainda uma combinação de todos esses, o uso de fluoretos é de extrema importância para a saúde bucal, tendo impacto na saúde geral e qualidade de vida da população [Tenuta e Cury, 2010; Whelton et al., 2019]. No entanto, os fluoretos são coadjuvantes e não são capazes de controlar o processo da doença por si só, portanto ainda há importância

do controle de consumo de açúcar na dieta para controlar a doença cárie, sendo ideal combinar a redução do consumo de açúcar com o acesso a fluoretos [Ha et al., 2021].

1.3   OBJETIVOS

1.3.1 Objetivo Geral

Avaliar a ocorrência de cárie entre as pessoas atendidas pelo Instituto Dom Orione, e o efeito dos tratamentos não-invasivos e invasivos combinados no controle da doença e no microbioma bucal.

1.3.2 Objetivos específicos

Artigo 1: Avaliar a influência da combinação do tratamento invasivo e não-invasivo da doença cárie na composição e na diversidade do microbioma bucal.

Artigo 2: Avaliar a ocorrência de cárie nos indivíduos atendidos pelo Instituto e comparar com dados clínicos de índice de placa.

1.4 REFERÊNCIAS

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2 MICROBIOME SHIFT AFTER COMBINED NON-INVASIVE AND INVASIVE TREATMENT FOR DENTAL CARIES

ABSTRACT

Dental caries is a result of a dysbiosis of the oral microbiome induced by diet. Longitudinal studies evaluating changes in the microbiome after caries treatment, particularly when the diet and sugar intake are controlled, are scarce. The aim of this longitudinal study was to assess the effect of caries treatment on the composition and diversity of the oral microbiome. Two groups (Residents and Visitors) from an institute of education were assessed for caries prevalence and extent (number of affected teeth) using the CAST index. The participants with caries activity received invasive (when necessary) and non-invasive treatments. Saliva samples were collected for microbiological analysis at baseline, 1 month (M1) and 6 months (M6) after treatment. The 16S rRNA partial sequencing (Illumina platform) identified 28 phyla, 55 classes, 95 orders, 171 families and 176 genera. *Firmicutes* (60.91%), *Actinobacteria* (12.07%) and *Proteobacteria* (10.95%) were the most abundant phyla in all samples. There was an enrichment of *Proteobacteria* and a reduction of *TM7* at M6. *Streptococcus* was the most abundant genera (36.14%), followed by *Actinomyces* (5.24%) and *Rothia* (3.26%). Member of the *Archaea* domain was identified in two samples from Residents group at M6. Alpha diversity analysis showed a drop in diversity of M6 samples, and higher diversity in Residents group than in Visitors group. All taxonomies from group Residents reduced in abundance after treatment. More pronounced changes in the composition of the salivary microbiome can be seen after 6 months of treatment more than after 1 month, suggesting that a long-term shift in diet and oral hygiene habits is necessary to impact the microbiota. The response to treatment seems to be accompanied by an enrichment of *Proteobacteria* and a reduction of *Lactobacillus.*

Keywords: Dental Caries; Microbiome; Dysbiosis.

2.1 INTRODUCTION

Dental caries is still highly prevalent in many countries, representing an important public health problem [Bernabe et al., 2017]. It has a cumulative aspect, as its frequency increases as the population ages and no interventions are made, therefore inferring it affects people of all ages, from children to the old-aged [Sheiham and James, 2015]. Despite decades of studies on dental caries, further work is needed to develop understanding with the hope of creating new more effective treatments to control it.

One relevant change in caries etiopathogenesis understanding occurred in the 90's, when the ecological plaque hypothesis was introduced [Marsh, 1994]. It was proposed that a change in the environment induced by diet leads to an ecological shift, favouring acidogenic and aciduric bacteria, and consequently inducing an imbalance in the de- and remineralization process towards demineralization. An extended ecological theory, also based in the knowledge that dysbiosis leads to oral diseases, was recently proposed by Nyvad and Takahashi. Their "integrated hypothesis" proposes that the high consumption of fermentable carbohydrates is a shared risk factor for both dental caries and periodontal diseases [Nyvad and Takahashi, 2020]. The common idea from both theories is that, in order to prevent/control disease, the focus should be on maintaining/restoring oral microbiome homeostasis by controlling the factors that can cause dysbiosis [Marsh, 2003; Nyvad and Takahashi, 2020], even though a large variation in individual-specific resilience and susceptibility also plays a part [Rosier et al., 2018]. However, no current strategies are available for microbial modulation to recover homeostasis, and the arsenal of treatments to treat dental caries is basically based on remineralization (such as fluoride use at the professional level), control of the biofilm, and diet [Meyer-Lueckel and Paris, 2016]. Recently, international consensus on how to intervene in the caries process in children and adults were proposed, suggesting the importance of continuous non-invasive interventions to control caries activity [Schwendicke et al., 2020; Splieth et al., 2020].

Clinical studies on dental caries treatment include restorations using different materials [Dorri et al., 2017], oral health instructions [Stein et al., 2017], professional fluoride applications [Gao et al., 2016], supervised toothbrushing [Hilgert et al., 2017], and others. These studies have helped to improve our knowledge of the different possibilities for treating dental caries and how they can affect the clinical development

of the disease. However, as aforementioned, dental caries is a disease that develops from dysbiosis, so it is important to understand how treatment affects the oral microbiome.

In terms of microbial diversity, microbiological studies had indicated *Streptococcus mutans* to be the main pathobiont for dental caries [Adler et al., 2017; Solbiati and Frias-Lopez, 2018]. With the development of next generation sequencing (NGS) methods, more in-depth characterization of microbial communities is possible. Differences in the relative abundance of identified taxonomies in different age groups have also been reported [Lee et al., 2021] as well as in different caries stages and activity [Jiang et al., 2014], which was later corroborated in metatranscriptomic studies [Simón-Soro and Mira, 2015; Corralo et al., 2021]. However, to the best of our knowledge, there is no such study using NGS data to explain the microbial shift after complete treatment for caries including non-invasive and invasive treatments.

All these studies contributed in a highly significant way to our understanding of dental caries development, but they do not confirm the difference that would happen after treatment. Longitudinal studies evaluating changes in the microbiome after caries treatment, particularly when the diet and sugar intake are controlled, are scarce. Little is known about the impact of cariogenic diet control in the oral microbiome, therefore this study aims to assess the effect of caries treatment on the composition and diversity of the oral microbiome.

2.2   MATERIALS AND METHODS

Study Design, Setting, Ethics and Participants
This longitudinal study was approved by The Committee for Ethics in Research (School of Health Sciences – UnB, process number 86836318.7.0000.0030). The consent form was obtained from all participants and their legal representatives. Subjects were recruited to participate in the study from March to June 2019. Their age ranged from 6 to 51 years-old and were enrolled at Dom Orione Institute, an institution located in the capital of Brazil, Brasilia - Federal District, that shelter adults and young adults with various disabilities, who are at risk or abandoned by their family. These adults are constantly under a professional care by a caregiver. Taking part in a social program called "Orioninho" project, the institute also offers free access to social assistance, educational activities, recreation, sport, and digital inclusion activities to children from

Distrito Federal and surrounding areas. Adults with disabilities live at the institute and have a controlled diet, with an average frequency of sucrose consumption of 2 times a day, whilst the children only stay half a day after their school activities, so there is no controlled diet. Data were collected, and the treatments described in the next section were conducted at the Institute.

Inclusion criteria: Individuals included in this project lived in the institute or were participating in "Orioninho" project. To be included in group Residents, the individuals should be living in the institute, while the participants of group Visitors were the ones attending the classes in the institute during the day. To be included, participants should also have at least one active carious lesion.

Exclusion criteria: people with cognitive conditions that did not allow the clinical evaluations and procedures to be carried out, such as severe intellectual disability and severe autism; children whose parents did not consent to participate in the study.

There were 40 individuals living in the institute, from which 21 fitted the inclusion and exclusion criteria, and 16 had caries activity, therefore received tretment. From the 62 children participating in "Orioninho" project, 59 fitted the inclusion and exclusion criteria and signed consent for participating in the study, and 33 had caries activity and received treatment (Figure 1). Two distinct unparalleled groups were then formed:

Group Residents: 16 subjects with intellectual disabilities (ID) aged 21-51 years old who live at the Institute, therefore have a controlled diet.

Group Visitors: 33 subjects with no disabilities aged 6-14 years old who spend half a day at the Institute and live with their parents, therefore do not have a controlled diet.

Clinical evaluation at baseline

Dental caries was examined using CAST instrument (Caries Assessment Spectrum and Treatment) [Leal et al., 2017] (Table 1) as a selection step of all potential participants after tooth brushing with a dental toothbrush provided to each participant. Besides CAST, caries activity was assessed, and the active lesions were identified. Visible plaque index (VPI) and gingival bleeding index (GBI) were also assessed.

Table 1: CAST instrument description.

Code	Characteristics	Description
0	Sound	No visible evidence of a distinct carious lesion is present
1	Sealant	Pits and/or fissures are at least partially covered with a sealant material
2	Restored	A cavity is restored with and (in)direct restorative material
3	Enamel	Distinct visual change in enamel only. A clear caries related discoloration in visible, with or without localized enamel breakdown
4	Dentine	Internal caries-related discoloration in dentine. The discolorated dentine is visible through enamel which may or may not exhibit a visible localized breakdown of enamel
5	Dentine	Distinct cavitation into dentine. The pulp chamber is intact
6	Pulp	Involvement of a pulp chamber. Distinct cavitation reaching the pulp chamber or only root fragments are present
7	Abscess/fistula	A pus containing swelling or a pus releasing sinus tract related to a tooth with pulpal involvement
8	Lost	The tooth has been removed because of dental caries
9	Other	Does not correspond to any of the other descriptions

On the presence of active carious lesions, the following interventions were taken:

- Oral hygiene instruction that included toothbrushing twice a day with fluoride toothpaste and flossing orientation. These instructions were given to the participants of the study and their caregivers at each treatment appointment.
- 72-hour recall interview and diet instruction. Food intake frequency was identified, as well as sucrose intake frequency and sucrose intake frequency between meals. For group Residents, the diet information was informed by the institute.
- Topic application of 2% Neutral Sodium Fluoride gel (4 weekly applications, 1 minute each) on the subjects with caries activity (CAST code 3).
- Atraumatic restorative treatment (ART) on cavitated teeth (CAST codes 4 and 5);
- Referral for more complex treatments (CAST codes 6, 7 and 8).

Initial instruction to reduce cariogenic diet was carried out with all the study subjects. Those with caries activity received additional individual instructions to reduce food intake frequency to less than 8 times/day, sucrose intake frequency 1 time/day during the meal, and sucrose intake frequency between meals 0 times/day, avoiding

sweetened beverages and sucrose-containing foods between meals, during the treatment period (4 weeks).

Reproducibility

The diagnostic was done in a reliable way, as only one examiner carried out the clinical assessments. Before the clinical evaluations, the examiner received a training on the CAST instrument with theoretical lecture and immediately afterwards, photographs were used for calibration. Afterwards, calibration was made in 10 patients that were assessed by the examiner and the trained clinician. The inter-examiner and intra-examiner Kappa coefficient were 0.85 and 0.94, respectively.

Dental treatment (invasive and non-invasive):

Caries lesions with indication for restorative treatment were handled by the dentist responsible for the study. All subjects with active caries lesion underwent conventional treatment, as follows: non-invasive (cariogenic diet control, oral hygiene instructions, fluoride applications) and, when necessary, invasive treatments were performed following the Atraumatic Restorative Treatment (ART) protocol [Frencken, 2017].

For the non-invasive treatment, teeth with initial non-cavitated lesions received four weekly one minute applications of topic 2% Neutral Sodium Fluoride gel [Holmen et al., 1987] (Flugel, DFL, Jacarepaguá, Brazil) under relative isolation using cotton roll in enough gel   quantity to cover all the lesion. During all clinical evaluations, subjects with caries activity received specific instructions as part of the non-invasive treatment. The instructions included toothbrushing twice a day with fluoride toothpaste, flossing orientation, and diet instructions to reduce the daily frequency of sucrose intake.

For the invasive treatments, the teeth were isolated with cotton rolls, and carious tissue was removed following the International Caries Consensus Collaboration recommendations [Schwendicke et al., 2016] using manual instruments. Then, the teeth were restored with a high-viscosity glass-ionomer (Equia Forte, GC America inc. IL, USA) following the manufacturer's instructions. When necessary, the subjects were referred to receive endodontic treatment.

Sample collection

Saliva samples were collected from all participants that received treatment. Before the sample collection, each participant received a code, so that the saliva collection tubes were not identified with the participant's name, maintaining the anonymity to ensure a blinding analysis for bias control. After brushing without toothpaste, saliva samples were collected for 5 minutes. The collection was done after stimulation with a rubber device for 1 minute and elimination of the first expectoration after stimulation. After collection, 1 ml of stimulated saliva was stored at -80ºC before conducting experiments for prokaryotes analysis. The collection was made pre-treatments as a baseline, 1 month after completion of invasive and non-invasive treatment (M1), when participants no longer had active carious lesions, and also 6 months after treatment for group Residents (M6). The 6-month sample collection was not possible for group Visitors, as the "Orioninho" project interrupted its activities during the COVID-19 pandemic.

16S rRNA sequencing

DNA was extracted using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) and quantified using the QuantiT PicoGreen dsDNA Assay Kit (Invitrogen, MA, USA) following the manufacturers' protocols. The regions V3-V4 from the 16S rRNA gene were amplified using the hot start NEBNext Ultra II Q5® Master Mix (New England BioLabs Inc. Life Technologies Inc, Ipswich, MA, USA) with the 564F (TCG-TCG-GCA-GCG-TCA-GAT-GTG-TAT-AAG-AGA-CAG-AYT-GGG-YDT-AAA-GNG) and 806R (GTC-TCG-TGG-GCT-CGG-AGA-TGT-GTA-TAA-GAG-ACA-GTA-CNV-GGG-TAT-CTA-ATC-C) primers (Eurogentec, Seraing, Belgium). The amplicons were purified using AMPure XP Beads (Beckerman Coulter, Inc., CA, USA). A second amplification was performed using Nextera® XT Index Kit v2 (Illumina, Inc., CA, USA), followed by the clean-up with AMPure XP beads (Beckerman Coulter, Inc., CA, USA) once again. Amplicon sizes were assessed with 2200 Tapestation System using 1 µl from each DNA sample, without dilution. Then, QuantiT PicoGreen dsDNA Assay Kit was used again to quantify the libraries following the manufacturer's protocol. The final multiplexed indexed library was pooled by adding equimolar concentration of the libraries 2.0 ml collection tube and then sequenced on the Illumina MiSeq platform.

Bioinformatics and data analysis

The demultiplexed paired-end reads were denoised with DADA2 using the Quantitative Insights into Microbial Ecology (QIIME) bioinformatics pipeline [Bolyen et al., 2019] and clustering was performed at 99% identity to create OTUs. The taxonomy was assigned to the sequences by matching against sequences in the Greengenes_13_8 database [McDonald et al., 2012]. The comparisons were made within the same group at the different time points when the saliva samples were collected. Alpha diversity was evaluated with Chao1 richness estimator and Shannon diversity index was calculated and compared between before and after treatment data for both groups. Bray-Curtis distance was also determined, and microbial structures compared using Unifrac between before and after treatment data. Unweighted Unifrac distances were used to perform a principal coordinate analysis in R software.

Participants were divided in "high caries" (caries extent – i.e. the number of affected teeth - percentage higher than 0.09) and "low caries" (caries extent percentage ranging from 0 to 0.08) at baseline for differential abundance analysis. Differential abundance was also calculated for M1 and M6 samples compared to baseline for group Residents.

2.3   RESULTS

Recruitment of participants is shown in figure 1.
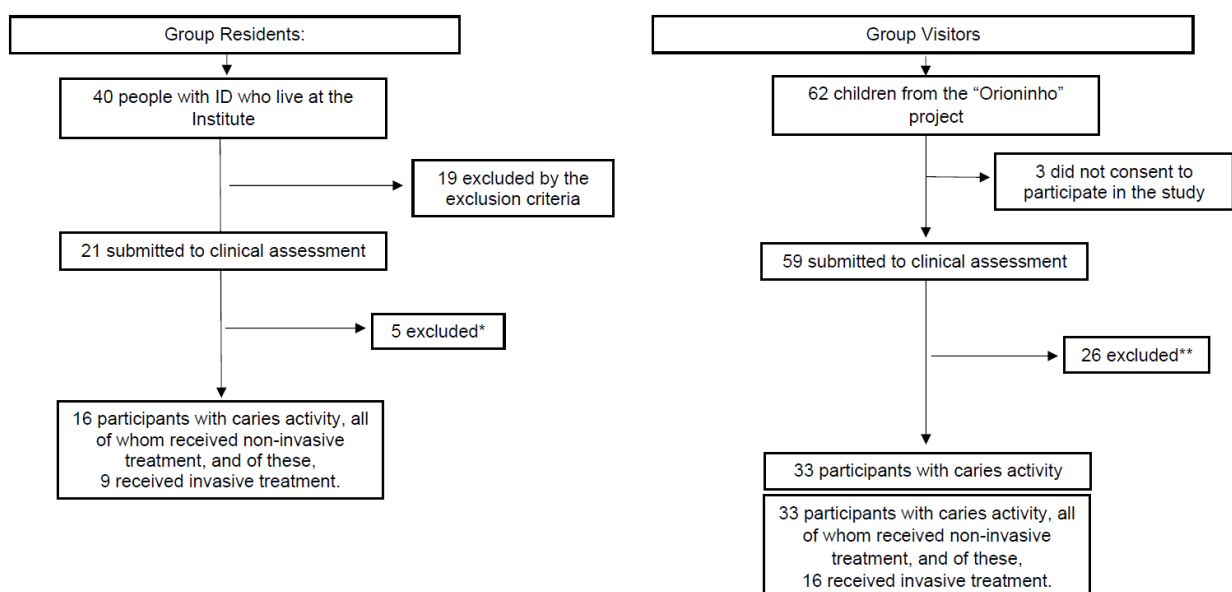
*Reasons for exclusion: patients lacking dental caries and who were unable to provide saliva samples.

**Reasons for exclusion: left the "Orioninho" project before the conclusion of the research, parents that did not give consent for treatment, could not collect the saliva samples or did not have dental caries.

Figure 1. Flow chart describing the participants recruitment to the study.

Six months after baseline dental assessment/treatment and sampling, our clinical assessment of group Residents revealed no active carious lesions. The VPI and GBI reduced approximately 13% in the Residents group, confirming the challenge of the plaque control. As previously stated, the assessment of group visitors was not possible due to restrictions related to the COVID-19 pandemic.

Sequencing output and the relative abundance of the salivary microbiota

The Operational Taxonomic Units (OTUs, 99% similarity) belonged to 28 phyla, 55 classes, 95 orders, 171 families and 176 genera. Of the 28 phyla, 12 had a relative abundance greater than 0.1%. The most abundant phylum in both groups was *Firmicutes* (60.91%) with a considerable higher relative abundance compared to the second and third most abundant phyla, namely *Actinobacteria* (12.07%) and *Proteobacteria* (10.95%) (Figure 2A for Residents, and 2B for Visitors). *Firmicutes* was the most abundant phylum at all collection times (baseline, 1 month after treatment and 6 months after treatment). Six months after treatment there was an enrichment of *Proteobacteria*, which became the second most abundant phyla, and a reduction of *TM7 (Saccharibacteria)* (Figure 2A). For the baseline and 1 month after treatment samples the second most abundant phylum was *Actinobacteria*. Eleven phyla had a ubiquity greater than 50%, which included less abundant phyla with relative abundance lower than 1%, namely *Spirochaetes* (0.78%), *Tenericutes* (0.51%) and *SR1* (0.26%) in both groups.
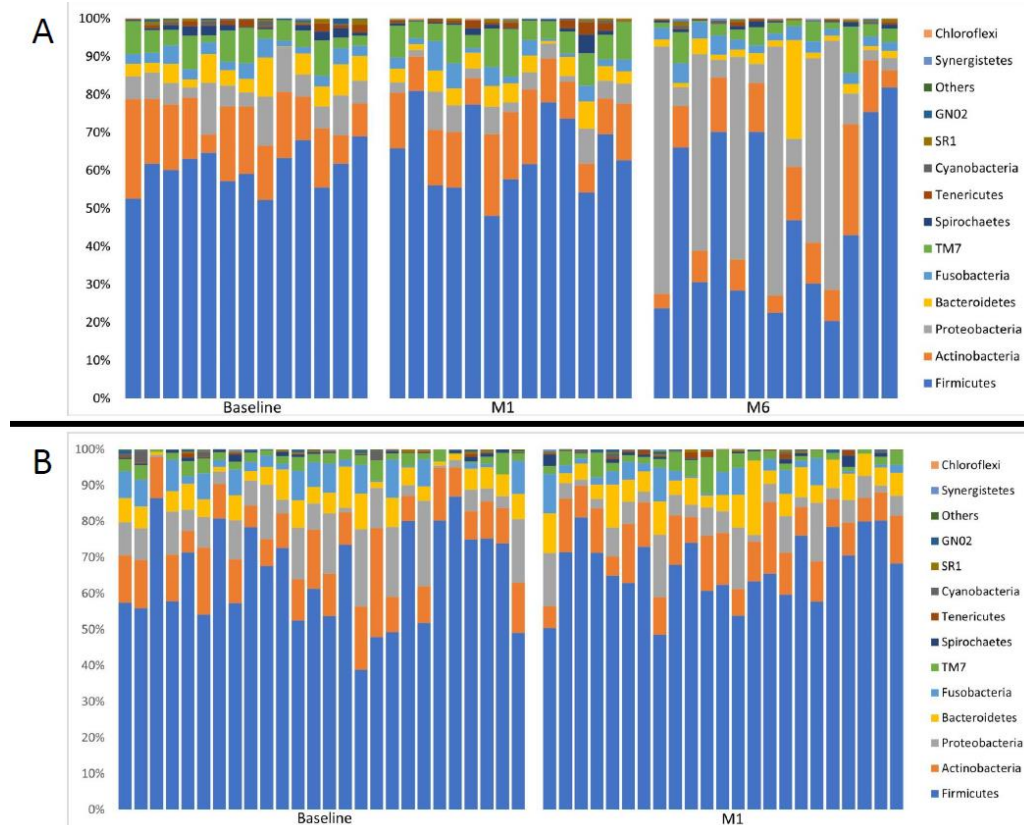
Figure 2. Relative abundance of the identified phyla in group Residents (A) and Visitors (B) at baseline, 1 month after treatment (M1) and 6 months after treatment (M6). Phyla with lower abundance (<0.01%) were grouped and identified as "Others" for ease of visualization. Each bar corresponds to the microbial profile of a participant.

Thirty-four of the identified genera had a ubiquity greater than 50%. The most abundant for all collection times in both groups was *Streptococcus* (36.14%), followed by *Actinomyces* (5.24%) and *Rothia* (3.26%) (Figure 3A for Residents and 3B for Visitors). Other genera with a relative abundance lower than 0.3% had a ubiquity greater than 50%, including *Peptococcus* (0.16%), *Mycoplasma* (0.19%) and *Campylobacter* (0.26%). *Streptococcus, Actinomyces and Rothia* were the only genera identified across all samples. As for the Residents, six months after treatment there was an enrichment of *Stenotrophomonas* and an important enrichment of *Staphylococcus* in one sample (Figure 3A). Interestingly, for those samples with the referred enrichment, a lower abundance of *Actinomyces* when compared to the others could be observed.
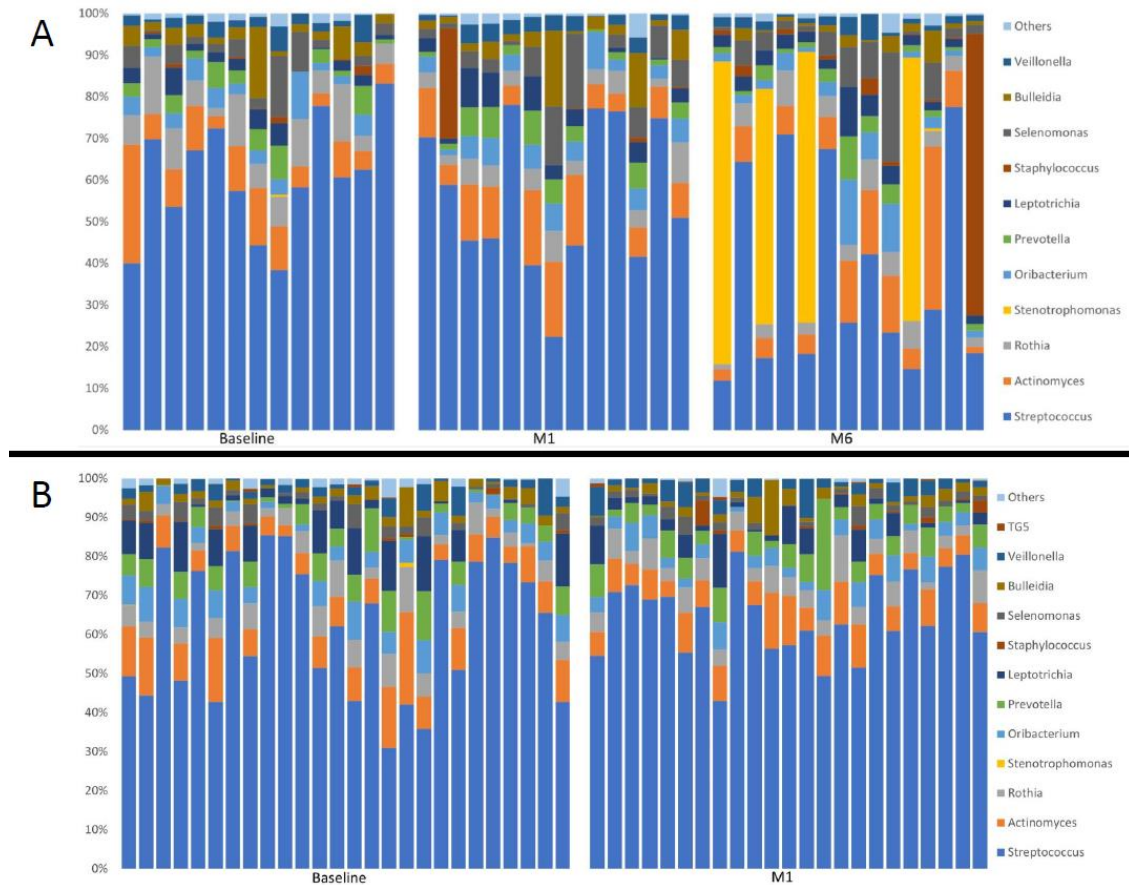
Figure 3. Relative abundance of the identified genera in group Residents (A) and Visitors (B) at baseline, 1 month after treatment (M1) and 6 months after treatment (M6). Genera with lower abundance (<0.01%) were grouped and identified as "Others" for ease of visualization. Each bar corresponds to the microbial profile of a participant.

Two samples from group Residents collected 6 months after treatment showed the presence of *Archaea* domain. Both detected taxa belong to the Euryarchaeota phylum and *Methanobrevibacter* spp. and it had a relative abundance of 0.12% in one sample and 0.22% in the other.

The abundance of most represented taxonomies in the samples is also presented in a heatmap (supplementary Figure 1).

Alpha diversity

To assess the diversity within the samples, alpha rarefaction diversity was calculated. The boxplots showing Chao1 and Shannon estimators for all collection times for groups Residents and Visitors are presented in Figure 4.

The diversity analyses of observed taxonomies, according to the collection time (baseline, M1 or M6) resulted in clear drop in diversity in the M6 samples (see figure 4A and 4B). In figure 4C and 4D it is possible to compare group Residents and Visitors, where a higher diversity could be observed for the Residents (older than the Visitors, presenting mental disabilities, and a controlled diet).
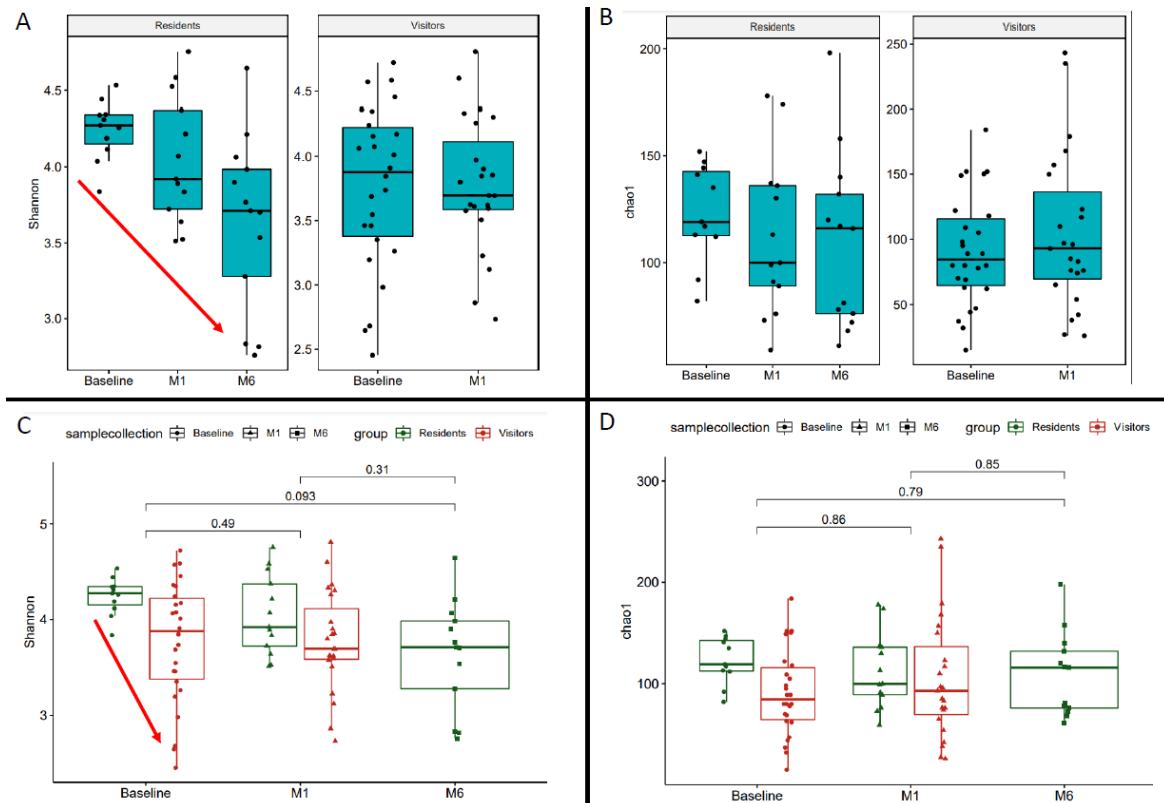

Figure 4. Group Residents and Visitors alpha diversity. A and B: Shannon's index and Chao1 index for samples collected at baseline, 1 month (M1) and 6 months after treatment (M6). Note the difference between baseline and M6 (red arrow). C and D: Shannon's index and Chao1 index for all collection times. Note the difference between group Residents and group Visitors (red arrow). Figure 4C also shows that there is almost statistical difference between baseline and M6 samples.

Beta diversity

To assess bacterial diversity between samples, beta diversity analysis was carried out. A principal coordinate analysis (PCoA) based on unweighted UniFrac plot is shown in Figure 5.
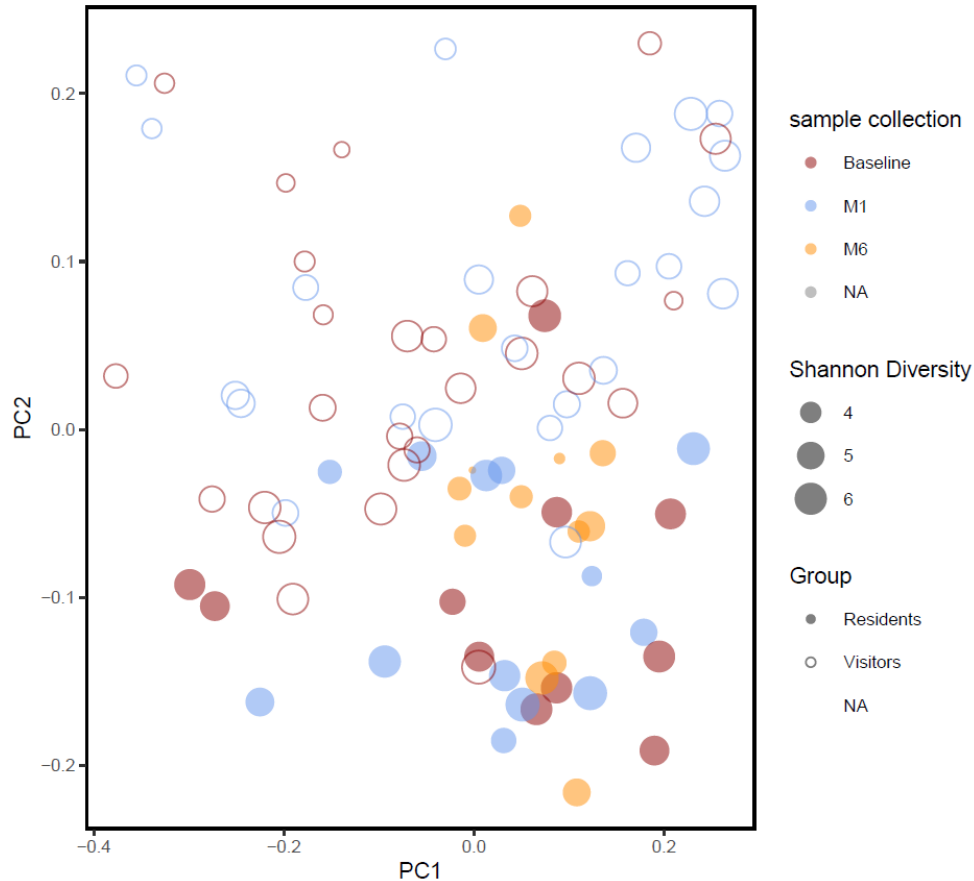
Figure 5. Principal coordinate analysis (PCoA) of Bray–Curtis similarity of bacterial communities. The analysis was based on square root-transformed proportions of OTUs and included all samples.

Differential abundance

To compare patients from the baseline regarding their caries extent, we analysed the differential abundance of samples of all participants classified as "low caries" (meaning 0-0.08% of affected teeth) and "high caries" (meaning >0.09% of affected teeth) at baseline and the result is presented in figure 6.
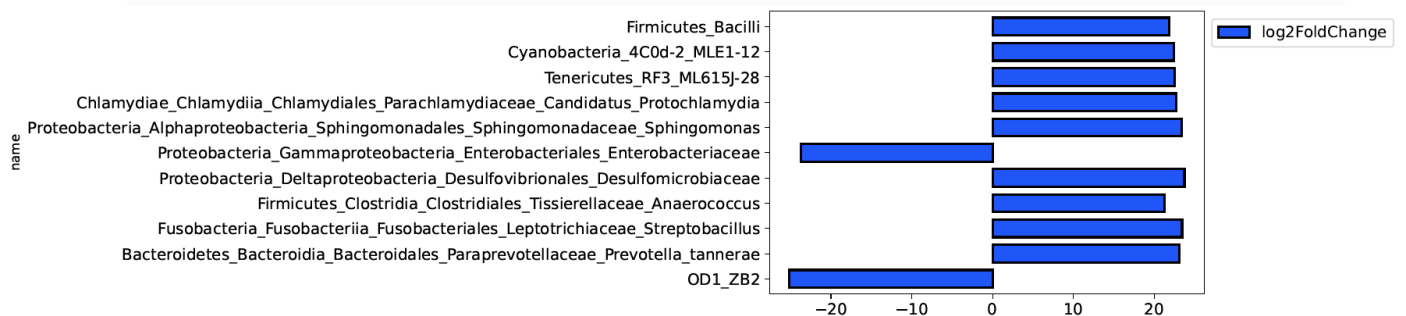

Figure 6. Low caries vs. high caries differential abundance. Negative values represent bacteria with higher abundance in high caries samples. Positive values represent bacteria with higher abundance in low caries samples.

When comparing only samples from group Residents, all taxonomies significantly reduced in abundance one month after treatment (Figure 7). From 11 taxonomies differentially abundant, 3 belonged to the *Lactobacillus* genus. Six months after treatment, a higher number of taxonomies were differentially abundant. There was a reduction in the abundance in some taxonomies, including *Lactobacillus,* TM7 and *Veillonella* (Figure 8). The *Methanobrevibacter spp.,* belonging to the *Archaea* domain, was enriched 6 months after treatment, as well as *Aggregatibacter.*
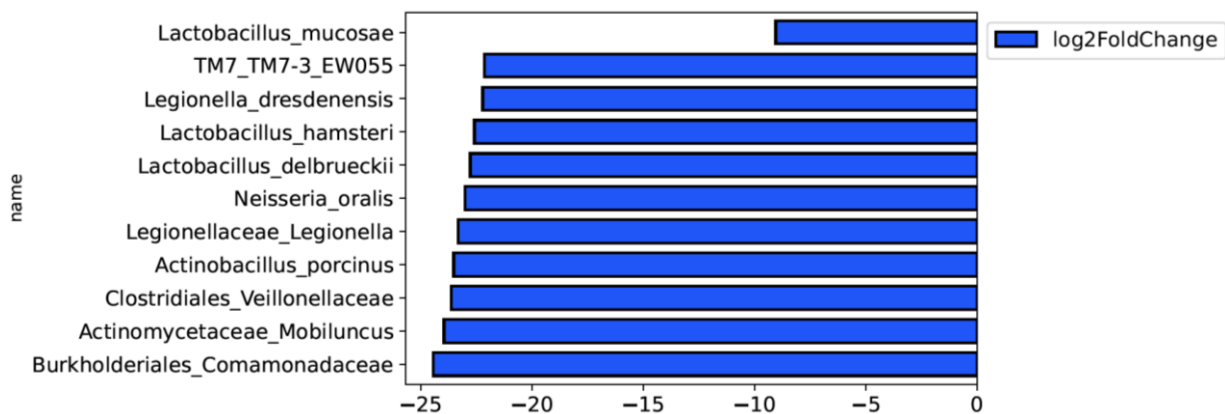
Figure 7. M1 vs. baseline differential abundance of Group Residents samples. Notice that all samples are less abundant in M1 (after treatment).
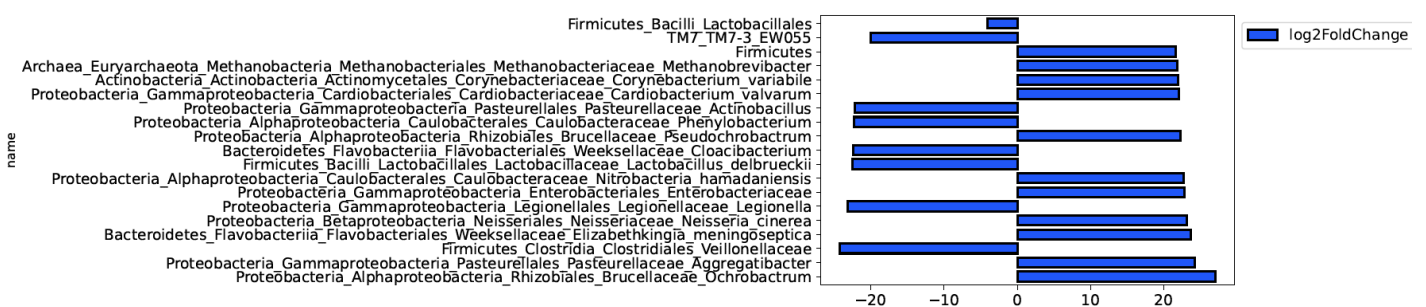

Fig. 8. M6 vs. baseline differential abundance of Group Residents samples. Negative values represent taxonomies less abundant in M6.

2.4   DISCUSSION

Dental caries treatment aims to arrest the progression of the disease. Different aspects of treatment include invasive treatment and non-invasive treatment, as previously

stated. Treatment of dental caries should focus on reversing the dysbiotic state rather than the eradication of a single pathobiont, as dental caries is a microbiologically complex disease [Zhan, 2018]. After treatment, we confirmed clinical changes in the carious lesions. As previously shown in a study on early childhood caries, these clinical changes are a result of a shift in the oral microbiota [Tanner et al., 2011]. This shift represents a change from a more acidogenic/aciduric bacterial environment to a microbiota with lower or no *Streptococcus mutans* and *Lactobacilli* [Tanner et al., 2011; Tanner et al., 2018; Zhan, 2018).

The structure of microbial communities includes high and low abundant microorganisms, and all of them somehow impact their community. As proposed by Hajishengallis, Darveau and Curtis [Hajishengallis et al., 2012], keystone species are species that impact the community in a significant way, despite their low abundance. These species may contribute to dysbiosis related diseases, therefore identifying keystone pathogen species would help understand the disease and create treatment alternatives [Cena et al., 2021; Hajishengallis et al., 2012]. In this study, some microorganisms found in lower abundance were identified in more than 50% of the samples, namely the ones belonging to the following phyla: *Spirochaetes*, *Tenericutes* and *SR1*. *Spirochaetes* and *SR1* are usually identified in patients with and without dental caries [Ling et al., 2010; Peterson et al., 2013; He et al., 2017]. *Tenericutes* have been reported in low abundance in saliva samples before [Ling et al., 2010]. Another microorganism found in low abundance was the member of the *Archaea* domain. *Archaea* presence in the oral microbiome is not yet well described in the literature, although there is some evidence of its presence in different oral niches and clinical conditions, suggesting potential roles in oral diseases [Belmok et al., 2020]. The genus identified in this study, *Methanobrevibacter*, has been described before as being associated with periodontal disease and endodontic infections [Aminov, 2013; Horz et al., 2015; Brzezińska-Błaszczyk et al., 2018]. Another study has reported the presence of *Archaea* in patients with dental caries, where the authors argue that *Archaea* could be part of changes the local environment, creating ideal conditions for the growth of other microorganisms such as acidogenic bacteria [Dame-Teixeira et al., 2020].

Different body sites have different microbial composition, as the local environment may favor the colonization of some microorganisms and at the same time be a hostile environment for others [Pitts et al., 2021]. Lifestyle factors such as diet, amount, and

frequency of sucrose intake, drinking habits and others may affect the oral microbiome balance, leading to dysbiosis and subsequently to the development of oral diseases. Therefore, to prevent or control the disease we should interfere in these factors [Pitts et al., 2021]. One other well-known modulating factor related to decreasing the risk of developing dental caries is fluoride. It has an important role in controlling dental caries as they lower the critical pH for mineral dissolution [Tenuta e Cury, 2010; Pitts et al., 2021], but the disease had been observed before even with low levels of sugar consumption, despite the use of fluoride [Peres et al., 2016]. In line with that, in this study it was possible to identify more pronounced changes after 6 months of treatment when there were no longer active carious lesions. Even if the clinical inactivation of the lesions is visible in a shorter period of time (1 month), our results on the small changes after a month support the importance of a long-term diet instruction and oral hygiene habits to effectively achieve the homeostatic situation at the microbiological level. Even though it was a borderline result at the taxonomic level, we believe this is an important finding that can corroborate somehow to previous data from Corralo et al., showing that the metatranscriptome of biofilms from inactive enamel caries lesion still hold differences of that from sound enamel surfaces [Corralo et al., 2021]. With that said, it is important to state that to assure a long-term oral health of the caries-active patients, fluoride and diet habits in caries control, in addition to the mechanical disruption of the biofilm are mandatory. It has been shown that oral hygiene alone, in the absence of fluoride, is not able to reduce the incidence of dental caries [Hujoel et al., 2018].

Although it was not our intention to compare the microbiome of groups Residents and Visitors, due to the heterogeneity between them, some differences could be observed. Several characteristics may have influenced this difference, but we cannot ignore that the presence of a controlled diet in one of the groups and the higher diversity in the diet of the other group might have played a role as well in the difference found between them. As aforementioned, diet composition and sucrose intake have an important effect in caries development, as the disease develops after dysbiosis caused by environmental change, such as frequent sucrose consumption [Tanner et al., 2018]. Therefore, we cannot ignore the controlled diet of one group in relation to the uncontrolled diet of the other. This result might give an idea of how sucrose control can influence the microbiome.

When comparing high caries and low caries samples, only eleven taxons were differentially abundant. This suggests that caries extent (number of affected teeth) is

not a strong factor changing the microbial composition. While *Enterobacteriaceae* and *CPR OD1_ZB2* were more abundant in high caries samples, *Bacilli, Cyanobacteria, Tenericutes, Protochlamydia, Sphingomonas, Desulfomicrobiaceae, Anaerococcus, Streptobacillus*, and *P. tannarae* were more prevalent in low caries samples.

When comparing only samples from group Residents, all taxonomies significantly reduced in abundance one month after treatment, and from 11 taxonomies differentially abundant, 3 belonged to the *Lactobacillus* genus. The reduction of this genus might be related to the restoration of cavitated lesions. After 6 months there was a reduction in the diversity with an enrichment of *Proteobacteria* and reduction of *TM7*. A study on early childhood caries showed a higher abundance of *Proteobacteria* in caries-free children compared to children with caries, which is in accordance with the results of this study [Yuan et al., 2017]. *TM7* have been related to disease before, usually in patients with gingivitis and periodontal disease, with a reduction on its abundance after treatment interventions [Huang et al., 2016; Bor et al., 2019]. The integrated hypothesis from Nyvad and Takahashi (2020) supports this finding, as it proposes that dental caries and periodontal diseases may be present in the same individual, and in that situation, it would be possible to identify the same bacteria in individuals with both diseases [Nyvad and Takahashi, 2020]. Our finding suggest that this phylum might be related to active dental caries as well, but further studies are needed to confirm this relationship. Furthermore, an enrichment of *Stenotrophomonas* was also observed 6 months after treatment. This genus has been related to oral health before [Jiang et al., 2014], which supports our finding.

As for bacterial diversity, dysbiosis is usually related to a reduction in the microbiome diversity whilst in a healthy and balanced environment we would find a higher diversity of microorganisms, as the local shifts favors the increase of some pathobionts and the decrease of beneficial microorganisms [Petersen and Round, 2014; Radaic and Kapila, 2021]. However, another recent study in individuals with periodontitis revealed a reduction in the diversity following treatment [Johnston et al., 2021], in agreement with our findings on dental caries. A higher diversity in individuals with dental caries have also been reported previously [Belstrøm et al., 2016]. These findings raise the discussion that disease might not always be accompanied by a reduction in bacterial diversity as previously believed. This could be further investigated with future longitudinal studies between health to diseased states.

It is important to stress that a balanced oral microbiome does not mean the same bacterial abundance of specific bacteria in all healthy individuals [Radaic and Kapila, 2021]. Studying the bacteriome and their interactions with the host is therefore not an exact science that can be generalized to all individuals in a category, whether healthy or diseased. For example, it has been shown that *Streptococcus mutans* is usually present in the oral microbiome of patients with dental caries, but it has also been shown in other studies that it is possible for a patient to have dental caries in the absence of this bacteria [Marsh, 2003; Radaic and Kapila, 2021].

In terms of bacterial abundance, it is expected that we find higher abundance when there is higher caries (more cavities and active carious lesions). In this study we could identify high bacterial abundance in low caries samples, which means that even when there is only one active carious lesion, the microbiome is already in dysbiosis so we can see a high abundance. Clinically, other modulating factors such as fluoride may justify the small number of carious lesions in a dysbiotic microbiome, but in terms of microbiological balance, it is already similar to what we see in high caries patients.

In summary, caries prevention should be based on keeping the oral microbiome balanced avoiding dysbiosis [Pitts et al., 2021], therefore, to achieve that, the focus should be on diet and oral hygiene instructions to reduce the daily frequency of sucrose intake and to promote the biofilm disruption along with the use of fluoride toothpastes. Educational programs are an effective way of influencing the habit shift specially when there is a follow-up, as shown is a previous systematic review [Avery et al., 2014]. Behavioral modification is difficult to achieve, so the more instruction people receive on a regular basis, the more effective it will be.

It is a great challenge to identify microorganisms associated with a particular disease and determine how they interfere the process. NGS studies aim to help identify bacteria and their abundance in different clinical situations, but to understand how these bacteria contribute to the development of disease, further work is needed.

2.5   CONCLUSION

More pronounced changes in the composition of the salivary microbiome can be seen after 6 months of treatment than after 1 month (immediately after finishing the non-invasive and invasive treatments), suggesting that long-term instructions in diet and oral hygiene habits are necessary to impact the microbiota. The return to homeostasis

seems to be accompanied by an enrichment of *Proteobacteria* and a reduction of *Lactobacillus.* More studies are needed to better understand the relationship between *TM7* and dental caries.

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SUPPLEMENTARY MATERIAL

Supplementary Figure 1. Heatmap describing the abundance of taxonomies. The percentage abundance is represented as a color gradient ranging from yellow to blue, with yellow being the most abundant features.

3   DENTAL CARIES OCCURRENCE AND TREATMENT IN GROUPS WITH POOR ORAL HYGIENE, WITH AND WITHOUT CONTROLLED DIET

ABSTRACT

Dental caries is a dynamic disease mediated by biofilm and modulated by diet. People with poor oral hygiene are therefore at higher risk of developing the disease if associated with a cariogenic diet. The aim of this study was to assess the dental caries occurrence and extent in two different risk groups comparing with their visual plaque index (VPI), as well as to assess their response to the non-invasive and invasive treatments. The groups (Residents and Visitors) were assessed at baseline, 1 month (M1) and 6 months (M6) after treatment using CAST index and the participants with caries activity received invasive and non-invasive treatment. Frequency of sucrose intake was assessed by an interview. Caries occurrence at baseline was 56.25% (SD 51.23) for group Residents and 65.7% (SD 48.16) for group Visitors. The VPI was 91.3% (SD 10.8) and 97.2% (SD 4.1) in caries-active participants of group Residents and Visitors, respectively. 28 participants from group Visitors had at least one tooth with cavitated lesions in dentin, and the total number of cavitated lesions was 41 for this group. The total number of cavitated lesion in Group Residents was 16. The participants with caries activity had a higher frequency of daily sucrose consumption and the dental caries activity was successfully controlled after treatment. Diet and oral hygiene control are effective to prevent and treat dental caries and should always be part of the non-invasive treatment, combined with fluoride applications.

Keywords: Dental Caries; Prevalence; Treatment.

3.1   INTRODUCTION

According to the European Organisation for Caries Research (ORCA) and Cariology Research Group of International Association for Dental Research (IADR) consensus, dental caries is defined as a dynamic disease mediated by biofilm and modulated by diet resulting in mineral loss of dental hard tissues, being also determined by biological, behavioral, psychosocial, and environmental factors [Fejerskov 1997; Pitts et al., 2017; Machiulskiene et al., 2020]. The ORCA and European Federation of Conservative Dentistry (EFCD) Expert Delphi Consensus recommends improvement of oral hygiene as part of the non-invasive strategy to promote remineralization as a prevention measure on the patient level [Paris et al., 2020]. A classic study from 1987 had already shown that the mechanic disruption of biofilm is able to arrest active carious lesions [Holmen et al., 1987]. Nonetheless, oral hygiene should be accompanied by fluoride, as a systematic review of the literature showed that there is not enough evidence to support the efficacy of oral hygiene in the prevention of dental caries in the absence of fluoride [Hujoel et al., 2018].

Risk factors for developing dental caries include sociobehavioural factors such as access to oral health services; socio-cultural factors including education, occupation and income; and environmental factors that include drinking water supply, hygiene habits, nutrition status, sanitation, and others [Petersen, 2005]. Other risk factors include frequency of carbohydrate intake, biofilm composition, saliva buffering capacity, hyposalivation, smoking (due to alterations in saliva), and medical conditions [Chapple et al., 2017]. Patients with intellectual disabilities (ID) may have difficulties in eating and performing oral hygiene, as well as resistance to accepting help for their oral care. Sometimes they might also have a diet poor in fibers, eating mainly food that are easy to swallow without chewing. All those factors may increase their risk of developing dental caries [Ningrum et al., 2020]. Children are also at increased risk of developing dental caries as they are developing ability to perform the oral hygiene and might not have the discipline to do it regularly [Aliakbari et al., 2020], in addition to generally having a more cariogenic diet [Paris et al., 2020]. For those groups of people, supervision in toothbrushing is recommended and diet control is an important measure to control the disease, when toothbrushing is not performed regularly and effectively, as we know how diet is the main factor associated with dental caries [Sheiham and James, 2015; Aliakbari et al., 2020; Cantoral et al., 2021].

For both groups, preventive care is of utmost importance and regular visits to the dentist are highly recommended. However, this is not a reality for most people. When they rely on the public health system, it might take a long time to get the necessary care, and when they get it, it might be too late for minimal invasive treatments or even to save the teeth. Besides, they usually do not make regular visits to the dentist for preventive care, and only look for professional dental care when the patients are already in pain or have difficulty to eat suggesting some problem with their teeth and oral health, which also ends up in the need of invasive treatments. Once again, the diet habits prove to be so important, because without the food source for the bacteria, it would be possible to control/prevent the disease and reduce the need for treatment [Sheiham and James, 2015], especially in groups of people with special needs, such as intellectual disabilities.

The aim for this study was to 1) assess the dental caries occurrence in two different risk groups comparing the prevalence with the visual plaque index of the participants, and 2) assess the response to the treatment given the diet and oral hygiene habits orientation. We also assessed the oral health habits, socioeconomical status and diet habits of the participants.

3.2 MATERIALS AND METHODS

This study was approved by The Committee for Ethics in Research (School of Health Sciences – UnB - n.86836318.7.0000.0030).

This was a longitudinal study of people assisted by the Dom Orione Institute in Brasilia, Federal District, Brazil. The recruitment of the participants was from March to June 2019. Consent for examination and treatment was obtained from all participants and their legal guardians. They also answered a socioeconomic questionnaire (Supplementary material 1).

1) *Occurrence study*

The inclusion criteria were patients that lived in the institute or participated in the "Orioninho" project, which provides educational and recreation activities. The exclusion criteria were people with cognitive conditions that did not allow the dental exams, such

as severe intellectual disability and severe autism; children whose guardians did not give permission to participate in the study.

All subjects that fitted the inclusion and exclusion criteria were invited to participate in the study. The data were collected, and the treatments were conducted at the Institute.

The participants were divided in 2 non-parallel groups. Group Residents comprised 21 subjects with intellectual disabilities that lived in the institute and fitted the eligibility criteria. They aged 21-51 years old. As they live in the institute, they have a controlled diet. Group Visitors comprised 59 subjects from the "Orioninho" project with no disabilities, who spent part of the day at the institute and were aged 6-14 years old. They spend half a day at the Institute and live with their parents, therefore do not have a controlled diet.

Visual-tactile examination was done with a periodontal probe and dental mirror using the CAST instrument (Caries Assessment Spectrum and Treatment) [Leal et al., 2017] (Table 1) after tooth brushing with a dental toothbrush provided to each participant. Besides CAST, caries activity was evaluated, and the active lesions were identified. The participants with caries activity received the treatment (16 from group Residents and 33 from group Visitors), and were assessed three times: baseline, 1 month after treatment (M1) and 6 months after treatment (M6). Visible plaque index and gingival bleeding were also evaluated at baseline and M6.

Reproducibility

The clinical assessments were carried out by a single examiner. Before the clinical evaluations, the examiner received a training on the CAST instrument with photographs of different dental caries lesions that were shown and explained to the examiner by a trained clinician. Afterwards, calibration was made in 10 patients that were assessed by the examiner and the trained clinician. The inter-examiner and intra-examiner Kappa coefficient were 0.85 and 0.94, respectively.

Table 1: CAST instrument description.

Code	Characteristics	Description
0	Sound	No visible evidence of a distinct carious lesion is present
1	Sealant	Pits and/or fissures are at least partially covered with a sealant material
2	Restored	A cavity is restored with and (in)direct restorative material
3	Enamel	Distinct visual change in enamel only. A clear caries related discoloration in visible, with or without localized enamel breakdown
4	Dentine	Internal caries-related discoloration in dentine. The discolorated dentine is visible through enamel which may or may not exhibit a visible localized breakdown of enamel
5	Dentine	Distinct cavitation into dentine. The pulp chamber is intact
6	Pulp	Involvement of a pulp chamber. Distinct cavitation reaching the pulp chamber or only root fragments are present
7	Abscess/fistula	A pus containing swelling or a pus releasing sinus tract related to a tooth with pulpal involvement
8	Lost	The tooth has been removed because of dental caries
9	Other	Does not correspond to any of the other descriptions

2) *Treatment study*

To be included in the treatment groups, participants should also have at least one active carious lesion. The exclusion criteria were people with cognitive conditions that did not allow the procedures, such as for the study 1.

Non-invasive and invasive treatment

All the participants and their caregivers received oral hygiene instructions. On the presence of active carious lesions, a 72-hour recall interview and diet instruction were also conducted to identify the daily frequency of food intake and frequency of sucrose intake between meals. Initial instruction to reduce cariogenic diet was carried out with all the study subjects. Those with caries activity received individual instructions to reduce food intake frequency to less than 8 times/day, sucrose intake frequency 1 time/day, and sucrose intake frequency between meals 0 times/day, during the treatment period (4 weeks). All participants and the institute received these instructions.

Active non-cavitated lesions (CAST code 3) received non-invasive treatment, that included 4 weekly one minute applications of topic 2% Neutral Sodium Fluoride gel

(Flugel, DFL, Jacarepaguá, Brazil) [Holmen et al., 1987], and all teeth that needed restorative (invasive) treatment (CAST codes 4 and 5) were treated following the Atraumatic Restorative Treatment (ART) protocol [Frencken, 2017]. The teeth were isolated with cotton rolls, and carious tissue was removed following the International Caries Consensus Collaboration recommendations [Schwendicke et al., 2016] using manual instruments. Then, the teeth were restored with a high-viscosity glass-ionomer (Equia Forte, GC America inc. IL, USA). Treatments were conducted by a single operator. When necessary, the subjects were referred to receive endodontic treatment. During all clinical evaluations, subjects with caries activity received specific instructions as part of the non-restorative treatment. The instructions included toothbrushing twice a day with fluoride toothpaste and flossing orientation, and diet instructions to reduce the frequency of sucrose intake (avoiding sweetened beverages and sucrose-containing foods between meals).

A descriptive analysis of subjects' characteristics was performed at baseline and at follow-up, as well as the Mann- Whitney test to compare groups.

3.3   RESULTS

1) *Occurrence study*

Study participants data is summarized in table 2. The mean age of the participants was 37.4 years old for group Residents, 100% male, and 9.2 years old for group Visitors, 54.54% male and 45.45% female. The number of participants in the Visitors group with a white spot lesion was 49, and 28 had one or more teeth with cavitated lesions in dentin. The total number of cavitated lesions that needed treatment for group Residents was 16. For group Visitors, the total number of cavities was 41. Clinical characteristics of Residents and Visitors are summarized in Tables 2 and 3.

Table 2: Clinical characteristics of the Residents according to the caries experience at the baseline.

	No caries	Caries	p-value (Mann-Whitney)
Age range (Years)	21-51		
mean % GBI*	93.8 (SD 0.062)	87.5 (SD 21.6)	1
mean % VPI*	65.2 (SD 38.4)	91.3 (SD 10.8)	0.3556
Cavitated caries prevalence (%) *	0	56.25 (SD 51.23)	

*GBI: Gingival bleeding index.
**VPI: Visual plaque index.
*** Proportion of individuals with caries activity (dentin caries)

Table 3: Clinical characteristics of the Visitors participants according to the caries experience at the baseline.

	No caries	Caries	p-value (Mann-Whitney)
Age range (Years)	06-14		
mean % GBI*	48.2 (SD 28.8)	45.0 (SD 22.6)	0.9169
mean % VPI*	93.9 (SD 6.5)	97.2 (SD 4.1)	0.1802
Cavitated caries prevalence (%) *	0	65.7 (SD 48.16)	

*GBI: Gingival bleeding index.
**VPI: Visual plaque index.
*** Proportion of individuals with caries activity (dentin caries)

Participants from group Residents had access to piped water, as they lived in the institute which is in an urban area. As for group Visitors, the legal guardians of the participants answered the socioeconomic questionnaire (Supplementary material 1), the results of which are summarized in Table 4.

Table 4: Demographic and socioeconomic characteristics of the legal guardians from the Visitors participants.

Variables	
Gender	
Male	54.54%
Female	45.45%
Habitation	
Owned	63.15%
Rented/borrowed	36.85%
Water source	
Piped	89.47%
Well	10.53%
Family income	
Up to 1 minimum wage	68.42%
More than 1 minimum wage	31.58%
Work hours	
More than 40/week	42.1
Up to 40/week	57.90%
Guardian's education level	
1st to 4th grade of Elementary school	21%
5th to 8th grade of Elementary school	42.10%
High school	26.30%
College/Postgraduate	10.50%

The water source of 10.53% of group Visitors' houses was not piped, which means they do not have access to fluoridated water at home. Most of the families have an income up to one minimum wage and only 10.5% of the children's guardians have a college or postgraduate degree. According to information collected from the recall interview, the daily frequency of sucrose intake at baseline ranged from 4-6 times/day in participants from group Visitors who had caries activity. The Residents group have a controlled diet, and the daily frequency of sucrose intake reduced from 3-4 times/day at baseline to once a day during the treatment period. The clinical assessment at M1 showed a decline in caries prevalence. There were no cavities as they had all been restored, and all white spots from group Residents showed clinical aspects of inactivity. At M6, only participants from group Residents were assessed due to restrictions related to the COVID-19 pandemic. The VPI and GBI reduced approximately 13% in the Residents group, confirming the challenge of the plaque control.

All participants from group Residents assessed showed no activity of dental caries at M6 (after study 2), confirming the success of the treatment.

2) Treatment study

Of all participants assessed initially (n = 87), 5 from group Residents were not included in the second study as they presented no dental caries activity and 33 from group Visitors were excluded for various reasons, such as leaving the institute, already undergoing treatment at a private dentist, the tooth with a carious lesion was deciduous and exfoliated by the time we started the treatments and/or parents did not authorize the treatment.

ART restorations assessment

The restorations of group Residents were also assessed at M6. Only one examiner carried out the clinical assessment, and it was not the same person that treated the participants. The criteria used to assess the restorations was a modification from the ART restoration assessment criteria [Farag et al., 2011] described in Table 5. The examiner received training on the mentioned criteria with photographs and calibration was also made using photographs. The Kappa coefficient was 0.83.

Table 5: Modified ART restoration assessment criteria.

Code	Criteria	Definition
1	Present, satisfactory	Successful
2	Present, slight deficiency at cavity margin of less than 0.5 mm - no repair is needed	Successful
3	Present, deficiency at cavity margin of 0.5 mm or more - repair is needed	Failed
4	Present, wear greater than 0.5mm - repair is needed	Failed
5	Secondary caries - repair is needed	Failed
6	Fracture in restoration and/or tooth - repair is needed	Failed
7	Not present - restoration has disappeared	Failed
8	Not present - other restorative treatment performed	Censored
9	Not present - tooth has been extracted	Censored
10	Painful symptomatology or pulpal involvement	Failed

Of the 16 participants in the Residents group, 9 received ART restorations, and a total of 16 ART restorations were performed in this group. After 6 months, 14 restorations survived with no need of repair or substitution. Six restorations were satisfactory (code 1), seven presented a slight deficiency with no need of repair (code 2), two needed repair (code 3) and only one was missing (code 7).

3.4 DISCUSSION

Preventive care has been increasingly the focus of current dentistry. A previous study showed that having access to public health system dental care had a positive impact on the increment of dental caries in children [Moraes et al., 2020]. Socioeconomical status has proven to be a factor that influence the risk of developing dental caries [Chankanka et al., 2011]. Access to piped water, for example, is a way of ensuring the daily access to fluoridated water, and therefore reducing the risk of developing dental caries. The family income, educational level and work hours of the legal guardians can also influence the risk of children to develop the disease, as if reflects the access to dental health services, information about the disease – that affects its development as it is a behavioral disease – and availability to guide and supervise the children's diet and hygiene habits [Costa et al., 2012]. More than 68% have a total family income of up to 1 minimum wage and 63.1% of the guardians stopped studying before high school. This might have influenced the caries prevalence of more than 50% in group Visitors. Moreover, most of the participants in this group have access to piped (therefore fluoridated) water, which could explain the prevalence of caries despite the visual plaque index over 90% in both participants with and without caries [Iheozor-Ejiofor et al., 2015].

Caregivers completed a questionnaire and were instructed on toothbrushing techniques and its importance, so that they could improve the daily oral healthcare of group Residents, as it is recommended by the ORCA and EFCD Expert Delphi Consensus [Paris et al., 2020]. The participants with better cognitive and manual skills should always be encouraged to do their own tooth brushing to help them improve their skills and be as independent as they can [King et al., 2016]. Nevertheless, they should always have the supervision and help of the caregivers, as many of them do not have the necessary manual skills to perform the adequate toothbrushing by themselves, or the cognitive ability to understand its importance [Ningrum et al., 2020].

People with ID might have behavioral characteristics that make their dental treatment a challenge, and the same might happen with children. A total number of 40 male adults with intellectual disabilities live at the institute but only 21 participated in the study. All others have more severe ID and did not allow any dental assessment or treatment. For these people it is difficult to guarantee that they receive adequate daily tooth cleaning or even that it is performed at all. Treatment of these patients is also

difficult, the same way it might be for children and adolescents with dental fear and/or anxiety [Grindefjord et al., 2018]. When they need treatment, they are taken to a hospital to be treated under sedatives and that usually happens at a late stage of the disease demanding more invasive treatments.

Likewise, children generally do not have adequate oral hygiene and are likely to have a more cariogenic diet [Paris et al., 2020] and it is important that their parents or guardians ensure they brush their teeth properly [Aliakbari et al., 2020]. In low-income families, especially when both parents work many hours a day and cannot always afford qualified care for their children, oral hygiene habits are usually neglected. Public health measures as water fluoridation and educational measures on oral hygiene with fluoride toothpaste are extremely important [Iheozor-Ejiofor et al., 2015; Ricomini et al., 2021].

Restoring cavitated lesions is frequently necessary as they retain dental plaque and it is not possible to remove it properly, therefore the caries process continue, putting the dental pulp at risk. ART restorations have been reported to have high survival percentages in the deciduous and permanent dentition and are a good treatment option, including for patients with special needs [de Amorim et al., 2018]. In this study, the ART restorations that failed after 6 months involved multiple surfaces, which have been reported to have lower survival percentages [de Amorim et al., 2018].

Although restoring cavitated lesions, oral hygiene instructions and fluoride application are important parts of caries treatment, diet control is often forgotten or overlooked by dental professionals despite the knowledge of sucrose role in the development of dental caries. Providing diet instructions is a helpful preventive measure, but the patient might not always know that some foods in their diet contain sucrose. The use of a diet recall interview might be a way to identify sucrose sources in the patient's diet, identify diet-disease relationships [Crowe et al., 2018] and then provide personalized and more effective instructions for each patient. For both groups diet control is crucial. When oral hygiene is not adequate, or not possible to achieve, removing the aetiologic factor of the disease should be even more expected to control and prevent it, reducing the need for treatment and improving oral health, hence the quality of life [Ricomini et al., 2021].

Therefore, measures to control dental caries should include professional fluoride applications and helping children and ID patients develop the skills to deal with their

own oral hygiene, but mainly diet instruction/diet control as a way to prevent the development of dental caries.

3.5   CONCLUSION

Participants with oral hygiene difficulties had high caries experience. The ones with caries activity had a higher frequency of daily sucrose consumption and the prevalence of dental caries decreased after reducing this frequency in addition to professional fluoride applications and ART restorations. Diet control combined with invasive treatment is effective to prevent dental caries and should always be part of the treatment.

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SUPPLEMENTARY MATERIAL

Socioeconomic Questionnaire

1. How many people live with you? (Including children, siblings, relatives, and friends)
   a) I live alone
   b) One to three
   c) Four to seven
   d) Eight to ten
   e) Over 10

2. The house where you live is:
   a) Owned by me
   b) Rented
   c) Borrowed (by friends, relatives, or the government)

3. Your house is located in:
   a) Rural area
   b) Urban area
   c) Indigenous community
   d) Quilombola community

4. Where you live, is the water piped or from an artesian well?
   a) Piped
   b) Well

5. What is your educational level?
   a) First half of elementary school
   b) Second half of elementary school
   c) High school
   d) College (University)
   e) Postgraduate

6. What is your family income? (Sum of all the people who live with you)
   a) None
   b) Up to 1 brazilian minimal wage (up to R$998,00)
   c) From 1 to 3 brazilian minimal wages (from R$998,00 to R$2.994,00)
   d) From 3 to 6 brazilian minimal wages (from R$ 2.994,00 to R$5.988,00)
   e) From 6 to 9 brazilian minimal wages (from R$ 5.988,00 to R$8.982,00)
   f) Over 9 brazilian minimal wages (over R$8.982,00)

7. Do you work or have you ever worked?
   a) Yes
   b) No

8. What is your occupation at the moment?
   a) Agriculture, in the fields, farm or fishing.
   b) Industry
   c) Construction
   d) Commerce, bank, transport, hospitality or other services
   e) Employee of the Federal Government, state or municipal.
   f) Self-employed or teacher (education)
   g) Outside informal activities such as painter, electrician, plumber, garbageman, etc.
   h) At home informal activities such as sewing, private lessons, cooking, handicraft, carpentry, etc.
   i) Housework in other people's homes (cook, maid, gardener, nanny, laundress, cleaner, elderly companion, etc).
   j) Housework at my home (with no wage).
   k) Other.
   l) I'm not working at the moment.

9. How many hours a week do you work?
   a) No fixed hours, up to 10 hours/week.
   b) From 11 to 20 hours/week.
   c) From 21 to 30 hours/week.
   d) From 31 to 40 hours/week.
   e) Over 40 hours/week.

10. How old were you when you first started to work?
    a) Before 14 years old.
    b) Between 14 and 16 years old.
    c) Between 17 and 18 years old.
    d) Over 18 years old.

4 DISCUSSÃO GERAL E CONCLUSÕES DA TESE

4.1 DISCUSSÃO GERAL

Diante dos estudos presentes na literatura, é possível observar que há uma grande variedade de aspectos e fatores que influenciam no desenvolvimento da doença cárie. Isso inclui aspectos individuais de saúde, coletivos e socioeconômicos [Pitts et al., 2017; Baltiner et al., 2018]. Apesar dos vários fatores considerados moduladores que podem influenciar no risco de cárie, o fator considerado determinante é a dieta rica em sacarose [Sheiham and James, 2015]. O acúmulo de biofilme cariogênico nos dentes resulta, então, no surgimento de lesões pela desmineralização dos tecidos dentários. Neste trabalho, avaliamos alguns dos fatores socioeconômicos que podem influenciar no risco de o indivíduo desenvolver a doença cárie, como o acesso a água fluoretada, renda familiar, nível de escolaridade dos responsáveis pelas crianças e número de horas trabalhadas por semana, que auxiliaram na compreensão dos resultados clínicos encontrados de prevalência de cárie.

A complexidade da doença reflete a complexidade da composição do microbioma em indivíduos com cárie, pois da mesma forma que há influência de diversos fatores no desfecho clínico da doença, o microbioma também é influenciado por diversos fatores, além de haver uma variação individual intrínseca a cada indivíduo [Anderson et al., 2018]. Ao avaliar os dados de abundância de microrganismos na cavidade bucal de pacientes com cárie, diversos pontos devem ser considerados. Um desses pontos é a presença de uma grande variedade de bactérias em baixa abundância. Neste estudo, mais da metade dos filos identificados tiveram abundância relativa menor que 0,1%. Estes acabam sendo descartados das análises, porém podem desempenhar um papel importante ainda desconhecido no desfecho da doença, pois a forma como os microrganismos interagem e influenciam no meio ainda não é totalmente esclarecida na literatura [de Cena et al., 2021].

Após o tratamento, alterações relevantes no microbioma somente foram observadas na análise após 6 meses de manutenção da saúde, o que mostra a importância da mudança de hábitos a longo prazo para que haja uma alteração a nível microbiológico. Este dado é importante pois pode direcionar a forma como os tratamentos da doença são conduzidos. A supervisão com maior frequência dos

pacientes para reforço das instruções e avaliação da manutenção da mudança de hábitos pode ser uma maneira de garantir o sucesso do tratamento. Como parte dessa supervisão, entrevistas motivacionais podem ser utilizadas, e identificação do risco de cárie do paciente para construção de um plano de tratamento individualizado. O acompanhamento do paciente a longo prazo mostra-se, então, essencial para a manutenção da saúde [Yu et al., 2018]. Novas pesquisas de avaliação microbiológica em pacientes submetidos a tratamento da doença cárie são necessárias com acompanhamento a longo prazo, para avaliar a microbiota bucal após um período de acompanhamento maior dos pacientes.

4.2   CONCLUSÕES

O tratamento com restauração atraumática, fluorterapia e instrução de hábitos alimentares e de higiene controla efetivamente a doença cárie, demonstrado através da modificação do microbioma salivar após tratamento. Uma modificação relevante do microbioma acontece após manutenção de hábitos de higiene bucal e controle do consumo de sacarose na dieta por certo tempo, maior do que 1 mês de manutenção da saúde, por isso essa alteração de hábitos deve ser mantida para que seja observado controle da doença a longo prazo.

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5   PRESS RELEASE

A Odontologia tem como um dos principais desafios o tratamento da doença cárie, que ainda é muito prevalente no mundo. Para que possamos identificar todos os aspectos importantes para um tratamento efetivo, precisamos entender bem sobre a doença, como ela altera o funcionamento normal do organismo, e como os tratamentos existentes conseguem interromper e reverter o processo da doença. Os resultados deste estudo mostraram a eficácia do tratamento que compreende medidas invasivas (restauração dos dentes que tem cavidades) e não invasivas (com aplicação tópica de flúor e instruções de higiene e de dieta) na composição dos microrganismos presentes na saliva e, consequentemente, no controle da doença. Com isso, tratamentos com foco nessas medidas devem ser realizados pelos cirurgiões-dentistas, de forma a impactar positivamente a saúde bucal da sociedade. Os resultados desta pesquisa podem contribuir para os cuidados de saúde bucal de pacientes com deficiências moradores de abrigos e casas de acolhimento, que costumam ter certa resistência ao tratamento odontológico (como um dos grupos de participantes desta pesquisa), assim como de toda a população.

APÊNDICE A - Residual bacteriome after chemomechanical preparation of root canals in primary and secondary infections

A análise bioinformática e escrita deste artigo foram desenvolvidas durante o programa de Doutorado-sanduíche financiado pela CAPES, e o artigo foi publicado na revista científica Journal of Endodontics (https://pubmed.ncbi.nlm.nih.gov/35381276/). A mesma etapa de análise bioinformática aprendida neste trabalho foi desenvolvida posteriormente com os dados do trabalho da tese, portanto este artigo teve grande importância como aprendizado para o desenvolvimento da tese de doutorado.

CLINICAL RESEARCH

Residual Bacteriome after Chemomechanical Preparation of Root Canals in Primary and Secondary Infections

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ABSTRACT

Introduction: Secondary infections may be linked to the presence of residual microorganisms within dental root canals. The purpose of this study was to investigate the bacterial composition of primary and secondary root canal infections before and after chemomechanical treatment. **Methods:** Samples were collected before chemomechanical preparation (S1) and before obturation (S2) from 19 subjects (10 primary and 9 secondary infections). DNA was extracted, and the V3/V4 region of the 16S ribosomal RNA gene was amplified using the 347 F/803R primers and paired-end sequenced using the MiSeq (Illumina, San Diego, CA) instrument. **Results:** Sequencing analysis yielded partial 16S ribosomal RNA gene sequences that were taxonomically classified into 10 phyla and 143 genera. The most prevalent phyla in the S1 and S2 samples were Firmicutes and Bacteroides; however, when comparing between sample groups, Proteobacteria seem to have been enriched in secondary infections. The dominant genera in the primary S1 samples were Bacillus, Streptococcus, and Prevotella, whereas Bacillus, Streptococcus, and Selenomonas dominated the secondary infection S1 samples. Bacillus and Marinilactibacillus were the most dominant genera in the primary and secondary S2 samples. The mean number of operational taxonomic units per sample was 32,656 (±12,124 SD) and 37,113 (±16,994 SD) in the S1 and S2 samples, respectively. Alpha and beta diversities presented the same pattern within samples from both groups. **Conclusions:** Great interindividual variations in the bacterial composition of the root canal biofilms were observed. There was no difference in the bacterial composition before and after treatment, although some genera survived and seem to be part of a residual microbiome. Our findings revealed a high diversity of the bacterial communities present in root canal infections after chemomechanical treatment, although the majority of the taxa detected were in low abundance. (J Endod 2022;■:1–9.)

KEY WORDS

16S ribosomal RNA sequencing; endodontic inflammation; microbiota; next-generation sequencing; pulpitis

SIGNIFICANCE

Our findings reveal great interindividual variations and high bacterial diversity in all root canal biofilm samples, indicating the persisting nature of microbiota in root canals after endodontic treatments. Further efforts in disinfection strategies are needed.

Apical periodontitis is an inflammatory oral disease characterized by contaminated dental pulp and apical tissues and necrotic root canals. Infection is triggered by oral opportunistic pathogens invading and colonizing the root canals due to carious lesions, trauma, tooth fracture, or disruptions by dental procedures exposing dental pulp[1–3]. Endodontic treatment is the recommended clinical approach, which typically consists of the removal of infected pulp tissue from the root canal system, chemomechanical disinfection, filling procedures, and tooth obturation to prevent reinfection[4]. A primary infection refers to the first ever infection of a root canal. Studies have reported variable rates of treatment success, from 70%[5] to as high as 95%[6]. A review based on 26 clinical studies reported a success rate around 80%[7].

However, endodontic treatment failures lead to reinfection and are considered secondary infections or persistent infections, which may be caused by persistent inflammation linked to the presence of residual microorganisms within the root canals after chemomechanical treatment[8–10]. Also, secondary

infections can be associated with the re-entry of isolated microorganisms or biofilms into the root canal, which may occur due to the delay in placing a definitive coronal restoration with adequate sealing[11–14]. Other reported causes of treatment failure include missed canals during treatment, insufficient enlargement of root canals, perforations, residual caries, and root fractures[15]. Indeed, dental root canal systems include an isthmus, lateral canals, and apical ramifications, which can be difficult to access during endodontic therapy, particularly when microorganisms have already colonized these areas and can remain viable after treatment procedures[12,16].

Residual bacteria surviving chemomechanical procedures have been investigated using cultural and molecular approaches[17,18]. It has been reported that apicocoronal seals may become ineffective and allow host glycoproteins to percolate into the root canal environment, therefore providing an endogenous nutrient source to residual bacteria allowing them to proliferate and cause periradicular lesions[13,19]. Bacterial genera isolated from necrotic root canals have been found to be mostly strict and facultative anaerobes, such as *Peptostreptococcus*, *Prevotella*, *Porphyromonas*, *Fusobacterium*, *Eubacterium*, *Actinomyces*, and streptococci[11]. Interestingly, secondary root canal infections have been reported to have distinct microbial populations compared with nontreated ones. Some of these bacteria have been found to be resistant to conventional antimicrobials used in endodontic treatment and are able to remain viable in root-filled teeth[11,20]. Studies found in the literature vary in methodologies and types of periapical diseases included. To the best of our knowledge, only 2 studies assessed the microbiome of primary and secondary endodontic infections including different periapical diseases using next-generation sequencing (NGS), but both studies only collected samples after chemomechanical treatment[21,22]. Although the presence of a residual bacterial community during the root filling procedure represents a poor prognosis, no specific species has been linked to it[23]. Irrespective of the individual distinctions in species composition, sophisticated molecular methods to detail the community's composition may help establish strategies for more effective and tailored antimicrobial treatments improving the success rates of endodontic treatments.

The understanding of the microbial diversity and ecology related to endodontic infections is important to help guide clinicians toward the ideal therapeutic approach[12,24]. The aim of this longitudinal study was to investigate the bacterial diversity of primary and secondary root canal infections using high-throughput sequencing to answer the following questions:

(1) What is the microbial composition and abundance of infected root canals?
(2) Does the microbiological diversity differ between primary and secondary infections? and
(3) Which bacterial species may persist after standard root canal treatment?

MATERIALS AND METHODS

Subjects

The sample size of this study was determined following statistical advice by a qualified biostatistician at the Centre of Epidemiology and Biostatistics, University of Leeds, Leeds, UK. There are significant differences in the anatomy, ecosystem, infection nature, and disease pathogenesis when comparing the root canal system with other body sites. Hence, a decision was made to conduct a pilot study. Three studies that used a similar NGS approach in the form of pyrosequencing[25–27] reported the recruitment of 7, 10, and 17 participants, respectively. The usual pilot study with a sample size of 30 does not apply here because our observed outcome was not expected to be normally distributed data. Therefore, based on this and previous literature as well as the time available for patient recruitment and sampling, which was limited, we proposed the recruitment of 20 participants with an expected dropout rate of <15%.

The study population included subjects who had nonvital infected teeth with evidence of chronic apical pathology confirmed by clinical signs and symptoms, such as tenderness to percussion, soft tissue palpation and/or the presence of a sinus tract, a negative response to (thermal and/or electrical) pulp testing, and apical radiographic changes that indicated an apical pathology in line with clinical signs and symptoms.

This clinical study included subjects with both primary and secondary infections. The demographic and clinical data of teeth included in the study are described in Supplemental Table S1 (available online at www.jendodon.com). Only 1 tooth per subject was included in this study. The research team included 2 experienced and trained dental nurses who were involved in the participants'

recruitment and clinical care. Only 1 trained dentist performed clinical diagnosis and endodontic treatments. Ethical approvals were obtained from the National Research Ethics Service Committee of Leeds East (Research Ethics Committee reference number: 13/YH/0035), and the Leeds Research and Development Directorate approval was obtained from Leeds Teaching Hospitals (Leeds Teaching Hospitals National Health Services Trust Research and Development number: DT 13/10723).

Eligibility Criteria

The inclusion criteria were individuals with teeth with primary (previously untreated) or secondary (previously root-filled) root canal infections, restorable teeth, a stable periodontal condition, and the absence of periodontal pockets >4 mm. The exclusion criteria were individuals under 18 years old, individuals with any immune deficiency such as human immunodeficiency virus or leukemia, pregnant patients, individuals who had antibiotics in the last month, teeth with severe anomalies, and cases in which microbiological sampling may not be optimum or compromised by an ineffective coronal seal (eg, teeth with post[s], teeth with a root curvature >15°, and teeth that fail to show radiographic evidence of patent canals). Clinical characteristics were balanced between groups (pain: 6 primary and 5 secondary, sinus: 2 patients per group, swelling: 3 primary and 2 secondary, and apical periodontitis with radiolucency >10 mm: 1 primary and 2 secondary) (Supplemental Table S1 is available online at www.jendodon.com).

Endodontic Treatment and Sample Collection

The research was conducted at the Leeds Dental Translational and Clinical Research Unit, Leeds Teaching Hospitals. The root canal (re)treatment was performed over 3 clinical visits in all cases according to the agreed protocol (Supplemental Fig. S1 is available online at www.jendodon.com).

Root canal biofilm samples were collected following the protocol described by Moller[28]. In total, 2 types of biofilm samples were collected from each subject. S1 samples were collected during the first visit before chemomechanical treatment (using 2.5% sodium hypochlorite, calcium hydroxide dressing, and manual instruments). S2 samples were collected during the third visit immediately before obturation of the root canal. Supplemental Figure S2 (available online

at www.jendodon.com) provides further details.

The root canal treatment procedures of this study were tailored to achieve this aim as well as to optimize the quality of the study in accordance with the ethics and regulations of the United Kingdom. Although the selected cases were of a chronic nature, the definitive diagnoses varied; hence, some details of the treatment needed to be personalized for each given case. In addition, other factors such as tooth morphology, the restorative status, or those related to the patient were vital when judging the most appropriate treatment choice. Despite all of this, the clinical protocol was designed to be as similar as possible for all patients. This, in addition to the collection of samples at exact time intervals, was aimed to obtain a more comparable, reflective picture of the microbiological status of the infected root canals.

The sample collection procedures were as follows: the canal was filled with about 0.5–2 mL sterile saline. A new sterile surgical glove was worn before sampling, and a sterile file (Dentsply Sirona, Weybridge, UK) of at least size 20 was introduced into the canal and moved with a gentle filing motion to disrupt the biofilm. The file was then placed in the sample collection tube (Bijou, Merck Life Science, Dorset, UK), which contained 1.5 mL reduced transfer fluid. A sterile paper point was then inserted in the canal to the full working length to absorb the canal contents and then transferred to the collection tube. This was repeated until all fluid and biofilm were absorbed. In multirooted teeth, the sample was collected from the canal with the apical pathology. Upon collection, the sample was immediately placed in a jar with an anaerobic sachet and immediately transferred to the oral microbiology laboratory. Upon arrival, the collection tube was vortexed for 30 seconds and then placed in the anaerobic workstation for further laboratory analyses.

16S Ribosomal RNA Sequencing

DNA was extracted using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. The regions V3–V4 from the 16S ribosomal RNA gene were amplified using the Q5 High Fidelity DNA polymerase kit (New England BioLabs Inc, Life Technologies Inc, Ipswich, MA) with the 347F and 803R primers (Eurogentec, Seraing, Belgium). The master mixture (Supplemental Table S2 is available online at www.jendodon.com) was distributed as aliquots of 23 μL plus 2 μL of the template DNAs. The polymerase chain reaction (PCR) tubes were then loaded onto the thermal cycler (Techne; Bibby

Scientific, Stone, Staffordshire, UK). (The thermal cycling conditions are described in Supplemental Table S3 [available online at www.jendodon.com]). The presence of PCR products was checked using agarose gel electrophoresis with 1 μL GelRed DNA stain (Biotium, Fremont, CA). MicroCLEAN (Microzone Ltd, West Sussex, UK) was used to purify the PCR product samples. The DNA pellet was resuspended in 55.5 μL Ambion nuclease-free water (Thermofisher Scientific, Warrington, UK). The cleaned DNA was used with the NEBNext Ultra DNA library preparation kit for Illumina (New England Biolabs, Hitchin, UK), and was added to 3.0 μL End Prep Enzyme Mix and 6.5 μL End Repair Reaction Buffer (10×) to yield a total volume of 65 μL. The mixture tube was then placed in the thermal cycler (Techne, Cole-Palmer, Staffordshire, UK). (Thermal cycling conditions are described in Supplemental Table S4 [available online at www.jendodon.com]). Next, 15 μL Blunt/TA Ligase Master Mix, 2.5 μL NEBNext Adaptor for Illumina, and 1 μL ligation enhancer were directly added to the End Prep reaction mixture.

AMPure XP beads (Beckerman Coulter, Inc, Brea, CA) were used for the cleanup, and the product was then eluted into 28 μL 0.1× Tris-EDTA buffer. Finally, 23 μL of the solution was mixed with 25 μL NEBNext High Fidelity 2× PCR Master Mix, 1 μL universal PCR primer, and 1 μL Primer Index 1-38 (1 unique index for each sample). After mixing by pipetting and a brief centrifuge, the mixture tubes were then placed in the thermal cycler for PCR amplification (see Supplemental Table S5 [available online at www.jendodon.com] for setting details). AMPure XP beads were used again for another cleanup. Amplicon sizes were assessed with the 2200 Tapestation System (Agilent, Santa Clara, CA) using 1 μL from each DNA sample without dilution. Qubit Assay Kits (Invitrogen, Life Technologies, Carlsbad, CA) were used to quantify the libraries. The final multiplexed indexed library was pooled by adding equimolar concentration of the libraries into a 2.0-mL collection tube and then sequenced on the MiSeq platform (Illumina, San Diego, CA).

Data Analysis

The demultiplexed paired-end reads were denoised with DADA2 using the Quantitative Insights into Microbial Ecology bioinformatics pipeline[29], and clustering was performed at 99% identity to create operational taxonomic units (OTUs). The taxonomy was using the Greengenes 13_8 database[30]. Alpha diversity was evaluated with the Chao1 richness estimator, and the Shannon diversity index

was calculated. Beta diversity was also determined and microbial structures compared using UniFrac. Unweighted UniFrac distances were used to perform a principal coordinate analysis in R software (https://www.r-project.org/).

RESULTS

The sample consisted of 19 participants, 14 women and 5 men, with an average age of 42.89 ± 13.05 years. The number of primary and secondary infected root canals were 10 and 9, respectively. Discomfort/pain was related from 11 subjects, and 3 sujects had radiolucency higher than 10 mm (Supplemental Table S1 is available online at www.jendodon.com).

The number of OTUs detected in each sample is shown in Supplemental Table S6 (available online at www.jendodon.com). Overall, the average number of OTUs detected in S1 samples was 32,656 (±144.8 SD) OTUs/sample compared with 37,113 (±140 SD) in S2 samples. A total of 13 bacterial phyla were assigned (Fig. 1). At lower classification levels, 27 different bacterial classes, 49 orders, and 86 families were identified in the root canal samples. On average, the 4 most abundant phyla were Firmicutes (55.1%), Bacteroidetes (15.7%), Proteobacteria (15.0%), and Actinobacteria (8.4%) (Fig. 1). Firmicutes, Bacteroides, Proteobacteria, and Actinobacteria were in the same order of abundance and had similar percentages in the primary and S1 samples (Fig. 2). The secondary infection and S2 samples showed a similar phyla profile. SR1 and Chloroflexi were found only in primary samples and Synergistetes only in 1 secondary sample. A detailed examination of the S2 samples revealed a notable increase in Actinobacteria and a decrease in Fusobacteria (Fig. 2).

The most abundant classes were Bacilli (36.7%) and Clostridia (18.2%), both with similar abundance in primary and secondary infection samples. Clostridia was more abundant in S1 than in S2 samples, whereas Bacilli showed a similar percentage of abundance in both S1 and S2 samples. Clostridiales was the most abundant order (18.6%) followed by Bacillales (16.5%) and Lactobacillales (17.6%). The most abundant family was Bacillaceae (22.6%).

With regard to genera, 135 different genera were found in the samples. Of these, only 20 were found at an abundance of >1% in the overall abundance (data not shown). Seventy percent of the top 10 genera belonged to the Firmicutes phylum.

On average, the most abundant genera (all 33 samples included) were *Bacillus* (22.5%),
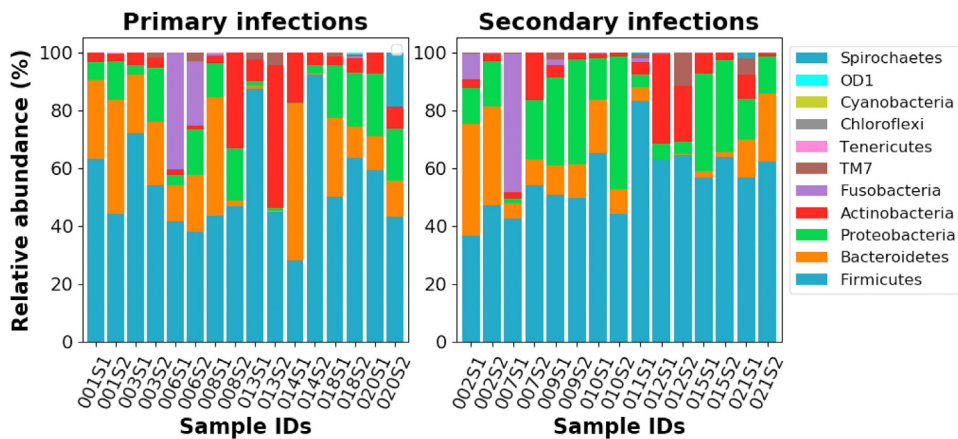
FIGURE 1 – The relative abundance of phyla in all samples. Eleven of the 13 identified phyla are displayed and listed in the legend (SR1 and Synergistetes abundance ranged between 0%–0.2% and 0%–0.1%, respectively, and were removed for ease of visualization).

Marinilactibacillus (9.2%), *Streptococcus* (7.3%), *Defluviitelea* (6.5%), and *Pseudomonas* (6.2%). The abundance of the main genera present in primary and secondary infection samples is shown in Figure 3. *Bacillus*, *Marinilactibacillus*, and *Pseudomonas* were more abundant in S2 samples than in S1 samples. The 10 most abundant genera also included *Clostridium*, *Selenomonas*, *Nonlabens*, *Anaerosinus*, and *Rothia*.

The most dominant genera in primary S1 samples were *Streptococcus* (4.4%), *Bacillus* (4.1%), and *Prevotella* (2.9%), whereas those in secondary S1 samples were *Bacillus* (6.2%), *Marinilactibacillus* (2%), and *Selenomonas* (1.9%). Interestingly, *Bacillus* and *Marinilactibacillus* were also the most dominant genera in primary S2 and secondary S2 samples. This may indicate the survival and resilience properties of these genera.

The 10 most abundant bacterial species were different between sample groups. Those from primary S1 samples are shown in

Supplemental Figure S3 (available online at www.jendodon.com). *Streptococcus agalactiae* was the most abundant bacterium in primary S1 samples, whereas *Marinilactibacillus psychrotolerans* was the most abundant in secondary S1 samples. *S. agalactiae*, *Defluviitalea saccharophila*, *Anaerosinus glycerini*, *Bacillus alkalinitrilicus*, *M. psychrotolerans*, and *Rheinheimera perlucida* were among the top 10 most abundant bacterial species in all 4 groups (Supplemental Figs. S2–S5 available online at www.jendodon.com). The abundance of most represented taxonomies in the samples is also presented in a heat map (Fig. 4).

Alpha Diversity

To assess the diversity within the samples, alpha rarefaction diversity was calculated and displayed in Supplemental Figures S6 and S7 (available online at www.jendodon.com). The diversity analyses of observed species according to infection type (primary or

secondary) and sample type (S1 or S2) resulted in a similar pattern. The box plots showing Chao1 and Shannon estimators for primary and secondary infections and the S1 and S2 samples are presented in Figure 5.

Beta Diversity

To assess bacterial diversity between samples, beta diversity analysis was performed. A principal coordinate analysis based on an unweighted UniFrac plot is shown in Figure 6. No distinct clustering between sample groups was observed, indicating that the samples had relatively similar microbial diversities.

DISCUSSION

High-throughput sequencing using Illumina's MiSeq was used to explore the diverse composition of endodontic infection samples before and after chemomechanical preparation of root canals in primary and secondary infections. In contrast with our study, some studies in the literature that assessed the microbiome in endodontic infections included only apical periodontitis samples[11,31], and 1 study did not make it clear if other periapical diseases were included[20]. Besides, this particular study used a checkerboard DNA-DNA hybridization to identify the microbiota present in root canal samples[20]. Two studies collected samples from extracted teeth[11,32], and in 1 of them the teeth were pulverized with a cryogenic grinder[32].

NGS data analysis indicated no significant difference in OTU abundance before and after root canal treatment (Supplemental Table S6 is available online at www.jendodon.com). Our study showed no difference in the bacterial composition before and after root canal treatment, although it was expected to
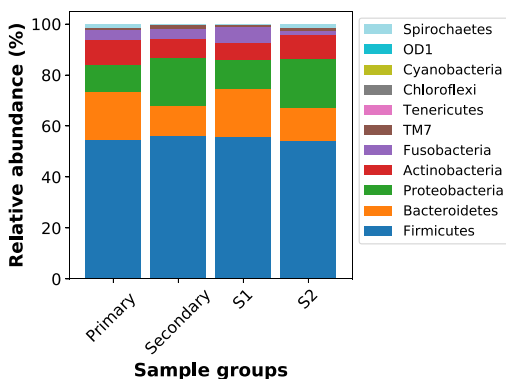

FIGURE 2 – The relative abundance of phyla in S1, S2, primary, and secondary infection samples. Eleven of the 13 identified phyla are displayed and listed in the legend (SR1 and Synergistetes abundance ranged between 0%–0.2% and 0%–0.1%, respectively, and were removed for ease of visualization).
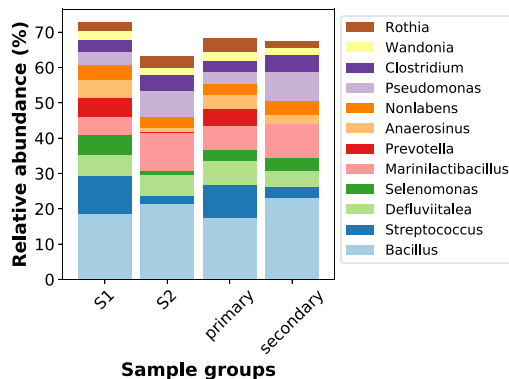
FIGURE 3 – Genera abundance in S1, S2, primary, and secondary infection samples.

observe a reduction in the bacterial load because of the chemomechanical treatment[33–36]. However, when comparing primary and secondary infections, a clear difference in the phylum composition was observed, with an enrichment of Proteobacteria in secondary infections. This result can indicate the resistance of taxa belonging to this phylum.

Our finding emphasizes the current knowledge that existing root canal preparation procedures usually fail to disinfect and clean large parts of the root canal system. It might reduce but not eliminate bacteria from the canal[33]. The presence of slightly higher proportions of OTUs in S2 samples compared with S1 samples may indicate clinical implications. These OTUs may have been present in low abundance in the primary samples. The change in the environment and the reduction in selection pressure after

treatment might have enriched these species. These OTUs may also come from viable and nonviable microorganisms that remained in the root canal because either they survived the treatment protocol or they were located and persisted in lateral canals that were not accessible to instrumentation and intracanal medication[12]. Some components from nonviable species remaining in the canal may serve as a nutrient source for the remaining microorganisms, leading to persisting or recurring infections. Moreover, other remnants of bacterial cells such as endotoxins may be involved in inflammatory reactions because they stimulate the release of cytokine and matrix metalloproteinases, which contribute to the inflammatory process[37,38].

Previous NGS studies support our findings related to the number of phyla and genera detected. One study using pyrosequencing detected 15 bacterial phyla

and 160 genera in 20 teeth[32], whereas in the apical root canal infections another study detected 84 genera and 10 phyla[27]. Other researchers[39] studied 23 extracted teeth and compared apical and coronal segments in which they detected 24 phyla. Other NGS studies detected between 9 and 18 phyla[11,22,31,40,41].

Firmicutes was the dominant phylum in primary and secondary infections, similar to the findings from a study using the Illumina HiSeq2000 instrument[41]. The results of other studies comparing primary and secondary infections may vary. Firmicutes was found to dominate secondary infections in 1 study[40], whereas Bacteroidetes was the most abundant phylum in primary infections in another study[33].

The enrichment of Proteobacteria in secondary infections has previously been described. A metagenomics study described similar findings to ours[24]. Furthermore, Keskin et al[32] showed a high abundance of this phylum in both primary and secondary infections. These reinforce the need of further studies on Proteobacteria's persistence into root canals.

Further evidence from Vengerfeldt et al[41] supports our findings of a high abundance of Firmicutes and Bacteroidetes in S1 samples. In a study from 2018, the Firmicutes phylum was also detected as being among the 5 most abundant phyla[11].

Streptococcus was the most dominant genus in primary S1 samples, and the same result was found in a number of culture, molecular, and pyrosequencing studies[40,42–44]. In secondary infection samples, genera such as
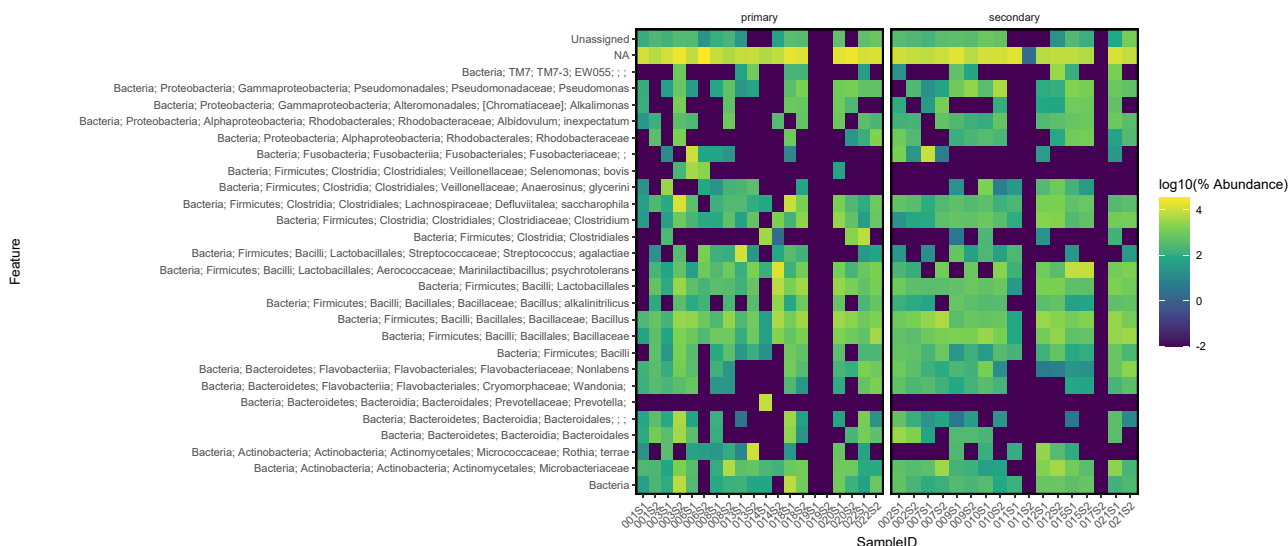

FIGURE 4 – The abundance of taxonomies described in a heat map. The percentage abundance is represented as log10 values and shown as a color gradient ranging from *yellow to blue*, with yellow being the most abundant features.
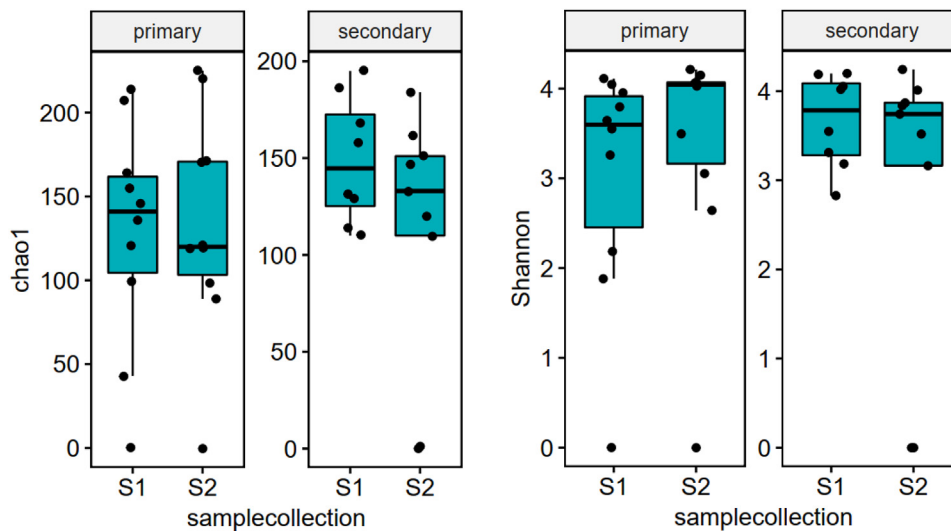
FIGURE 5 – Alpha diversity indices (Chao1 and Shannon) for primary and secondary infections. The *box plots* display indices grouped by S1 (before canal treatment) and S2 (before obturation) samples.

Fusobacterium, *Streptococcus*, and *Actinomyces* identified in this study also appeared in other studies as was described in a recent review[13].

In the primary infection samples, the dominant genera remained mostly the same before and after chemomechanical treatment of the root canal (*Streptococcus* and *Bacillus* in S1 samples and *Bacillus and Marinilactibacillus* in S2 samples) (Supplemental Fig. S8 is available online at www.jendodon.com). Similarly, in secondary infection samples, the dominant genera detected in both the S1 and S2 samples were *Bacillus* and

Marinilactibacillus, which gives evidence of their resilience.

Primary and secondary S1 samples had a similar OTU count. However, the dominant genera were found to be different; *Streptococcus*, *Bacillus*, and *Prevotella* were dominant in primary samples, whereas *Bacillus*, *Marinilactibacillus*, and *Selenomonas* were dominant in secondary infection samples. Nevertheless, most of the assigned bacteria were found in both primary and secondary infection samples although at a different abundance. Some studies have also found varied bacterial communities in primary

and secondary endodontic infections[11], but other studies found no difference between the 2 types of infection[32]. One consensus between these studies is that endodontic infections are polymicrobial and complex with some predominant genera but may still remain variable between individuals[10,11]. The differences in the dominant phyla and/or genera observed in these studies might be due to several aspects. These include variations in clinical conditions and anatomic locations, sampling methods, NGS platforms, and the read lengths used for analysis. In addition, site-specific endodontic bacterial communities can also contribute to variations[24].

Because of the change in the environmental conditions after endodontic treatment, bacteria persisting in the root canal and identified in secondary infections are usually the ones that can survive harsh conditions, such as a wide pH range and low nutrient availability[10]. Some studies have detected species such as *Enterococcus faecalis*[23] in secondary infections, which was not detected in this study. A recent systematic review showed that studies found *E. faecalis* mostly in secondary infection samples, sometimes in high abundance[45]. However, other studies, including ours, have not found *E. faecalis* in secondary infections[46] or have detected it in low abundance[20,36]. Reasons for its absence or low detection include sample selection, patient condition, and the detection method used[46]. It might also be due to the fact that in this study the samples were taken from inside the entire root canal and not from the root surface or different thirds of the root canal (apical or cervical). Elucidating the ecology and pathogenicity of microbial communities
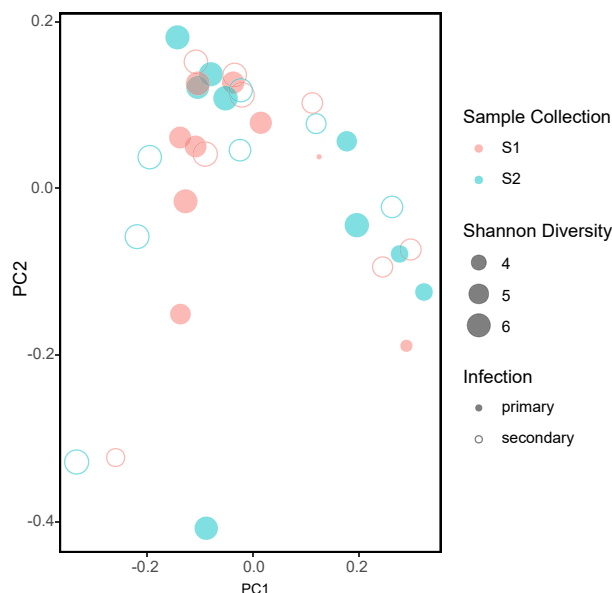

FIGURE 6 – Principal coordinate analysis of Bray-Curtis similarity of bacterial communities. The analysis was based on square root–transformed proportions of OTUs and included all samples.

requires the thorough identification of site-specific microbial species present in low abundance[47]. We especially recognize that the dental pulp was initially a sterile environment[26]. This aspect might be important in treatment strategies aiming for better rates of long-term success and reducing the need for expensive, unnecessary additional interventions.

S. agalactiae, the most abundant species in primary S1 samples, is a Gram-positive, facultative anaerobic bacterium. It can be commensal but is usually considered an opportunistic pathogen because it has been associated with systemic infections[48].

Marinilactibacillus is a relatively new gram-positive, facultative anaerobic genus with only 2 species described so far[49,50]; therefore, there is currently sparse information in the literature about the species *M. psychrotolerans* and its association with endodontic infections. More research is necessary to confirm our findings and better understand this microorganism.

One of the secondary objectives of this study was to investigate the prokaryote microorganisms that can resist after chemomechanical preparation. Opting for a multiple root canal treatment visit approach allowed for this investigation as well as for comparison with prepreparation samples. Although a Cochrane review[51] detected no significant differences in the effectiveness of root canal treatment between single and multiple visits, it concluded that the former is associated with a higher frequency of symptoms. In addition, for teeth with necrotic pulps and apical disease, as in this study, multiple-visit root canal treatment is the traditional treatment option because it allows

the use of interappointment medication, which may be beneficial for the cases with more established infections.

Limitations of this study include the lack of discrimination from dead and live microorganisms; hence, all genetic materials were assessed, which may have overestimated the bacterial load[52]. However, it is argued that an assessment of both live and dead microorganisms is important because these bacteria may have been predominant in the early phases of disease or played a part in biofilm formation[53]. Besides, targeting fragments of 16S ribosomal RNA variable regions using short-read sequences (up to ∼300 bp) instead of the full gene or a shotgun sequencing approach does not provide the same level of accuracy for identification to the species level[54]. Such profiling also lacks the necessary details required for a full understanding of the microbiota including nonbacterial microorganisms. About 37% of the reads could not be assigned to any taxa at the phylum level; this may be due to the PCR artifact, sequencing errors, or possibly unknown bacterial phyla[45]. The results should be interpreted with caution because of the heterogeneous samples regarding clinical variables such as pain and swelling, teeth with a sinus tract, and chronic apical periodontitis, although this was minimized by balancing these characteristics within groups. Further investigations are needed to complete a thorough profiling of endodontic biofilms.

CONCLUSIONS

Secondary infections have been shown to have similar diversity to primary infections but

with different bacterial abundance. Similar diversity was also found before and after chemomechanical preparation of the root canal, although some bacteria such as *Bacillus* and *Marinilactibacillus* were the most dominant genera in primary and secondary S2 samples and seem to be part of a residual microbiome. This is an indication that specific bacteria are able to survive the standard root canal disinfection procedure. Therefore, strict aseptic procedures; a more specific, targeted disinfection technique; irrigation; and washing time may be recommended. Further studies are essential to further explore the understanding of the ecology within the infected root canal and apical regions and guide strategies for treatment improvement.

SUPPLEMENTARY MATERIAL

Supplementary material associated with this article can be found in the online version at www.jendodon.com (https://doi.org/10.1016/j.joen.2022.03.008).

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APÊNDICE B - Enrichment of sulphate-reducers and depletion of butyrate-producers may be hyperglycaemia signatures in the diabetic oral microbiome

A etapa de sequenciamento do gene 16S rRNA deste artigo, parte da dissertação de mestrado da colega Camilla Pedrosa Vieira Lima (desse programa de pós-graduação) foi desenvolvida durante o programa de Doutorado-sanduíche financiado pela CAPES e o artigo foi publicado na revista científica Journal of Oral Microbiology (https://pubmed.ncbi.nlm.nih.gov/35694216/)

Enrichment of sulphate-reducers and depletion of butyrate-producers may be hyperglycaemia signatures in the diabetic oral microbiome

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ABSTRACT
Objectives: This study aimed to investigate oral microbial signatures associated with hyperglycaemia, by correlating the oral microbiome with three glycaemic markers. Potential association between clinical parameters and oral bacterial taxa that could be modulating the hyperglycaemic microbiome was also explored.
Methods: Twenty-three individuals diagnosed with type 2 Diabetes Mellitus (T2D) and presenting periodontitis were included, as well as 25 systemically and periodontally healthy ones. Fasting blood glucose, glycated haemoglobin, salivary glucose, periodontitis classification, caries experience and activity and salivary pH were evaluated. The V4 region of the *16S rRNA* gene was amplified from total salivary DNA, and amplicons were sequenced (Illumina MiSeq).
Results: Hyperglycaemia was correlated with proportions of *Treponema*, *Desulfobulbus*, *Phocaiecola* and *Saccharimonadaceae*. *Desulfobulbus* was ubiquitous and the most enriched organism in T2D individuals (log2FC = 4). The *Firmicutes/Bacteroidetes* ratio was higher at alkali salivary pH than acidic pH. In the network analysis, *Desulfobulbus* was clustered in a negative association with caries-associated and butyrate-producing bacteria.
Conclusion: The salivary microbiome is shaped by systemic hyperglycaemia, as well as changes in the salivary pH, which may be linked to local hyperglycaemia. The enrichment of predictive biomarkers of gut dysbiosis in the salivary microbiome can reflect its capacity for impairment of hyperglycaemia.

Introduction

Several oral manifestations of Diabetes Mellitus (DM) can be explained by the hyperglycaemia state that directly favours the enrichment of microbial pathogens, promoting damage of cellular function, and consequently, local inflammatory responses. This occurs due to interactions between the increased concentration of advanced glycation end-products and the increased proinflammatory cytokine levels. A well-established oral manifestation of DM is periodontitis, which may also impair the systemic glycaemia control [1,2]. A reduced salivary flow is also commonly observed in individuals suffering from this metabolic disorder [3,4]. The oral health is deprecated, essentially when the glycaemic levels are uncontrolled [5,6]. The poor glycaemic control can make adults with type 2 DM (T2D) more prone to dental caries, although the reasons behind this association are not yet explained [7]. A potential hypothesis is that the hyperglycaemia may increase the glucose levels in the saliva of patients, changing the oral microbial environment and promoting salivary acidification [8].

A growing body of literature recognizes the importance of salivary glucose as a biomarker of blood glucose levels [9–12]. Salivary glucose may be accountable for reducing the pH of the oral cavity, since oral bacteria can use glucose as a substrate in fermentative pathways, releasing acids as final metabolites. If these changes in the availability of metabolic substrates linger, the so-called 'dynamic stability stage' of the oral microbiome can be lost [13]. The acidification would facilitate acidogenic bacterial growth, shifting the ecological balance of the microbiota [8,14]. Furthermore, individuals with uncontrolled DM frequently present ketoacidosis increasing the ketone bodies (acetone, acetoacetic acid, and β-hydroxybutyric acid) in blood and urine [15], and probably in saliva. The potentially altered pH [4] can represent a selective pressure over the diabetic oral microbiome. If the pH-balance of the microbial community is disrupted by severe environmental pressures, the microbiome may collapse into an 'acidogenic stage' (increase in the acidogenic microorganisms) that initiates dental caries or into an "inflammatory stage" (increase of

inflammophilic anaerobic microorganisms) leading to periodontitis [13].

The impact of DM on the salivary microbial biodiversity has been investigated [14,16–19]. *Bacteroidota* and *Proteobacteria* are enriched in the salivary microbiome of DM patients, somehow reflecting the pattern seen in the gut microbiome [20], and suggesting a potential correlation of gut and oral microbiomes in diabetic subjects. Indeed, the imbalance observed in the gut microbiota might be a main contributor of local and systemic diseases [18,21]. Microbial communities along mucosal surfaces throughout the digestive tract are hypothesized as risk factors for impaired glucose regulation. Since some gastric bacteria are introduced through the oral cavity, it is possible that a decreased salivary pH due to hyperglycaemia may act as a filter to inhibit replenishment of gastric *Bacteroidota*, while more easily transmitting gastric *Firmicutes* [8]. This can also be explained by the communication through secondary metabolites of different microbiota of the human body [22]. Nevertheless, it is not yet clear if dysbiosis in the oral microbiome is a typical feature of hyperglycaemia and a potential contributor to progression of hyperglycaemia itself. Microbial metabolites from the oral cavity could serve as crosstalk mediators between host and microbiome, impacting glucose metabolism. So far, the oral microbial signatures associated with hyperglycaemia, as well as its oral manifestations, are still undetermined.

The oral dysbiosis–diabetes relationship is to be elucidated. A fundamental need is understanding how the oral microbiome shifts from homeostatic to dysbiotic condition, altering the oral health status of DM patients. Understanding this process would allow more efficient treatments for oral manifestations of DM. This study aimed to investigate oral microbial signatures associated with systemic and local hyperglycaemia, by correlating the oral microbiome with three glycaemic biomarkers (glycate haemoglobin (HbA1c), fasting blood glucose (FBG) and salivary glucose). We also aimed to explore a potential association between biological markers and oral bacterial taxa that could be modulating the hyperglycaemic microbiome, such as the salivary pH, T2D and periodontitis diagnosis, and levels of dental caries.

Subjects and methods

Ethics

The study was approved by the Research Ethics Committee of the School of Health Sciences of the University of Brasilia (process number 87962818.4.0000.0030) in accordance with the declaration of Helsinki. All patients signed a formal consent form and received basic dental treatment.

Healthy participants received oral hygiene instruction and professional prophylaxis.

Study design, setting and participants

This study was nested in a cross-sectional study [23], and it was performed and reported following the STROBE checklist [24]. Eligible individuals (>18 years old) were enrolled in the Diabetes Dental Clinic at the University Hospital of Brasilia (Federal District, Brazil). Patients were recruited from June 2018 to March 2020.

Individuals with and without a diagnosis of T2D were included in order to ensure a broad range of glycaemic levels. Cases of T2D were defined through a previous medical diagnosis. All patients in this group were using hypoglycaemic medication, either insulin or another hypoglycaemic drug. Only individuals diagnosed with any level of periodontitis were included in this group to assure its homogeneity (interdental clinical attachment loss ≥3 mm detectable at ≥2 non-adjacent teeth) [25]. Another group of systemically and periodontally healthy individuals was included (named as no-T2D), which were selected among family members and other individuals under treatment at the university clinics. All individuals, either T2D or no-T2D, went through blood and saliva glucose levels measurements (as described below). Individuals with type 1 DM were excluded, as well as those with severe systemic comorbidities, pregnant or puerperia, transplanted patients, individuals with a history of epilepsy, or with systemic conditions that may influence the physiology of the salivary gland, such as hypothyroidism, radiotherapy or chemotherapy treatment that preceded 3 months.

Based on a pilot study [26], a minimum number of 14 samples is required to detect a correlation of 0.7 with power of 80% in an alpha of 5% (Fisher's Z test) between bacteria taxa and clinical parameters. For a mean difference of 2 (standard deviation of 1.5 and 3.1) in the *Firmicutes/Bacteriodota* ratio between T2D and no-T2D microbiomes, a minimum of 48 samples is required, to which was added a loss rate of 10%, resulting in a sample size of 52 individuals.

Salivary sampling

Stimulated and passive salivary flow samplings were performed in the morning (8–10 am) to minimize the effect of circadian rhythms. Individuals were asked to refrain from drinking, eating, and performing physical activities at least 2 h before salivary collection. The salivary collection was carried out for 5 min of passive drooling. Upon collection, 500 μL of the saliva samples were aliquoted into microtubes and pellets stored at −20°C until further DNA extraction and sequencing.

Stimulated saliva was also collected for 5 min, while participants chewed a rubber device (1 x 1 cm, free of flavor). They were tied to a piece of dental floss so that there was no danger of swallowing by the patient during chewing.

Glycaemic markers

Fasting blood glucose (FBG) (hexokinase method; mg/dL) and glycated haemoglobin (HbA1c) (turbidimetric inhibition immunoassay; %) tests were carried out at the university's partner-certified centre of diagnosis (Sabin labs, Brasilia – Distrito Federal, Brazil). Salivary glucose was measured from the stimulated saliva using the Labtest Glucose liquiform® kit (Labtest Diagnóstica S.A – Minas Gerais, Brazil), according to the manufacturer's instructions with an adaptation for the saliva volumes, as follows: after centrifugation, 150 µL of the supernatant was added to 500 µL of the kit reagent 1 (phosphate buffer 30 mmol/L, pH 7.5; phenol 1 mmol/L; glucose oxidase 12,500 U/L; peroxidase 800 U/L; 4-aminoantipyrine 290 mol/L; azide sodium 7.5 mmol/L; and surfactant). A glucose standard was added to the experiment. After homogenization and incubation at 37°C for 10 min, 250 µL of the reaction was transferred to the 96-wells plate, in duplicates, and read at 505 nm. Blood and glucose levels were analysed as continuous variables, and the salivary glucose was also categorised as high ($\geq$0.35 mg/dL) and low (<0.35 mg/dL), according to the data distribution.

Sucrose frequency intake

A 24-h diet recall was performed to determine the frequency of sucrose intake.

Periodontitis classification

All patients underwent periodontal examination and evolution of panoramic x-rays. The stage and extension of the periodontitis were then classified by the same examiner, with broad experience as a periodontist, based on the International Classification of Periodontal Diseases [2017, 25].

Dental caries detection

Dental caries examinations were performed by trained and calibrated dental students (Kappa > 0.7), as described elsewhere [23]. Briefly, the presence of caries was observed and recorded by thorough dental examination under artificial light, in a supine position, using clinical mirrors, WHO probes, and tooth isolation with cotton rolls. After tooth cleaning and drying, the visual-tactile inspection was performed to record active and inactive coronal caries

lesions, based on the Nyvad criteria [27]. Caries activity (the number of surfaces with either non-cavitated or cavitated caries) and the traditional DMFS (WHO criteria; at the cavity level, representing the past caries experience) were evaluated.

Salivary pH

The salivary pH was tested on the stimulated saliva using the pH-Fix® indicator strips (Macherey-Nagel GmbH & Co. KG- Düren, Germany). After 1 min of immersion in the saliva, the result was compared to the standard table, as indicated by the manufacturer. The buffering capacity was used for adjustment in the multivariate analysis. It was measured from 1 mL of stimulated saliva; then 3 mL of 0.005 M hydrochloric acid was added, and the pH was measured with an indicator strip after 2 min.

Salivary DNA extraction and sequencing

DNA was extracted from saliva using the QIAamp DNA Mini Kit (Qiagen), following the manufacturer's protocol. The V4 region of the *16S rRNA* gene was amplified using the Q5 High Fidelity DNA polymerase kit (New England BioLabs Inc., Life Technologies Inc., MA) and the 564F (TCG-TCG-GCA-GCG-TCA-GAT-GTG-TAT-AAG-AGA-CAG-AYT-GGG-YDT-AAA-GNG) and 806R (GTC-TCG-TGG-GCT-CGG-AGA-TGT-GTA-TAA-GAG-ACA-GTA-CNV-GGG-TAT-CTA-ATC-C) primers (Eurogentec, Belgium). PCR generated amplicons with approximately 242 bp length and products were checked in agarose gel electrophoresis. Amplicons were then purified using MicroCLEAN (Microzone ltd, UK). The Nextera XT kit was used for library preparation and adaptor ligation, followed by clean-up with AMPure Beads (Beckerman Coulter, Inc). Amplicon sizes were assessed with the 2200 Tapestation System, and the QuantiT PicoGreen dsDNA Assay Kit was used to quantify the libraries. Amplicons were then paired-end sequenced on the Illumina MiSeq platform (Illumina, San Diego, CA).

Bioinformatics and statistics analysis

The amplicon sequence variants (ASVs) were generated through the DADA2 pipeline v.1.12.1 [28] in R version 3.6.1 [29]. Reads were trimmed in 15 nt on left side, and the identified Phi-x sequences were removed. Datasets were filtered allowing a maximum of two expected bases errors per read, N called bases were not permitted, and reads were truncated at 220 nt for the forward and 200 nt for the reverse fragments. Error rates were estimated using a training set of reads and inferred to the whole dataset, and sequences were denoised. Denoised reads were merged, and

chimeras identified by method consensus were removed. Qualified sequence variants had an average length of 246 bp and were assigned using the Silva v.138 database [30]. Before performing downstream analysis, ASVs assigned to Eukaryote, Chloroplast, and Mitochondria were removed using the Phyloseq package (version 1.34.0) [31].

The Spearman's correlation was performed to determine the correlation between the taxa and explanatory variables using the Microbiome R package (version 1.12.0) [32]. The taxa presenting a mean relative abundance higher than 0.001% and significant association ($p < 0.01$) to the variables tested were plotted in a heatmap. The Canonical Correlation Analysis (CCA) was performed using the vegan R package (version 2.5–7) [33] and plotted using the ggrepel package (version 0.9.1) [34]. The significance of correlation between canonical axes and explanatory matrix was tested with 10,000 permutations.

The alpha diversity was estimated for a dataset of sequence variants rarefied to 35,000 sequences per sample, by the rarecurve function from the vegan R package (version 2.5–7) [33]. The Shannon's index, Chao1′s index and the Pielou index of samples were determined using the Microbiome R package (version 1.12.0) [32], for univariated comparison between groups and bivariate comparisons between groups and pH (alkali – pH 8, neutral – pH 7 or acidic – pH 6) or salivary glucose (≥0.35 or <0.35 mg/dL). The square-root transformed relative abundances of sequence variants combined at the genus level (or the highest taxonomic level annotated) were used to build matrices of similarity based on the Bray-Curtis dissimilarity. The ordination distance was plotted in a non-metric multidimensional scaling (nMDS) using the Phyloseq package (version 1.34.0) [31].

Microbial taxa with differential abundance between groups were identified by DESeq algorithm with the Benjamini-Hockenberg (BH) correction test. Results were obtained using DESeq2 package (version 1.30.1) [35].

Mean and standard deviations were calculated for clinical parameters. The relative abundances at different taxonomic levels were used to evaluate comparisons within and between groups regarding the salivary pH and salivary glucose. Pearson's correlation, Mann–Whitney and Kruskal–Wallis non-parametric tests were applied for data comparison using SPSS (SPSS Inc. version 26, Chicago, IL).

Network analysis was performed for the modified centered log ratio (mclr) normalized data, using the spring association method from the NetCoMi package (version 1.0.2) [36]. Differences into the taxa association between sample groups were tested with the cluster fast greedy method.

Results

Clinical characteristics

Saliva samples were obtained from 52 individuals who underwent dental examinations. Samples from four individuals were excluded from analysis due to missing data. From the remaining sample, 23 individuals had a clinical diagnosis of T2D, from which 10 were insulin users and the remaining used other hyperglycemic medication. The same individuals had periodontitis: n = 6 had stage 4 generalized periodontitis, n = 11 had stage 4 localized periodontitis; n = 6 had stage 3 localized periodontitis. Their HbA1c and FBG levels varied from 6 to 14.2% and 47 to 310 mg/dL, respectively, confirming a great range of glycaemia levels. Twenty-five other individuals were systemically and periodontally healthy, all of them presenting HbA1c lower than 6% and FBG lower than 120 mg/dL. Glycaemic markers, either from saliva or blood, were significantly higher in T2D individuals. However, the local hyperglycaemia varied more, and six subjects had T2D diagnosis but salivary glucose levels were below 0.35 mg/dL, while seven subjects had salivary glucose >0.35 mg/dL and had no diagnosis of T2D (Table 1). Patients with T2D (average age = 58 ± 8) were slightly older than patients with no-T2D (average age = 43 ± 13) ($p < 0.001$). Besides their hyperglycaemic state, their frequency of 24 h-sucrose intake was higher than for no-T2D, similar to their caries experience (DMFS). This pattern was not observed for active caries (active D-S component). The salivary pH and the salivary glucose had a weak negative correlation ($r = -0.3$; $p = 0.04$).

Sequencing output

The dataset from saliva samples sequencing, after screening and optimization, resulted in 2,281 ASVs. Seventy-eight ASVs belonged to the Archaea domain and 2,203 ASVs to the Bacteria domain. Archaea represented 0.01% of the reads, and 33 samples presented at least one taxon belonging to the Archaea domain. The overall salivary microbiota was composed of 33 phyla, 61 classes, 130 orders, 194 families, 332 genera and 407 different taxa annotations. A total of 47 samples were included in downstream analyses after quality checking.

Correlation of taxa with glycaemic markers and clinical parameters

There were 119 taxa significantly correlated ($p < 0.05$) to at least one of the analysed clinical parameters, including the three glycaemic markers and 38 out of 119 taxa with p-value < 0.01 (see Supplementary Table 1 for correlation values). What stands out from this result is the positive correlation between both blood glucose

Table 1. Clinical characteristics of the samples. No-T2D (systemically and periodontally healthy individuals); T2D (individuals with type 2 diabetes mellitus and periodontitis).

	no-T2D N = 25		T2D N = 23		
	Average	SD	Average	SD	p
Sociodemographic and habits					
Age	43.13	12.98	58.52	8.5	<0.001*
Sex – N(%) female	19 (76%)		35 (72%)		0.748**
24-h sucrose frequency****	0.7	0.9	2.1	1.6	0.005*
Glucose levels					
Salivary glucose (mg/dL)	0.37	0.5	0.84	0.65	0.002*
<0.35 mg/dL (N)	14		6		0.008**
≥0.35 mg/dL (N)	7		17		
HbA1c (%)	5.25	0.41	8.83	2.02	<0.001*
FBG (mg/dL)	89.67	13.07	148.13	65.43	<0.001*
Dental caries					
DMFS (WHO criterium)	36.06	21.01	83.41	31.41	<0.001*
Caries active extent (active D-S)	2.00	3.9	1.14	1.69	0.637*
Other salivary characteristics					
Unstimulated salivary flow – N (%)					
Assialia	3 (12%)		3 (13%)		0.440**
Hypossalivation	10 (40%)		13 (56.5%)		
Ideal	12 (48%)		7 (30.4%)		
Salivary pH	7.08	0.49	7.04	0.64	0.850*
Acidic – pH 6 (N)	2		4		0.483***
Neutral – pH 7 (N)	19		14		
Alkali – pH 8 (N)	4		5		

*U Mann–Whitney test; **Fisher exact test; SD = Standard deviation; WHO = World Health Organization criteria for caries detection; ***Chi-square test; ****Missing data = 6 individuals

levels (FBG and HbA1c) with *Treponema, Desulfobulbus* and *Phocaicola*. The salivary pH had the highest number of negatively correlated taxa: *Actinobacillus, Haemophilus, Kingella, Mannheimia, Neisseriaceae, Prevotellaceae* UCG.004, T2WK15B57, and TM7a. *Abiotrophia* and *Oceanivirga* were positively correlated while *Desantisbacteria* was negatively correlated with both caries variables (active caries extent and DMFS, representing caries activity and past caries experience, respectively), with strength of correlations between 0.4 and 0.6 (Figure 1(a)).

The CCA multivariate analysis under-reduced model confirmed statistical significance for salivary pH (p = 0.04) and HbA1c (p = 0.02) (53 taxa with abundance >1%) (Table 2, ANOVA multivariate analysis). Buffer capacity, salivary glucose, FBG and caries (activity and experience) were not significantly affecting the microsystem. Caries indices (active caries extent and DMFS) were concurrent with all blood indexes (HbA1c, FBG and salivary glucose), and *Desulfobulbus* and *Saccharimonadaceae* followed the increase of all those parameters (Figure 1(b)).

Diversity and relative abundances of the salivary microbiome

The diagnosis of T2D was used to compare diversity and relative abundances, so as the salivary pH that represented the clinical parameter with the highest significance in the CCA multivariate analysis. The salivary glucose was also tested, as the glycaemic marker that varied most independently of the T2D diagnosis, and represented the local hyperglycaemia. There was no difference for alpha-diversity in the

salivary microbiome regarding the diagnosis of T2D (Figure 2(a), Supplementary Figure 1, Supplementary Table 2), although both groups presented differences in clinical characteristics that should substantially shape the oral microbiome (age, hyperglycaemia, diagnosis of T2D, periodontitis, caries experience). The alpha-diversity was calculated for samples rarefied at 35,000 reads. Two samples (one with no-T2D and another with T2D) were removed from the set of analysis due to lower counts of reads (Supplementary Figure 2). A borderline result showed higher diversity in individuals without a diagnosis of T2D when they had low salivary glucose (Figure 2(a)).

The compositional dissimilarity among samples was calculated by the PERMANOVA test, for 10,000 permutations. It showed significant variability in community composition regarding the diagnosis of T2D (F = 1.93; p = 0.02), with respect to pH (F = 1.58; p = 0.01), and the salivary glucose (F = 2.16; p = 0.01) (Table 3). In this analysis, the systemic hyperglycaemia was categorised (HbA1c < 6.0%/>6.0%; FBG = <100 mg/dL/>100 mg/dL), so that periodontitis (localised/generalised), showed no significant differences. A cluster of samples was observed regarding the salivary glucose, and the pH 6 was slightly far from the others (Figure 2(b)).

Twenty-three out of 33 phyla presented very low abundance and prevalence. The average number of phyla was 22 in T2D and 25 in no-T2D samples. Regarding the archaeal content, the *Euryarchaeota* phylum (which includes methanogenic organisms) was detected in only two samples, while *Halobacterota, Chrenarchaeota* and *Nanoarchaeota* were more prevalent archaeal phyla, but representing no more than 0.001% of the total reads (Supplementary Figure 3).
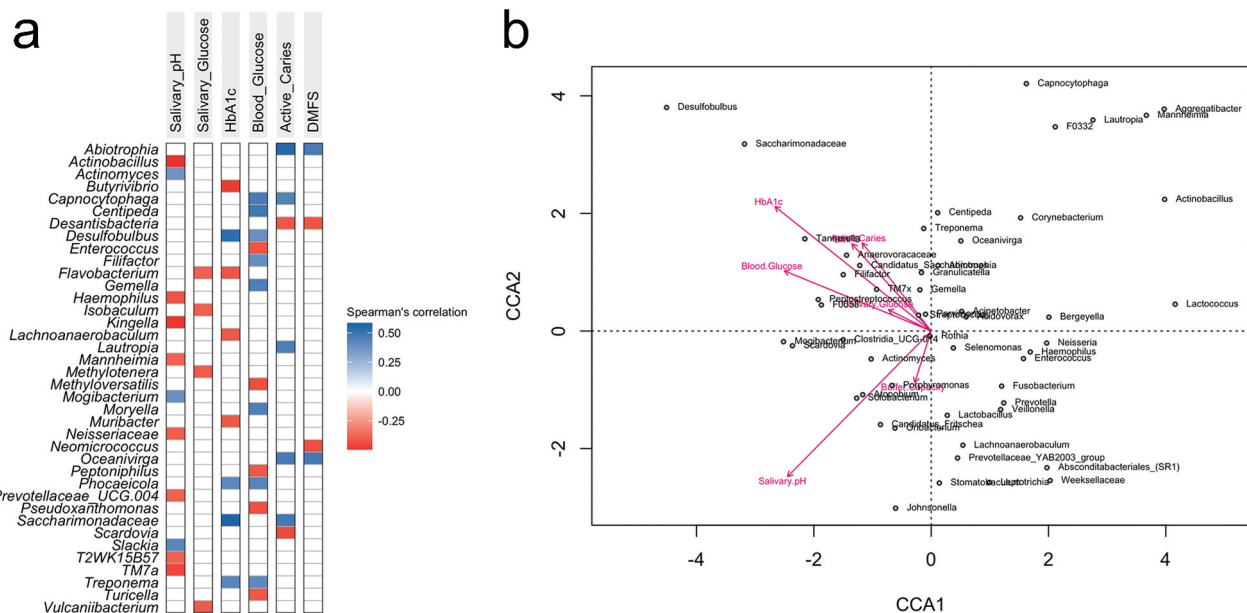
Figure 1. (a) Spearman correlation between taxa with glycaemic markers and clinical variables (p < 0.01). (b) PERMANOVA test plot (number of permutations: 10,000) for CCA under reduced model (53 taxa with abundance higher than 1%, N = 47).

Table 2. ANOVA CCA permutation test under reduced model for glycaemic markers and clinical parameters.

Factor	F value	p – value
Salivary pH	**1.6218**	**0.0447**
Buffer capacity	0.4605	0.9776
Salivary glucose	1.1879	0.2518
HbA1c	**1.8194**	**0.022**
Fasting blood glucose	0.7376	0.8054
Active caries	0.8324	0.6562
DMFS	0.6739	0.8581

The 10 most abundant phyla presented no differences in their relative abundance regarding the diagnosis of T2D (Figure 2(c)). The increase of the salivary pH was followed by a clear reduction of *Proteobacteria* and an increase in *Firmicutes* and *Actinobacteriota*. The relative abundance of *Actinobacteriota* was also affected by the salivary glucose (Figure 2(c)), which was confirmed when the taxa average differences were compared. The *Actinobacteriota* phylum showed higher abundance in the group of individuals with salivary glucose <0.35 mg/dL than in the group with salivary glucose ≥0.35 mg/dL (17.6 ± 6.8% vs. 13.1 ± 5.1%; p = 0.01))(Table 4, p = 0.08). The *Firmicutes/Bacteroidota* ratio significantly increased with the alkalinisation of the salivary pH (p = 0.03). The opposite was observed for *Proteobacteria* that seemed to be increased in abundance through saliva acidification (pH 6; p = 0.003). *Bacteroidota* (p = 0.02), and *Spirochaetota* (p = 0.01) were in low abundance, butwere most likely affected by the salivary pH. Regarding the genus level, it is worth to mention that *Veillonella* was enriched in the acidic saliva (p = 0.01). These estimations were performed based on non-parametric calculations, considering the

small size of the saliva samples at acidic and alkali pHs (Table 4).

Fifty-three genera had abundance higher than 1% (Supplementary Figure 4). Several organisms in abundance lower than 0.01% were detected in at least 50% of the samples, such as *Aggregatibacter* (m = 0.001%, 0–0.09%), *Bifidobacterium* (m = 0.0001%, 0–0.012%), *Capnocytophaga* (m = 0.002%, 0–0.064%), *Lactobacillus* (m = 0.0001%, 0–0.013%), *Verrucomicrobiales* (m = 0.0002%, 0–0.008%), amongst others (Figure 3). Some of these microorganisms were ubiquitous taxa in individuals with the diagnosis T2D, such as *Desulfobulbus* (m = 0.0002%, 0–0.002%). Others, such as *Brevundimonas* (m = 0.0001%, 0–0.0008%) were ubiquitous taxa in no-T2D samples.

Deseq2 analysis showed significant differential abundance for nine taxa (p < 0.05; BH correction) out of 390 taxa (Figure 4). *Desulfobulbus*, described above as being ubiquitous in T2D samples, correspondingly had the greatest enrichment in individuals with T2D (*Desulfobaterota* phylum, log2FC = 4), followed by *Bacteroidota* (*Capnocytophaga* and *Tannerella* genus, log2FC = 2). *Actinobacteriota*, (*Neomicrococcus* genera, log2FC = −6) and *Proteobacteria* (the *Methyloversatillis* and *Brevundimonas* genus) were the most significantly enriched organisms in T2D samples. *Patescibacteria* (*Saccharimonadaceae* GTL1 and *Butyvibrio*), as well as *Fusobacteriota* (*Leptotrichia*) were also significantly more abundant in the samples from individuals diagnosed with T2D.

As a complementary analysis, the average abundances of deliberately selected genera (usually linked to dental caries) were compared to test the hypothesis of the enrichment of typical acidogenic/acidophilic
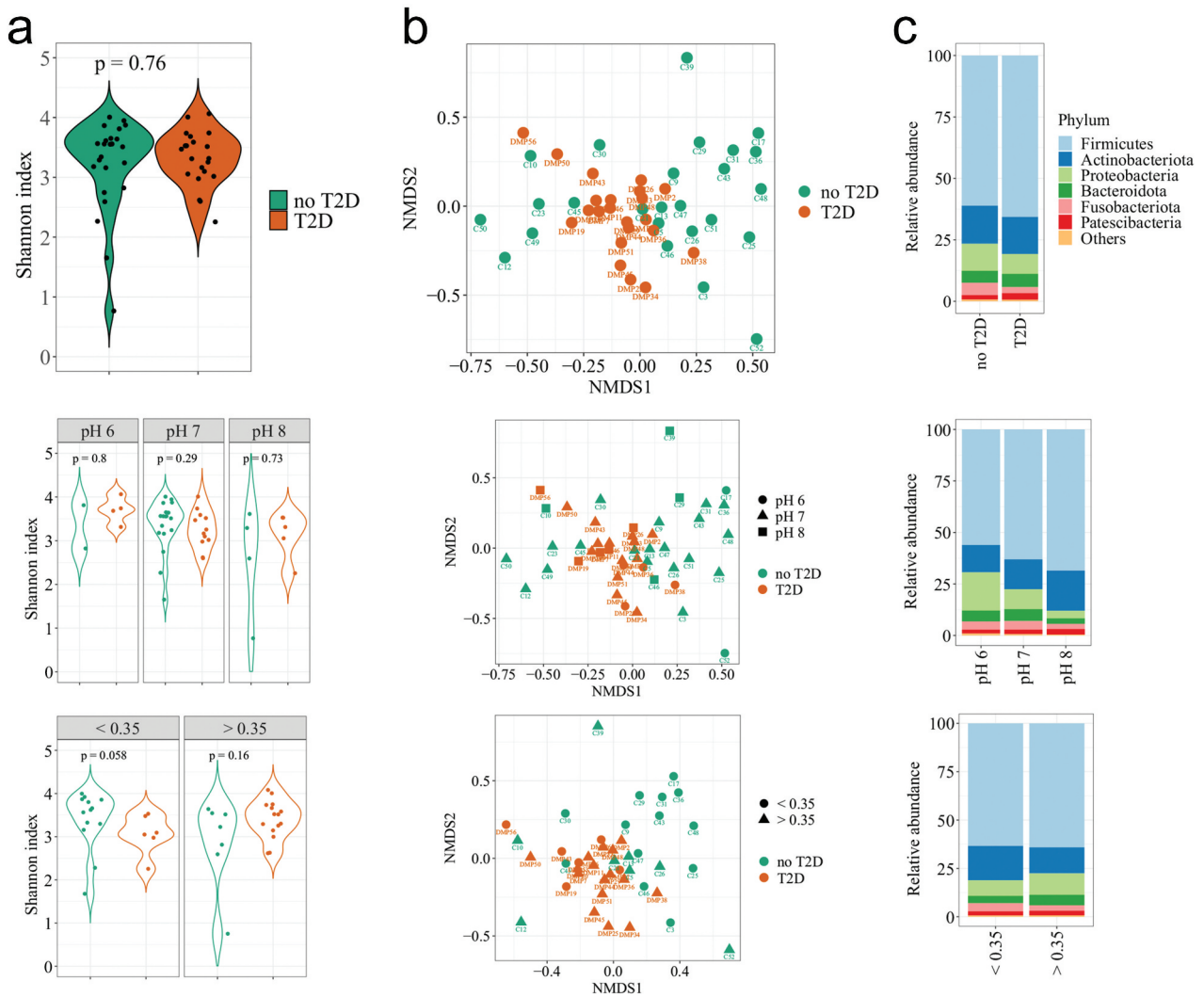
Figure 2. Diversities of the salivary microbiome according to the T2D diagnosis, salivary pH, and salivary glucose (<0.35 mg/dL or >0.35 mg/dL). (a) Alpha-diversity index (Shannon index). B = Beta diversity measured by the Bray-Curtis distance on nMDS plot (n = 390 taxa); C = Most abundant phyla in the salivary microbiome.

Table 3. PERMANOVA multivariate test, showing the influence of the glycaemic markers and clinical variables in the salivary microbiome. T2D = individuals with diagnosis of type 2 diabetes mellitus, no-T2D = individuals without type 2 diabetes mellitus.

Factor	Group	F value	p – value
Diagnosis of T2D	**T2D/no-T2D**	**1.9265**	**0.0241**
Salivary pH	**pH 6/pH 7/pH 8**	**1.5815**	**0.0098**
Salivary glucose	**<0.35 mg/dL/>0.35 mg/dL**	**2.1604**	**0.0097**
HbA1c	<6.0%/>6.0%	0.9949	0.3372
Fasting blood glucose	<100 mg/dL/>100 mg/dL	1.5547	0.0871
Periodontitis extent	Localised/Generalised	1.2732	0.1874

Table 4. Relative abundance (%) of salivary microbiome taxa significantly influenced by the salivary pH and/or salivary glucose.

|	Acidic salivary pH (n = 6)	Neutral salivary pH (n = 32)	Alkali salivary pH (n = 9)	p*	Salivary glucose < 0.35 mg/dL (n = 21)	Salivary glucose ≥ 0.35 mg/dL (n = 26)	p
Rate *Firmicutes/ Bacteriodota*	16.83 ± 10.12	25.06 ± 32.04	81.88 ± 120.41	0.03	49.94 ± 89.29	23.89 ± 26.16	> 0.05
Proteobacteria	0.16 ± 0.12	0.11 ± 0.10	0.04 ± 0.04	0.003	0.09 ± 0.07	0.12 ± 0.12	> 0.05
Actinobacteriota	0.13 ± 0.04	0.15 ± 0.05	0.20 ± 0.09	0.08	0.18 ± 0.07	0.13 ± 0.05	0.01
Bacteroidota	0.05 ± 0.03	0.06 ± 0.04	0.02 ± 0.02	0.02	0.04 ± 0.03	0.05 ± 0.04	> 0.05
Spirochaetota	0.01 ± 0.01	0.00 ± 0	0	0.01	0.001 ± 0.002	0.002 ± 0.004	> 0.05
Veillonella spp.	0.03 ± 0.02	0.04 ± 0.03	0.01 ± 0.01	0.01	0.03 ± 0.02	0.03 ± 0.03	>0.05
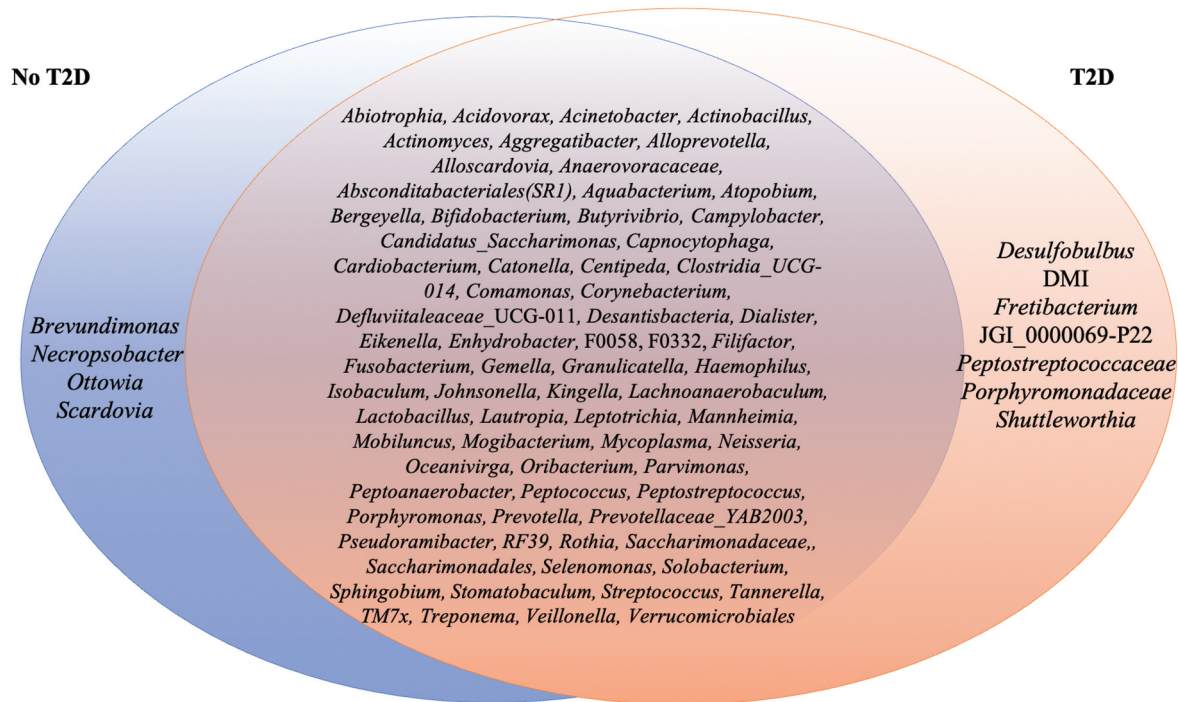
Figure 3. List of members of the ubiquitous microbiome (present in at least 50% of the samples). Blue represents no-T2D, while Orange represents T2D. The intersection represents the ubiquitous taxa present in all samples.
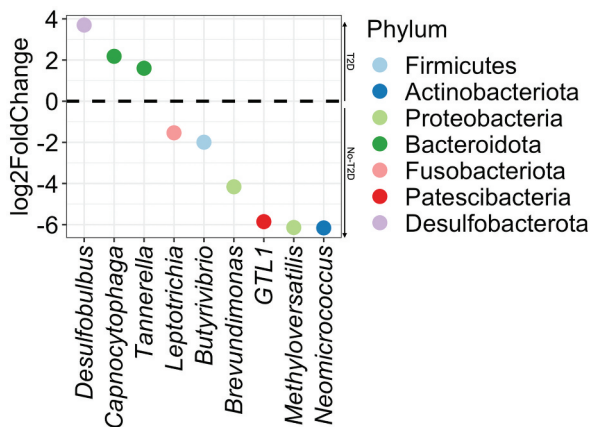

Figure 4. Differential abundance plot calculated by DESeq2. Taxa with positive log2 fold-change values are significantly more abundant (enriched) in T2D and negative log2 fold-change values are enriched in no-T2D.

taxa due to the hyperglycaemia state. No differences were observed for the five acid-related genera in T2D and no-T2D samples (from 390 taxa from 47 samples) (Figure 5(a), Supplementary Table 3). Interestingly, the same was observed for the proteolytic pathobiont taxa, except for *Treponema*, that was significantly more abundant in T2D samples (Figure 5(b)).

Network analysis

The microbial network profiles of samples from individuals diagnosed with T2D and no-T2D considered 110

taxa that were prevalent in at least 10 out of 47 samples (Figure 6). Distance of centralities (degree, betweenness, eigenvector, and closeness) were tested using the Jaccard index. Degree ($p < 0.05$), betweenness ($p < 0.01$), and eigenvector ($p < 0.001$), but not for closeness ($p > 0.1$) presented significant differences between groups (Supplementary Table 4). Keystone taxa were not identified by the centrality values.

The node pairs were connected by the shortest path in T2D samples compared to those in no-T2D, indicating that the T2D microbiota taxa had better interconnected clusters. This can be particularly relevant for taxa nodes associated with *Acidovorax* (sp82) and *Acinetobacter* (sp32) (dark red cluster in T2D) or for nodes associated with *Actinomyces* (sp6), *Granulicatella* (sp7) and *Solobacterium* (sp8) (red cluster in T2D) and for nodes associated with *Eikenella* (sp104) and *Haemophilus* (sp5) (orange cluster in T2D) (see Supplementary Table 5 for the network taxa ID annotation).

Methyloversatilis (sp345), significantly enriched in the no-T2D samples, clustered with *Saccharimonadaceae* (sp71), *Actinomycetaceae* F0332 (sp85), *Ottowia* (sp161), and *Aquabacterium* (sp127) (blue cluster in T2D). An interdomain network was observed between archaea-bacterial taxa. *Aenigmarchaeum* (sp840) and *Woesearchaeales* SCGC_AAA286-E23 (sp869) comprised unconnected taxa (grey nodes) in the T2D, however in no-T2D were positive and directly associated with *Saccharimonadaceae* GTL1 (sp382), and a *Saccharimonadales* species (sp95), respectively.
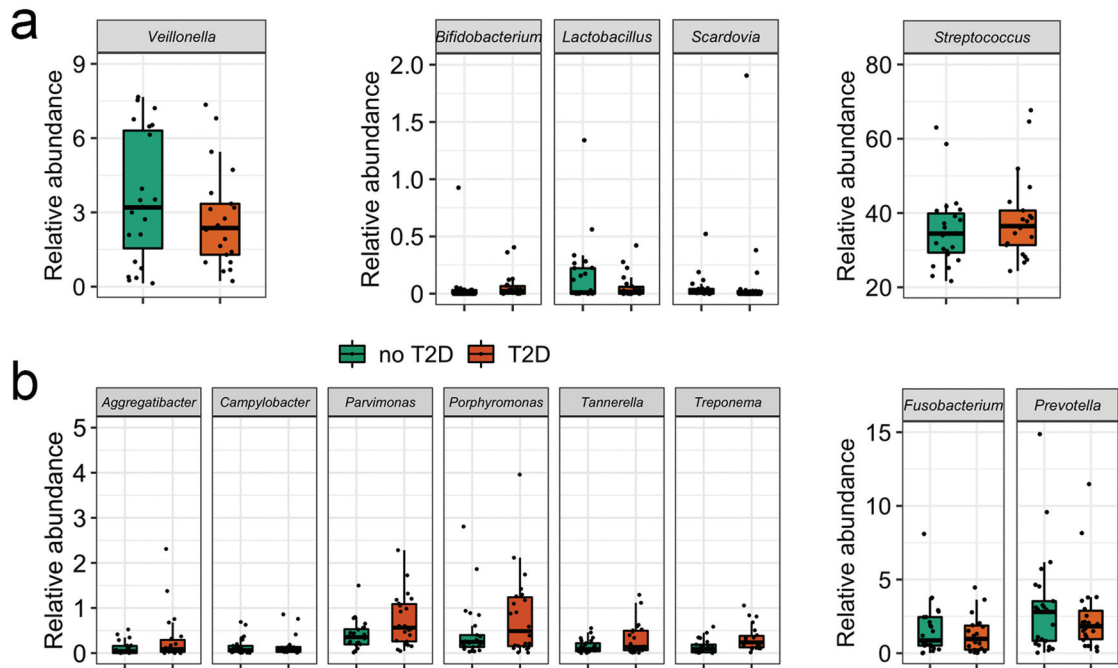
Figure 5. Relative abundance (%) in T2D and no-T2D groups of (a) acidogenic caries-associated taxa, and (b) proteolytic periodontitis-associated taxa. Wilcoxon test (significance level <0.05).
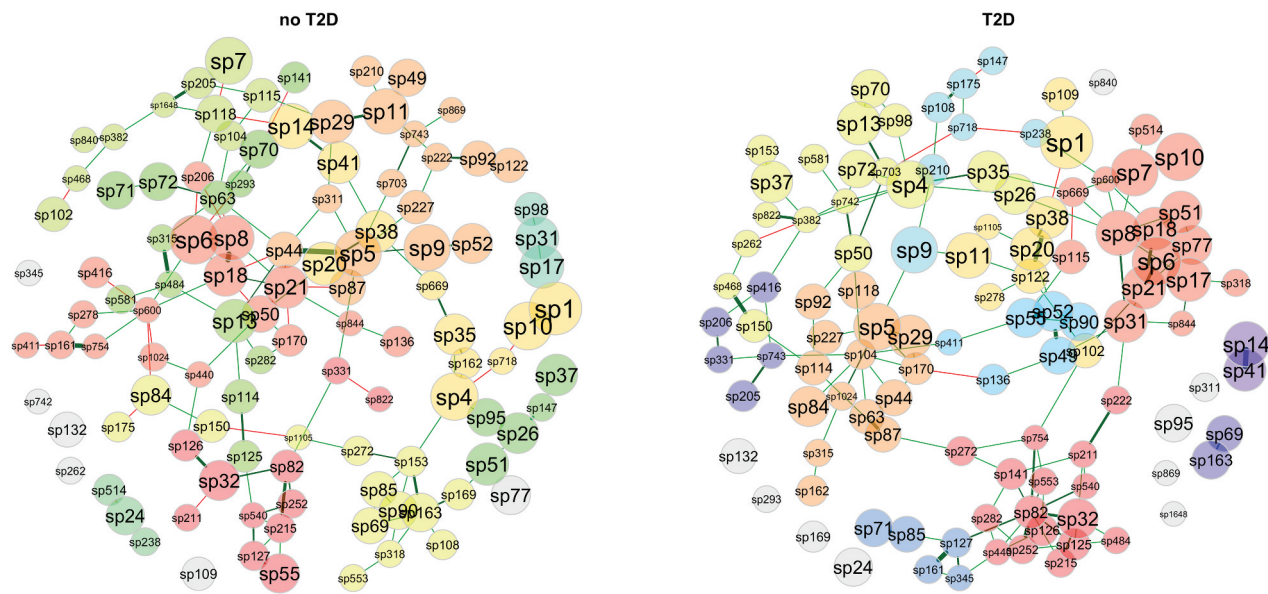

Figure 6. Network analysis (clusters are differentiated by colours; node size based on mclr values). Green edges correspond to positive estimative association and red edges correspond to negative association between taxa. Similarity between clusterings – Adjusted Rand index = 0.038 (p-value = 0.007).

Regarding the taxa with significant differential abundance, *Desulfobulbus* (sp147) stood out in the network analysis. It was within a cluster of no-T2D samples, in positive association with *Peptostreptococcus* (sp26), *Saccharimonadales* species (sp95), *Parvimonas* (sp37) and *Anaerovoracaceae* species (sp51) (green cluster). In the T2D, *Veillonella* (sp9), *Bifidobacteria* (sp175), and *Scardovia* (sp108), amongst others (light blue cluster) are clustered in direct or indirect negative association with *Desulfobulbus*. This indicates an important

opposition to the caries-associated and butyrate-producing bacteria.

A central role of *Veillonella* (sp9) confirmed its importance in the T2D microbiome, when combined with the previously described higher relative abundances in acidic than alkali pHs. *Veillonella* appears as a connector node linking three clusters in positive association with *Dialister* (sp210) (light blue cluster), with *Haemophilus* (sp5) (orange cluster) and with *Prevotella* (sp11) (gold cluster). In the no-T2D

samples, we cannot see *Veillonella* as a connector node taxon, but it is clustered with direct or indirect positive association with *Haemophilus* (sp5), *Dialister* (sp210) and *Prevotella* (sp11). It suggests *Veillonella* as a multifunctional taxon capable of maintaining the bacterial network structure of the oral microbiota in T2D samples.

Butyrivibrio (sp102) was differentially more abundant in no-T2D samples and also seemed to have a role as cluster connector within the T2D samples. In no-T2D, it was negatively linked to the *Anaerovoracacea* family XIII UCG-001 (sp468), while in T2D it comprised the same cluster of *Prevotella* (gold cluster) and positively associated with *Atopobium* (sp21) (red cluster), *Novosphingobium* (sp754) (dark red cluster), and *Catonella* (sp122) (gold cluster). Another differentially abundant bacterium *Leptotrichia* (sp20) was enriched in no-T2D, and in the network analysis was positively and directly associated with *Stomatobaculum* (sp38) in both groups. Differentially abundant bacteria in T2D, *Tannerella* (sp90), was positively associated with *Johnsonella* (sp55) and clustered with *Selenomonas* (sp52), *Prevotellaceae* YAB2003 group (sp49), *Alloscardovia* (sp136) and *Peptostreptococcales-Tissierellales* W5053 (sp411) (blue cluster). *Tannerella* is known as an abundant component in periodontitis sites, and it may reflect its importance as a link in the network of T2D, where most individuals had stages 3/4 localised periodontitis.

Pondering the strongest positive links in the T2D, some correlations may be highlighted: *Pseudoramibacter* (sp222) with *Parascardovia* (sp211) and *Bifidobacterium* (sp175) with *Scardovia* (sp108). *Pseudoramibacter* represents a very common microorganism in root canals, while all others belong to the family *Bifidobacteriaceae*. Meanwhile, strong positive association in no-T2D samples included the fermenter and nitrate-reducer *Actinobacillus* (sp44) and *Haemophilus* (sp5), a bacterial genus found in all oral cavity sites of patients. Also, in no-T2D samples, there was a positive association of *Desulfobulbus* (sp147) and the infection-associated *Peptostreptococcus* (sp26).

Discussion

Some oral microbial signatures such as *Desulfobulbus* were associated with all hyperglycaemia indices tested. Members of this genus were favoured by the hyperglycaemia state showing significant positive correlation with FBG and HbA1c levels. *Desulfobulbus* was ubiquitous and highly abundant in individuals with T2D. Moreover, *Desulfobulbus* seemed to be an antagonist to caries-associated and butyrate-producing bacteria. The increase in blood glucose levels was significantly linked to the increase in *Treponema*, *Phocaiecola* and *Saccharimonadaceae* species. On the other hand, *Actinobacteriota* may be

depleted in the hyperglycaemia state, as taxa belonging to this phylum were highly abundant in no-T2D samples and in individuals with salivary glucose <0.35 mg/dL. A notable influence of the salivary pH on the oral microbiome was observed through changes in the proportions of the *Firmicutes/Bacteriodota* ratio (increasing in the saliva with alkali pH). In addition, we found the highest number of negatively correlated taxa between all the explanatory variables (Figure 1(a), showing eight taxa that were significantly negatively correlated with the salivary pH).

Our observations are consistent with the broader hypothesis that microbial communities along the digestive tract might be risk factors for diabetic regulation in diabetic individuals, such as the increased abundance of sulphate-producers in the hyperglycaemic state. The microbiota may also be involved in the early onset of diabetes development [37]. It was shown elsewhere that the dysbiosis index explained 6% of the variation in longitudinal glucose change, predicting 2 year glucose change among diabetes-free individuals [38]. Likewise, the glycaemic control led to a shift in the oral microbial population resembling that of healthy individuals, which are complex and biodiverse [19]. In this context, the salivary microbiome of individuals with T2D had higher abundance of *Desulfovibrionaceae*, a taxonomic order associated with dyslipidaemia, obesity [39] and DM [40]. This is a sulphate-reducer that can perform anaerobic respiration utilizing sulphate as a terminal electron acceptor. Sulphate-reducing bacteria are the main H_2S generators in the gut microbiome, with a potential role in the individual's metabolic condition and complication related to DM. A relationship between H_2S and gut microbial dysbiosis signalling and function has been suggested [41,42], as increased H_2S levels disturb the pancreatic β-cell function and decrease insulin secretion [41]. Furthermore, *Desulfovibrio desulfuricans* are trimethylamine oxide generators, which is similarly correlated with the risk of metabolic syndromes [41]. Another sulphate-reducer was found to be a protagonist in this study, *Desulfobulbus*, which has previously been referred to as a periodontal pathobiont, because it induces proinflammatory response and secretes potential protein toxins [43]. Although in low abundance, it was significantly enriched in subgingival sites with periodontitis [44]. Therefore, the presence of this sulfidogenic microorganism in hyperglycaemic and periodontitis microbiomes is easily understandable. Systemically, a significantly higher abundance of sulphate-reducing bacteria in the oral microbiome can indicate saliva as a potential biomarker of DM-dysbiotic gut microbiome.

Gut and oral diabetic microbiomes may be more connected than we expected. We found a significantly

negative association between *Desulfobulbus* and butyrate-producing bacteria from the *Bifidobacteriaceae* family in the diabetic oral microbiota. Locally, members of the *Bifidobacteriaceae* family are strongly associated with caries as their fermentation end products are mainly organic acids. These metabolic products can reduce the pH, leading to critical acidity levels for tooth demineralisation, indicating a potential link to the increased prevalence of caries in diabetic individuals [7]. Organic acids can be converted into short-chain fatty acids (SCFAs) by butyrate-producing bacteria through cross-feeding interactions [45]. Systemically, the SCFAs including butyrate serve as key mediators of microbial-host signalling and are linked to a better insulin response [46]. For instance, *Bifidobacterium* spp. have anti-inflammatory properties and protect the epithelial barrier by reducing lipopolysaccharides and the trimethylamine N-oxide (TMAO) influx into the blood [21]. Our results on the salivary microbiota are in line with the decreased butyrate-producing bacteria levels in the gut microbiome of DM individuals [47]. This is the case of the butyrate-producer *Butyrivibrio*, belonging to the *Clostridiales* order, that was significantly depleted in the T2D samples and presented a central role in the network analysis. Changes in the SCFAs metabolism in the diabetic gut microbiome are linked to the enrichment of *Bacteriodota*, which was also found to be enriched in our data. A biomarker of this event is the *Firmicutes/Bacteriodota* ratio. It has been positively linked with blood glucose levels [46], and we showed its significant reduction in the salivary microbiota among individuals with lower salivary pHs.

Other oral phyla were reduced in the hyperglycaemia state. *Actinobacteriota* was significantly lower in salivary glucose >0.35 mg/dL, while *Proteobacteria* (*Methyloversatillis* and *Brevundimonas* genus) were the most significantly depleted organisms in T2D samples. Many pathobionts are members of the *Proteobacteria* phylum, and a high proportion of such organisms may have a pro-inflammatory effect in diabetic subjects. This observed imbalance in the microbial composition can be a result of the gut dysbiosis and potentially impair the insulin resistance. *Leptotrichia* was likewise associated with samples without T2D and is a representative of the core microbiome, present in almost all individuals [48,49], as a bridge between early and late colonizers within oral biofilms. New studies investigating microbial functions are necessary to explain connections with *Stomatobaculum*, as observed in our network analysis, independently of the sample group status.

The altered blood sugar status can disrupt homeostasis, providing a more profound change on the microbiota profile particularly when combined with periodontitis [19,50]. It is essential to point out that

genera strongly associated with periodontitis, *Tannerella* and *Treponema*, demonstrated a connection with increased pH, the diagnosis of T2D, and the blood glycaemic levels. TM7, *Neisseriaceae* [G-1] bacterium HMT-174 (F0058) and *Tannerella* demonstrated a positive correspondence with HbA1c, FBG, and salivary glucose in the CCA multivariate analysis. Additionally, *Tannerella* had several links in the T2D-associated microbial network. Since all diabetic individuals included in the present study were also diagnosed with some level of periodontitis, it was not possible to clarify if the higher level of some periodontal-related taxa was influenced by the T2D condition or by periodontitis, although the periodontitis extent was included in the PERMANOVA multivariate test, showing no significant impact in the analysis. Despite this potential limitation, it is important to highlight both the results of the Spearman correlation, as well as of the canonical correlation analysis, demonstrating that all the glucose parameters profoundly impacted the salivary microbiota changes. Hence, it is the glycaemic status rather than the T2D diagnosis that perhaps should be considered a biomarker related to salivary dysbioses.

Another obvious factor influencing the oral ecosystem of individuals with T2D was the significant salivary dysfunctions, such as pH changes. Goodson et al. evaluated changes in abundance of some bacterial species in the saliva of adolescents with high concentrations of salivary glucose, showing that the higher the salivary glucose, the lower the pH of the saliva [8], which we confirmed here. As glucose is a well-known energy source for many oral bacteria, changes in its concentration would lead to reduced overall bacterial diversity, favouring acidic and acidogenic bacterial species. We showed an enrichment of the *Abiotrophia* and *Oceanivirga* in the oral microbiota with the increase of active caries extent and DMFS. For instance, *Oceanivirga* has been found in pharyngeal infections [51], while *Abiotrophia* was enriched in adolescents from a community with high caries prevalence when compared to the ones from a low caries prevalence community [52]. Meanwhile, *Desantisbacteria* was negatively correlated with both caries variables and significantly affected by the salivary pH. Indeed, the salivary pH had the highest number of negatively correlated taxa and significantly changed the beta diversity and the CCA multivariable analysis. This confirmed the relevance of the pH changes in the diabetic microbiome, even though the results did not directly change the proportion of the typical acidogenic microbiota regarding the diabetes status. Furthermore, there was a central role of *Veillonella* spp. in the bacterial network of the diabetic salivary microbiome (Figure 6), and they were significantly enriched in the acidic saliva (Table 4). Members of this genus

are linked to the classical Socransky's purple-complex [53], and their lactate metabolism might facilitate the pH neutralization in biofilms [54]. They have been already related to hyperglycaemia elsewhere [55] and dental caries [56]. These characteristics might explain the significant higher proportions of these organisms in lower pH environments. Furthermore, they are health-associated organisms in periodontal sites [57].

Our results confirmed the importance of analysing not only the main taxa present but also the microorganisms in low abundance, as these may be impacted by clinical parameters. Current research on the salivary microbiome has mainly been restricted to the identification of the most abundant microbiota associated with health or disease. We believe that this strategy could cause an incomplete misunderstanding of the ecology and environment as metabolic functions exerted by low-abundant microorganisms can be linked to the dysbiotic microhabitats in a sort of 'butterfly effect' [58]. This can be clearly observed by the inclusion of *Desulfobulbus* in the analysis, even at very low relative abundance. Although representing a minority taxon, its ubiquity and association with clinical parameters were found to be consistent. Besides, the network analysis indicated its important role in the microbiome, as discussed above.

The analysis comparing the diagnosis of T2D vs. no-T2D should be interpreted with caution, considering confounding aspects affecting all differences between samples. Those factors represent additional selective pressure over the microbiota composition in the diabetic group. Also, in general, the dichotomization of individuals did not separate the ones with controlled from uncontrolled glucose levels, those with long-term diagnostic of DM and use of hypoglycaemic drugs were not taken into account either. To overcome this issue, we performed several analyses without considering the diagnostic of T2D, instead taking into account the glycaemic status using HbA1c, FBG and salivary glucose as continuous variables. Salivary glucose showed some influence in the salivary microbiome, and this trend should be further studied using a more sensitive test for salivary glucose measurement. Although it is not possible to confirm that salivary glucose plays an important role in disturbing the microsystem, perhaps it favours microorganisms that influence the pH balance. In this case, the salivary pH would be indirectly influenced by the salivary hyperglycaemia, although it is not the microbiota typically acidogenic that is enriched in the hyperglycaemic state. Other clinical parameters, not evaluated here, could also be involved in the imbalance of the diabetic microbiome, such as smoking and adiposity. Future perspectives in this field include the development of a longitudinal study to confirm these associations, and hence the potential of targeting the oral microbiome as an approach to detect and treat T2D.

In conclusion, the salivary microbiome was shaped by systemic hyperglycaemia, as well as changes in the salivary pH, which may be linked to local hyperglycaemia. Locally, these changes might be related to the oral manifestations of T2D, including their higher caries experience. Systemically, the enrichment of predictive biomarkers of gut dysbiosis in the salivary microbiome can reflect its capacity of impairment of the hyperglycaemia. More than leading to local changes in the oral cavity, the oral microbiome may harbour important biomarkers for the early diagnosis of T2D due to the enrichment of sulphate-reducers and depletion of butyrate-producers. In the context of the integrated hypothesis of caries and periodontal diseases [13], due to the link of sugar-driven hyperglycaemia and inflammation in periodontal tissues, there is a potential to control caries and periodontal diseases by stabilization of blood sugar levels.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Author's contributions

CPVL: Contributed to conception, design, data acquisition and interpretation, drafted and critically revised the manuscript; DCG: Contributed to conception, design, data acquisition and interpretation, drafted and critically revised the manuscript; MCMG: Contributed to conception, design, data acquisition and interpretation, and critically revised the manuscript; LPS: Contributed to conception, design, data acquisition and interpretation, and critically revised the manuscript; PCK: Contributed to data acquisition and critically revised the manuscript; TD: Contributed to conception, design, data acquisition and interpretation, and critically revised the manuscript; LGAB: Contributed to conception, design, data acquisition and interpretation, performed statistical analysis, drafted and critically revised the manuscript; NDT: Contributed to conception, design, data acquisition and interpretation, performed statistical

analysis, main supervision, drafted and critically revised the manuscript.

Data availability statement

The sequences were deposited at the National Biotechnology Information Center (NCBI) in BioProject PRJNA807496 (http://www.ncbi.nlm.nih.gov/bioproject/807496).

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ANEXO – Aprovação pelo Comitê de Ética

UNB - FACULDADE DE CIÊNCIAS DA SAÚDE DA UNIVERSIDADE DE BRASÍLIA
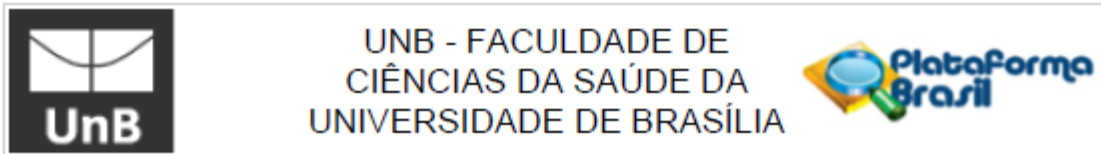
PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Cárie dentária em crianças institucionalizadas com dieta controlada: um estudo coorte prospectivo

Pesquisador: Paula de Castro Kruly

Área Temática:

Versão: 2

CAAE: 86836318.7.0000.0030

Instituição Proponente: FACULDADE DE SAÚDE - FS

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 2.690.398

Apresentação do Projeto:

"Resumo:

Introdução: A exposição ao açúcar pode ser considerada um fator determinante para o desenvolvimento da doença cárie. Atualmente, com o acesso ao flúor, tem se observado que a relação direta entre açúcar e cárie não é tão forte quanto costumava ser, devido à sua capacidade de reduzir a perda mineral dos tecidos dentários. Objetivo: avaliar a atividade de cárie entre os alunos atendidos pelo Instituto Dom Orione durante o período de dois anos, comparando alunos internos com dieta exclusiva e acompanhada por nutricionista do Instituto com alunos que frequentam o Instituto durante o dia, mas voltam para casa à noite. Metodologia: os alunos serão divididos em 2 grupos (G1: 40 alunos internos e G2: 100 alunos não internos) e avaliados com exame visual-tátil por 2 anos com intervalo de 12 meses entre as avaliações. A saliva total estimulada será coletada e armazenada para futuras análises. Além disso, um questionário será aplicado aos cuidadores para avaliação dos conhecimentos sobre cuidados com saúde bucal. Hipótese nula: não haverá correlação entre a dieta e a atividade de cárie."

"Introdução:

A dieta é o fator causador principal da doença cárie, o que não é um conceito novo. Evidências de que na ausência de açúcar não havia cárie são definitivas. Antes da revolução industrial que deu

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Continuação do Parecer: 2.690.398

início ao consumo de açúcares fermentáveis, observava-se que cárie não era um agravo prevalente. Um exemplo disso foi a observação de uma população isolada na ilha de Tristão da Cunha. Outro exemplo é um estudo que avaliou esqueletos de indivíduos de quatro sítios arqueológicos no sul da França, em que foi observado menor quantidade de dentes cariados na população que morava em um local e época em que o consumo de carboidratos e alimentos cozidos era baixo, e o cultivo de cereais era menos comum comparado às outras populações avaliadas. De semelhante forma, o estudo de Costa Junior (1980) concluiu que os baixos índices de dentes cariados observados nos esqueletos de moradores do Alaska são atribuídos à dieta totalmente desprovida de açúcar refinado e amidos. Já o estudo de Hopehood House mostrou que o tipo de alimentação está diretamente ligado ao desenvolvimento de lesões de cárie, mesmo na ausência de cuidado odontológico ou instrução de higiene bucal. Ainda em 1954, Gustafsson et al. observaram uma relação positiva in vivo de consumo de açúcar e cárie no famoso estudo de Vipeholm. Neste estudo, concluiu-se que o consumo de açúcar em maior frequência durante o dia tem relação direta com maiores índices de cárie. Mais recentemente, revisões como a de Sheiham & James e Hujoel & Lingström retomaram essa relação. A exposição ao açúcar pode ser considerada um fator determinante para o desenvolvimento da doença cárie, e sendo esse açúcar a sacarose a cariogenicidade é ainda maior, pois ao metabolizar a sacarose a bactéria, além de produzir ácido, produz polissacarídeos extracelulares que aumentam a cariogenicidade do biofilme. Atualmente, com o acesso ao flúor, tem se observado que a relação direta entre açúcar e cárie não é tão forte quanto costumava ser, devido à sua capacidade de reduzir a perda mineral dos tecidos dentários. Quando o flúor está presente na saliva ou no biofilme na sua forma solúvel, ele induz a precipitação de minerais em forma de fluorapatita na estrutura dentária, o que não reduz a produção de ácidos pelas bactérias ou o acúmulo de biofilme, mas é capaz de reduzir a perda mineral do dente. O consumo de açúcar tem se tornado o fator mais importante para o risco de cárie em pessoas que não têm uma exposição frequente ao flúor. Porém, os fluoretos são coadjuvantes e não são capazes de controlar o processo por si só, justificando assim que ainda há importância do controle de consumo de açúcar da dieta para controlar cárie. A teoria da placa ecológica proposta por Marsh explicou a relação dinâmica que acontece no biofilme dentário, onde uma mudança ambiental (pH baixo), causada pelo alto consumo de açúcares fermentáveis gera uma mudança no equilíbrio da microbiota do biofilme, que causa desmineralização da estrutura dental. Dessa forma, para controlar a doença cárie uma medida efetiva seria a redução do consumo de açúcar ou a utilização de substitutos do açúcar, como o aspartame, o xilitol e a sacarina. Além disso, o acompanhamento nutricional por um profissional bem informado sobre a

relação dieta-cárie é fator de extrema importância no controle da doença. A simples instrução nutricional pode reduzir o índice de cárie e a severidade da doença em crianças, o que mostra a importância do trabalho multiprofissional para um correto controle da dieta e, consequentemente, controle da doença cárie. Existem muitas pesquisas sobre o desenvolvimento da cárie em populações de crianças sem deficiência. Pesquisas com pacientes com deficiência são mais raras, havendo uma necessidade de estudos com essa população. Os pacientes com deficiência requerem atendimento odontológico diferenciado. Dificuldades motoras e mentais estão relacionadas à uma pior higiene bucal, sendo necessária atenção especial no atendimento à esses pacientes. Estudos já mostraram a necessidade da criação de políticas públicas para essa população, com foco no controle e também tratamento da doença cárie. Estudos que comparem o desenvolvimento da doença cárie nesta população, em comparação à pessoas que não têm deficiência podem ajudar a esclarecer as necessidades dos pacientes especiais relacionadas aos cuidados de higiene bucal, e dessa forma ajudar a desenvolver atividades de educação em saúde voltadas para este grupo de pessoas. O Instituto Dom Orione, localizado em Brasília, abriga jovens e adultos portadores de deficiências em situação de risco ou abandonados pela família, e presta assistência e educação a menores não portadores de deficiência no seu contraturno escolar. O instituto oferece aos alunos acompanhamento escolar, atividade física, alimentação, tratamento odontológico, dentre outras atividades. Dentre os portadores de deficiências, há pessoas com síndrome de Down, esquizofrenia e autismo. Tais escolares ficam internos no instituto, portanto com dieta controlada, enquanto as demais crianças sem deficiência voltam para casa à noite e, portanto, têm dieta familiar."

Metodologia Proposta:

"GRUPO 1: n=40 alunos portadores de deficiência (idade entre 18 a 50 anos) e que são internos na escola, recebendo dieta controlada. GRUPO 2: n=100 alunos não portadores de deficiência (idade entre 6 a 12 anos) e que não tem dieta controlada fora da escola (dieta da família). As avaliações serão realizadas por um único operador durante o período de dois anos, com intervalo de 12 meses. No total serão realizadas 3 avaliações (inicial, após 1 ano e após 2anos). O exame visual-tátil será utilizado. Para igualar os grupos, eliminando as diferenças em relação à higiene bucal, a cada 6 semanas será realizada profilaxia em todos os alunos.Dessa forma, somente a ação da alimentação será avaliada.Para as avaliações serão utilizados kits de exame clínico, em dentes limpos e secos. As lesões iniciais ou cavitadas detectadas clinicamente serão anotadas para cálculo do índice ICDAS modificado. Um questionário será feito com os pais, para coletar dados de nível

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socioeconômico e de higiene bucal.Apenas um examinador irá realizar todas as avaliações clínicas. Previamente ao estudo, o examinador realizará treinamento com fotografias e calibração com um padrão-ouro em 10 pacientes. Um kappa acima de 0,7 será buscado para calibração intra e inter examinador.Durante o estudo, será realizada mais uma calibração intra examinador, avaliando duplamente 10 crianças com intervalo de 7 dias.A avaliação da dieta dos alunos que residem no instituto será feita com colaboração da nutricionista que trabalha no local. Todos os alimentos que os alunos consomem em cada horário do dia serão anotados em um diário alimentar para avaliação.Para os alunos que não residem no instituto, em cada dia de avaliação clínica será entregue um diário alimentar do mesmo modelo para ser preenchido em casa pelos responsáveis por um período de três dias, no qual deve ser anotado todo alimento que o aluno consumir durante o dia, com os respectivos horários de consumo. Os pais serão instruídos sobre a forma de preencher os formulários, que deverão ser entregues no instituto ao final dos três dias.O diário será repetido para reprodutibilidade em 10 alunos, com período de 7 dias.A saliva total estimulada será coletada durante 5 minutos, por expectoração em um recipiente de plástico, após os pacientes mastigarem um dispositivo feito de silicone por 1minuto. Após a coleta, a 1mL da saliva estimulada sera armazenado e congelado à -20 o C para futuras análises.O DNA microbiano será extraído e analisado por técnicas moleculares, como a amplificação e sequenciamento do gene 16SrRNA.As lesões de cárie que tiverem indicação para tratamento restaurador serão tratadas pelo dentista responsável pelo estudo. Na necessidade de tratamentos mais complexos, os participantes serão encaminhados para atendimento odontológico pelo Sistema Único de Saúde.Durante todas as avaliações da pesquisa, todos os alunos receberão instruções de higiene bucal e de dieta cariogênica. A nutricionista da instituição também receberá informações sobre cariogenicidade dos alimentos. Alunos com atividade de cárie terão orientação específica como parte do tratamento não restaurador.Os

alunos do instituto que possuem deficiência recebem a ajuda diária de cuidadores nos cuidados pessoais e de saúde. Um questionário de múltipla escolha será aplicado para avaliação da percepção dos cuidadores relativo a saúde bucal dos pacientes com deficiência que estão sob os seus cuidados, e seus próprios conhecimentos sobre saúde bucal. Será realizada uma prática educativa com os cuidadores, através de uma palestra sobre saúde bucal, levando o conhecimento de noções básicas de prevenção de doenças da cavidade bucal."

"Critério de Inclusão:
Grupo 1: Alunos que passam dia e noite no instituto; Alunos com dieta 100% controlada por

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nutricionista.

Grupo 2: Alunos que ficam meio período no instituto, portanto tem dieta não controlada 100% por nutricionista.

Critério de Exclusão:

Grupo 1: Pessoas não colaboradoras, nas quais não for possível realizar o exame odontológico e as limpezas regulares;

Alunos que possuem algum acesso à alimentação externa ao instituto.

Grupo 2: Pessoas não colaboradoras, nas quais não seja possível realizar o exame odontológico e as limpezas regulares;

Alunos que não tenham expectativa de permanecer ligados ao instituto durante todo o período da pesquisa."

"Metodologia de Análise de Dados:

Regressões logísticas serão aplicadas para avaliar o risco relativo das crianças, com análises uni e multivariadas. Variáveis como idade, sexo, nível socioeconômico (escolaridade da mãe), acesso à fluoretos, higiene bucal (autorreferida e índice de sangramento gengival) e fluxo salivar serão utilizadas como controle. Os dados coletados pelo questionário com os cuidadores serão processados e analisados através da análise estatística de Friedman e teste de Dunnett.

Desfecho Primário:

Espera-se que os alunos com dieta controlada apresentem menos lesões de cárie ao longo dos dois anos de avaliação clínica."

"Tamanho da Amostra no Brasil: 140"

Objetivo da Pesquisa:

"Objetivo Primário:

O objetivo deste estudo é avaliar a prevalência, incidência e atividade de cárie entre os alunos atendidos pelo Instituto Dom Orione durante o período de dois anos, comparando alunos internos com dieta exclusiva e acompanhada por nutricionista do Instituto com alunos que frequentam o Instituto durante o dia, mas voltam para casa à noite.

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Objetivo Secundário:

Testar as seguintes hipóteses nulas:

1-Não haverá correlação entre o consumo de açúcar e a atividade de cárie;

2-Não haverá diferença entre atividade de cárie entre as crianças com deficiência e as crianças sem deficiência;

3- Não haverá correlação entre o tipo de dieta (controlada x família) e a atividade de cárie;

4-Não haverá alteração na atividade da cárie em relação ao tempo (2 anos)."

Avaliação dos Riscos e Benefícios:

"Riscos:

Algumas pessoas podem sentir algum constrangimento ao mostrar a boca durante a avaliação, quando há lesões de cárie. Alguns participantes poderão ter dificuldade no exame para coleta de saliva, tais como dificuldade para "cuspir" no recipiente. Com relação ao questionário socioeconômico, alguns poderão ficar constrangidos ao responder perguntas relacionadas ao grau de instrução, e renda familiar

Benefícios:

Os alunos receberão instruções de controle da doença cárie e profilaxias regulares, realizadas pelo pesquisador, a cada 6 semanas, e os cuidadores receberão palestra educativa sobre saúde bucal."

Comentários e Considerações sobre a Pesquisa:

Trata-se de Projeto de Pesquisa de Doutorado de Paula de Castro Kruly, Programa de Pós-Graduação em Odontologia da UnB, tendo como Orientadora Fernanda Cristina Pimentel Garcia.

O estudo que será realizado no Instituto Dom Orione em Brasília.

Constam como membros da equipe Naile Dame Teixeira, Mariana Souza Fidelis de Oliveira e Fernanda Cristina Pimentel Garcia.

O Cronograma apresenta atividades com participantes da Pesquisa para o período de maio de 2018 a maio de 2020.

O Projeto de Pesquisa possui orçamento de R$ 16.005,00 com aquisição de materiais e consta que os reagentes para realização de testes que envolvem biologia molecular serão solicitados a FAP/DF (R$ 10.000,00).

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Considerações sobre os Termos de apresentação obrigatória:

Documentos analisados para emissão do presente parecer:

1. "PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_1062882.pdf", postado em 28/05/2018 - documento com informações básicas do projeto de pesquisa "Cárie dentária em crianças institucionalizadas com dieta controlada: um estudo coorte prospectivo", Versão 2, da Pesquisador principal Paula de Castro Kruly, tendo como Instituição Proponente a Faculdade de Ciências da Saúde da UnB e como local de realização do projeto o Instituto Dom Orione/Brasília.

2. "termodeassentimentolivreeesclarecido.pdf", postado em 24/05/2018 – Termo de Assentimento a ser oferecido aos participantes da Pesquisa, em versão PDF.

3. "termodeassentimentolivreeesclarecido.docx", postado em 24/05/2018 - Termo de Assentimento a ser oferecido aos participantes da Pesquisa, em versão WORD.

4. "tcleresponsaveis.pdf", postado em 24/05/2018 - documento a ser apresentado ao responsável pelo aluno e participante da pesquisa contendo as informações obrigatórias e necessárias para a tomada de decisão, em versão PDF.

5. "tcleresponsaveis.doc", postado em 24/05/2018 - documento a ser apresentado ao responsável pelo aluno e participante da pesquisa contendo as informações obrigatórias e necessárias para a tomada de decisão, em versão WORD.

6. "tclecuidadores.pdf", postado em 24/05/2018 - documento a ser apresentado ao participante da pesquisa – Cuidador contendo as informações obrigatórias e necessárias para a tomada de decisão, em versão PDF.

7. "tclecuidadores.docx", postado em 24/05/2018 - documento a ser apresentado ao participante da pesquisa – Cuidador contendo as informações obrigatórias e necessárias para a tomada de decisão, em versão WORD.

8. "tcleadultosindepentendes.pdf", postado em 24/05/2018 - documento a ser apresentado ao participante da pesquisa – aluno do Instituto contendo as informações obrigatórias e necessárias para a tomada de decisão quanto a participação na pesquisa, em pdf.

9. "tcleadultosindepentendes.doc", postado em 24/05/2018 - documento a ser apresentado ao participante da pesquisa – aluno do Instituto contendo as informações obrigatórias e necessárias para a tomada de decisão quanto a participação na pesquisa, em WORD.

9. "questionariosocioeconomico.pdf", postado em 24/05/2018 – instrumento da pesquisa a ser apresentado aos pais ou responsáveis legais dos alunos do Instituto.

10. "questionariosocioeconomico.docx", postado em 24/05/2018 - instrumento da pesquisa a ser apresentado aos pais ou responsáveis legais dos alunos do Instituto.

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11. "questionariocuidadores.pdf", postado em 24/05/2018 – instrumento da pesquisa a ser apresentado aos cuidadores, em PDF.

12. "questionariocuidadores.docx", postado em 24/05/2018 - instrumento da pesquisa a ser apresentado aos cuidadores, em WORD.

13. "projetodepesquisa.pdf", postado em 24/05/2018 - documento que apresenta o projeto de pesquisa "Cárie dentária em crianças institucionalizadas com dieta controlada: um estudo coorte prospectivo", com cronograma de atividades, planilha de orçamento, Anexo 1 – Diário Alimentar e Anexo 2 – Ficha de Exame Clínico.

14. "projetodepesquisa.docx", postado em 24/05/2018 - documento que apresenta o projeto de pesquisa "Cárie dentária em crianças institucionalizadas com dieta controlada: um estudo coorte prospectivo", com cronograma de atividades, planilha de orçamento, Anexo 1 – Diário Alimentar e Anexo 2 – Ficha de Exame Clínico, em WORD.

15. "orcamentopesquisa.pdf", postado em 24/05/2018 - Planilha de Orçamento da Pesquisa, apresentando descrição de materiais e custo final de R$ 16.005,00, parte com financiamento próprio e parte com solicitação a FAP/DF.

16. "orcamentopesquisa.doc", postado em 24/05/2018 - Planilha de Orçamento da Pesquisa, apresentando descrição de materiais e custo final de R$ 16.005,00, parte com financiamento próprio e parte com solicitação a FAPDF, em WORD.

17. "curriculonailedameteixeira.pdf", postado em 24/05/2018 - Currículo da Plataforma Lattes de Nailê Damé Teixeira, com última atualização em 15 abr 2018. Professora Adjunta do Departamento de Odontologia e do PPG de Odontologia da Universidade de Brasília (UnB). Possui mestrado e Doutorado em Clínica Odontológica - ênfase em Cariologia/Dentística pela Universidade Federal do Rio Grande do Sul (UFRGS). Atuação em Cariologia/Dentística, com foco na cárie radicular. Utiliza Epidemiologia e Microbiologia Oral/biologia molecular (análises de comunidades microbianas, técnicas de DNA-seq e RNA-seq) para o estudo da cárie dentária.

18. "curriculomarianafidelis.pdf", postado em 24/05/2018 - Currículo da Plataforma Lattes de Mariana Souza Fidelis de Oliveira, com última atualização em 12 abr 2017. Acadêmica de Odontologia da FS/UnB (2014).

19. "cartaresppendencias.pdf", postado em 24/05/2018 – Carta de respostas às pendencias apontadas pelo Parecer Consubstanciado CEP/FS nº 2.623.931, de 26 abr 2018, assinada pela Pesquisadora e datada em 24 maio 2018.

cartaresppendencias.doc", postado em 24/05/2018 - Carta de respostas às pendencias apontadas pelo Parecer Consubstanciado CEP/FS nº 2.623.931, de 26 abr 2018, sem assinatura e datada em

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24 maio 2018, em WORD.

20. "analisemicrobiologica.pdf", postado em 24/05/2018 – documento assinado pela Profa. Nailê Damé Teixeira , datado em 23 maio 2018, trazendo informações sobre as análises microbiologias a serem realizadas no material biológico coletado dos participantes da pesquisa.

21. "analisemicrobiologica.docx", postado em 24/05/2018 - documento assinado pela Profa. Nailê Damé Teixeira , datado em 23 maio 2018, trazendo informações sobre as análises microbiologias a serem realizadas no material biológico coletado dos participantes da pesquisa, em WORD.

22. "amostrasaliva.pdf", postado em 24/05/2018 - documento assinado pela Profa. Nailê Damé Teixeira , datado em 21 maio 2018, que traz informações sobre onde ficarão armazenadas as amostras biológicas coletada dos participantes da pesquisa e sob a responsabilidade pela guarda deste material.

Recomendações:

Uniformizar orçamento constante dos documentos "projetodepesquisa.pdf" e "projetodepesquisa.doc" com a revisão do total de custo do projeto de pesquisa para R$ 16.005,00, como apresentado nos documentos PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_1062882.pdf" e "orcamentopesquisa.pdf".

Conclusões ou Pendências e Lista de Inadequações:

Análise das Resposta às Pendencias apontadas no Parecer Consubstanciado No. 2.623.931:

1 – Solicita-se informar os critérios de inclusão e de exclusão dos participantes da Pesquisa, em especial aqueles relacionados aos Participantes alunos do Instituto, quer sejam internos ou não. A idade e a característica de ser portador ou não de necessidade especial podem não ser suficientes para a delimitação de Participante da Pesquisa. Esta informação deve constar do Projeto de Pesquisa e Projeto da Plataforma Brasil.

RESPOSTA – "Os critérios de inclusão e exclusão dos participantes da pesquisa foram inseridos no documento "projeto de pesquisa" página 4, dentro da seção de Metodologia, subseção "grupos"; e no projeto da Plataforma Brasil, na seção "metodologia proposta"."

ANÁLISE – Os critérios de inclusão e exclusão foram definidos e incluídos nos documentos solicitados.

PENDÊNCIA ATENDIDA

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2 – Solicita-se revisar a Análise de Riscos e Benefícios para cada categoria de participante da Pesquisa, incluindo as atividades a serem realizadas com cada grupo de participantes, desde o exame bucal, coleta de saliva, até a resposta a questionários, quer sejam ALUNOS DO INSTITUTO, RESPONSÁVEIS LEGAIS PELOS ALUNOS E CUIDADORES. Tais informações devem constar dos documentos Projeto de Pesquisa, Projeto da Plataforma Brasil, modelo de Termo de Assentimento e modelo de TCLE para cada categoria de participante.

RESPOSTA – "A análise de riscos e benefícios foi revisada, e atualizada nos documentos: "tcleadultosindependentes" parágrafo 4, linha 19. "tclecuidadores" parágrafo 4, linha 15. "tcleresponsaveis" parágrafo 4, linha 19. "termodeassentimentolivreeesclarecido" parágrafo 4, linha 20. "projetodepesquisa" página 6, seção "Riscos e benefícios". Projeto da Plataforma Brasil, seção "Riscos" e "Benefícios"."

ANÁLISE – Foi refeita a Análise de Riscos e Benefícios e as informações incluídas dos documentos explicitados.

PENDÊNCIA ATENDIDA

3 – Quanto à coleta/armazenamento de saliva para realização de exames posteriores, que consta no documento "projetodepesquisa.docx", no item "Metodologia", subitem "Avaliação microbiológica salivar", pág. 5 de 13, lê-se: "Após a coleta, a 1mL da saliva estimulada será armazenado e congelado à -80oC para futuras análises. O DNA microbiano será extraído e analisado por técnicas moleculares, como a amplificação e sequenciamento do gene 16SrRNA." . Ainda, foi citado no modelo de Termo de Assentimento e modelo de TCLE: "uma análise da saliva que será coletada somente em uma situação". Solicita-se:

3.1 – Informar no documento "projetodepesquisa.docx" o local onde ficarão armazenadas as amostras coletadas e quem ficará responsável pelo armazenamento e controle. Solicita-se, ainda, apresentar documento comprobatório do local e do responsável na Plataforma Brasil, com nome/assinatura e data (Norma Operacional CNS 001/2013, Anexo II);

RESPOSTA – " "projetodepesquisa" página 5, item "avaliação microbiológica salivar".O local de armazenamento das amostras foi incluído no texto, e o documento comprobatório do local e responsável foi anexado na plataforma Brasil."

ANÁLISE – Foi atendida a solicitação com a inclusão a informação no documento projetode pequisa, pág. 5, e a inclusão do documento amostrasalivapdf constando o armazenamento da amostra biológica no Laboratório de Microbiologica da FS/UnB, sob a responsabilidade da Profa.

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Nailé Damé Teixeira, devidamente assinado e datado em 21 maio 2018.
PENDÊNCIA ATENDIDA

3.2 – Fazer constar nos documentos "tclealunosindepentendes.doc" e "tcleresponsaveis.docx", o armazenamento da amostra de saliva coletada , bem como a informação de que exame/análise será realizada com a amostra biológica coletada e que se trata de andamento da mesma pesquisa;
RESPOSTA – "Nos seguintes documentos foi incluído o armazenamento da amostra salivar, e o exame que será realizado com a amostra: "tclealunosindependentes" parágrafo 4, linha 19. "tcleresponsaveis" parágrafo 4, linha 19."
ANÁLISE – Foi atendida a solicitação com a inclusão da informação nos documentos requeridos.
PENDÊNCIA ATENDIDA

3.3 – Fazer previsão das análises nas amostras biológicas/saliva na Planilha Orçamentária. Deverão constar os custos com os itens para a realização destas análises ou quem ou qual Laboratório se responsabilizará por esta etapa da pesquisa. Assim sendo, deve ser incluído na Plataforma Brasil o documento de compromisso e capacidade técnica do local de realização destas análises com nome do responsável, sua

assinatura e período de realização.
RESPOSTA – A previsão de custos com análise das amostras biológicas foi incluída na planilha orçamentária. O recurso será solicitado em edital da FAP-DF. Foi também anexado na plataforma o documento "analisemicrobiologica" de compromisso e capacidade técnica do pesquisador responsável por esta etapa da pesquisa, local onde serão realizadas as análises e período de realização.
ANÁLISE – Foi realizada a previsão de recursos financeiros como solicitado e a inclusão no Orçamento Financeiro do documento da Plataforma Brasil PB_INFORMAÇÕES_ BÁSICAS_DO_PROJETO_1062882.pdf. Como citado pela Pesquisadora foi incluído o documento analisemicrobiologica.pdf com o termo de compromisso e qualificação técnica da Profa. Nailé Damé Teixeira que realizará os testes, em documento devidamente assinado e datado em 23 maio 2018.
PENDÊNCIA ATENDIDA

3.4 – Realizar correção no projeto da Plataforma Brasil quanto ao item "Haverá retenção de amostras para armazenamento em banco? Não". Tal reposta deverá ser "sim".

Continuação do Parecer: 2.690.398

RESPOSTA – "O item "Haverá retenção de amostras para armazenamento em banco? Teve a resposta alterada para "Sim"."

ANÁLISE – Foi realizada a alteração.

PENDÊNCIA ATENDIDA

4 - No documento "projetodepesquisa.docx", no item "Metodologia", subitem "Avaliação clínica", pág. 4 de 13, lê-se: "Um questionário será feito com os pais, para coletar dados de nível socioeconômico e de higiene bucal.". Na pág. 5 de 13, subitem "Questionário com os cuidadores", lê-se: "Um questionário de múltipla escolha será aplicado para avaliação da percepção dos cuidadores relativo a saúde bucal dos pacientes com deficiência que estão sob os seus cuidados, e seus próprios conhecimentos sobre saúde bucal.". Os instrumentos da Pesquisa a serem aplicados nos participantes PAIS ou RESPONSÁVEIS e CUIDADORES devem estar incluídos no documento Questionário com os cuidadores ou apresentados em arquivos separados na Plataforma Brasil.

RESPOSTA – "O questionário a ser aplicado com os pais ou responsáveis foi anexado na plataforma Brasil. (documento: questionariosocioeconomico)"

ANÁLISE – Foram incluídos na Plataforma Brasil os documentos questionário cuidador.pdf. e questionariosocioeconomico.

PENDÊNCIA ATENDIDA

5 - Quanto ao documento "termodeassentimentolivreeesclarecido.doc":

5.1 - Não deve conter termos ou expressões que dificultem o entendimento. Deve ser elaborado em linguagem acessível para os menores ou para os legalmente incapazes, proporcionando um fácil entendimento. Assim: "retirar seu assentimento", "dieta exclusiva e acompanhada por nutricionista", "apresentam dieta controlada", "Não existem danos relacionados às avaliações além dos associados ao tratamento restaurador", dentre outras, devem ser substituídas ou esclarecidas. Solicita-se adequação.

RESPOSTA – "Quanto ao documento "termodeassentimentolivreeesclarecido"

5.1 O termo: "retirar seu assentimento" foi substituído por "cancelar esta autorização"; (linha 28) "dieta exclusiva e acompanhada por nutricionista" foi substituído por "alimentação com comidas só dentro do instituto" (linha 6) "apresentam dieta controlada" foi substituído por "se alimentam só dentro do instituto" (linha 8) "Não existem danos relacionados às avaliações além dos associados ao tratamento restaurador" foi substituído por "Você participar da pesquisa não vai te trazer algum

risco. Quando você precisar de uma restauração, você pode sentir dor com a anestesia, ou algum incômodo enquanto recebe a restauração". (linha 19) "dieta controlada por uma nutricionista" foi substituído por "alimentação só dentro do instituto" (linha 3) "Se você precisar de tratamento restaurador" foi substituído por "Se você tiver cárie e precisar de uma restauração" (linha 16) "profilaxias" (linha 25) foi substituído por "limpezas"."

ANÁLISE – As alterações foram realizadas.

PENDÊNCIA ATENDIDA

5.2 – Da mesma forma, a expressão "Caso haja algum dano direto ou indireto decorrente de sua participação na pesquisa, você deverá buscar ser indenizado, obedecendo-se as disposições legais vigentes no Brasil." não tem lugar neste documento, devendo constar no TCLE dos responsáveis. Solicita-se adequação.

RESPOSTA – "A expressão "Caso haja algum dano direto ou indireto decorrente de sua participação na pesquisa, você deverá buscar ser indenizado, obedecendo-se as disposições legais vigentes no Brasil." foi removida deste documento, sendo mantida no TCLE dos responsáveis."

ANÁLISE – A modificação foi realizada.

PENDÊNCIA ATENDIDA

6 - Quanto à apresentação do documento "tclealunosindepentendes.doc" e o "projetodepesquisa.docx", deve ser esclarecido no projeto para que nível de Participante de Pesquisa esse TCLE será apresentado (ou seja, critérios de inclusão e/ou exclusão). A inclusão de aluno desta Instituição Coparticipante no critério de oferecimento de TCLE - Termo de Consentimento Livre e Esclarecido ou TALE - Termo de Assentimento não pode estar ligado somente a idade do Participante da Pesquisa ou ao critério de ser ou não residente no Instituto ou ter ajuda de Cuidador. Está sim ligado à capacidade de compreensão do participante de pesquisa. Solicita-se adequação.

RESPOSTA – "No documento "projetodepesquisa" página 6, foi incluída uma seção "Termo de consentimento", na qual foi descrita como serão utilizados os termos de consentimento."

ANÁLISE – Foi incluída no citado documento as informações solicitadas.

PENDÊNCIA ATENDIDA

7 – No documento "PB_INFORMAÇÕES_BÁSICAS_DO_ PROJETO_1062882.pdf" deve ser incluído como Instituição Coparticipante, o Instituto Dom Orione.

RESPOSTA – "O instituto Dom Orione (CNPJ: 00.102.921.0001-65) foi incluído como instituição

coparticipante no projeto da Plataforma Brasil."

ANÁLISE – Foi atendida a solicitação.

PENDÊNCIA ATENDIDA

8 - O documento "PB_INFORMAÇÕES_BÁSICAS_DO_ PROJETO_1062882.pdf" informa como membros da Equipe de Pesquisa NAILE DAME TEIXEIRA e MARIANA SOUZA FIDELIS DE OLIVEIRA. Solicita-se informar quais as atividades a serem desenvolvidas pelas citadas pesquisadoras e incluir o currículo delas na Plataforma Brasil.

RESPOSTA – "A pesquisadora Nailê Damé Teixeira participará da etapa de avaliação microbiológica salivar. A pesquisadora Mariana Souza Fidelis de Oliveira aplicará o questionário com os cuidadores, e fará com eles a atividade de educação em saúde bucal.

Os currículos das duas pesquisadoras foram anexados na Plataforma Brasil."

ANÁLISE – A solicitação foi atendida, bem como foram anexados os currículos das duas pesquisadoras.

PENDÊNCIA ATENDIDA

Todas as pendências foram atendidas.

Não há óbices éticos para a realização do presente projeto de pesquisa.

Considerações Finais a critério do CEP:

Conforme a Resolução CNS 466/2012, ítens X.1.- 3.b. e XI.2.d, os pesquisadores responsáveis deverão apresentar relatórios parcial semestral e final do projeto de pesquisa, contados a partir da data de aprovação do protocolo de pesquisa.

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_1062882.pdf	28/05/2018 12:49:03		Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	termodeassentimentolivreeesclarecido.pdf	24/05/2018 14:11:49	Paula de Castro Kruly	Aceito
TCLE / Termos de Assentimento /	termodeassentimentolivreeesclarecido.docx	24/05/2018 14:11:39	Paula de Castro Kruly	Aceito

Continuação do Parecer: 2.690.398

Justificativa de Ausência	termodeassentimentolivreeesclarecido.docx	24/05/2018 14:11:39	Paula de Castro Kruly	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	tcleresponsaveis.pdf	24/05/2018 14:11:28	Paula de Castro Kruly	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	tcleresponsaveis.doc	24/05/2018 14:11:16	Paula de Castro Kruly	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	tclecuidadores.pdf	24/05/2018 14:10:52	Paula de Castro Kruly	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	tclecuidadores.docx	24/05/2018 14:10:40	Paula de Castro Kruly	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	tcleadultosindepentendes.pdf	24/05/2018 14:10:22	Paula de Castro Kruly	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	tcleadultosindepentendes.doc	24/05/2018 14:10:10	Paula de Castro Kruly	Aceito
Outros	questionariosocioeconomico.pdf	24/05/2018 14:09:03	Paula de Castro Kruly	Aceito
Outros	questionariosocioeconomico.docx	24/05/2018 14:08:49	Paula de Castro Kruly	Aceito
Outros	questionariocuidadores.pdf	24/05/2018 14:08:32	Paula de Castro Kruly	Aceito
Outros	questionariocuidadores.docx	24/05/2018 14:08:17	Paula de Castro Kruly	Aceito
Projeto Detalhado / Brochura Investigador	projetodepesquisa.pdf	24/05/2018 14:07:48	Paula de Castro Kruly	Aceito
Projeto Detalhado / Brochura Investigador	projetodepesquisa.docx	24/05/2018 14:07:38	Paula de Castro Kruly	Aceito
Orçamento	orcamentopesquisa.pdf	24/05/2018 14:07:01	Paula de Castro Kruly	Aceito
Orçamento	orcamentopesquisa.doc	24/05/2018 14:06:44	Paula de Castro Kruly	Aceito
Outros	curriculonailedameteixeira.pdf	24/05/2018 14:06:18	Paula de Castro Kruly	Aceito
Outros	curriculomarianafidelis.pdf	24/05/2018	Paula de Castro	Aceito

Continuação do Parecer: 2.690.398

Outros	curriculomarianafidelis.pdf	14:05:58	Kruly	Aceito
Parecer Anterior	cartaresppendencias.pdf	24/05/2018 14:05:29	Paula de Castro Kruly	Aceito
Parecer Anterior	cartaresppendencias.doc	24/05/2018 14:05:18	Paula de Castro Kruly	Aceito
Declaração de Manuseio Material Biológico / Biorepositório / Biobanco	analisemicrobiologica.pdf	24/05/2018 14:04:51	Paula de Castro Kruly	Aceito
Declaração de Manuseio Material Biológico / Biorepositório / Biobanco	analisemicrobiologica.docx	24/05/2018 14:04:44	Paula de Castro Kruly	Aceito
Declaração de Manuseio Material Biológico / Biorepositório / Biobanco	amostrasaliva.pdf	24/05/2018 14:04:23	Paula de Castro Kruly	Aceito
Declaração de Manuseio Material Biológico / Biorepositório / Biobanco	amostrasaliva.docx	24/05/2018 14:04:15	Paula de Castro Kruly	Aceito
Projeto Detalhado / Brochura Investigador	cartaencaminhprojeto.pdf	03/04/2018 15:11:18	Paula de Castro Kruly	Aceito
Projeto Detalhado / Brochura Investigador	cartaencaminhprojeto.doc	03/04/2018 15:10:41	Paula de Castro Kruly	Aceito
Declaração de Pesquisadores	termorespecompromdopesquisadorcepfs.doc	03/04/2018 15:09:40	Paula de Castro Kruly	Aceito
Declaração de Pesquisadores	termorespcompromorientadorcepfs.doc	03/04/2018 15:09:05	Paula de Castro Kruly	Aceito
Declaração de Instituição e Infraestrutura	termoconcord.doc	03/04/2018 15:04:40	Paula de Castro Kruly	Aceito
Declaração de Pesquisadores	termorespcompromorientadorcepfs.pdf	22/02/2018 11:07:07	Paula de Castro Kruly	Aceito
Outros	curriculoorientadora.pdf	22/02/2018 11:05:04	Paula de Castro Kruly	Aceito
Outros	curriculoorientadora.docx	22/02/2018 11:04:24	Paula de Castro Kruly	Aceito
Outros	curriculopaula.pdf	22/02/2018 11:03:33	Paula de Castro Kruly	Aceito

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Outros	curriculopaula.docx	22/02/2018 11:03:16	Paula de Castro Kruly	Aceito
Cronograma	cronograma.pdf	22/02/2018 11:02:51	Paula de Castro Kruly	Aceito
Cronograma	cronograma.docx	22/02/2018 11:02:40	Paula de Castro Kruly	Aceito
Declaração de Pesquisadores	termoresponsecomprdopesquisadorcepfs.pdf	17/02/2018 15:00:05	Paula de Castro Kruly	Aceito
Declaração de Instituição e Infraestrutura	termodeconcordancia.docx	17/02/2018 14:58:52	Paula de Castro Kruly	Aceito
Declaração de Instituição e Infraestrutura	termodeconcordancia.pdf	17/02/2018 14:56:59	Paula de Castro Kruly	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	termoautorizacaoimagemsomcepfs.docx	17/02/2018 14:54:54	Paula de Castro Kruly	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	termoautorizacaoimagemsomcepfs.pdf	17/02/2018 14:53:47	Paula de Castro Kruly	Aceito
Folha de Rosto	folhaderosto.pdf	17/02/2018 14:44:21	Paula de Castro Kruly	Aceito

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não

BRASILIA, 04 de Junho de 2018

_____

Assinado por:
Marie Togashi
(Coordenador)

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