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TAYANE DA ROCHA COSTA COELHO

AVALIAÇÃO DO PERFIL MICROBIOLÓGICO DO BIOFILME SUBGENGIVAL EM PACIENTES COM COVID-19

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Tese apresentada ao Programa de Pós-graduação da Faculdade de Odontologia da Universidade Federal da Bahia, como requisito para obtenção do grau de Doutora em Odontologia e Saúde

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Inclui referências e anexos.

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Se as coisas são inatingíveis... ora!
Não é motivo para não querê-las...
Que tristes os caminhos, se não fora
A presença distante das estrelas!

Mario Quintana

APRESENTAÇÃO

Seguindo o Manual de trabalhos acadêmicos do Programa de Pós-Graduação em Odontologia e Saúde da Universidade Federal da Bahia, a presente tese será apresentada em formato de dois artigos científicos.

SUMÁRIO

INTRODUÇÃO

A composição do microbioma oral apresenta alta complexidade pois varia de acordo com a região avaliada, como múltiplas superfícies dentárias e diferentes tipos de mucosa; bem como, depende da maturidade do biofilme avaliado e da presenca de doenças orais, incluindo condições inflamatórias periodontais (Ximénez-Fyvie, Haffajee e Socransky, 2000; Yamashita e Takeshita, 2017). A adaptação dos biofilmes bacterianos orais aos mais diversos estímulos e ambientes, também ocorre como resposta a infecções, de maneira que, a associação entre a infecção pelo SARS-CoV-2 e alterações no microbioma oral é biologicamente plausível (Xiang et al., 2020) e foi previamente acessada.

Diversos estudos avaliaram a associação entre alterações no microbioma da orofaringe e diagnóstico de COVID-19, mostrando impactos importantes na diversidade e complexidade desse microbioma; sintetizações desses estudos demonstram disbiose (alterações no perfil da microbiota) com redução da diversidade em indivíduos com COVID-19 (Ganesan et al, 2023 e Zhou et al, 2023). Em indivíduos com COVID-19, há também, relatos de redução da diversidade da microbiota bacteriana do dorso lingual, (Ren et al., 2021), aumento de espécies específicas, na saliva, de acordo com a carga viral do SARS-CoV-2 (Miller et al., 2021) e alterações na microbiota bacteriana da língua (Haran et al., 2021). Não foram encontrados estudos que avaliaram a associação entre COVID-19 e alterações no perfil microbiológico do biofilme oral de outras regiões da cavidade bucal. Partindo do pressuposto que a boca humana abriga o segundo maior microbioma humano e desempenha um papel fundamental na manutenção da saúde do hospedeiro (Bao et al., 2020), frisa-se a importância de entender o impacto dessa infecção viral nos mais diversos nichos dessa região.

A microbiota e o sistema imune do hospedeiro estabelecem interações moleculares de extensa magnitude, de maneira que, essas interações parecem guiar o desenvolvimento da resposta imune e, por sua vez, o sistema imune molda a composição da microbiota (Round e Mazmanian, 2009). Nessa linha, há descrições de que os impactos locais e sistêmicos da infecção por SARS-CoV-2 no microbioma oral parecem estar associados à resposta imune frente às endotoxinas bacterianas (Iebba et al., 2021) e ao aumento da expressão de mediadores pró-inflamatórios

devido à seleção de bactérias patogênicas (Ren et al., 2021). É esperado, então, que o processo de disbiose perturbe a interação entre a microbiota e o sistema imune humano, resultando em diversos distúrbios inflamatórios (Round e Mazmanian, 2009). Essa conexão entre disbiose e o desenvolvimento de inflamação, é bastante conhecido na relação entre o biofilme dental e condições inflamatórias periodontais, como a gengivite e a periodontite (Chapple et al, 2018).

Nesse ínterim, estudos anteriores demonstraram que o biofilme dental, supra e subgengival, foi previamente caracterizado como reservatório do SARS-CoV-2 em indivíduos com COVID-19; no entanto, a presença do vírus e sua quantificação não foram associados a condições periodontais (Gomes et al., 2021 e Gomes et al. 2022). Por outro lado, há relatos que pacientes com má condição oral, como presença de cáries e doença periodontal, apresentam maior risco de agravamento da COVID-19 (Sirin et al., 2021; Costa et al. 2022); e sabe-se que ambas as patologias são condições infecciosas associadas ao processo de disbiose do biofilme dental (Marsh e Zaura, 2017). Esses resultados reforçam a existência de uma ligação entre a COVID-19, seu patógeno SARS-CoV-2 e o biofilme dental, mas não esclarecem o mecanismo dessa interação.

Diante do que foi exposto, levantam-se as seguintes hipóteses: 1- a presença do SARS-CoV-2 no biofilme dental pode resultar em mudanças no perfil microbiológico desse ecossistema; 2- existe uma associação entre a presença do SARS-CoV-2 no biofilme dental e a condição inflamatória periodontal, neste caso, sendo possível que a inflamação periodontal interfira na instalação do vírus no biofilme subgengival, mas também que o vírus modifique o microbioma local, o que poderia resultar em inflamação periodontal; e 3- a COVID-19 pode alterar o microbioma do biofilme subgengival. Assim sendo, a confirmação da detecção do SARS-CoV-2 nesse biofilme e a compreensão da interação entre o microbioma desse ecossistema com o SARS-CoV-2 e a COVID-19, na saúde periodontal e na presença de inflamação local, pode ajudar a esclarecer o papel dos vírus no perfil microbiológico oral, bem como, predizer o tipo de resposta do aglomerado bacteriano a partir da avaliação da concentração de espécies. Dessa maneira, o presente estudou objetivou avaliar a presença do SARS-CoV-2 no biofilme subgengival em indivíduos com COVID-19 e sua correlação com os achados clínicos periodontais e avaliar, pela primeira vez,

através do sequenciamento do gene 16S, o microbioma do biofilme subgengival de indivíduos com COVID-19, associado à saúde periodontal e à gengivite.

REVISÃO DA LITERATURA

SARS-CoV-2

O SARS-CoV-2 é um vírus de RNA de cadeia simples, envelopado, de sentido positivo, do gênero Betacoronavirus (Lu et al, 2020).

O vírus do coronavírus apresenta envelope viral que consiste em uma bicamada lipídica que contém as proteínas estruturais nucleocapsídeo (N), membrana (M), envelope (E) e spike (S). A glicoproteína S é mediadora da entrada das partículas virais, a partir da ligação à membrana da célula hospedeira e consequente fusão (Jackson et al, 2022).

A biologia estrutural da proteína S do SARS-CoV-2 avançou muito rapidamente desde o surto inicial de COVID-19, no entanto, é sabido que a enzima convertora de angiotensina 2 (ECA2) tem papel fundamental na entrada viral às celulas humanas por apresentar especificidade ao domínio de ligação de receptor (RBD) da proteína S (Shang et al, 2020).

As mutações naturais no SARS-CoV relacionados à estrutura e ligação regulam a infecciosidade, a patogênese e as transmissões virais.

Enzima Conversora de Angiotensina tipo 2 (ECA2)

A ECA2, assim como a ECA, é uma enzima secretada e associada à membrana, expressa predominantemente no endotélio. ECA2 possui função catalisadora na clivagem de Angiotensina 1 em Angiotensina 1-9; enquanto a protease transmembrana serina 2 (TMPRSS2) realiza a função de ativação da proteína S, fortalecendo assim a ligação entre o SARS-CoV-2 e a ECA2.

Estruturalmente, a ECA2 possui um peptídeo sinal aparente, um único sítio ativo de metaloprotease e um domínio transmembrana (Donoghue et al, 2020). Os receptores ECA2 servem como entrada para o acesso de coronavírus, como HoV-NL63, SARS-CoV e SARS-CoV-2, às células humanas; as células epiteliais orais e outras áreas do trato respiratório apresentam expressão substancial de ECA2, explicando sua suscetibilidade à entrada viral (Shirbhate et al, 2021).

Sakagushi et al (2020) detectaram a expressão de ECA2 e TMPRSS2 em tecidos orais, língua, glândulas salivares, língua, tecidos gengivais e papilas

fungiformes humanas. O que sugere que múltiplas células orais são suscetíveis à infecção por SARS-CoV-2.

Vírus e Periodonto

A associação entre vírus e doenças peridodontais não está totalmente esclarecida. No entanto, diversos estudos associaram a presença de herpesvírus (Epsteinn Barr, Herpes vírus simples e Cytomegalovirus Humano) com o desenvolvimento e manutenção da periodontite (Maulani et al, 2021).

Hipotetiza-se que os vírus podem participar da patogênese da periodontite alterando as defesas imunológicas, ativando reações destrutivas do hospedeiro ou por meio de efeitos líticos diretos nos tecidos periodontais (Tsuchida, 2020).

Herpesvírus periodontais que se disseminam através da circulação sistêmica para locais não orais podem representar um importante elo entre a periodontite e as doenças sistêmicas (Slots J., Slots H et al, 2019).

A possível interação entre vírus e doenças periodontais, salienta a necessidade de entender o comportamento do SARS-CoV-2 no biofilme dental. Se não pela possível potencialização de condições inflamatórias locais, pela capacidade de disseminação sistêmica, principalmente na presença de inflamação.

SARS-CoV-2 e cavidade bucal

A presença do vírus SARS-CoV-2 foi substancialmente estudada nos mais diversos nichos da cavidade bucal (A *Tabela 1* descreve os estudos e seus principais achados). Enquanto a descrição da associação entre COVID-19, e/ou presença do vírus no periodonto e regiões anexas, e condições periodontais, apesar de bastante especulada em revisões sistemáticas e de literatura (Brandini et al, 2021; Campisi et al, 2021; Baima et al, 2022), até o momento, se restringiu a três principais estudos.

Gomes et al (2021) detectaram a presença do vírus SARS-CoV-2 no biofilme dental, coletado da margem gengival, de pacientes sintomáticos com COVID-19. Embora a avaliação peridodontal não tenha sido realizada pelos pesquisadores, o relato da presença do vírus no microbioma local refletiu a necessidade de uma investigação mais apurada. Por conseguinte, Gomes et al (2022) avaliaram a

presença do SARS-CoV-2 no biofilme dental, supra e subgengival, de indivíduos hospitalizados por consequência da COVID-19, e incluíram a avaliação periodontal desses indivíduos. Apesar de reforçar o resultado positivo para presença do SARS-CoV-2 no biofilme dental, os autores não conseguiram estabelecer relação entre condição periodontal e presença ou carga viral. De maneira que, indivíduos periodontalmente saudáveis e doentes apresentaram resultados semelhantes para presença ou ausência do vírus no biofilme, bem como para a carga viral detectada no biofilme dental.

Por outro lado, Marouf et al (2021) preconizaram um estudo em indivíduos com complicações da COVID-19 e avaliaram a associação entre a presença de periodontite (avaliada exclusivamente pela existência de perda óssea radiográfia) e o desenvolvimento dessas complicações. Os autores encontraram associação entre a presença de periodontite e o desenvolvimento de complicações, com achados que demonstraram uma chance 3,7 vezes maior de desenvolver complicações da COVID-19 em pacientes com diagnóstico radiográfico de periodontite.

Não há dados na literatura, de estudos clínicos, que objetivem descrever o impacto da presença da COVID-19 ou do SARS-CoV-2 no perfil microbiano do biofilme dental.

Tabela 1. Características de estudos que avaliaram a associação entre a cavidade bucal e a presença do vírus SARS-CoV-2 em pacientes com COVID-19.

Autor(es), ano	Tamanho da amostra/ desenho do estudo	Tipo de avaliação	Perfil dos pacientes	Resultados principais
To et al, 2020	12/ Estudo transversal	Presença do vírus SARS-CoV-2 na saliva de pacientes com COVID-19, através do método de RT-PCR	Pacientes hospitalizados	Resultado positivo para SARS-CoV-2 na saliva em 91.7% dos pacientes (n=11)
Chen et al., 2020	31/ Estudo transversal	Presença do vírus SARS-CoV-2 na saliva de pacientes com COVID-19, através do método de RT-PCR; e avaliação da orofaringe	26 pacientes caracterizados como caso leve a severo e 5 pacientes em suporte ventilatório	13 casos positivos para detecção em swab orofaríngeo e, dentre esses, 30,7% (n=04) foram positivos para saliva (onde 03 casos correspondiam a pacientes em suporte ventilatório)
Matuck et al., 2020	07/ Estudo transversal	Análise de tecidos periodontais de pacientes com COVID-19 (post-mortem), atraves de histologia e RT-PCR	Post-mortem	Histologicamente: alteração de queratinócitos no epitélio juncional e, em alguns casos, pleomorfismo nuclear. Análise molecular: 71,43% (n=5) apresentaram resultado positivo para SARS-CoV-2 no tecido periodontal
Gomes et al., 2020	70/ Estudo transversal	Presença do virus SARS-CoV-2 no biofilme dentário de pacientes com COVID-19, via RT-PCR	Pacientes com sintomas leves de COVID-19	18.6% (n=13) dos casos foram positivos para SARS-CoV-2 no biofilme dentário
Berton et al., 2021	12/ Estudo transversal piloto	Presença do virus SARS-CoV-2 no cálculo dentário de pacientes com COVID-19, via RT-PCR	05 pacientes com COVID-19, com intervalo de 2 meses até a pesquisa; 2 com suspeita de COVID-19 (negativados) e 5 saudáveis	SARS-CoV-2 foi detectado no cálculo dentário de todos os indivíduos curados, nos indivíduos suspeitos e em 1 indivíduo saudável
Gupta et al., 2021	33/ Estudo transversal	Presença do virus SARS-CoV-2 no fluido crevicular gengival (FCG) e na saliva de pacientes com COVID-19, atraves do metodo de RT-PCR	20 pacientes assintomaticos e 13 com sintomas leves de COVID-19. 14 pacientes foram diagnosticados com gengivite	Detecção de SARS-CoV-2 no FCG em 63.64% dos casos (n=21) e detecção de SARS-CoV-2 na saliva em 64.52% (n=20); duas amostras de saliva foram perdidas. 02 casos foram positivos apenas para FCG, enquanto 03 casos foram positivos apenas para saliva
Gomes et al., 2021	52/ Estudo transversal	Presença do virus SARS-CoV-2 no biofilme dentario, supra e subgengival, de pacientes com COVID-19, via RT-PCR	Pacientes hospitalizados	50% (n=26) dos casos foram positivos para SARS-CoV-2 no biofilme dentário supra e/ou subgengival. Dentro desse grupo, 01 paciente foi positivo apenas para o biofilme supragengival
Marouf et al, 2021	568/ Caso-controle	Associação entre doenças periodontais e o desenvolvimento de complicações referentes à COVID-19	Pacientes com histórico de COVID-19 curados ou falecidos	258 pacientes diagnosticados com doença periodontal, 33 sofreram complicações da COVID-19; enquanto 310 indivíduos eram periodontalmente saudáveis e, desses, 7 apresentaram complicações por COVID-19
Pasomsub et al., 2021;	200/ Estudo transversal	Presença do virus SARS-CoV-2 na saliva de pacientes com COVID-19, atraves do metodo de RT-PCR; simultaneamente a avaliação da orofaringe	Pacientes com sintomas leves de COVID-19. [antidepressivos, esteroides])	9,5% (n=19) dos casos foram positivos para detecção em swab orofaríngeo enquanto 9,0% (n=18) foram positivos para saliva. Houve concordancia em 97,5% dos casos

Análise do microbioma oral

A abundante microbiota da cavidade bucal impacta na dificuldade em descrever a comunidade microbiona devido às limitações de cultivo fora do habitat natural. De maneira que, as inovações da tecnologia de sequenciamento e plataformas baseadas em Sequenciamento da Nova Geração (NGS) tornaram possível o acesso e descrição dessa enorme diversidade de espécies (Verma et al, 2018).

O sequenciamento é baseado na conservação evolutiva de marcadores, como o gene 16S do RNA ribossômico de bactérias e archaea, os quais são frequentemente usados para caracterizar a composição taxonômica e a diversidade filogenética de amostras ambientais (Langille et al, 2013). A distinção de espécies é possível devido à existência de regiões hipervariáveis, sendo as regiões V1, V2, V3 e V4 as mais preconizadas. A avaliação dessas regiões é capaz de fornecer o quadro completo dos filos bacterianos presentes em um nicho (Chakravorty et al, 2007).

Youssef et al (2009) avaliaram a capacidade de geração de resultados, a partir de fragmentos do gene 16S, e concluíram que a região V4 é caracterizada por apresentar as descrições mais precisas, garantindo a acurácia das atribuições filogenéticas e estimativas de riqueza de espécies. Os autores também relataram que as regiões V3 e V4 fornecem desempenhos eficazes na determinação da riqueza de espécies, mesmo tratando-se de fragmentos curtos.

A aplicabilidade das regiões V3-V4 está descrita na literatura em estudos relacionados à detecção do SARS-CoV-2 na cavidade oral e orofaringe. Wu et al (2021) avaliaram a microbiota oral e intestinal, através do sequenciamento do gene 16S, regiões V3 e V4, previamente à infecção ao SARS-CoV-2 e após finalização do ciclo viral e mostraram que a infecção por SARS-CoV-2 foi associada a alterações na comunidade do microbioma desses pacientes, através da comparação entre alfa e beta diversidades. Enquanto Ventero et al (2021), ao realizar sequenciamento do gene 16S, regiões V3 e V4, de amostras coletadas da região nasofaríngea de pacientes com e sem COVID-19, encontraram diferenças quanto às espécies presentes e complexidade da microbiota. Ambos estudos demonstram a capacidade e versatilidade do sequenciamento do gene 16S e reforçam a escolha das regiões V3-V4.

Em adição, é sabido que o processo de disbiose, associado ao biofilme dental, tem como consequência a inflamação dos tecidos periodontais (Murakamami et al, 2007). De maneira que, o entendimento da microbiologia local desse ecossistema a partir de análises apuradas, tem impacto no delineamento de tratamentos e estabelecimento do prognóstico associado às doenças periodontais.

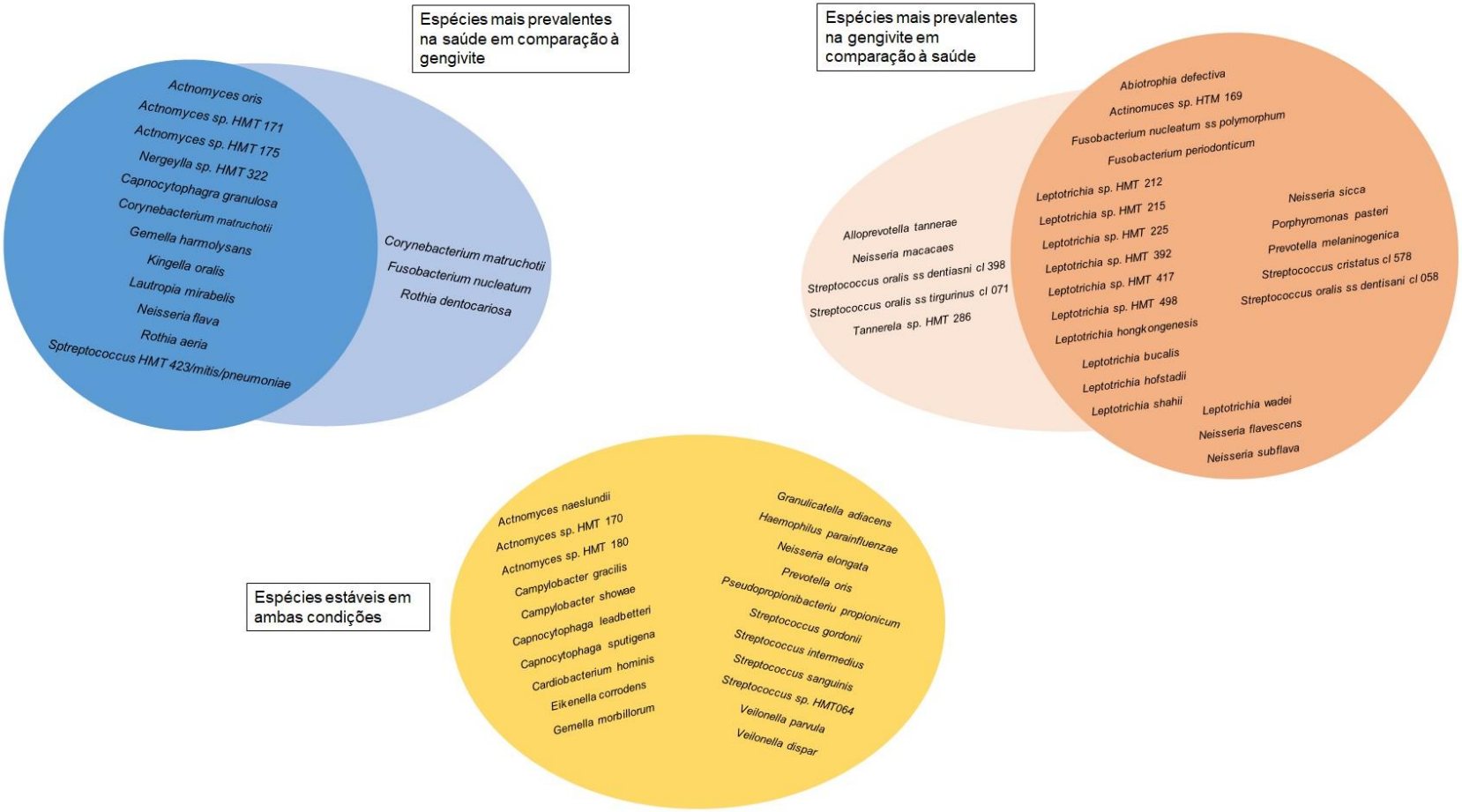
Microbiota associada à gengivite

A gengivite é considerada como o estágio que precede o desenvolvimento da periodontite (Loe et al, 1965). O desenvolvimento da gengivite mediada por placa está relacionada ao déficit de remoção do biofilme dental, o qual gera seu acúmulo, podendo ser revertida após aplicação dos métodos de higiene oral (Loe et al, 1965). Não está esclarecido, no entanto, de qual maneira a microbiota associada à gengivite contribui no desenvolvimento da periodontite, o que reforça a necessidade de entendimento desse ecossistema (Abusleme et al, 2021).

Abusleme et al (2021) realizaram uma meta-análise incluindo estudos relacionados ao microbioma periodontal na saúde, gengivite e periodontite e descreveram que as comunidades com gengivite são enriquecidas principalmente com espécies anaeróbias gram-negativas, embora consumidores de oxigênio também possam estar presentes. Huang et al (2014) notaram a presença de um aspecto funcional relacionado às espécies mais prevalentes na gengivite: a presença de flagelos que podem auxiliar na invasão de tecidos do hospedeiro, bem como promover motilidade para escapar da fagocitose. Os autores descreveram, também, uma maior presença de espécies dos gêneros Tannerella e Treponema, bem como, do filo TM7 (Saccharibacteria) em pacientes com gengivite caracterizada como severa. Enquanto Schincaglia et al (2017) relataram uma grande proporção dos gêneros *Prevotella* e *Selenomonas* na presença de gengivite.

Algumas espécies bacterianas são consideradas centrais e não sofrem alterações em sua proporção no processo saúde-doença, mas podem exercer função mediadora no processo de formação da placa, auxiliando o processo de disbiose (Abusleme et al, 2021). Diaz et al (2016) descreveram as espécies *Veillonella parvula*, *Veillonella dispar* e *Campylobacter gracilis* como espécies centrais na comparação entre saúde e gengivite.

Figura 1 - Visão geral integrada das espécies bacterianas que definem saúde e gengivite.



Fonte: Adaptado de Abusleme et al (2021, p. 74).

ARTIGO 1

SARS-CoV-2 in subgingival biofilm among COVID-19 patients and periodontal health profile assessment

ABSTRACT

OBJECTIVE: The present study aimed to evaluate the presence of SARS-CoV-2 in the subgingival biofilm of patients with COVID-19 and to correlate SARS-CoV-2 with periodontal inflammation.

METHODS: In this cross-sectional study, subgingival biofilm was collected from 28 healthy individuals and 67 individuals diagnosed with COVID-19. They were categorized into periodontal health [absence of bleeding on probing (BoP) and clinical attachment loss (CAL); n = 48]; gingivitis [BoP + probing depth (PD) < 4mm + absence of CAL; n = 37); or periodontitis (PD ≥ 5mm + CAL; n = 10). One pool of dental biofilm was obtained for each individual (shallow subgingival) independently of periodontal state, in periodontitis cases, another sample was collected from the deepest site. All samples were tested for the presence of SARS-CoV-2 RNA using the qRT-PCR technique. Chi-square analysis and t-test were applied to evaluate associations between the presence of SARS-CoV-2 RNA and its quantification and periodontal diagnosis ($p \leq 0.05$).

RESULTS: SARS-CoV-2 RNA was detected in subgingival biofilm of 35.8% (n=24) of individuals with COVID-19; whereas there was no detection in individuals without COVID-19. SARS-CoV-2 was detected in 27.9% (n=12) of participants with periodontal health, 50% (n=12) with gingivitis, and none with periodontitis. It was not possible to detect any association between periodontal diagnosis and SARS-CoV-2 RNA presence ($p \geq 0.09$ or quantification ($p \geq 0.31$).

CONCLUSION: SARS-CoV-2 RNA was detected in the shallow subgingival biofilm, but not in the subgingival biofilm of deep periodontal pockets of patients with COVID-19. The present findings confirm the presence of SARS-CoV-2 in subgingival biofilm, independently of

periodontal status. Anywise, this highlights the critical need to unravel the virus interaction with local microbiota.

INTRODUCTION

The understanding of how viruses spread through the oral cavity is an ongoing focus. The potential for viruses to transmit via particles and aerosols, coupled with their presence on various mouth surfaces and sites (Hoffmann, 2023), raises significant concerns about the oral cavity. It's crucial to explore how the oral cavity contributes to SARS-CoV-2 infection and its effects on oral health.

Saliva, known to harbor various viruses including SARS-CoV-2, play a significant role in COVID-19 transmission (Baghizadeh, 2020). Studies indicate a SARS-CoV-2 positivity rate in saliva of over 91%, with live virus cultivation possible from saliva samples (To et al., 2020; Costa et al, 2021). Additionally, SARS-CoV-2 has been detected in gingival crevicular fluid (GCF) (Gupta et al., 2021). Considering the multifaceted nature of the oral cavity, encompassing teeth, gums, tongue, and other mucosa, it serves as a pivotal environment for hosting microorganisms and understanding the behavior of SARS-CoV-2.

Angiotensin-converting enzyme 2 (ACE2), found in the respiratory tract and salivary gland duct epithelium, serves as a receptor for SARS-CoV. This may clarify why there's a notable concentration of SARS-CoV-2 virus in saliva (Letko and Marzi, 2021; Meng, Hua, and Bian, 2020). ACE2 expression has been observed in both tongue and gingival surfaces (Sakaguchi et al., 2020). This presence of the receptor facilitates virus entry, replication, and potential for infection, suggesting the oral cavity also as a significant pathway for the virus.

Another potential reservoir for SARS-CoV-2 resides in oral biofilms, particularly dental biofilm, constituting a diverse microorganism community on the tooth surface within a polymer-based extracellular matrix (Costerton et al., 1995). Previous reports suggest that the presence of virus in dental biofilm might influence its behavior (Marsh, 2005). This interaction,

combined with pathogenic bacteria, could contribute to inflammatory conditions such as gingivitis and periodontitis (Slots, 2010).

Two studies detected SARS-CoV-2 RNA in dental biofilm (Gomes et al., 2021; Gomes et al., 2022), while another found the virus in dental calculus (Berton et al., 2021). However, the study examining the link between SARS-CoV-2 RNA detection and periodontal conditions in severe hospitalized patients did not establish any correlation (Gomes et al., 2022). While the presence of the virus in dental biofilm was assessed, its potential to interact with periodontal disease sites, in mild to moderate COVID-19, is yet to be fully understood. Seeking to clarification, this study aims to assess SARS-CoV-2 RNA presence in subgingival biofilm, through qRT-PCR, among both mild COVID-19 patients and healthy individuals, and to investigate the relationship between this presence and periodontal clinical findings.

MATERIAL AND METHODS

The Ethics Board of the Dental School of the Federal University of Bahia approved this study (Protocol: 52185521.8.0000.5024). All patients signed the informed consent.

Study design, setting, and participants

This observational clinical study recruited patients with and without COVID-19 symptoms who attended Public Health Service Units in Salvador, Bahia, Brazil, between 2021 and 2022. Individuals with flu-like symptoms were previously, on the same day, submitted to rapid antigen testing by an immunochromatographic method (Vida Biotecnologia®, Belo Horizonte-MG, Brazil) using nasopharyngeal swab samples. Individuals with positive results were invited to take part in this study. The primary outcome was the presence/load of SARS-CoV-2 in subgingival biofilm, while the periodontal diagnosis was considered the predictor.

After all the COVID-19 patients were evaluated, individuals with similar social-demographic characteristics (sex, age, race, and ethnicity) and without flu-like symptoms were invited to participate.

The following inclusion criteria were applied: age ≥ 18 years, dentate participants, and systemic condition adequate to periodontal exam. For the COVID-19 population were also required the presence of flu-like symptoms for 3 to 7 days and a positive test for SARS-CoV-2, including patients with mild to moderate symptoms.

Data collection

Demographic data were collected from the Public Health Service's records to define the medical condition of all the individuals enrolled in the study. The following data were obtained: age, sex, and COVID-19 symptoms (fever, cough, tiredness, anosmia or dysgeusia, sore throat, headache, body aches and pains, diarrhea, red or irritated eyes, difficulty breathing or shortness of breath, chest pain, and others).

Clinical evaluation and biofilm sampling

Two trained periodontists performed all the clinical examinations. Clinical attachment loss (CAL), probing depth (PD) and bleeding on probing (BOP) were evaluated at all teeth' interproximal sites (mesio-buccal, mesio-lingual, distal-buccal, and distal-lingual). Health periodontal condition was characterized as absence of BoP and CAL; gingivitis was defined as presence of BoP in association with PD < 4mm and absence of CAL; whilst periodontitis was characterized as PD ≥ 5mm in association with CAL (Lang et al., 2018; Murakami et al., 2018).

After cotton-roll isolation, one pool of subgingival biofilm was obtained with a sterilized periodontal curette from the shallow subgingival area of all teeth (P1). In participants with PD ≥ 5.0mm, one sample of the deepest site was also obtained (P2), using another sterilized

periodontal curette. In such a way that P1 always precedes P2, to avoid the contamination of the deep subgingival sample.

One conical centrifuge cube tube containing 3 mL of Phosphate-Buffered Saline (PBS) was used for each pooled sample. Therefore, there were one or two tubes for each participant.

All samples were stored at -80°C until analysis in the laboratory.

SARS-CoV-2 qRT-PCR

At first, the samples were vortexed, and three aliquots of 1 mL were extracted. Two of the aliquots were stored at -80°C as reserve samples. Viral nucleic acid was extracted from 150 μL diluted dental biofilm and eluted to 55 μL with elution buffer, according to manufacturer's instructions (High Pure Viral Nucleic Acid Kit, Roche,Mannheim, Germany). RNA templates were amplified by qRT-PCR (CDC, 2020), targeting nucleocapsid genes N1 and N2 and the RNAse P gene as an internal control (QuantStudio 3 Real-Time PCR System, Applied Biosystems).

According to the protocol of the US Centers for Disease Control and Prevention, CT < 40 for viral and control genes were positive controls for SARS-CoV-2. The samples in which only one of the viral genes was amplified were classified as inconclusive, and the RT-qPCR was repeated. The samples that did not show amplification of either gene were considered inadequate, and the RNA extraction and RT-qPCR were repeated. A total of three samples required RT-qPCR repetition.

2.5. Data analysis

The percentage of BOP was obtained based on the number of positive sites in relation to the multiplication of the total number of teeth examined per participant by a factor of 4 (mesio-buccal, mesio-lingual, distal-buccal, and distal-lingual). The PD and CAL were reported

in millimeters. The delta-delta Ct method was applied to compare the virus quantification between samples.

Means and standard deviations were calculated for each parameter. The homogeneity of variance was verified, and Kendall rank correlation coefficient was applied to evaluate the correlation between the delta-delta Ct and the variables. Regarding the qualitative variables, the chi-square was used. The level of significance was set at $p \leq 0.05$. Data were analyzed using the SPSS Statistics 13.0 program (SPSS Inc., Chicago, IL, USA).

RESULTS

From the COVID-19 positive patients, 372 were potentially eligible individuals, after applying the exclusion criteria, 150 individuals were invited, 67 agreed to participate and were enrolled in the study. The COVID-19 negative patients were filled with the invitation of 28 individuals without flu-like symptoms, all the invited patients agreed to participate. A total of 95 individuals were included in this study.

The mean age of the patients was 37 years (18-74 years) and most of them were female (n = 65). *Table 1* shows age, sex, and periodontal characteristics of all the participants.

Patients with COVID-19 described the following symptoms: headache (53.57%); fever (48.21%); cough (32.14%); congestion or runny nose (30.36%); body aches (28.57%); sore throat (26.79%); anosmia and/or dysgeusia (19.64%); prostration (16.07%); eye pain (8.93%); dysphonia (3.57%); nausea and/or diarrhea (1.79%). From the patients without COVID-19, 25% (n=07) described history of COVID-19; with an average time interval of 14 months from the disease and this study.

The clinical exam showed gingivitis in 37 patients. Periodontitis was diagnosed in 10 patients, with 25 compromised sites. The SARS-CoV-2 was detected in subgingival biofilm of 35.8% of COVID-19 group (n=24). None deep subgingival sample was positive for SARS-CoV-2 presence and none of the patients without COVID-19 presented positive result for SARS-CoV-2 in subgingival biofilm. *Table 2* shows the association of the presence of SARS-CoV-2 in subgingival biofilm with the periodontal condition and the assessment of viral quantification via delta delta CT and gingivitis is described in *Table 3*.

DISCUSSION

At the outset, this study examined the presence of SARS-CoV-2 RNA in subgingival biofilm using qRT-PCR among individuals with and without COVID-19. Subsequently, it aimed to establish a link between positive SARS-CoV-2 detection and the occurrence of periodontal inflammation. The findings confirmed the presence of SARS-CoV-2 RNA in subgingival biofilm throughout the duration of COVID-19. However, the absence of SARS-CoV-2 detection in subgingival samples and the lack of a correlation between the presence or concentration of SARS-CoV-2 and gingivitis contradict the idea that the virus has a heightened interaction with compromised periodontal sites. It's important to note that dysbiosis, while not directly assessed in this study, remains a potential factor. These results underscore the coexistence of viruses and bacteria in subgingival biofilm, irrespective of inflammatory conditions.

The detection of SARS-CoV-2 RNA in 35.8% of the COVID-19 group aligns with existing literature. In a prior study, 18.6% of mildly symptomatic COVID-19 patients (n=70) exhibited SARS-CoV-2 detection in supragingival biofilm (Gomes et al., 2021). Conversely, among intensive care unit patients (n=52), a higher prevalence of SARS-CoV-2 was observed,

with the virus detection in 50% of cases across supragingival and subgingival samples (Gomes et al., 2022). While these findings do not confirm its viability, they underscore the virus' resilience in spreading within the oral cavity and coexisting with other microorganisms.

The absence of SARS-CoV-2 RNA in subgingival biofilm of previously infected yet currently healthy patients, suggests a potentially transient presence of the virus in this environment. In contrast, the delayed detection (two months post-COVID-19 recovery) of SARS-CoV-2 in dental calculus revealed positive results in all COVID-19 positive patients (n=5) (Berton et al., 2021). This raises the possibility that the mineralization process might contribute to sustaining the presence of SARS-CoV-2. Besides its importance in describing the microbiology of calculus, it's understood that the impact on surrounding tissues relates more to non-mineralized dental biofilm (Waerhaug, 1956). The limited research on the effects of rapid-cycle viral diseases on oral microbiota complicates discussions. To date, there hasn't been an assessment of any virus' presence in dental biofilm post complete recovery, further contributing to the existing gaps in understanding its correlation.

Conversely, various viruses linked to persistent viral diseases — including herpesviruses, enterovirus, HIV, and HPV — have been identified in the oral cavity, leading to pathological outcomes like mucosal lesions, oral ulcerations, and periodontitis (Ray, 2023). Considering these observations, it prompts the question of whether the presence of SARS-CoV-2 can alter the microbiology of dental biofilm or escalate the damage to adjacent tissues, both of which could significantly impact the progression of inflammatory periodontal conditions. Supporting this premise, there's evidence linking SARS-CoV-2 to the induction of fibrotic pathogenic traits in periodontal ligament fibroblasts and a decrease in mitochondrial β-oxidation (Gao et al., 2023). However, the potential alteration in dental biofilm microbiology due to SARS-CoV-2 remains unexplored in current literature.

The current study did not establish a connection between gingivitis and increased expression of SARS-CoV-2 RNA in subgingival biofilm. This aligns with a prior investigation where the cycle quantification values of SARS-CoV-2 PCR from dental biofilm samples remained unaffected by periodontal parameters (Gomes et al., 2022). Furthermore, the viral load of SARS-CoV-2 doesn't reliably to predict the severity of COVID-19 (Le Borgne et al., 2021). Consequently, potential damages resulting from the presence of SARS-CoV-2 in dental biofilm and tissues might occur independently of the detected virus quantity.

The primary limitation of this study is the absence of molecular analysis, which could have revealed the interaction between SARS-CoV-2 and the subgingival biofilm ecosystem. Despite using cotton isolation, to minimize saliva contamination, there remains a possibility of cross-contamination or contamination from the gingival crevicular fluid. Additionally, a limitation arises from the extended interval between the COVID-19 recovery period and the sample collection from healthy patients, potentially influencing the negative results. Nevertheless, this study emphasizes the critical need for discussions on the presence and consequences of significant viruses in the oral cavity, highlighting the existing gap in understanding this domain.

CONCLUSION

The present study concludes that SARS-CoV-2 can be detected in shallow subgingival biofilms throughout the duration of COVID-19. The virus detection was not correlated with periodontal health, gingivitis, and periodontitis. Nevertheless, there's a pressing need to further explore the effects of virus presence within the oral cavity, particularly in sites with intimate interaction with blood circulation, such as subgingival biofilms.

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Table 1 - Description of the study sample (N = 95).

Variables	N (%)
Age (Years)	
0–36	45 (47.4)
≥37	50 (52.6)
Sex	
Female	65 (68.4)
Male	30 (31.6)
Gingivitis	
No	58 (61.1)
Yes	37 (38.9)
Periodontitis	
No	85 (89.5)
Yes	10 (10.5)

Table 2 - Association of the presence of SARS-CoV-2 in the subgingival biofilm with periodontal health, gingivitis, and periodontitis in the bivariate analysis, in COVID-19 group (N = 68).

Variables	SARS-CoV-2 (%)		P value
	No	Yes	
Periodontal health			0.09
	27 (69.2)	12 (30.8)	
Gingivitis			
	12 (50)	12 (50)	
Periodontitis			
	4 (100)	0 (0)	

Table 3. Relationship between the ΔΔCT of cycle quantification values and periodontal condition in the univariate analysis (N = 24).

ΔΔCT (log)		Odds Ratio (IC 95%)	P value
Periodontal health	**Gingivitis**		
3.28	6.86	1.09 (-1.07-3.26)	0.31
7.81	8.93		
8.12	9.38		
9.37	9.38		
10.67	5.59		
8.43	4.22		
2.85	8.87		
7.49	3.82		
7.09	8.23		
7.74	3.05		
9.44	7.51		
6.39	2.76		

ARTIGO 2

COVID-19 and subgingival biofilm: microbiome alterations and clinical implications

ABSTRACT

OBJECTIVE: The present study aimed to describe and compare subgingival biofilm profile of COVID-19 patients in the presence or absence of SARS-CoV-2 in this biofilm, in periodontal health and gingivitis condition.

MATERIAL AND METHODS: In this cross-sectional study, subgingival biofilm collections were carried out on symptomatic individuals diagnosed with COVID-19 and on healthy individuals, covering 60 patients. Clinical data was collected and periodontal examination was performed. Subgingival biofilm samples from all individuals included in the study were tested for the presence of SARS-CoV-2 using the qRT-PCR technique. Individuals were grouped according to combinations of presence or absence of COVID-19, presence or absence of SARS-CoV-2 in subgingival biofilm, and presence or absence of gingivitis. The biofilm samples were sequenced for the 16S gene, regions V3-V4. Species were evaluated when they were present in at least 10% of the samples with a minimum count of 10 readings. Logistic models were applied to assess profile associations between certain groups ($p \leq 0.05$).

RESULTS: 11 phyla, 93 genera and 258 species were detected. Differences in α-diversity and β-diversity were found, with notorious upper-regulation in richness in COVID-19 groups and down-regulation in diversity in the presence of gingivitis and COVID-19; while the local presence of SARS-CoV-2 did not generate expressive impacts. The presence of COVID-19 in association with gingivitis showed the most expressive results, with emphasis on the increase of bacteria from genera Saccharibacteria ($p \leq 0.04$) and Streptococcus ($p \leq 0.01$). The genera Saccharibacteria was also dominant in periodontal health, in association with an increase of periodontopathic bacteria (including *Parvimonas micra* and *Prevotella intermedia*).

CONCLUSION: COVID-19 was related to changes in subgingival biofilm microbiota, independently of detecting SARS-CoV-2 in the local ecosystem. The genera Saccharibacteria

and Streptococcus were repeatedly found and demands attention. COVID-19 patients should emphasize oral hygiene to reduce the risk of local dysbiosis and its consequences.

1 INTRODUCTION

COVID-19, caused by severe acute respiratory syndrome Coronavirus 2 (SARS-CoV-2) (Ochani et al., 2021), has been linked to various oral manifestations such as xerostomia, anosmia, mucosal lesions, and inflammation (Sharm et al., 2022). Moreover, speculation surrounds the potential association between COVID-19 and periodontal conditions, whereas periodontitis has been linked to heightened susceptibility to COVID-19, an increased risk of COVID-19 exacerbation, and elevated mortality rates (Meng et al., 2023; Marouf et al., 2021).

Some studies have observed the presence of SARS-CoV-2 RNA in diverse oral sites, including gingival crevicular fluid, and both supragingival and subgingival biofilm (Gupta et al., 2021; Gomes et al., 2021; Gomes et al., 2022). Similarly, research has highlighted the coexistence of herpesviruses with periodontopathogenic bacteria in dental biofilm, stablishing their ability to influence periodontal destruction (Slots, 2007). While direct evidence linking SARS-CoV-2 presence in the dental biofilm environment to an interaction with periodontopathogenic bacteria remains underexplored, the analogy drawn from herpesviruses suggests a potential parallel. The detected SARS-CoV-2 in dental biofilm may hint at a potential impact on local microbiota, potentially contributing to periodontal inflammatory conditions.

The accumulation of microbial biofilm exerts a major role in instigating plaque-induced gingivitis and periodontitis, seeing that dysbiosis triggers the host's immune-inflammatory response (Murakamami et al., 2007). This intricate immune reaction within the local environment can be significantly impacted by broader systemic factors, including various diseases and potential hyperinflammatory states (Kinane, 1999; Brandini et al., 2021). The linkage between the initial microbial biofilm-induced local response and the subsequent modulation by systemic compromises highlights the complexity of periodontal diseases. Moreover, beyond the impact of SARS-CoV-2 presence on dental biofilm and its connection

to periodontal conditions, the systemic and hyperinflammatory nature of COVID-19 could further contribute to the modulation of periodontal state.

Identifying the microbial composition of subgingival biofilm in COVID-19 patients through high-throughput sequencing could unveil the influence of viral diseases on periodontal conditions. To date, molecular assessments of subgingival biofilm in relation to SARS-CoV-2 presence remain unexplored. Hence, sequencing the 16S rRNA gene, this study aims to delineate and compare the subgingival biofilm profiles in healthy and COVID-19 patients, in the presence or absence of SARS-CoV-2 within the subgingival biofilm, encompassing both periodontal health and the presence of local inflammation (gingivitis condition).

2 MATERIAL AND METHODS

This cross-sectional observational study was approved by the Ethics Board of the Dental School of the Federal University of Bahia (CAAE: 52185521.8.0000.5024) and by the Institutional Review Board of the University of Kentucky (Number: 78126). All patients signed the informed consent.

2.1 Participants

Individuals with and without COVID-19 were from an observational study performed at the Federal University of Bahia, between 2021 and 2022.

The inclusion criteria for COVID-19 subjects were as follows:

1. Age ≥ 18 years;
2. Dentate participants;
3. Systemic condition adequate to periodontal exam;
4. Presence of flu-like symptoms for 3 to 7 days;

5. Positive nasopharyngeal antigen test for SARS-CoV-2 in the same day of performing the present study.

The inclusion criteria for non-COVID-19 subjects were as follows:

1. Age ≥ 18 years;
2. Dentate participants;
3. Systemic condition adequate to periodontal exam;
4. Absence of flu-like symptoms for the last 7 days;

2.2 Clinical evaluation and biofilm sampling

Clinical examination was performed before sample collection by two trained periodontitis. Clinical periodontal examinations included probing depth (PD), clinical attachment loss (CAL) and bleeding on probing (BoP). The samples were pools of subgingival biofilm collected by sterile curettes from shallow subgingival sites of all present teeth, excluding third molars.

2.3 SARS-CoV-2 detection

The samples were aliquoted 1 mL aliquots and stored in -80ºC, all samples were evaluated for the presence of SARS-CoV-2 RNA through qRT-PCR (QuantStudio 3 Real-Time PCR System, Applied Biosystems) detection, applying viral nucleic acid extraction (High Pure Viral Nucleic Acid Kit, Roche, Mannheim, Germany) and targeting nucleocapsid genes N1 and N2 and RNAse P gene as an internal control (CDC, 2020). CT < 40 for viral and control genes were considered positive for SARS-CoV-2 RNA presence.

2.4 Group allocation

Six groups, with 10 samples each, were analyzed according to the following criteria: positive or negative for COVID-19, positive or negative for SARS-CoV-2 RNA in subgingival biofilm, and presence or absence of gingivitis (BoP, PD < 5,0mm, and absence of CAL) (illustrated in Table 1): 1) GROUPc+s+g+ positive for COVID-19, positive for SARS-CoV-2 in the biofilm and presence of gingivitis; 2) GROUPc+s-g+ positive for COVID-19, negative for SARS-CoV-2 in the biofilm and presence of gingivitis; 3) GROUPc+s+g- positive for COVID-19, positive for SARS-CoV-2 in the biofilm and health; 4) GROUPc-s+g+ negative for COVID-19, positive for SARS-CoV-2 in the biofilm and presence of gingivitis; 5) GROUPc-s-g+ negative for COVID-19, negative for SARS-CoV-2 in the biofilm and presence of gingivitis; 6) GROUPc-s-g- negative for COVID-19, negative for SARS-CoV-2 in the biofilm and health.

2.5 DNA isolation

According to the manufacturer's instructions, 1ml of each selected sample was used to perform DNA isolation using MasterPure Complete DNA and RNA Purification Kit (Biosearch Technologies, Wisconsin, USA). DNA concentration was measured through spectrophotometry (Thermo Scientific™ NanoDrop™ One Microvolume UV-Vis Spectrophotometer) and concentration of DNA between 13.67 and 482.71 ng/μL was obtained per sample.

2.6 Sequencing and bioinformatic analysis

The V3–V4 regions of the 16S rRNA gene were sequenced using the Illumina Miseq platform, with 10 ng of DNA used per sequencing run. The raw sequences were deposited in the Sequence Read Archive (SRA) database under registration number PRJNA1017523 (ncbi.nlm.nih.gov/sra). The sample processing protocol library preparation and sequencing protocol have been described in a previous study (Gantner et al., 2011).

Microbiome analysis was conducted by Mothur and R pipeline software using the following packages from the last one: vegan, phyloseq, tidyverse, patchwork, agricolae, FS, companion, lefse, and microbiome Marker. Only OTU with a number higher than 10 in at least 10% of the sample was maintained in the assessment. For data normalization, abundance was adopted. At first, analyses were made based on the division in 6 groups (previously described). The groups were united to form 4 groups based on the presence of COVID-19 and gingivitis: GROUPc+g+, GROUPc+g-, GROUPc-g+, and GROUPc-g-. In addition, 4 different groups based on the presence of SARS-COV-2 in subgingival biofilm and gingivitis were considered: GROUPs+g+, GROUPs+g-, GROUPs-g+, and GROUPs-g-.

The Chao 1 and the Shannon index were used α-diversity estimators, and differences between α-diversities group-wise were measured using the Wilcoxon test (per pair). Intragroup dispersion homogeneity was tested using the PERMDISP test. After this assessment, the unweighted UniFrac distance (Bray-Curtis) was applied to evaluate the β-diversity and the differences between groups were analyzed using principal coordinate analysis (PCoA) and tested using PERMANOVA test (PERMIDISP $p>0.05$). ANOSIM test was carried out in case of intragroup dispersion heterogeneity (PERMIDISP $p<0.05$).

Intergroup differences in microbiomes' composition between COVID(+)/gingivitis (+) vs COVID(-)/gingivitis(+) and COVID(+)/gingivitis(-) vs COVID(-)/gingivitis(-) were executed by the Wilcoxon test (per pair). In addition, a Linear Discriminant Analysis - Effect

Size (LEfSe) was performed to determine the most important bacteria in each periodontal condition (gingivitis and health) associated with COVID diagnosis (+/-). Only LDA score (log10) foldchange>2 and p-value≤0.05 was considered relevant in this analysis.

2.7 Demographic and clinical analysis

Demographic and clinical data were compared considering the COVID-19 presence, SARS-CoV-2 detection, and presence or absence of gingivitis. The chi-square test was applied to evaluate these variables. The level of significance was set at $p \leq 0.05$. Data were analyzed using the SPSS Statistics 13.0 program (SPSS Inc., Chicago, IL, USA).

3 RESULTS

A total of 60 patients were included in this study and a total of 11 phyla, 93 genera and 258 species were detected. Clinical and demographic data of all participants are described in *Table 2*. There was no statistically significant difference between groups concerning sex and age. The samples of all patients were distributed according to the proposed groups (described above).

First, the overall characteristics of the included patients' subgingival biofilm microbiome (Figure 1 and Figure 2) were investigated. *Figure 1a* shows the β-diversity. When analyzing the 6 groups, a statistically significant difference between the following groups was found: GROUPc+s+g+ and GROUPc+s-g- (p = .015); GROUPc+s+g+ and GROUPc-s-g+ (p = .045); GROUPc+s+g+ and GROUPc-s-g- (p = .03); GROUPc+s+g- and GROUPc+s-g- (p = .015); GROUPc+s+g- and GROUPc-s-g+ (p = .03); GROUPc+s+g- and GROUPc-s-g+ (p = .015); and GROUPc+s-g- and GROUPc+s+g- (p = .01). In the 4 groups analysis, based on COVID-19 condition and gingivitis, a statistically significant difference for β-diversity in the PCoA was found between GROUPc+g+ and GROUPc-g+ (p = .012); between GROUPc+g+

and GROUPc-g- (p = .04); and GROUPc-g- and GROUPc+g- (p = .01). The similar analysis conducted with 4 groups, based on SARS-CoV-2 detection in subgingival biofilm and gingivitis, showed a statistically significant difference for β-diversity in the PCoA between GROUPs+g- and GROUPs-g- (p = .006); and a trend towards significance between GROUPs+g+ and GROUPs-g+ (p = .06).

In addition, the α-diversity related to Chao1 demonstrated differences when analyzing 6 groups with upper-regulation in COVID-19 groups, including GROUPc-s-g+ and GROUPc+s-g+ (p = .002); GROUPc-s-g- and GROUPc+s-g+ (p = .037); GROUPc-s-g+ and GROUPc+s+g+ (p = .028); and GROUPc+s-g- and GROUPc+s+g+ (p = .02). Whilst α-diversity related to Shannon showed down-regulation in groups related to the presence of COVID-19 (not statistically significant). The 4 groups' analysis, in reference to COVID-19 condition and gingivitis, showed differences in α-diversity in Chao1 between GROUPc-g+ and GROUPc+g+ (p = .002), with an upper-regulation for the COVID-19 positive group. In this second analysis, the α-diversity related to Shannon also showed down-regulation in groups related to the presence of COVID-19 (statiscally non-significant). Considering the SARS-CoV-2 detection in the biofilm and gingivitis, no statistically significant difference for α-diversity between any of the 4 groups was found. The results are shown in *Figure 2*.

3.1 Analyses at the species level

Table 2 describes the findings related to the differences between groups when considering 6 groups. Statistical significance was found between the groups without gingivitis GROUPc+s-g- and GROUPc+s+g- in 6 different species where *Aggregatibacter sp*, *Saccharibacteria (TM7) [G-1] bacterium HMT 346*, *Prevotella intermedia* and *Corynebacterium matruchotii* were more prevalent in the group positive only for COVID-19

(c+s-g-); while the *species Neisseria Oralis* and *Haemophilus parainfluenzae* were more prevalent in the group positive for COVID-19 and SARS-CoV-2 in subgingival biofilm (c+s+g). Differences between species in the groups with gingivitis GROUPc+s+g+ and GROUPc-s-g+ were significant in 12 described species, where *Granulicatella adiacens*, *Porphyromonas catoniae*, *Gemella sp*, *Parvimonas micra*, *Gemella morbillorum*, *Atopobium parvulum*, *Porphyromonas pasteri*, *Streptococcus salivarius* and *Schaalia sp* were more present in the positive group for COVID-19 and SARS-CoV-2 in subgingival biofilm (c+s+g+).

Table 3 describes the findings related to the differences between 4 groups based on COVID-19 condition and gingivitis. The same species that showed significance when comparing 6 groups in the absence of gingivitis, presented alteration when analyzing the COVID-19 positive and negative groups without gingivitis, independently of SARS-CoV-2 detection in subgingival biofilm. Regarding to groups with gingivitis, a total of 37 species showed difference between groups, where 9 species where more present in samples from patients without COVID-19, while 28 species were more present in the COVID-19 positive group (including *Streptococcus sp*, *Saccharibacteria_(TM7)_[G-1] bacterium_HMT_346*, *Gemella morbillorum,*, *Porphyromonas pasteri*, *Granulicatella adiacens*, *Parvimonas micra*, *Porphyromonas catoniae*, *Porphyromonas sp*, *Aggregatibacter sp._HMT_898*, *Streptococcus intermedius*, *Abiotrophia defectiva*, *Parvimonas sp*, *Gemella sp*, *Ruminococcaceae_[G-1] bacterium_HMT_075*, *Lactobacillales sp*, *Schaalia sp._HMT_180*, *Solobacterium moorei*, *Saccharibacteria_(TM7)_[G-1] bacterium_HMT_348*, *Arachnia propionica*, *Bacilli sp*, *Saccharibacteria_(TM7)_[G-1] bacterium_HMT_347*, *Porphyromonas sp._HMT_278*, *Streptococcus salivarius*, *Schaalia sp*, *Granulicatella elegans*, *Atopobium parvulum*, *Schaalia sp._HMT_178*, *Absconditabacteria_(SR1)_[G-1] bacterium_HMT_345*).

3.2 Linear discriminant analysis Effect Size (LEfSe)

The LEfSe analysis (*Figure 4*) was used to compare the biofilm microbiota taxa that were significantly different between the groups based on the COVID-19 condition in association with gingivitis or periodontal health. LEfSe analysis showed that both evaluations had significant differences in the microbiome composition when the four groups were considered.

Health (without gingivitis) groups showed different microbial profiles regarding COVID-19 condition. When COVID-19 was not present, the health microbiota was predominantly composed of gram-negative bacteria, and included mostly the genera Aggregatibacter, Capnocytophaga, and Veillonella. Conversely, in the presence of COVID-19, the health microbiota was mainly gram-positive and had a considerable amount of Saccharibacteria, Parvimonas, and Schaalia.

Differences were also found when describing the microbial profile of patients with gingivitis. In the absence of COVID-19, a high number of gram-negative bacteria were described with a great presence of Selenemonas and Treponema genera. The presence of COVID-19 showed an important concentration of gram-positive bacteria, and the genera Streptococcus, Sacharibacteria, and Porphyromonas were the more frequent.

4 DISCUSSION

This study aimed to characterize the microbial profile of subgingival biofilm samples in COVID-19 patients, emphasizing alterations in microbiota associated with the presence of the disease, local detection of the causative pathogen SARS-CoV-2, and the occurrence of gingivitis. Findings revealed marked differences among analyzed samples, mostly attributed to

the concurrent presence of COVID-19 and gingivitis. This suggests a potential amplification of microbial profile disturbances due to co-infection.

The present findings highlighted alterations in microbial diversity associated with the presence of COVID-19 and local SARS-CoV-2, demonstrating decreased species diversity and increased species richness. Recent meta-analysis examining the relationship between oral and oropharyngeal microbiome with SARS-CoV-2 infection have shown reduced diversity in the oral and oropharyngeal microbiome (Ganesan et al., 2023). These results further emphasize that dysbiosis resulting from both local and systemic infections can disrupt the delicate balance of microbial communities (Lederberg and McCray, 2001).

4.1 Microbial profile of gingival health in the absence or presence of COVID-19

When assessing the microbiota in gingival health, in the absence of COVID-19, the enriched species observed mostly consisted of aerobes and facultative aerobes. These species are recognized for their contributions to early biofilm colonization, aligning with previous findings (Abusleme et al., 2021). Upon individual analysis, these identified species are often linked to a healthy status and have not been notably associated with respiratory diseases.

The assessment of local health in COVID-19 revealed distinct characteristics within the subgingival biofilm. Notably, an abundance of Saccharibacteria (TM7) species and periodontal pathogens from the orange complex, such as *Prevotella intermedia* and *Parvimonas micra*, was observed. While the orange complex bacteria are commonly found in the interdental spaces of individuals with periodontal health (Carrouel et al., 2016), dysregulation of Prevotella has been associated with SARS-CoV-2 infection (Gupta et al., 2022), and Parvimonas micra has been linked to anaerobic pneumonia in children (Zhijun et al., 2023). Saccharibacteria species, described as obligate episymbionts thriving on the surface of host bacteria (Chipashvili et al.,

2021), have been detected in elevated quantities within the nasal and oropharyngeal microbial community of COVID-19 patients (Rueca et al., 2021). The presence of SARS-CoV-2 in subgingival biofilm introduced species like Neisseria Oralis, associated with ventilator-associated pneumonia (Carvalho et al., 2018), and Haemophilus parainfluenzae, which has been described as more present in healthy conditions when comparing COVID-19 patients with a control group (Nath et al., 2018).

4.2 Microbial profile of gingivitis in the absence of COVID-19

In the context of gingivitis, the significant presence of gram-negative bacteria, including Selenemonas, Treponema, Fretibacterium, Bacteroidete, Prevotella, and Campylobacter genera, aligns with previous findings in individuals with gingivitis (Abusleme et al., 2021; Schincaglia et al., 2017; Bertelsen et al., 2022; Huang et al., 2014). Interestingly, similar microbial signatures have been reported in distinct COVID-19-related responses. For instance, low responders to COVID-19 vaccination exhibited a higher concentration of Campylobacter genera in their saliva (Ghorbani et al., 2022). Additionally, the presence of *Dialister pneumosintes*, a species prominent in individuals recovered from COVID-19 compared to those infected, adds to the similarity between these microbial profiles (Meng et al., 2022). The observed resemblance between microbial profiles in individuals with gingivitis and various responses related to COVID-19, such as post-vaccination or recovery, suggests a potential link warranting deeper exploration into their underlying associations and implications.

4.3 Microbial profile of gingivitis in the presence of COVID-19

Subgingival biofilm's microbiota in gingivitis and COVID-19 was abundant and diverse. Upon examining specific species, a notable prevalence of Saccharibacteria and Streptococcus was observed. Although both groups have been previously associated with gingivitis (Abusleme et al., 2021), their high concentrations were observed in the COVID-19 group, unlike in the non-COVID-19 gingivitis group, where their prevalence was less pronounced. Additionally, Saccharibacteria were highly represented in gingival health and COVID-19 association.

The Streptococcus genus is commonly found in human mucous membranes, serving as part of the normal flora or potentially acting as an opportunistic pathogen (Krzyściak et al., 2013). This genus has been notably identified in various COVID-19 microbial studies, exhibiting enrichment in the oral and nasal microbiomes of COVID-19 patients (Gupta et al., 2022; Mat et al., 2021; Liu et al., 2021). Additionally, Streptococcus species have been associated with ventilator-associated pneumonia (Lu et al., 2014). On the other hand, Saccharibacteria (TM7) has been linked to inflammatory mucosal diseases, showing increased abundance in disease states, including various stages of periodontal disease (Brining et al., 2003; Bor et al., 2019). Furthermore, two studies have described the prevalence of the Candidatus Saccharibacteria phylum in both asymptomatic and symptomatic cases infected with SARS-CoV-2 (Liu et al., 2021; Rueca et al., 2021).

4.4 Local and systemic impacts of COVID-19 on subgingival biofilm microbiota

This study reveals notable differences in the microbiota profile of subgingival biofilm among individuals with and without COVID-19 These findings highlight the potential impact of co-infections on the local microbiota and reiterates that the oral cavity might serve as a

reservoir for respiratory pathogens that can breach the defense barriers of the lower respiratory tract, leading to respiratory diseases (Pathak et al., 2021). Reciprocally, respiratory diseases have been linked to dysbiosis in the oral microbiome (El-Solh et al., 2004), further emphasizing the intricate relationship between respiratory health and oral microbial balance.

The observed changes in bacterial diversity and increased pathogen presence in COVID-19 patients align with multiple studies (Iebba et al., 2021; Gupta et al., 2022; Rueca et al., 2021). Given that oral dysbiosis may facilitate SARS-CoV-2 infection by affecting respiratory epithelium, enhancing adhesion of respiratory pathogens, and heightening local inflammation (Baghbani et al., 2020), the significance of oral health becomes evident. These specific findings underscore the link between poor oral health, pathogenic bacterial accumulation, and respiratory diseases (Gomes-Filho et al., 2020), highlighting the potential for oral plaque control to mitigate inflammation and minimize microbial imbalances.

The primary limitation in this study pertains to the number of included participants. Furthermore, integrating immunological parameters could significantly enhance the comprehension of the interactions between COVID-19 and the microbiome. Despite the study's limitations, its contribution remains pivotal in expanding the understanding of the microbial profile within subgingival biofilms concerning health, gingivitis, and their association with COVID-19 and SARS-CoV-2.

5 CONCLUSION

The present study revealed that subgingival biofilm of COVID-19 patients exhibits unique microbial signatures with increased abundance of specific bacteria, notably from the Saccharibacteria (TM7) and Streptococcus genera. When associated to gingivitis the microbial profile presented the most pronounced changes. To attenuate these alterations observed in the

subgingival biofilm of COVID-19 patients and the accentuated changes noted in association with gingivitis, a multifaceted approach to oral hygiene management is recommended.

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Table 1 – Group allocation (N = 60).

GROUP TITLE	GROUPc+s+g+	GROUPc+s+g-	GROUPc+s-g+	GROUPc+s-g-	GROUPc-s-g+	GROUPc-s-g-
DISTRIBUTION	10 COVID + SARS-CoV-2 PCR + GINGIVITIS +	10 COVID + SARS-CoV-2 PCR + GINGIVITIS -	10 COVID + SARS-CoV-2 PCR - GINGIVITIS +	10 COVID + SARS-CoV-2 PCR - GINGIVITIS -	10 COVID - SARS-CoV-2 PCR - GINGIVITIS +	10 COVID - SARS-CoV-2 PCR - GINGIVITIS -

+ positive/presence

- negative/absence

Table 2 - Description of the study sample (N = 60).

Variables	All patients (%)	COVID-19 (%)		SARS-CoV-2 in SB (%)	
		No	Yes	No	Yes
Age (Years)					
0–36	29 (48.3)	9 (45)	20 (50)	21 (52.5)	8 (40)
≥37	31 (51.7)	11 (55)	20 (50)	19 (47.5)	12 (60)
Sex					
Female	42 (70)	16 (80)	26 (65)	29 (27.5)	13 (65)
Male	18 (30)	4 (20)	14 (35)	11 (72.5)	7 (35)
Gingivitis					
No	30 (50)	10 (50)	20 (50)	20 (50)	10 (50)
Yes	30 (50)	10 (50)	20 (50)	20 (50)	10 (50)

SB = subgingival biofilm

Table 3 – Comparison between the relative abundances of species when analyzing 6 groups.

Species	**GROUP**	**GROUP**	**P value**
	COVID(-)/SARS-COV-2(-)/GINGIVITIS(-)	COVID(+)/SARS-COV-2(+)/GINGIVITIS(-)	
Aggregatibacter sp	0.24	0.07	0.04
Saccharibacteria (TM7) [G-1] bacterium HMT 346	4.09	2.59	0.04
Prevotella intermedia	1.07	0.73	0.04
Corynebacterium matruchotii	0.57	0.19	0.04
Neisseria Oralis	0.10	1.57	0.04
Haemophilus parainfluenzae	0.17	0.44	0.04
	COVID(-)/SARS-COV-2(-)/GINGIVITIS(+)	COVID(+)/SARS-COV-2(+)/GINGIVITIS(+)	
Granulicatella adiacens	0.11	1.37	0.01
Campylobacter sp	0.04	0.00	0.02
Porphyromonas catoniae	0.03	0.70	0.03
Gemella sp	0.02	0.21	0.04
Parvimonas micra	0.54	3.71	0.05
Gemella morbillorum	0.29	1.68	0.05
Streptococcus oralis	6.84	1.78	0.05
Atopobium parvulum	0.01	0.05	0.04
Porphyromonas pasteri	0.07	1.01	0.03
Campylobacter rectus	0.44	0.03	0.03
Streptococcus salivarius	0.00	0.03	0.05
Schaalia sp	0.00	0.03	0.04

Table 4 – Comparison between the relative abundances of species when analyzing 4 groups based on the COVID-19 condition and gingivitis.

Species	**GROUP**	**GROUP**	**P value**
	COVID(+)/GINGIVITIS(-)	COVID(-)/GINGIVITIS(-)	
Saccharibacteria_(TM7)_ [G-1] bacterium_HMT_346	0.65	3.31	0.04
Prevotella intermedia	0.02	0.89	0.04
Haemophilus parainfluenzae	3.05	0.31	0.04
Aggregatibacter sp	1.40	0.15	0.04
Corynebacterium matruchotii	0.69	0.37	0.04
Neisseria Oralis	1.56	0.23	0.04
	COVID(+)/GINGIVITIS(+)	COVID(-)/GINGIVITIS(+)	
Streptococcus sp	3.28	8.56	0.01
Streptococcus oralis_subsp._dentisani_clade_058	6.75	1.75	0.02
Selenomonas sp	6.18	0.66	0.03
Saccharibacteria_(TM7)_[G-1] bacterium_HMT_346	0.77	3.16	0.04
Gemella morbillorum	0.29	2.46	0.05
Treponema socranskii	1.80	0.22	0.05
Porphyromonas pasteri	0.07	1.31	0.05
Granulicatella adiacens	0.11	1.61	0.04
Parvimonas micra	0.53	4.09	0.03
Porphyromonas catoniae	0.03	0.56	0.03

Porphyromonas sp	0.16	1.06	0.05
Campylobacter gracilis	0.97	0.13	0.04
Aggregatibacter sp._HMT_898	0.00	0.06	0.01
Streptococcus intermedius	0.00	0.57	0.01
Abiotrophia defectiva	0.01	0.63	0.02
Parvimonas sp	0.00	0.18	0.01
Campylobacter rectus	0.43	0.04	0.01
Gemella sp	0.02	0.20	< 0.001
Ruminococcaceae_[G-1] bacterium_HMT_075	0.13	0.38	0.03
Lactobacillales sp	0.00	0.021	0.01
Schaalia sp._HMT_180	0.02	0.15	0.01
Solobacterium moorei	0.05	0.32	0.01
Saccharibacteria_(TM7)_[G-1] bacterium_HMT_348	0.03	0.14	0.04
Arachnia propionica	0.00	0.02	0.01
Aggregatibacter sp._HMT_512	0.25	0.03	0.03
Bacilli sp	0	0.01	0.02
Campylobacter sp	0.04	0.00	< 0.001
Saccharibacteria_(TM7)_[G-1] bacterium_HMT_347	0.00	0.06	0.01
Porphyromonas sp._HMT_278	0	0.01	0.02
Streptococcus salivarius	0.00	0.02	0.01
Schaalia sp	0.00	0.03	0.01
Granulicatella elegans	0.00	0.11	0.03
Fretibacterium sp._HMT_361	0.59	0.00	0.04
Atopobium parvulum	0.00	0.04	0.01
Schaalia sp._HMT_178	0.00	0.03	0.02

Absconditabacteria_(SR1)_[G-1] bacterium_HMT_345	0.00	0.01	0.03
Dialister pneumosintes	0.26	0.01	0.04

Figure 1 - (a) α-diversity: Chao1 index for all 6 groups (b) Chao1 index for 4 groups.

Figure 1a

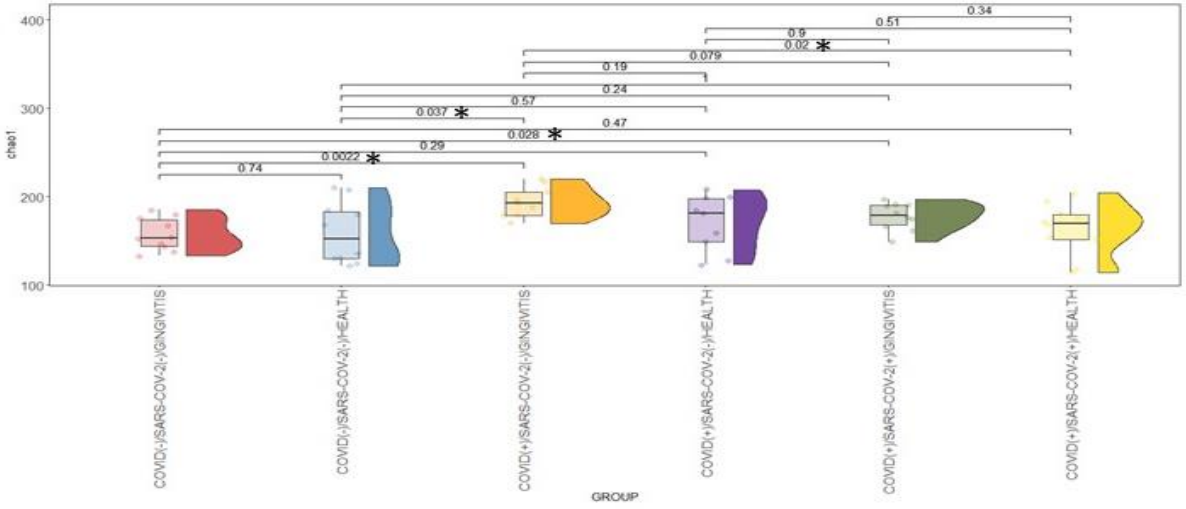


Figure 1b

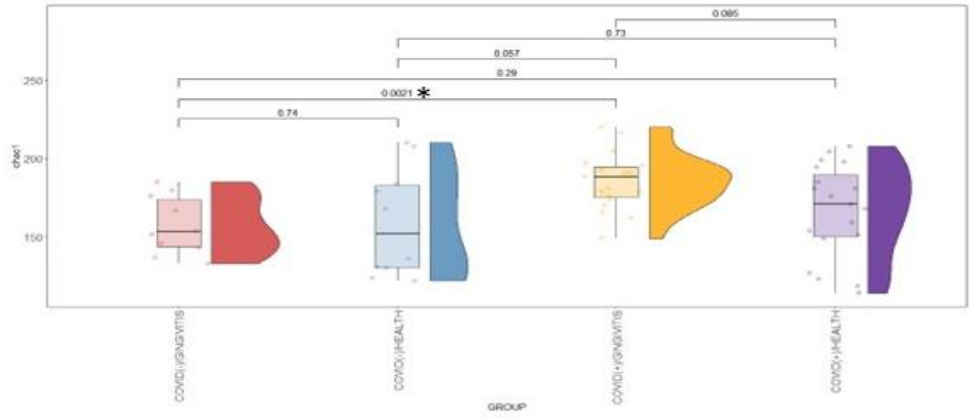


Figure 2 - (a) β-diversity for all 6 groups (b) β-diversity for 4 groups.

Figure 1a

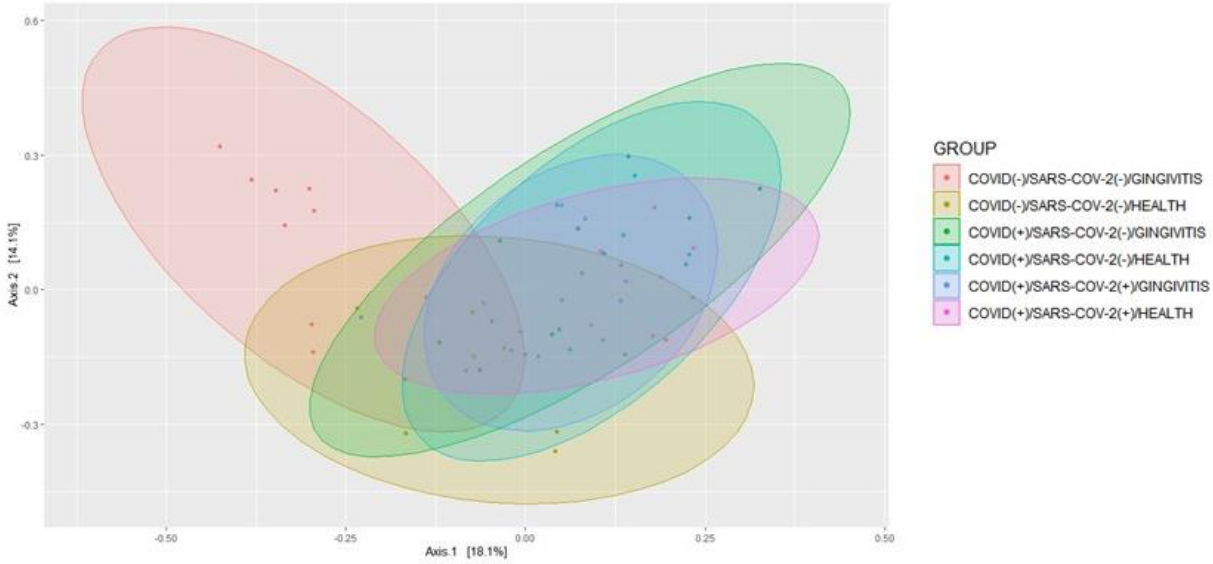


Figure 1b

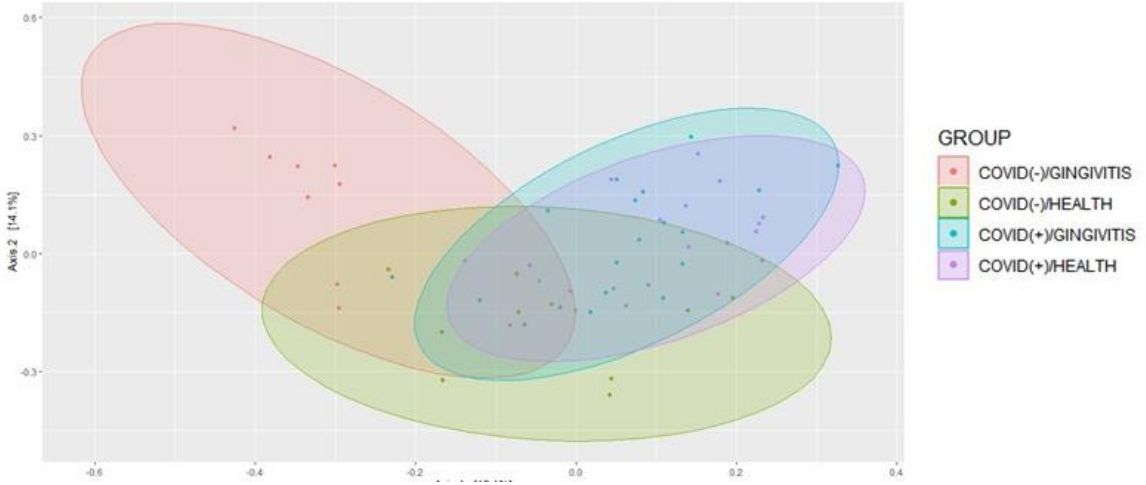
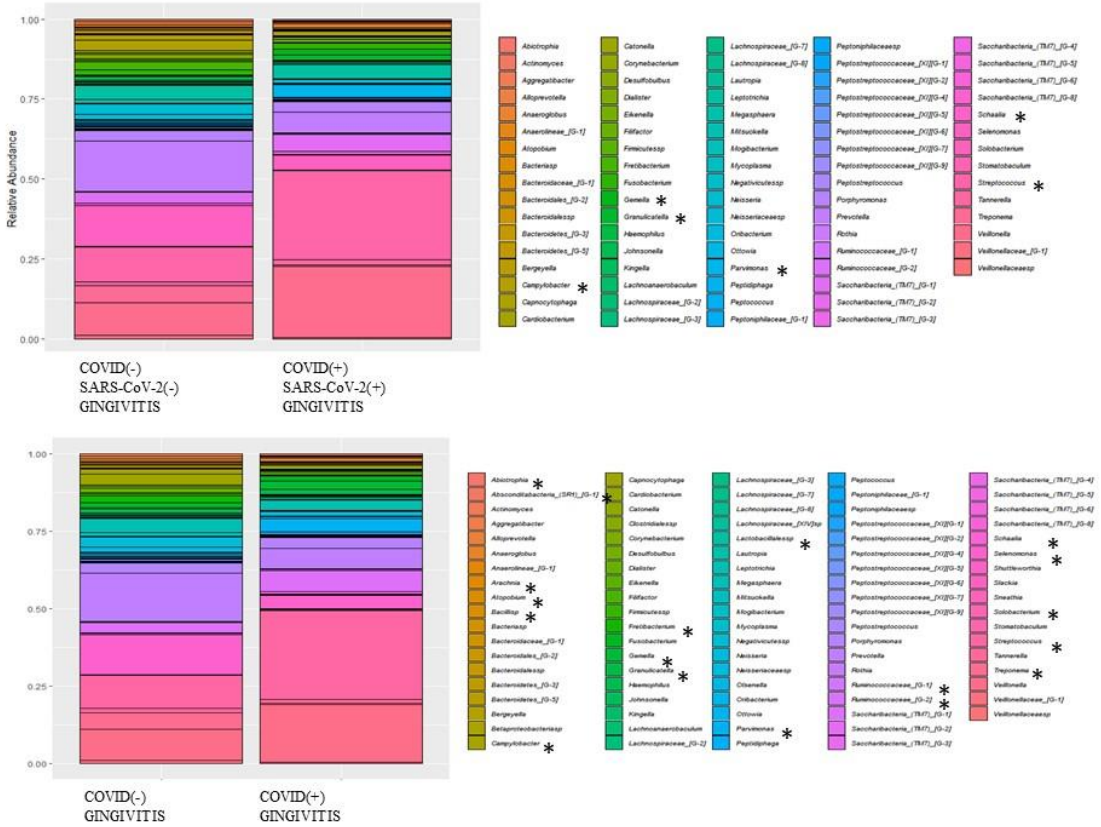


Figure 3 - Bacterial community composition considering the genus (a) when analyzing gingivitis in association with COVID-19 and SARS-CoV-2 in all 6 groups (b) when analyzing gingivitis in association with COVID-19 in all 4 groups.



*p<0,05

Figure 4 - Histogram of LDA logarithmic scores of species found by LEfSe comparing COVID-19 positive and negative groups in periodontal health.

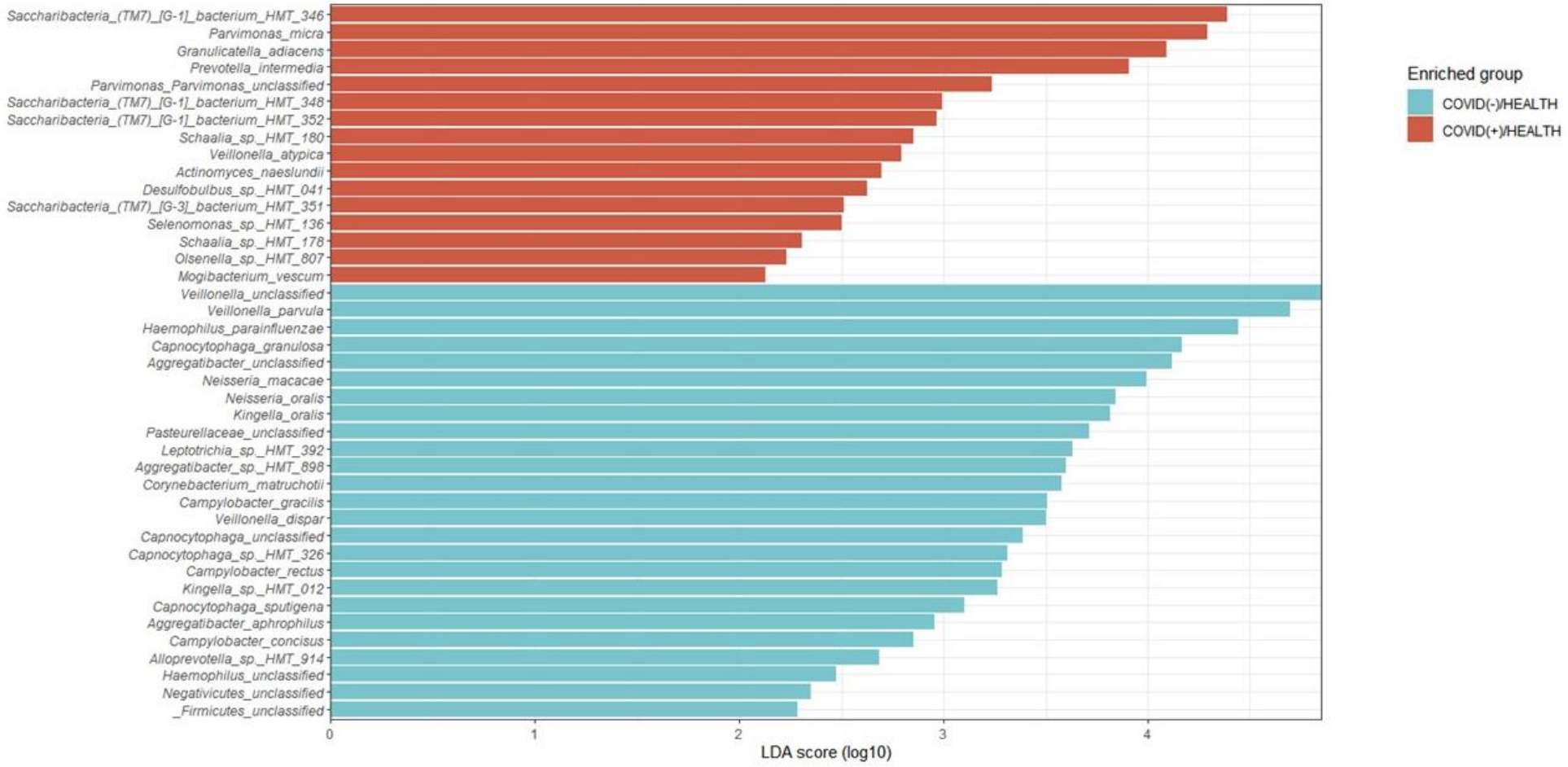
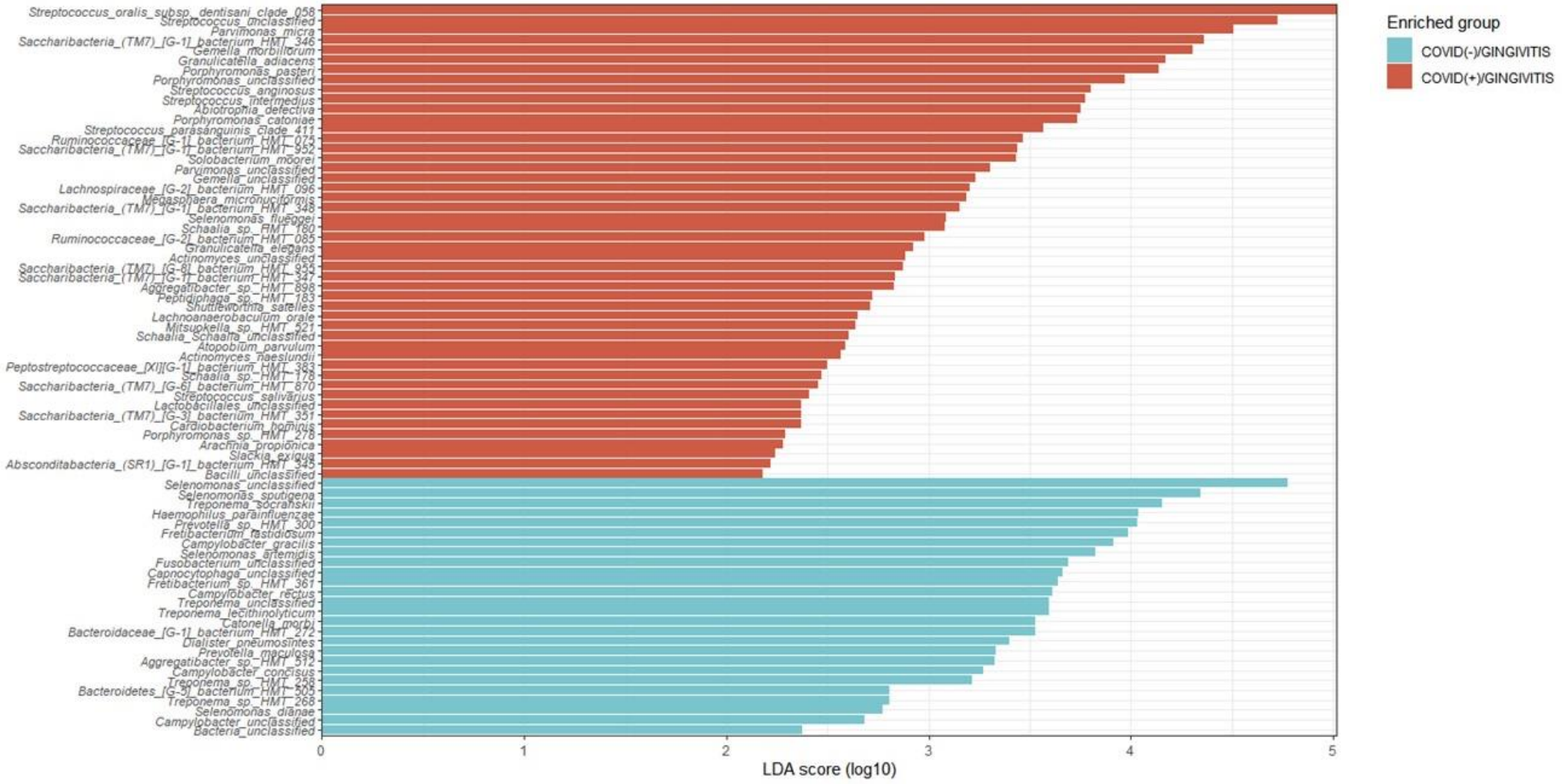


Figure 5 - Histogram of LDA logarithmic scores of species found by LEfSe comparing COVID-19 positive and negative groups in gingivitis.



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UNIVERSIDADE FEDERAL DA BAHIA
FACULDADE DE ODONTOLOGIA
COMITÊ DE ÉTICA EM PESQUISA

APROVAÇÃO

Os membros do Comitê de Ética em Pesquisa da Faculdade de Odontologia da UFBA, em sessão ordinária no dia 17 de março de 2022, através do Parecer Consubstanciado nº 5.297.177, resolveram aprovar o projeto de pesquisa "***Avaliação da condição de saúde bucal e carga viral do biofilme de pacientes positivos para o Sars-CoV-2",*** da pesquisadora **TAYANE DA ROCHA COSTA COELHO**, Área 4, registro no CONEP: 52185521.8.0000.5024.

Salvador, 04 de abril de 2022.

Profa. Dra. Fabiola Bastos de Carvalho
Coordenadora do CEP FOUFBA

ANEXO B

University of Kentucky | Office of Research Integrity
IRB, RDRC

EXEMPTION CERTIFICATION

IRB Number: 78126

TO: Luciana Shaddox
Dentistry Oral Health Practice
PI phone #: +1(352) 262 4215

PI email: lshaddox@uky.edu

FROM: Chairperson/Vice Chairperson
Medical Institutional Review Board (IRB)

SUBJECT: Approval for Exemption Certification

DATE: 7/29/2022

On 7/29/2022, it was determined that your project entitled "*Clinical and microbiological evaluation of the oral cavity of patients with Covid-19*" meets federal criteria to qualify as an exempt study.

Because the study has been certified as exempt, you will not be required to complete continuation or final review reports. However, it is your responsibility to notify the IRB prior to making any changes to the study. Please note that changes made to an exempt protocol may disqualify it from exempt status and may require an expedited or full review.

The Office of Research Integrity will hold your exemption application for six years. Before the end of the sixth year, you will be notified that your file will be closed and the application destroyed. If your project is still ongoing, you will need to contact the Office of Research Integrity upon receipt of that letter and follow the instructions for completing a new exemption application. It is, therefore, important that you keep your address current with the Office of Research Integrity.

For information describing investigator responsibilities after obtaining IRB approval, download and read the document "PI Guidance to Responsibilities, Qualifications, Records and Documentation of Human Subjects Research" available in the online Office of Research Integrity's IRB Survival Handbook. Additional information regarding IRB review, federal regulations, and institutional policies may be found through ORI's web site. If you have questions, need additional information, or would like a paper copy of the above mentioned document, contact the Office of Research Integrity at 859-257-9428.

UNIVERSIDADE FEDERAL DA BAHIA
FACULDADE DE ODONTOLOGIA
PROGRAMA DE PÓS-GRADUAÇÃO EM ODONTOLOGIA E SAÚDE

TERMO DE APROVAÇÃO

C.D. TAYANE DA ROCHA COSTA COELHO

"AVALIAÇÃO DO PERFIL MICROBIOLÓGICO DO BIOFILME DENTAL NA PRESENÇA DO VÍRUS SARS-COV-2"

BANCA EXAMINADORA:

Profa. Dra. Patricia Ramos Cury **(Orientador)**
Professora da Universidade Federal da Bahia – Faculdade de Odontologia

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Prof. Dr. Carlos Brites **(Examinador Interno)**
Professor da Universidade Federal da Bahia

Profa. Dra. Luciana Salles Branco de Almeida **(Examinador Externo**)
Professora da Universidade Federal do Maranhão

Prof. Dr. Renato Corrêa Viana Casarin **(Examinador Externo)**
Professor da Universidade Estadual de Campinas

Profa. Dra. Luciana Machion Shaddox (**Examinador Externo)**
Professora da University of Kentucky