UNIVERSIDADE FEDERAL DO CEARÁ
FACULDADE DE FARMÁCIA, ODONTOLOGIA E ENFERMAGEM
FACULDADE DE ODONTOLOGIA
PROGRAMA DE PÓS-GRADUAÇÃO EM ODONTOLOGIA

SARAH FLORINDO DE FIGUEIREDO GUEDES

EXPRESSÃO DE GENES DE VIRULÊNCIA DE *Streptococcus mutans* EM BIOFILME *IN VIVO* DE LESÕES CARIOSAS ATIVAS E INATIVAS DE ESMALTE E DENTINA

FORTALEZA
2019

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Orientadora: Profª. Dra. Lidiany Karla Azevedo Rodrigues Gerage

FORTALEZA

2019

Dados Internacionais de Catalogação na Publicação
Universidade Federal do Ceará
Biblioteca Universitária
Gerada automaticamente pelo módulo Catalog, mediante os dados fornecidos pelo(a) autor(a)

G959e Guedes, Sarah Florindo de Figueiredo.
Expressão de genes de virulência de Streptococcus mutans em biofilme in vivo de lesões cariosas ativas e inativas de esmalte e dentina / Sarah Florindo de Figueiredo Guedes. – 2019.
98 f. : il. color.

Tese (doutorado) – Universidade Federal do Ceará, Faculdade de Farmácia, Odontologia e Enfermagem, Programa de Pós-Graduação em Odontologia, Fortaleza, 2019.
Orientação: Profa. Dra. Lidiany Karla Azevedo Rodrigues Gerage.

1. Biofilme dentário. 2. Streptococcus mutans. 3. Expressão gênica. I. Título.

CDD 617.6

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Aprovada em: ___/___/_____.

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A **Deus**, por sua eterna presença em minha vida.

Aos meus pais, **Luiz e Izabel**, pela imensa dedicação à minha formação, por todo amor, pelo apoio incondicional durante toda a minha trajetória e por serem os meus maiores exemplos de vida. Obrigada por tudo! Amo vocês!

Aos meus irmãos, **Luizinho e Isabela**, por todo amor, amizade e respeito que tanto nos une. A vida é bem mais fácil porque sei que tenho vocês. Amo vocês!

Ao meu marido, **Anderson**, por todo amor, apoio nos momentos difíceis, compreensão da minha ausência e, principalmente, por vibrar com cada conquista minha. Sou bem mais forte com você, meu amor. Obrigada por tornar a minha vida mais leve. Amo você!

Ao meu tio e padrinho, **Gerardo**, *in memoriam*, por ter acreditado, quando eu ainda era criança, que a minha curiosidade me faria estudar muito. Tenho certeza que o senhor me acompanhou durante toda essa trajetória. Amo você!

AGRADECIMENTOS ESPECIAIS

À minha orientadora, **Profª. Dra. Lidiany Karla Azevedo Rodrigues Gerage**, pelo exemplo profissional que sempre foi, desde a minha graduação. Tenho muito orgulho de ser a sua primeira aluna de Iniciação Científica a concluir o Doutorado, e espero fazer jus a todos esses anos investidos na minha formação acadêmica. Foram anos de muito aprendizado que, para a minha sorte, não ficaram somente no âmbito profissional, e que guardarei com imenso carinho; carinho esse que, hoje, estende-se a toda a sua família. Obrigada por tudo!

À **Profª. Dra. Iriana Carla Junqueira Zanin dos Santos**, por ter me concedido a oportunidade de realizar um sonho, o estágio de doutorado sanduíche nos Estados Unidos da América (EUA), e por todos os ensinamentos ao longo da minha trajetória acadêmica.

À **Profª. Dra. Simone Duarte**, por ter me recebido com tanto carinho na *New York University* (NYU) e na *Indiana University-Purdue University Indianapolis* (IUPUI). Obrigada por todo o aprendizado dentro do laboratório, mas, principalmente, pelos ensinamentos além do âmbito profissional. Guardo com muito carinho todos esses ensinamentos e todas as ótimas lembranças desse período.

À minha amiga **Beatriz Gonçalves Neves**, por toda a amizade e pelo companheirismo ao longo de toda essa jornada, e que jornada! Tanta coisa a gente viveu, entre pesquisas, congressos, viagens, alegrias e tristezas, nosso vínculo de amizade só cresceu. Agradeço por todo o seu empenho na realização desse trabalho e das várias outras pesquisas. Foi um prazer muito grande trabalhar com você.

À **Profª. Dra. Adriana da Fonte Porto Carreiro**, por toda a amizade, pelo carinho e por ter me recebido tão bem na NYU. Aprendi muito com você, minha amiga, e guardo com todo carinho todos os momentos vividos ao seu lado e de toda a sua família.

AGRADECIMENTOS

À Universidade Federal do Ceará (UFC), por meio do seu Magnífico Reitor **Henry de Holanda Campos** e do vice-reitor **Custódio Luís Silva de Almeida**.

À Faculdade de Farmácia, Odontologia e Enfermagem da Universidade Federal do Ceará, na pessoa de sua diretora, **Profª. Dra. Lidiany Karla Azevedo Rodrigues Gerage** e da vice-diretora **Profª. Dra. Ana Karina Bezerra Pinheiro**.

Ao Coordenador do Programa de Pós-Graduação em Odontologia (PPGO) da Universidade Federal do Ceará, **Prof. Dr. Vicente de Paulo Aragão Saboia** e sua vice-coordenadora **Profª. Dra. Cristiane Sá Roriz Fonteles**.

A todos os docentes e servidores técnico-administrativos do **Programa de Pós-Graduação em Odontologia (PPGO)** da Universidade Federal do Ceará, pelos ensinamentos e serviços concedidos.

À Coordenadora do Curso de Odontologia da Universidade Federal do Ceará **Profª. Dra. Ana Karine Macedo** e sua vice-coordenadora **Profª. Dra. Alynne Vieira de Menezes**.

À **Coordenação Nacional de Desenvolvimento Científico e Tecnológico (CAPES)** pela concessão de bolsa durante o período de doutorado, bem como pela bolsa no período de estágio de doutorado sanduíche.

A todos os docentes, alunos e servidores da ***New York University*** e ***Indiana University-Purdue University Indianapolis,*** por todo o acolhimento durante o estágio de doutorado sanduíche. Em especial, as meninas do meu querido "*Biofilm team*" **Ramille Araújo Lima**, **Beatriz Helena Dias Panariello**, **Cecília Atem Gonçalves de Araújo Costa** e **Paula Ventura da Silveira**, por toda amizade e companheirismo.

Ao **Laboratório de Biotecnologia e Biologia Molecular** da Universidade Estadual do Ceará, na pessoa da **Profª. Dra. Maria Izabel Florindo Guedes** e toda a sua equipe, por terem sido sempre tão receptivos e disponíveis todas as vezes que precisei.

Ao cunhado **Eridan Orlando Pereira Tramontina Florean,** por toda a amizade e incentivo ao longo desses anos.

Ao amigo **Pedro Henrique Acioly Guedes Peixoto Vieira,** por toda a amizade, ao longo desses anos, e por todo o empenho para a realização deste estudo. Eu prometi por muitas vezes que seria a última triagem de pacientes e o último dia de atendimento clínico, e não cumpri nenhuma delas, mas prometo a mesma dedicação para a realização das suas pesquisas.

À amiga **Myrna Maria Arcanjo Frota Barros,** por toda a amizade e parceria que me acompanham desde a graduação.

Às amigas **Daniela da Silva Bezerra** e **Juliana Paiva Marques Lima Rolim,** pela amizade e por terem contribuído imensamente para a minha formação acadêmica.

À amiga **Wanessa Fernandes Matias Regis Pinheiro,** por toda a amizade, pelo carinho e apoio durante esses anos. Sua amizade foi um grande presente desse período.

Ao amigo **Paulo Goberlânio de Barros Silva**, pela amizade desde a graduação e pelo auxílio na interpretação dos resultados deste trabalho.

Ao amigo **Abelardo Barbosa Moreira Lima-Neto**, pela amizade e pela parceria na realização de vários estudos durante essa jornada.

À amiga **Ernanda Maria de Araújo Sales,** por toda a amizade e pelo apoio durante esse período.

Aos meus amigos **Francisco Wilker Mustafa Gomes Muniz**, **Renata Guerra Cavalcante e Marília de Carvalho Rolim**, por toda a amizade e pelo carinho durante esses anos e, hoje, mesmo distantes fisicamente, continuam apoiando todos os momentos da minha vida.

À amiga **Valquíria Conceição Araújo Lima**, pela amizade e por todos os cuidados a mim dispensados.

À ex-funcionária da Clínica de Dentística da UFC **Marta Ferreira da Silva**, por toda a amizade, pelo afeto e por estar sempre disponível a colaborar com os atendimentos clínicos.

Aos **pacientes e seus responsáveis**, pela compreensão, contribuição e participação na execução deste estudo. O atendimento dessas crianças resultou em grande aprendizado.

Às **Escolas Municipais José Batista de Oliveira, Padre Félice Pistoni, São Raimundo e Vicente Fialho**, pela atenção dispensada, viabilizando a triagem de pacientes para este estudo.

Às ex-funcionárias da secretaria da PPGO-UFC **Janaíne Marques Leal** e **Lúcia Ribeiro Marques Lustosa**, e aos atuais secretários **Rafael Maia Reis** e **Joana Karla de Assis Pinheiro**, por terem sido sempre tão solícitos.

Ao técnico do laboratório de Pesquisa do PPGO-UFC **David Queiroz de Freitas**, pela disponibilidade em ajudar sempre que necessário.

A todos os auxiliares de serviços gerais, em especial ao **Rui Lino de Sousa**, pelo zelo com que mantém a Clínica de Dentística durante todos os atendimentos clínicos.

A todos os **funcionários** e **servidores** do Curso de Odontologia da UFC, por sempre estarem dispostos a ajudar.

E a todos que, direta ou indiretamente, contribuíram para a realização deste trabalho, o meu sincero agradecimento.

“Tudo posso nAquele que me fortalece.”

(Filipenses 4:13)

RESUMO

A cárie dentária é uma desordem biofilme-açúcar-dependente. O *Streptococcus mutans* é considerado um microrganismo cariogênico presente na microbiota bucal, pois é acidogênico, tolerante a um pH ácido e capaz de formar um biofilme tridimensional tenaz e bem-estruturado, sendo relacionado às diferentes formas de progressão da doença cárie. Uma melhor compreensão dos vários mecanismos de virulência e do papel ecológico de *S. mutans* no biofilme de lesões cariosas ativas e inativas pode ajudar no desenvolvimento de novas estratégias de prevenção da cárie. Assim, objetivou-se investigar a proporção de *S. mutans* (SM) em relação ao total de estreptococos (ST) e bactérias totais (BT) e o perfil de expressão de genes relacionados às principais características de virulência do SM: gtfB e gtfC (adesão); atpD, aguD, nox e fabM (acidogenicidade e aciduricidade) em biofilme *in vivo* de lesões cariosas ativas e inativas de esmalte e dentina. Para tal, biofilme oral foi coletado de crianças alocadas por conveniência em cinco grupos (n = 8): livres de cárie (LC), cárie de esmalte ativa (CEA) e inativa (CEI), cárie dentinária ativa (CDA) e inativa (CDI). Extração e purificação do RNA total e obtenção do DNA complementar (cDNA) foram realizadas no biofilme. Reações em cadeia da polimerase quantitativa da transcrição reversa (RT-qPCR) foram executadas em todas as amostras. Os dados foram analisados pelo teste Kruskal-Wallis seguido do pós-teste de Dunn (α=5%). Os resultados mostraram que SM foi detectado em todas as amostras. A proporção de SM relacionada à quantidade de ST e BT foi significativamente maior no biofilme de lesões dentinárias (grupos CDA e CDI) ($p < 0,001$) ($p < 0,001$). O gene gtfB foi mais expresso nos grupos cariados (grupos CDA, CDI, CEA e CEI) quando comparados ao grupo livre de cárie (grupo LC) ($p = 0,023$), enquanto os genes gtfC, atpD e nox foram expressos em níveis mais elevados nos grupos de cárie dentinária (CDA e CDI) quando comparados aos grupos de cárie em esmalte (CEA e CEI) e livre de cárie (LC) ($p = 0,001$; $p = 0,002$ e $p = 0,005$). O gene fabM foi mais expresso nos grupos CDA, CDI e CEI do que nos grupos CEA e LC ($p = 0,004$). Não foram encontradas diferenças estatisticamente significativas na expressão do gene aguD entre os diferentes grupos ($p = 0,209$). Conclui-se que, nas condições avaliadas, *S. mutans* faz parte da comunidade microbiana viável do biofilme de lesões ativas e inativas de esmalte e dentina. A expressão elevada dos genes gtfC, atpD, fabM e nox em grupos de cárie dentinária sugere a relação desses genes com a progressão de lesões cariosas, e a maior expressão do gene gtfB no biofilme de todos os grupos cariados sugere a relação desse gene com a presença de biofilme. Nas condições testadas, os genes estudados parecem não ter associação com a atividade de cárie.

Palavras-chave: Biofilme dentário. *Streptococcus mutans*. Expressão gênica.

ABSTRACT

Streptococcus mutans is an oral pathogen considered to play a major role in dental caries development. It has been considered the most cariogenic microorganism of the oral microbiota, because it is acidogenic, tolerance to acid pH and capability of forming a firm and well-structured three-dimensional biofilm. A better understanding of the various virulence mechanisms and the ecological role of *S. mutans* in the biofilm of active and inactive caries lesions may help in the development of new caries prevention strategies. The aim of this study was to investigate the S. mutans proportion in relation to total streptococci (TS) and total bacteria (TB) and the expression profile of genes related to the main virulence characteristics of *S. mutans*: gtfB and gtfC (adhesion); atpD, aguD, nox and fabM (acidogenicity and aciduricity) in *in vivo* biofilm of active and inactive caries lesions of enamel and dentin. For this, oral biofilm was collected from specific-sites from children allocated for convenience in 5 groups (n = 8): caries-free sites (CF), active enamel caries (AEC) and inactive (IEC), active dentin (ADC) and inactive (IDC) caries. Total RNA extraction, purification and cDNA synthesis were performed in all biofilm samples. Quantitative reverse transcriptase polymerase chain reactions (RT-qPCR) were performed for all samples. Data were analyzed by the Kruskal-Wallis test followed by the Dunn post-test ($\alpha = 5\%$). The results showed that *S. mutans* was detected in all samples. The SM proportion related to TS and TB quantity was significantly higher in biofilm on dentine lesions (ADC and IDC groups) ($p < 0.001$). The gtfB gene was more expressed in the caries groups (ADC, IDC, AEC and IEC groups) when compared to the caries-free group (CF group) ($p = 0.023$) while the gtfC, atpD and nox genes were expressed at higher levels in the (ADC and IDC) and caries-free (CF) groups ($p = 0.001$, $p = 0.002$ and $p = 0.005$). The fabM gene was more expressed in the DCA, DCI and ECI groups than in the ECA and CF groups ($p = 0.004$). No statistically significant differences were found in the expression of the aguD gene between the different groups ($p = 0.209$). It is concluded that, under the conditions evaluated, *S. mutans* is part of the viable microbial community of the biofilm of active and inactive lesions of enamel and dentin. The high expression of the gtfC, atpD, fabM and nox genes in dentin caries groups suggests the relationship of these genes with the progression of carious lesions, and the greater expression of the gtfB gene in the biofilm of all the carious groups suggests the relation of this gene with the presence of biofilm. The studied genes do not appear to be associated with caries activity.

Keywords: Oral biofilm. *Streptococcus mutans*. Gene expression.

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AGUD	Agmatina Desiminase D
aguD	Gene codificador da Agmatina Desiminase D
ATCC	*American Type Culture Collection*
ATP	Adenosina trifosfato
ATPD	Subunidade beta da ATP sintase
atpD	Gene codificador da subunidade beta da ATP sintase
β-Me	Betamercaptanol
BT	Bactérias Totais
cDNA	DNA complementar
CA	Califórnia
CAPES	Coordenação Nacional de Desenvolvimento Científico e Tecnológico
CDA	Cárie de Dentina Ativa
CDI	Cárie de Dentina Inativa
CEA	Cárie de Esmalte Ativa
CEI	Cárie de Esmalte Inativa
DE	*Delaware*
DP	Desvio Padrão
CEP	Comitê de Ética em Pesquisa
CO_2	Dióxido de Carbono
DNA	Ácido Desoxirribonucleico
DNAse	Desoxirribonuclease
EUA	Estados Unidos da América
FABM	Trans-2-cis-3-decenoil-ACP isomerase
fabM	Gene codificador da trans-2-cis-3-decenoil-ACP isomerase
GTF	Glicosiltransferase
GtfB	Glicosiltransferase B
gtfB	Gene codificador da glicosiltransferase B
GtfC	Glicosiltransferase C
gtfC	Gene codificador da glicosiltransferase C
GtfD	Glicosiltransferase D
H^+	Átomo de Hidrogênio ionizado

H_2O	Água
H_2O_2	Peróxido de hidrogênio
ICDAS II	*International Caries Detection and Assessment System*
IUPUI	*Indiana University-Purdue University Indianapolis*
LC	Livre de Cárie
LDH	Lactato desidrogenase
NAD^+	Dinucleotídeo de Adenina Nicotinamida oxidado
NADH	Dinucleotídeo de Adenina Nicotinamida reduzido
NOX	NADH oxidase
nox	Gene codificador da NADH oxidase
NTCC	*National Type Culture Collection*
NYU	*New York University*
O_2	Oxigênio
OK	Oklahoma
PCR	Reação em Cadeia da Polimerase
PEC	Polissacarídeo extracelular
pH	Potencial Hidrogeniônico
PPGO	Programa de Pós-Graduação em Odontologia
RJ	Rio de Janeiro
RNA	Ácido Ribonucleico
RNAse	Ribonuclease
RT-qPCR	Reação em Cadeia da Polimerase Quantitativa em tempo real da transcrição reversa
S. mutans	*Streptococcus mutans*
SM	*Streptococcus mutans*
SP	São Paulo
SPSS	*Statistical Package for the Social Sciences*
ST	Estreptococos Totais
TX	Texas
UFC	Universidade Federal do Ceará
3D	Matriz Tridimensional

LISTA DE SÍMBOLOS

µL	Microlitro
µM	Micromolar
mL	Mililitro
min	Minuto
mm	Milímetro
n	Número total
-	Menos
±	Mais ou menos
s	Segundo
%	Porcentagem
β	Beta
°C	Grau Celsius
ng	Nanograma
g	Grama
G	Força g (força centrífuga relativa)
®	Marca Registrada
p	Significância estatística

SUMÁRIO

1 INTRODUÇÃO

A cárie dentária é uma desordem progressiva caracterizada pela desmineralização dos tecidos dentários. Essa desmineralização é resultado da frequente produção de ácido pelas bactérias do biofilme dental durante a fermentação de açúcares da dieta, sendo considerada, portanto, uma doença biofilme-açúcar-dependente (BAKRY *et al*., 2014; CURY *et al*., 2009; TENUTA *et al*., 2010).

Os principais fatores etiológicos envolvidos no processo carioso são a formação de biofilme e a presença de sacarose (BOWEN & KOO, 2011; MARSH, 2010). O biofilme é considerado um ecossistema dinâmico e extremamente complexo composto por uma comunidade estruturalmente organizada de células microbianas aderidas a uma superfície úmida e aglomeradas por uma matriz de polissacarídeos (DIGE *et al*., 2014; MARSH, 2006). Já a sacarose é considerada o principal catalisador para o desenvolvimento de lesões cariosas, uma vez que serve como substrato para a produção de ácidos e polissacarídeos extracelulares (PECs) (NGUYEN *et al*., 2014). Para microrganismos associados à cárie, a produção de uma matriz de biofilme rica em PECs, a acidificação do meio e a manutenção do pH ácido nas proximidades do esmalte dentário são os principais fatores de virulência associados à patogênese da doença (NGUYEN *et al* ., 2014).

O *Streptococcus mutans* é considerado o microrganismo mais cariogênico da microbiota bucal sendo relacionado às diferentes formas de progressão da doença cárie (BEZERRA *et al*., 2016; LIU *et al*., 2013; YIN *et al*., 2017). Além de ser acidogênico e tolerante a um pH ácido, o *S. mutans* tem a capacidade de formar um biofilme tridimensional tenaz e bem-estruturado na presença de sacarose, já que essa bactéria sintetiza rapidamente os PECs através da atividade de exoenzimas (por exemplo, glicosiltransferase - GTFs) (GARCIA *et al*., 2016; NGUYEN *et al*., 2014; VISZWAPRIYA *et al*., 2017).

A síntese de PECs via GTFs de *S. mutans* é crítica para a formação de biofilme cariogênico, uma vez que os glucanos produzidos a partir da reação do metabolismo da sacarose promovem acúmulo bacteriano local, incorporando bactérias em uma matriz limitadora de difusão. Esses processos criam biofilmes altamente coesivos e adesivos que estão fortemente aderidos às superfícies e são de difícil remoção (FUJIWARA *et al*., 2002; KRETH *et al*., 2008; NGUYEN *et al*., 2014). *S. mutans* secreta três GTFs: GtfB (sintetiza glucano insolúvel em água), GtfC (sintetiza glucano solúvel e insolúvel em água) e GtfD (sintetiza glucano solúvel em água) que são codificadas pelos genes gtfB, gtfC e gtfD, respectivamente (BOWEN & KOO, 2011; VISZWAPRIYA *et al*., 2017).

As estratégias utilizadas por *S. mutans* para a produção de ácidos, sobrevivência e adaptação às condições de estresse ambiental em biofilmes orais têm sido atribuídas a algumas enzimas que são responsáveis direta ou indiretamente por essas funções. Por exemplo, a proteína transportadora de membrana denominada F_1F_0-ATPase (codificada pelo gene atpD) é responsável pela expulsão de prótons H^+ para fora da célula, agindo como ATP-sintase em condições de baixo pH, produzindo ATP para o crescimento e a persistência da colônia (LEMOS & BURNE, 2008). A enzima NADH oxidase, uma enzima contendo flavina e codificada pelo gene nox, contribui para o metabolismo dos substratos de açúcar em ácido láctico através da regeneração do NAD^+ e para a manutenção dos índices NAD^+/NADH (HIGUCHI *et al*., 1999). A enzima denominada FabM (trans-2-cis-3-decenoil-ACP isomerase, codificada pelo gene fabM) está envolvida no aumento da proporção de ácidos graxos monoinsaturados da membrana plasmática em resposta à acidificação do meio ambiente (FOZO & QUIVEY, 2004a). Outra característica protetora importante encontrada em *S. mutans* é a atividade do sistema enzimático de agmatina desiminase (AgDS, codificada pelo gene aguBDAC), que cataboliza agmatina para produzir putrescina, amônia, CO_2 e ATP. AgDS é sugerido para reforçar a aptidão competitiva de *S. mutans*, contribuindo para a sua persistência e patogênese no processo carioso (BURNE & MARQUIS, 2000; GRISWOLD *et al*., 2004, 2006).

Análises quantitativas e qualitativas de ecossistemas polimicrobianos, como o biofilme dentário, são complicadas porque eles podem consistir em centenas de diferentes espécies bacterianas, muitas das quais não são identificáveis. Uma grande limitação de estudos utilizando meios de cultura é que muitas bactérias da microbiota oral não crescem em meio de cultura artificial convencional em laboratório (AAS *et al*., 2005; PASTER *et al*., 2001). Assim, o advento de novos métodos moleculares para identificação e quantificação bacteriana tem tornado possível reavaliar a patogênese de infecções orais de forma mais precisa (BECKER *et al*., 2002; MUNSON *et al*., 2004).

Uma melhor compreensão dos vários mecanismos de virulência e do papel ecológico de *S. mutans* no biofilme de lesões cariosas ativas e inativas pode ajudar no desenvolvimento de novas estratégias de prevenção da cárie. Assim, os objetivos deste estudo foram: investigar a prevalência de *S. mutans* em relação ao total de estreptococos (ST) e bactérias totais (BT), e a expressão de genes selecionados relacionados às principais características de sobrevivência e virulência de *S. mutans*, como gtfB e gtfC (adesão), atpD, aguD, nox e fabM (acidogenicidade e aciduricidade) em biofilme associado à atividade cariosa de lesões de esmalte e dentina de crianças.

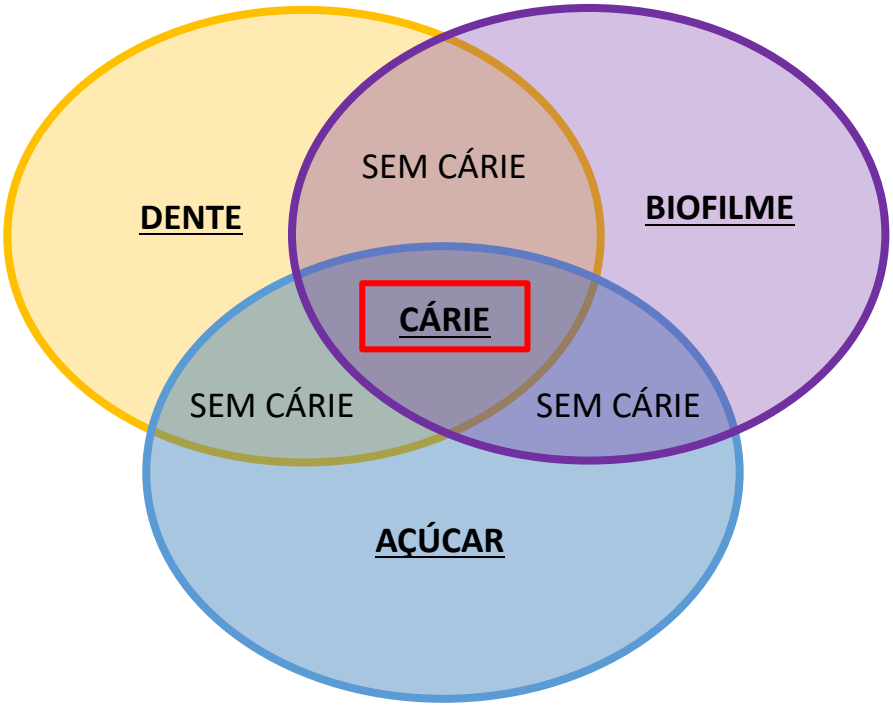
2 REFERENCIAL TEÓRICO

2.1 Cárie dentária

O termo cárie dentária foi originalmente usado, em 1634, para descrever buracos nos dentes com pouco conhecimento da etiologia e da patogênese da doença (BOWEN, 2016). Com a descoberta dos microrganismos, no final do século XIX, conceitos e crenças sobre a etiologia da cárie dentária começaram a evoluir (CONRADS & ABOUT, 2018).

Atualmente, é considerada uma desordem progressiva caracterizada pela desmineralização dos tecidos dentários. Essa desmineralização é resultado da frequente produção de ácido pelas bactérias do biofilme dental durante a fermentação de açúcares da dieta, sendo considerada, portanto, uma doença biofilme-açúcar-dependente v(CURY *et al*., 2009; TENUTA *et al*., 2010; BAKRY *et al*., 2014) (FIGURA 1). É uma das doenças dependentes de biofilme mais prevalentes na infância em todo o mundo, podendo causar dor e infecção, resultando em extensas lesões cariosas e redução na qualidade de vida, especialmente em populações socialmente desfavorecidas (BERKOWITZ, 2003; GROSS *et al*., 2012; NEVES *et al*., 2018).

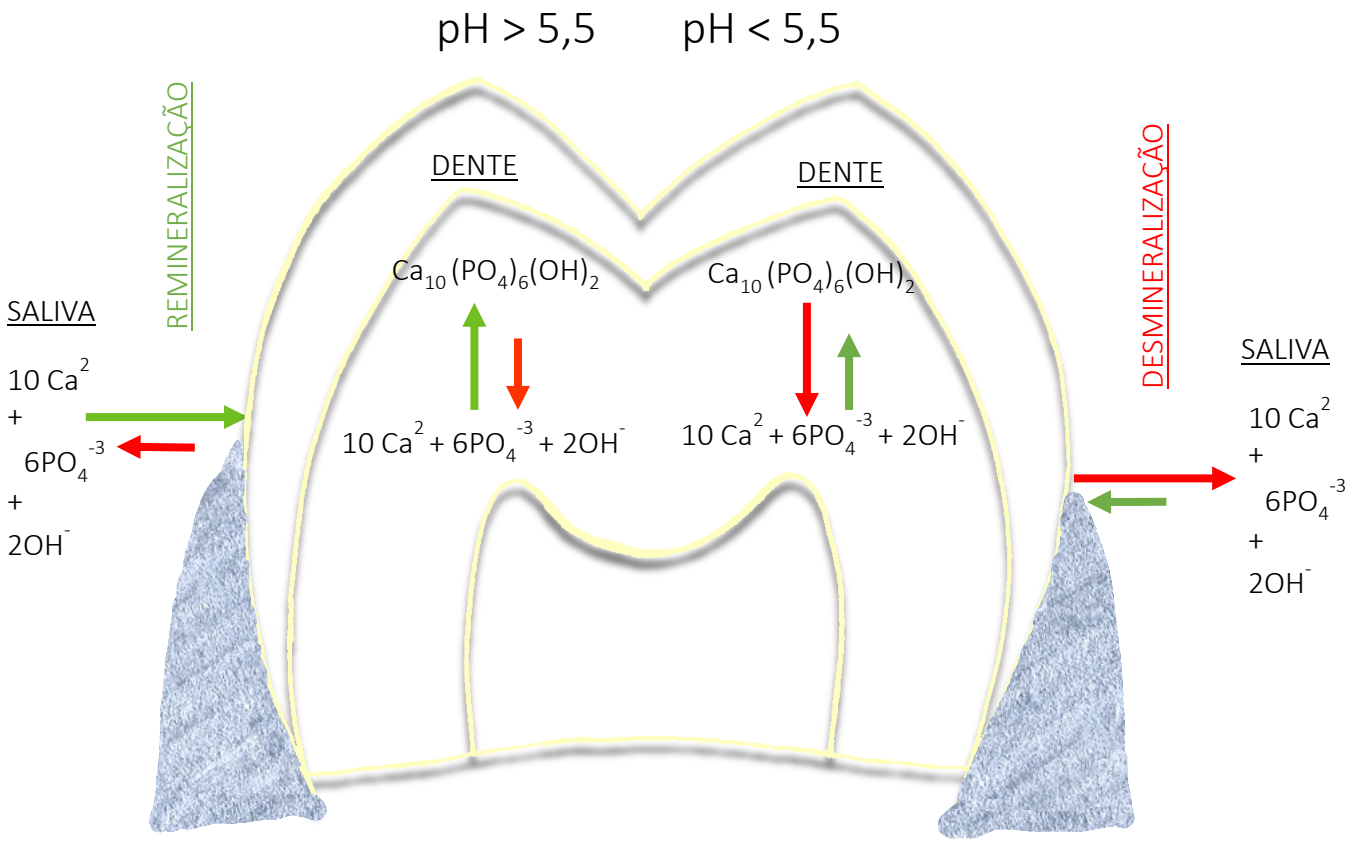
Figura 1 – Principais fatores envolvidos com a formação das lesões cariosas.



Fonte: adaptado de Fejerskov & Kidd, (2008).

Na cavidade oral, há uma troca constante de metabólitos e íons, tais como cálcio e fosfato, entre a superfície mineral dos dentes e os compostos salivares. Em condições normais, os processos de desmineralização e remineralização são equilibrados. No entanto, quando o pH derivado do metabolismo de microrganismos que fazem parte do biofilme dental é inferior a um certo limite, normalmente estabelecido em torno de 5,5, a perda mineral é maior do que a remineralização, iniciando o processo carioso (FIGURA 2) (FEJERSKOV & KIDD, 2008).

Figura 2 – Esquema do processo de remineralização e desmineralização que ocorre nos dentes durante a formação das lesões de cariosas.

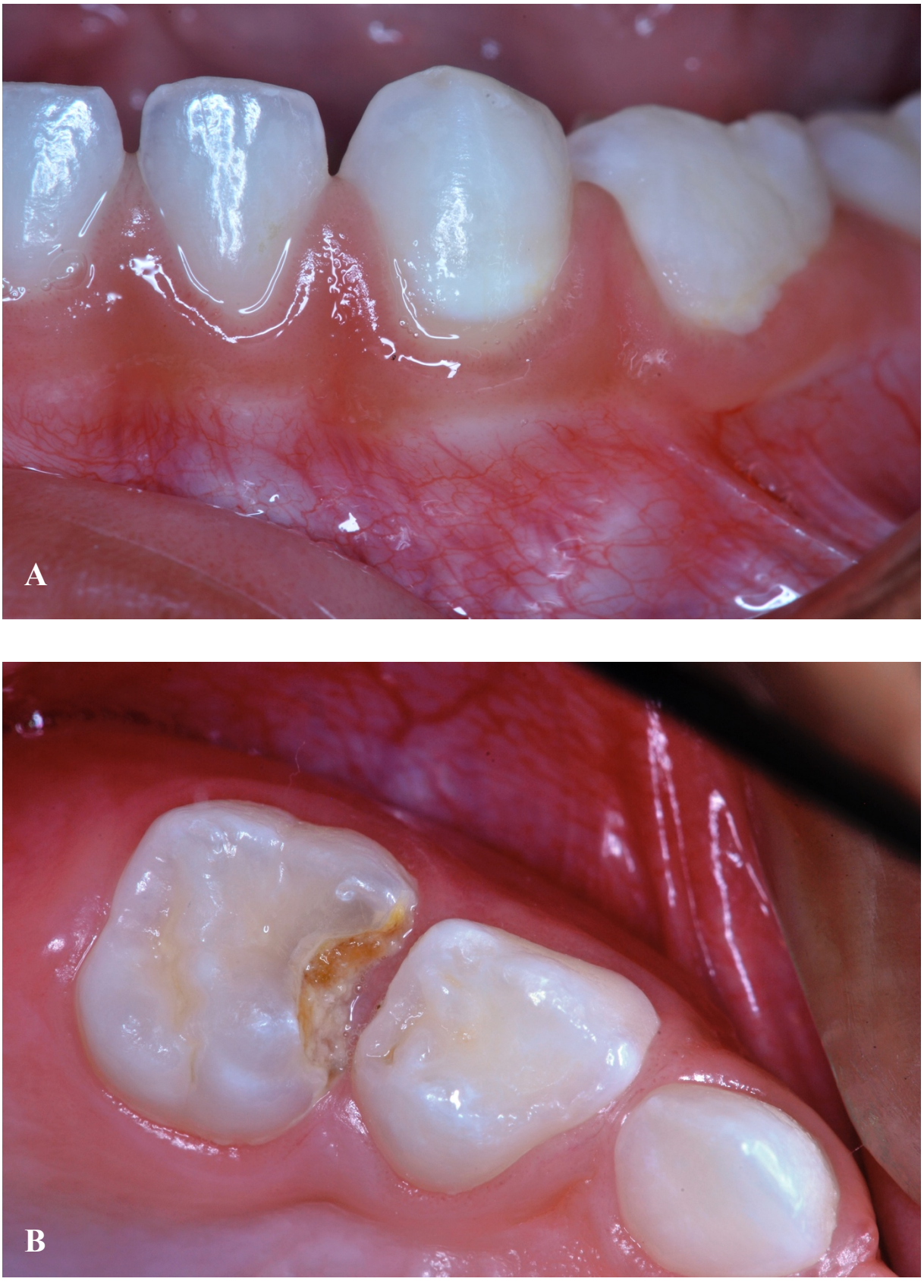


Fonte: adaptado de Baratieri *et al*., (1989).

De acordo com a Organização Mundial de Saúde (OMS), a contabilização de lesões de cárie é recomendada apenas a partir do nível de cavidade devido à dificuldade de se manter a confiabilidade entre os examinadores com a inclusão de lesões de cárie não cavitadas (GUILBERT, 2003). No entanto, a detecção precoce de lesões de cárie, especialmente em crianças, torna-se essencial, uma vez que a cárie progride rapidamente e, ocorrendo um atraso no diagnóstico da doença, muitos dentes decíduos poderão ser destruídos ou perdidos (PITTS, 2004). A inspeção visual-tátil é o primeiro e mais importante método para detectar e diagnosticar a cárie (BAELUM *et al*., 2012; NEUHAUS & LUSSI, 2018). Para a realização dessa inspeção, um eficaz sistema de detecção visual de cárie deve ser utilizado para que todas as fases de desenvolvimento da cárie sejam englobadas, e não apenas aquelas lesões que atingiram um estágio de cavitação. Os principais sistemas disponíveis paro o uso tanto na prática clínica como em pesquisas clínicas e estudos epidemiológicos são o *International Caries Detection and Assessment System* (ICDAS II) (ISMAIL *et al*., 2007) e o *Nyvad System* (NYVAD *et al*., 1999). Além da detecção de uma lesão cariosa, a avaliação da sua atividade, ou seja, se está em progressão ou estagnada, deve ser considerada (NYVAD *et al*., 1999; PITTS, 2004). Essa avaliação é um fator crucial, pois o seu resultado contribui para o planejamento da abordagem clínica a ser realizada (NYVAD *et al*., 2003).

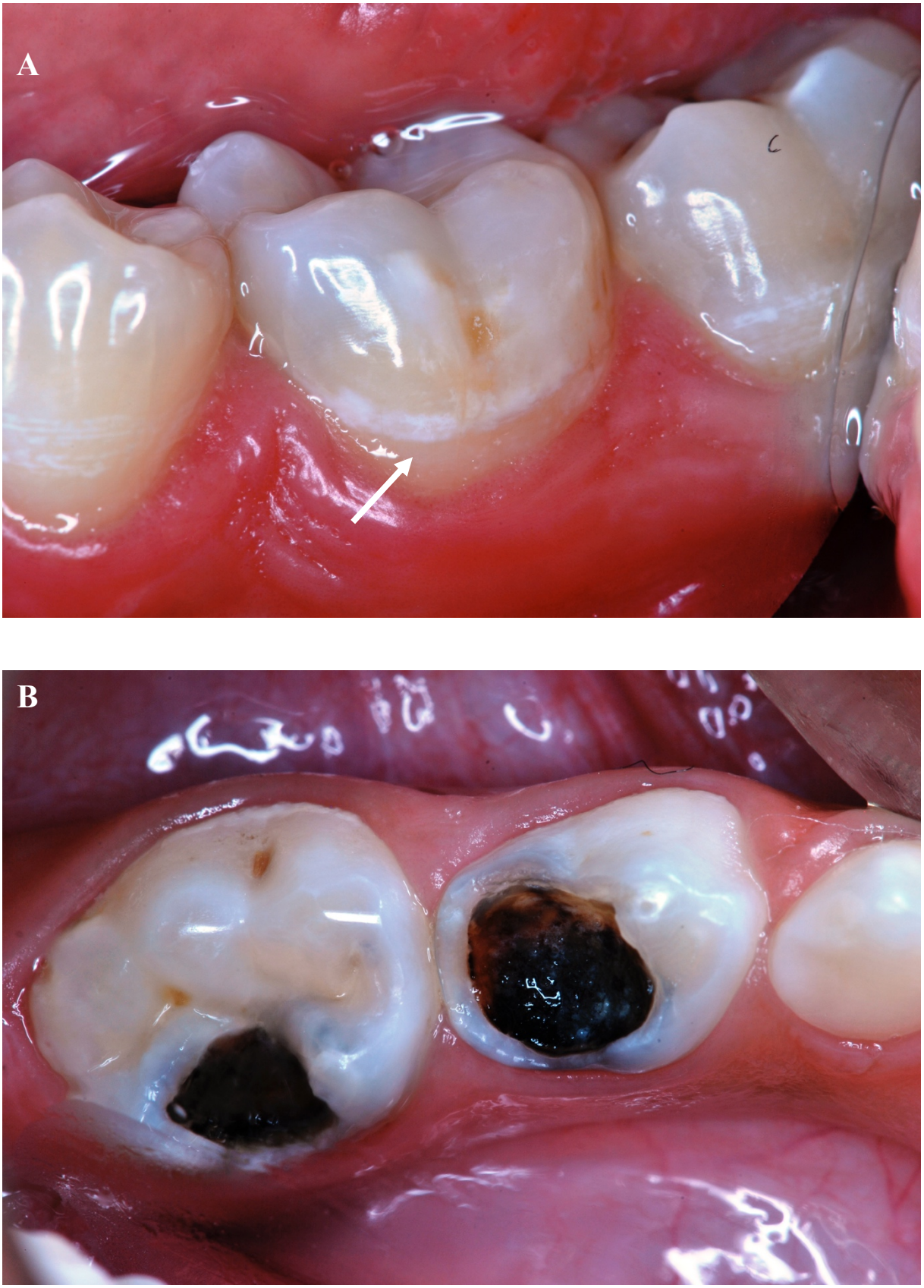
Usando critérios táteis-visuais, lesões cariosas ativas possuem aparência esbranquiçada (lesões em esmalte) (FIGURA 3 - A) ou amarelada (lesões em dentina) (FIGURA 3 - B), fosca e opaca, além de se apresentarem ásperas a uma leve sondagem, e geralmente estão cobertas por placa dentária; quando as lesões são em superfícies lisas, apresentam-se localizadas geralmente próximas à margem gengival (FIGURA 3 - A); quando são em superfícies oclusais, estendem-se ao longo das paredes da fissura. Já as lesões cariosas inativas apresentam-se com coloração esbranquiçada (lesões em esmalte) (FIGURA 4 - A) ou amarronzada/preta (lesões em dentina) (FIGURA 4 - B), brilhantes e textura lisa e dura quando levemente sondadas. As lesões localizadas em superfícies lisas apresentam-se localizadas e geralmente longe da margem gengival com uma faixa de substrato sadio entre a lesão e a margem gengival, como mostrado na FIGURA 4 – A através de uma seta; quando são em superfícies oclusais, também se estendem ao longo das paredes da fissura. A evolução nesse processo gera descontinuidade de superfície, podendo chegar a cavitações em esmalte e/ou dentina facilmente visíveis a olho nu; quando sondadas, a textura é macia ou de couro (lesões ativas) ou duras (lesões inativas), podendo haver ou não envolvimento pulpar (NYVAD *et al*., 1999; NYVAD *et al*., 2003).

Figura 3 – Lesões cariosas ativas em esmalte (A) e dentina (B).



Fonte: arquivo pessoal.

Figura 4 – Lesões cariosas inativas em esmalte (A) e dentina (B).



Fonte: arquivo pessoal.

2.2 Biofilme oral

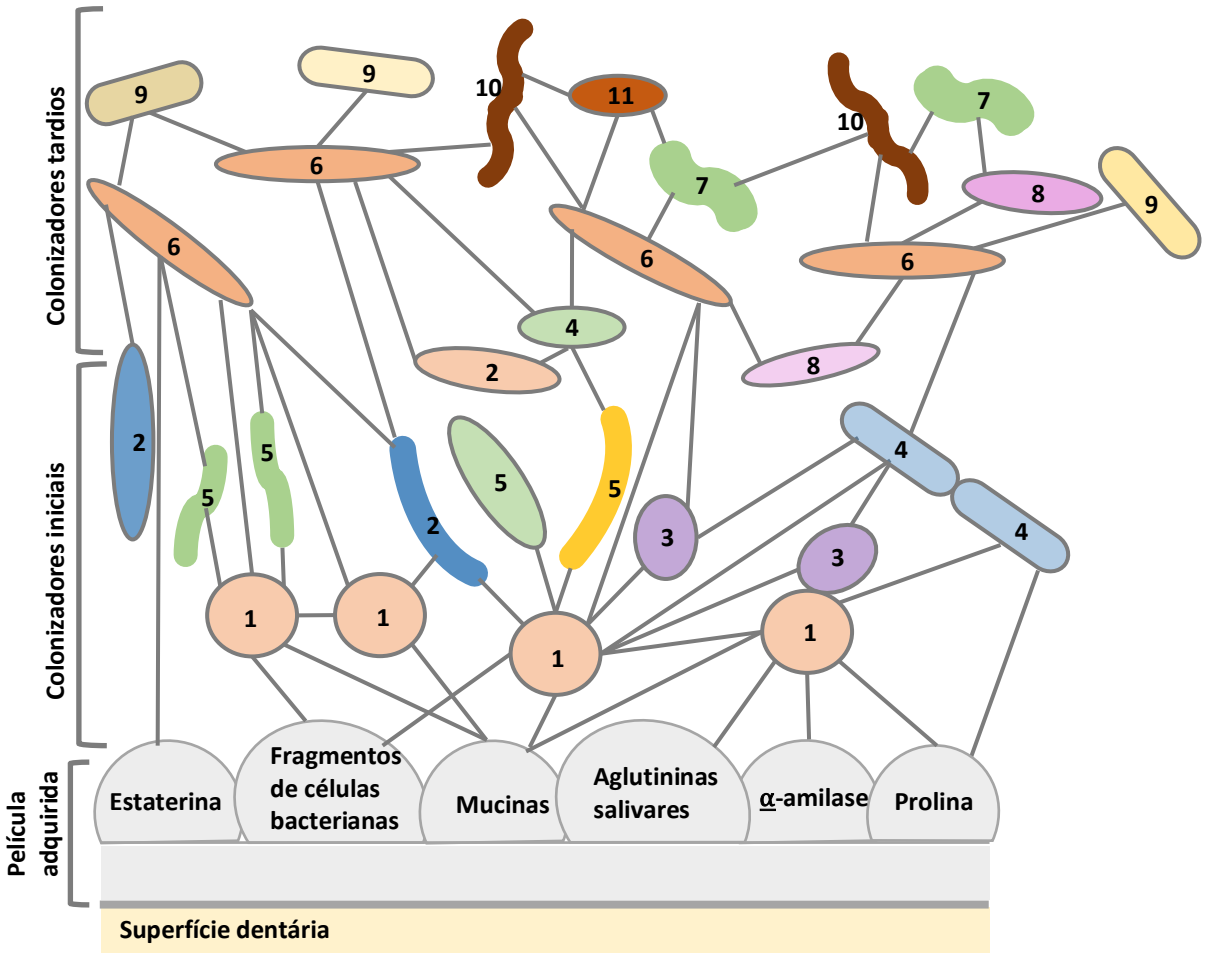
Um dos principais fatores etiológicos envolvidos no processo carioso é a formação de biofilme (MARSH, 2010), o qual é composto por uma comunidade estruturalmente organizada de células microbianas aderidas a uma superfície úmida e aglomeradas por uma matriz de polissacarídeos (FLEMING & WINGENDER, 2010; NASCIMENTO *et al*., 2014). O biofilme é considerado um ecossistema dinâmico e extremamente complexo e a sua relação com o aparecimento e a progressão da cárie dentária é inquestionável (MARSH, 2006; DIGE *et al*., 2014).

O desenvolvimento de um biofilme ocorre através de uma sucessão de microrganismos, os quais são classificados de acordo com o momento de envolvimento na sua formação como colonizadores iniciais, intermediários e tardios. O primeiro passo para o seu desenvolvimento é a formação da película adquirida, que ocorre segundos após a erupção do dente ou após a profilaxia profissional, uma combinação de proteínas ativas e glicoproteínas, como estatinas, mucinas, aglutininas, alfa-amilases e prolinas, oriundas da saliva e do fluido crevicular gengival, que se ligam à superfície do dente. Essas moléculas são mantidas reversivelmente próximas à superfície do dente por ligações físico-químicas. Inicialmente, apenas espécies principalmente aeróbicas dos gêneros *Streptococcus, Capnocytophaga, Veillonella ou Actinomyces spp*. (conhecidas como colonizadoras iniciais), produtoras de adesinas são capazes de aderir-se a receptores na película. Elas se multiplicam e formam a primeira camada do biofilme. Uma segunda camada de biofilme desenvolve-se quando outros microrganismos são ligados aos colonizadores iniciais através da adesão de suas superfícies, mecanismo conhecido como coagregação ou coadesão. Nessa fase, o biofilme torna-se, principalmente, anaeróbico, por meio de colonizadores como o *Fusobacterium nucleatum*, e colonizadores tardios, como *Porphyromonas gingivalis*, *Treponema denticola*, *Tannerella forsythia*, *Prevotella spp*., *Lactobacillus spp*., e a composição do biofilme torna-se mais diversificada (sucessão microbiana) (KOLENBRANDER *et al*., 2010). As bactérias aderidas produzem polissacarídeos extracelulares (PECs) que formam a matriz da placa e consolidam a fixação do biofilme. A matriz é mais do que um andaime para o biofilme, já que pode ligar e reter moléculas, incluindo enzimas, e também retardar a penetração de moléculas carregadas no biofilme (FIGURA 5) (KOLENBRANDER *et al*., 2010; MARSH, 2010).

A cavidade oral fornece condições necessárias para o desenvolvimento de biofilmes microbianos complexos, já que mais de 700 espécies de microrganismos já foram identificadas nesse habitat (FLOREZ SALAMANCA & KLEIN, 2018). A composição microbiana do

biofilme varia em locais distintos em um dente (fissuras, superfícies proximais e sulco gengival) e reflete as diferenças inerentes à sua anatomia e biologia (AAS *et al*., 2005; PAPAIOANNOU *et al*., 2009). A microbiota normal das fissuras é esparsa, e os organismos apresentam um metabolismo sacarolítico (isto é, sua energia é derivada do catabolismo da sacarose); as bactérias predominantes são estreptococos e há poucos gram-negativos ou organismos anaeróbicos. Em contraste, o sulco gengival tem uma microbiota mais diversificada, incluindo muitas espécies anaeróbicas e proteolíticas gram-negativas, enquanto as superfícies têm uma microbiota que é intermediária na composição (MARSH, 2010).

Figura 5 – Modelo de desenvolvimento de um biofilme oral.



Nesse modelo de desenvolvimento de biofilme oral, os colonizadores iniciais, como bactérias dos gêneros *Streptococcus* (1), *Capnocytophaga* (2), *Veillonella* (3), *Actinomyces spp*. (4) ou de outras espécies (5), ligam-se à película adquirida na superfície dentária e coagregam-se com outras espécies bacterianas de forma sequencial. *Fusobacterium nucleatum* (6) parece ter múltiplas funções e foi proposto como uma "ponte" entre os colonizadores iniciais e tardios, como *Porphyromonas gingivalis* (7), *Prevotella spp*. (8), *Lactobacillus spp*. (9), *Treponema denticola* (10) e *Tannerella forsythia* (11).

Fonte: adaptado de Kolenbrander *et al*., (2010).

Uma vez estabelecido o biofilme oral, também chamado placa dentária, a sua composição de espécies em um determinado local é caracterizada por um grau de estabilidade ou equilíbrio entre as espécies componentes. Essa estabilidade, denominada homeostase microbiana, reflete um estado altamente dinâmico em que as proporções individuais das espécies estão em equilíbrio devido às muitas interações sinérgicas e antagônicas (MARSH, 2006). Em qualquer ecossistema, a homeostase microbiana pode ser quebrada ocasionalmente, devido a alguma mudança substancial em determinado parâmetro que é crítico para manter a estabilidade ecológica daquele local, resultando no aumento do número de componentes anteriormente menores da comunidade. Uma consequência clínica disso na boca pode ser doença (BOWEN *et al*., 2016; MARSH, 2010).

Na cárie dentária, as interações entre as bactérias orais, a saliva do hospedeiro e os carboidratos da dieta modulam a transição de uma condição saudável para um estado de doença, promovendo o estabelecimento de biofilmes cariogênicos na superfície dentária (MARSH, 2006). O biofilme cariogênico consiste em uma microbiota altamente acidogênica e acidúrica envolta por uma matriz rica em PECs, que cria um meio adesivo, coesivo e ácido (BOWEN *et al.*, 2011; PAES LEME *et al.*, 2006). O meio ácido eventualmente leva ao início clínico da cavitação através da desmineralização ácida do esmalte adjacente (KLEIN *et al.*, 2012).

Os PECs são formados pelas glicosiltransferases (Gtfs) de *S. mutans* presentes na película adquirida e ligados a bactérias na superfície do dente (MATTOS-GRANER *et al.*, 2000; TAKAHASHI & NYVAD, 2011). Os polissacarídeos formados na superfície fornecem sítios de ligação para posterior colonização e acumulação de *S. mutans* e de outros organismos na superfície dentária (BOWEN *et al.*, 2011). Se a sacarose estiver frequentemente disponível, a produção contínua de PECs aprofunda as células microbianas que formam microcolônias, resultando em uma matriz tridimensional (3D) altamente estruturada em biofilmes cariogênicos virulentos (KOO *et al*., 2010; XIAO *et al*., 2010; XIAO *et al*., 2012). Em paralelo, as células bacterianas embutidas na matriz e agrupadas em microcolônias 3D podem converter sacarose (e outros carboidratos fermentáveis) em ácidos, que por sua vez ficam retidos nesse meio rico em PECs (XIAO *et al*., 2012), deixando o microambiente com baixo pH, o que resulta numa seleção de microrganismos acidúricos. Portanto, *S. mutans* e sacarose têm sido como os principais moduladores para a evolução de biofilmes cariogênicos (KLEIN *et al*., 2012).

2.3 *Streptococcus mutans* e os seus mecanismos de virulência

Streptococcus mutans, uma bactéria gram-positiva, é uma das principais espécies bacterianas associadas com a formação da cárie (LOESCHE, 1986; MATTOS-GRANER *et al*., 2014), embora microrganismos adicionais possam contribuir com a sua patogênese (NYVAD *et al*., 2013). Esse microrganismo (1) utiliza sacarose da dieta, quebrando-a para sintetizar PECs através da ação de glicosiltransferases (Gtfs), (2) adere-se firmemente a superfícies revestidas com glucano, (3) é acidogênico e acidúrico (LOESCHE, 1986).

A sacarose é considerada o catalisador primário para o desenvolvimento de lesões cariosas, pois serve de substrato para produção de ácidos e dos PECs (BOWEN & KOO, 2011; LYNCH *et al*., 2013). O *S. mutans* secreta três GTFs: GtfB, GtfC e GtfD, que são codificados pelos genes gtfB, gtfC e gtfD, respectivamente. As Gtfs quebram a molécula de sacarose em glicose e frutose. A GtfB sintetiza principalmente glucano insolúvel em água, a GtfC sintetiza glucanos solúveis e insolúveis em água, e a GtfD sintetiza glucanos solúveis em água. Os glucanos sintetizados promovem adesão e coesão de células bacterianas, levando à formação de microcolônias e modulando os processos iniciais de formação de um biofilme cariogênico (KOO *et al*., 2010; NGUYEN *et al*., 2014).

O *S. mutans* possui uma via glicolítica completa e, como produtos de fermentação, pode produzir lactato, formato, acetato e etanol. A distribuição precisa dos produtos da fermentação depende das condições de crescimento do microrganismo, sendo lactato o produto principal quando a glicose é abundante (HILMAN, 2002). Cepas deficientes em lactato desidrogenase (LDH), a enzima que converte o piruvato (produto final da glicólise quando o oxigênio está ausente ou disponível em pequenas quantidades) em lactato, reduzem a sua cariogenicidade (FITZGERALD *et al*., 1989), e a ausência dessa enzima é considerada letal para o microrganismo (BANAS, 2004; HILMAN *et al*., 1996). Para que essa conversão de piruvato em lactato ocorra, é necessária também a oxidação de NADH em NAD^+. O gene nox codifica a enzima NADH oxidase (LEMOS *et al*., 2005), responsável por tal conversão.

A velocidade com que *S. mutans* produz ácido numa variação de pH de 7,0-5,0 excede a de outros estreptococos orais (SOET *et al*., 2000). A acidogenicidade relativa de *S. mutans* pode variar entre as diferentes cepas, e há uma correlação positiva entre a ausência de acidogenicidade com a ausência de cárie (KÖHLER *et al*., 1995). Geralmente a acidogenicidade desse microrganismo leva a mudanças ecológicas na microbiota da placa, que inclui uma elevação na proporção de *S. mutans* e outras espécies acidogênicas e acidúricas. Esta microbiota cariogênica reduz o pH da placa para níveis mais baixos do que o de placa saudável

após a ingestão de carboidrato fermentável, e a recuperação para um pH neutro será prolongada. Os valores de pH abaixo de 5,5 favorecem a desmineralização do esmalte e, consequentemente, o desenvolvimento de lesões cariosas (BANAS, 2004; LOESCHE, 1986).

Adicionalmente à sua acidogenicidade, *S. mutans* mantém sua capacidade glicolítica mesmo em níveis baixíssimos de pH, como 4,4, que são níveis inibitórios para o crescimento de muitas espécies microbianas (BANAS, 2004). A sua tolerância ao ambiente ácido é amplamente mediada por uma bomba de prótons, a F_1F_0-ATPase, mas também envolve mecanismos adaptativos através de alterações na expressão gênica e proteica desse microrganismo. *In vitro*, a resposta ácido-tolerante do *S. mutans* mostrou proteger o microrganismo de um pH sub-letal, e um choque ácido ou crescimento em ambientes ácidos é associado com alterações na expressão de mais de 30 proteínas (KLEIN *et al*., 2012; WILKINS *et al*., 2002).

Evidências mostram que essa tolerância ao ácido pode ser auxiliada pela síntese de glucanos insolúveis em água e consequente formação de um biofilme. Células de *S. mutans* dentro de um biofilme possuem uma maior capacidade de sobreviver em um ambiente ácido do que bactérias planctônicas (FLOREZ SALAMANCA & KLEIN, 2018; McNEILL & HAMILTON, 2003). Isso ocorre devido a sistemas de detecção de *quorum sensing* que são induzidos pela resposta ácido-tolerante e pelas características físicas do biofilme. Estudos relataram que a velocidade de difusão de íons H^+ é proporcional à quantidade de produção de glucanos por *S. mutans* (KLEIN *et al*., 2012; NGUYEN *et al*., 2014). Esses resultados demonstram a conexão entre os diferentes mecanismos de virulência de *S. mutans* e indicam que o papel do glucano estende-se além de promover a adesão (BANAS, 2004).

A acidificação do ambiente é uma consequência da excreção de derivados ácidos do metabolismo bacteriano. Prótons externos são capazes de permear a membrana de estreptococos e acidificar o seu citoplasma, inibindo, assim, enzimas glicolíticas e outras funções sensíveis ao baixo pH intracelular. Portanto, a atividade da F_1F_0-ATPase, codificada pelo gene atpD, é crítica para estabelecer e manter um gradiente de pH ao longo da membrana citoplasmática. À medida que o pH diminui, há aumento da atividade da F_1F_0-ATPase do *S. mutans,* que ajuda a manter um pH intracelular viável para a atividade de diferentes enzimas (KLEIN *et al*., 2012; XU *et al*., 2011). Além do sistema F_1F_0-ATPase, o sistema agmatina desiminase (AgDS), codificado por alguns genes, como o aguD, também é capaz de aumentar a competitividade e aptidão de *S. mutans*, contribuindo para a persistência e patogênese desse organismo (XU *et al*., 2011). Esse sistema converte agmatina, um inibidor do crescimento de *S. mutans*, em putrescina, amônia e CO_2. A produção de amônia aumenta o pH citoplasmático

e gera ATP, que pode ser usado para o crescimento bacteriano ou para extrusão de prótons H^+ (GRISWOLD *et al*., 2006). Adicionalmente, o perfil de ácidos graxos da membrana muda, diminuindo a permeabilidade a prótons, e a excreção de produtos finais ácidos aumenta. O gene fabM é responsável pela síntese de ácidos graxos monoinsaturados e a sua expressão é essencial para a sobrevivência de *S. mutans* em baixo pH (FOZO & QUIVEY, 2004b; KLEIN *et al*., 2012). O estudo de Fozo & Quivey (2004b) mostrou que uma cepa mutante de *S. mutans* sem a expressão de fabM apresentou membrana com composição alterada, gerando efeitos significativos sobre a glicólise, fosfotransferase, permeabilidade da membrana e atividade da F_1F_0-ATPase.

3 OBJETIVO

Investigar a prevalência de *S. mutans* em relação ao total de estreptococos (ST) e bactérias totais (BT) e a expressão de genes selecionados relacionados às principais características de sobrevivência e virulência de *S. mutans*, como gtfB e gtfC (adesão), atpD, aguD, nox e fabM (acidogenicidade e aciduricidade) em biofilme formado *in vivo* sobre substratos de esmalte e dentina com diferentes *status* de atividade cariosa em crianças.

4 MATERIAIS E MÉTODOS

4.1 Aspectos éticos

O desenho do estudo e o consentimento informado foram aprovados pelo Comitê de Ética em Pesquisa da Universidade Federal do Ceará (CEP/UFC) com parecer número 3.092.796 (ANEXO A). Um consentimento informado por escrito e assinado foi obtido dos pais ou responsáveis legais de cada criança inscrita no estudo. As amostras foram coletadas somente após obter a aprovação dos pais ou responsáveis.

4.2 Sujeitos do estudo

A população do estudo foi constituída por crianças de cinco a nove anos de idade de escolas públicas e residentes na cidade de Fortaleza, Ceará, Brasil. As escolas foram selecionadas por conveniência e estão localizadas na periferia da cidade. Uma triagem inicial sobra a situação de cárie das crianças foi realizada em escolas públicas de Fortaleza-CE, um total de 220 crianças foi examinado na nessa fase do estudo, na escola para selecionar a população do estudo.

Amostras foram coletadas de uma população constituída por crianças livres de cárie e crianças diagnosticadas com lesões cariosas ativas e/ou inativas em esmalte e/ou dentina. A assinatura do termo de assentimento das respectivas crianças e do termo de consentimento livre e esclarecido dos respectivos responsáveis, assim como a disposição das crianças a participarem do estudo foram usadas como critérios de inclusão. O estudo excluiu crianças com histórico de doença médica significativa, que usaram antibióticos ou antifúngicos no período inferior a três meses da data do exame de seleção, e aqueles que não cooperaram durante o exame clínico ou durante a coleta das amostras. Nenhuma das crianças apresentava distúrbios nas glândulas salivares.

Para o estudo, só foram consideradas as lesões de cárie presentes em dentes decíduos. As lesões de cárie dentinária que apresentaram sinais clínicos ou sintomas de pulpite irreversível foram excluídas do estudo.

4.3 Diagnóstico e determinação da atividade das lesões cariosas

Os exames clínicos, para confirmar a situação de condição oral relacionadas à cárie

dentária acessadas na escola na fase inicial, foram realizados na clínica de Dentística da Faculdade de Farmácia, Odontologia e Enfermagem da Universidade Federal do Ceará por um examinador previamente treinado e calibrado (kappa intra-examinador = 0,72). Das 220 crianças que participaram da triagem inicial, 53 compareceram a clínica acompanhada dos seus responsáveis e foram incluídas no estudo. As crianças foram acomodadas na cadeira odontológica, e uma remoção profissional de biofilme com uso de taça de borracha e pasta profilática foi realizada. Em seguida, os dentes foram secos com jato de ar, e o diagnóstico das lesões foi realizado. O índice utilizado para o diagnóstico das lesões de cárie foi o *International Caries Detection and Assessment System* (ICDAS II) (ISMAIL *et al*., 2007) associado ao *Nyvad System* para diferenciação entre lesões cariosas ativas e inativas (NYVAD *et al*., 1999).

Inicialmente, como preconizado pelo ICDAS II, o examinador determinou, após limpeza e secagem, se o dente era hígido, selado ou restaurado, com coroa ou ausente. Em seguida, as superfícies foram classificadas em relação à cárie, usando uma escala ordinal, que vai de superfície hígida (escore 0) à cavitação extensa (escore 6). No ICDAS II, todas as faces de cada dente (mesial, distal, vestibular, lingual e oclusal) são avaliadas (ISMAIL *et al*., 2007) (TABELA 1).

Em um segundo momento, após o diagnóstico, o *Nyvad System* foi utilizado para a avaliação da atividade das lesões cariosas. Esse sistema baseia-se em critérios táteis e visuais e classifica a lesão cariosa com escores de 1- 6 (NYVAD *et al*., 1999) (TABELA 2). O examinador avaliou a aparência clínica dos sítios selecionados com base na cor, opacidade, e na presença de descontinuidades superficiais ou cavidades.

Tabela 1 – Critérios de classificação do ICDAS II.

Escores	Critérios de classificação
0	Nenhuma ou sutil alteração na translucidez do esmalte após secagem prolongada (5s).
1	Opacidade dificilmente visível na superfície úmida, mas notável após secagem prolongada.
2	Opacidade visível sem secagem.
3	Cavidade localizada em esmalte opaco ou pigmentado.
4	Sombreamento da dentina subjacente.
5	Cavitação em esmalte opaco ou pigmentado com exposição de dentina subjacente.
6	Cavitação em esmalte opaco ou pigmentado com exposição da dentina subjacente, envolvendo mais de metade da superfície.

Fonte: adaptado de Ismail *et al*., (2007).

Tabela 2 – Critérios de classificação do *Nyvad System*.

Escore	Classificação	Critérios de classificação
1	Cárie ativa (superfície intacta)	A superfície do esmalte apresenta-se com coloração amarelada/esbranquiçada, opaca e com perda de brilho; quando sondada, a textura é áspera; geralmente está coberta por placa; não há perda de tecido clinicamente detectável. Superfície lisa: lesão de cárie localizada geralmente próxima à margem gengival. Fissura: morfologia apresenta-se intacta; lesão estende-se ao longo das paredes da fissura.
2	Cárie ativa (descontinuidade de superfície)	Mesmos critérios do escore 1. Microcavidade localizada apenas em esmalte.
3	Cárie ativa (cavidade)	Cavidade em esmalte e dentina facilmente visível a olho nu; quando sondada, a textura é macia ou de couro; pode ou não haver envolvimento pulpar.
4	Cárie inativa (superfície intacta)	A superfície do esmalte apresenta-se com coloração esbranquiçada, amarronzada ou preta, e com brilho; quando sondada, a textura é dura e lisa; não há perda de tecido clinicamente detectável. Superfície lisa: lesão de cárie localizada e longe da margem gengival. Fissura: morfologia apresenta-se intacta; lesão estende-se ao longo das paredes da fissura.
5	Cárie inativa (descontinuidade de superfície)	Mesmos critérios do escore 4. Microcavidade localizada apenas em esmalte.
6	Cárie inativa (cavidade)	Cavidade em esmalte e dentina facilmente visível a olho nu; brilhante; quando sondada, a textura é dura; não há envolvimento pulpar.

Fonte: adaptado de Nyvad *et al*., (1999).

4.4 Amostragem

Com base na diferença da expressão média de genótipos associados à modificação do pH mantidos em biofilmes a base de água (3.90±0.06) e sacarose (3.70±0.10), observado por Arthur *et al*. (2011), estimou-se necessária uma amostra de oito unidades por grupo de estudo para, com um poder de 90% e uma confiança de 95%, obter uma amostra que rejeite a hipótese de nulidade desse estudo (teste t, www.openepi.com/samplesize/ssmean.htm).

Tabela 3 – Delineamento do estudo.

Grupos (n=8)	**Diagnóstico das lesões cariosas (ICDAS II)**	**Avaliação da atividade das lesões cariosas (*Nyvad System*)**
Livre de cárie (LC)	Escore 0	-
Cárie de esmalte ativa (CEA)	Escores 1, 2 ou 3	Escores 1, 2 ou 3
Cárie de esmalte inativa (CEI)	Escores 1, 2 ou 3	Escores 4, 5 ou 6
Cárie de dentina ativa (CDA)	Escores 5 ou 6	Escore 3
Cárie de dentina inativa (CDI)	Escores 5 ou 6	Escore 6

Fonte: elaborado pelo autor.

4.5 Coleta das amostras

A coleta das amostras foi realizada em uma nova consulta, aproximadamente sete dias após a realização da consulta inicial. As crianças selecionadas foram orientadas a não escovar os dentes por 24 horas e não ingerir alimentos duas horas antes da coleta das amostras. Após a classificação das lesões cariosas, como previamente relatado, as amostras de biofilme foram coletadas com a utilização de curetas estéreis (*SS White Duflex*, Rio de Janeiro, RJ, Brasil). Para o grupo LC, as amostras foram coletadas das faces vestibulares de todos os dentes das crianças selecionadas. Para os grupos CEA e CEI, as amostras foram coletadas da superfície das lesões cariosas de esmalte ativas e inativas, respectivamente. Já para os grupos CDA e CDI, as amostras foram coletadas da superfície das lesões cariosas de dentina ativas e inativas, respectivamente. Para os grupos cariados, não foi realizado *pool* das amostras.

Após a coleta, as amostras foram imediatamente colocadas em microtubos de tampa de rosca estéreis livres de RNAse (*Axygen*, Union City, CA, EUA) contendo 1 mL de solução estabilizadora de RNA (*RNAprotect Bacteria Reagent*®, Qiagen, Valencia, CA, EUA). Os tubos foram centrifugados (10.000 G/ 1 min/ 4 °C), e o sobrenadante foi descartado. Em seguida, os microtubos foram mantidos a – 80 ºC até a extração do RNA (DO *et al*., 2015). Após o

procedimento de coleta das amostras, os pacientes foram submetidos aos procedimentos clínicos de acordo com a necessidade de cada caso.

- Pacientes livres de cárie e com lesões cariosas inativas em esmalte:

1. profilaxia;
2. instrução de higiene bucal.

- Pacientes com lesões cariosas ativas em esmalte:

1. profilaxia;
2. instrução de higiene bucal;
3. aplicação tópica de flúor.

- Pacientes com lesões cariosas dentinárias ativas e inativas:

1. profilaxia;
2. instrução de higiene bucal;
3. isolamento do campo operatório com dique de borracha;
4. limpeza do dique de borracha com clorexidina 2%;
5. remoção da dentina cariada;
6. selamento da cavidade com cimento de ionômero de vidro *Riva Light Cure* (SDI, São Paulo, SP, Brasil).

Um total de 121 amostras foram coletadas, nove amostras para o grupo LC, 17 amostras para o CEA, 23 amostras para o CEI, 50 amostras para o CDA e 22 amostras para o CDI.

4.6 Extração e purificação do RNA

Inicialmente, a ruptura mecânica das células foi realizada com a adição de 400 µL de tampão RLT (*RNeasy Kit*®, *Qiagen*, Valencia, CA, EUA) a 1% de β-mercaptoetanol (β-ME) e 0,16 g de esferas de zircônia com 0,1 mm de diâmetro (*Biospec Products*, Bartlesville, OK, EUA) através do processamento das amostras no *MiniBeadbeater* (*Biospec Products*, Bartlesville, OK, EUA) na potência máxima (dois ciclos de 30 s com 1 min de repouso no gelo). Após a centrifugação (16.000 G/ 2 min/ 4 °C), uma alíquota de 350 µL do sobrenadante foi submetida à extração de RNA, enquanto a outra parte foi armazenada, caso houvesse necessidade de uma nova extração. Aos 350 µL removidos inicialmente, foram adicionados 250 µL de etanol puro, e o microtubo foi agitado em vórtex. Em seguida, o conteúdo foi transferido para uma coluna do kit (*RNeasy Kit*®, *Qiagen*, Valencia, CA, EUA) e centrifugado (16.000 G/ 30 s/ 20 ºC). Após a centrifugação, o conteúdo que passou pela coluna foi descartado, 700 µL

de RW1 foram adicionados à coluna, e o microtubo foi centrifugado novamente (16.000 G/ 30 s/ 20 ºC). Em seguida, o conteúdo que passou pela coluna foi descartado novamente, 500 µL de RPE foram adicionados à coluna, e o microtubo foi centrifugado (16.000 G/ 30 s/ 20 ºC). Esse passo foi realizado duas vezes, e uma nova centrifugação (16.000 G/ 2 min/ 20 ºC) sem adição de reagentes foi realizada com intuito de remover todo o álcool presente na coluna. Posteriormente, a eluição da amostra foi realizada e, para isso, a coluna foi posicionada em um microtubo sem tampa, 40 µL de água livre de RNAse (*Qiagen*, Valencia, CA, EUA) foram adicionados, e uma centrifugação foi realizada (16.000 G/ 1 min/ 20 ºC). O eluído foi armazenado em um novo microtubo devidamente identificado.

Após a extração, a concentração e a pureza do RNA, através da razão de absorbância A_{260}/A_{280}, foram verificadas no espectrofotômetro Nanodrop 2000c (*Thermo Scientific*, Wilmington, DE, EUA). Todas as amostras que apresentaram concentração inferior a 20 ng/ µL e/ ou razão de absorbância inferior a 2 foram excluídas do estudo: uma amostra do grupo LC, nove amostras do grupo CEA, 15 amostras do grupo CEI, 42 amostras do grupo CDA e 14 amostras do grupo CDI.

Para a remoção do DNA genômico, cada amostra foi tratada com 5 µL do tampão e 5 µL da enzima Turbo DNAse® (*Applied Biosystems/ Ambiom,* Austin, TX, EUA), vortexada e mantida a 37 ºC durante 15 min.

Para a purificação das amostras, o protocolo de extração de RNA, detalhado anteriormente, foi utilizado sem o passo de ruptura mecânica das células. Após a purificação, a concentração e a pureza do RNA foram novamente verificadas no espectrofotômetro Nanodrop 2000c (*Thermo Scientific*, Wilmington, DE, EUA). Todas as amostras que não apresentaram concentração superior a 20 ng/ µL e/ ou razão de absorbância superior a 2 foram excluídas do estudo. Em seguida, a integridade desse RNA foi avaliada por meio da corrida em gel de eletroforese, e as bandas 16S e 23S de RNA ribossômico foram visualizadas para todas as amostras do estudo (BEZERRA *et al*., 2016).

4.7 Transcrição reversa e PCR quantitativa em Tempo Real da transcrição reversa (RT-qPCR)

A conversão de RNA extraído para cDNA foi conduzida usando o Kit de Síntese de cDNA *iScript*® (*Bio-Rad Laboratories*, Hercules, CA, EUA), de acordo com as instruções do fabricante. Após a adição de todos os reagentes, as amostras foram vortexadas, e um *spin* foi realizado. Em seguida, as amostras foram mantidas a 25 ºC durante 5 min, aquecidas a 42 ºC

durante 60 min e 85 ºC durante 5 min para que a reação da transcriptase reversa fosse concluída. Após a reação da transcriptase reversa, a concentração de todas as amostras foi ajustada para 10 ng/µL.

Curvas padrão utilizadas em cada corrida de RT-qPCR foram preparadas usando DNA purificado de *S. mutans* UA159 para os testes dos genes de *S. mutans*, de *Streptococcus oralis* ATCC 35037 para ST e de *Streptococcus mitis* ATCC 49456 - NTCC 12261 para BT. Diluições seriadas a partir de 300 a 0,0003 ng (10 vezes) de DNA purificado foram utilizadas como padrões e controles positivos para quantificação relativa de bactérias. Uma curva de amplificação padrão e uma curva de produto de ponto de fusão foram obtidas para cada par de *primers*. Os ensaios de RT-qPCR foram realizados em duplicata no *StepOne® Real Time PCR* (*Applied Biosystems,* Foster City, CA, EUA). As reações foram realizadas usando uma placa de reação de 48 poços *MicroAmpFast Optical* (*Applied Biosystems*, Foster City, CA, EUA) coberta com filme adesivo óptico (*Applied Biosystems*, Foster City, CA, EUA). Os *primers* utilizados neste estudo para a quantificação de SM, ST e BT foram listados na Tabela 4 (BEZERRA *et al*., 2016). Os *primers* utilizados para análise de expressão gênica de *S. mutans* [gtfB e gtfC (FUJIWARA et al., 2002), aguD, nox, fabM e atpD (BEZERRA et al., 2016)] foram listados na Tabela 5. Uma mistura de 5 µL de *Master SYBR® Green* PCR *Master Mi*x (*Applied Biosystems*, Foster City, CA, EUA), 3,4 µL de água sem nuclease, 0,6 µL de cada *primer* F/R 10 µM e 1 µL de cDNA (10 ng/µL) foi adicionada em cada poço de uma placa de 48 poços. As análises finais foram baseadas na média das duas reações. O controle negativo incluiu reações sem o cDNA. As curvas padrão foram usadas para transformar a quantificação dos valores de ciclo para a massa de cDNA amplificado.

Tabela 4 – *Primers* para identificação bacteriana usados na RT-qPCR.

Gene	Bactéria	Sequência do *primer* (5'→ 3')	Tamanho da amplificação (pb)	Número de ciclos	Temperatura de anelamento (ºC)
Sm F5/R4	SM	F: AGCCATGCGCAATCAACAGG R: CGCAACGCGAACATCTTGATCAG	415	40	64
23S rRNA	ST	F: AGCTTAGAAGCAGCTATTCATTC R: GGATACACCTTTCGGTCTCTC	308	40	60
16S rDNA	BT	F: TCCTACGGGAGGCAGCAGT R: GGACTACCAGGGTATCTAATCCTGTT	466	40	57

SM = *S. mutans*; ST = estreptococos totais; BT = bactérias totais; F = *forward*; R = *reverse*. Sm F5/R4: sequência do *primer* retirada de Yano *et al*., (2002); 23S rRNA: sequência do *primer* retirada de Sakaguchi *et al*., (2010); 16S rDNA: sequência do *primer* retirada de Nadkarni *et al*., (2002).

Fonte: retirada do artigo de Bezerra *et al*., (2016).

Tabela 5 – *Primers* para análise da expressão gênica de *S. mutans* usados na RT-qPCR.

Gene	Sequência do *primer* (5'→ 3')	Tamanho da amplificação (pb)	Número de ciclos	Temperatura de anelamento (ºC)
gtfB	F: AGCAATGCAGCCAATCTACAAAT R: ACGAACTTTGCCGTTATTGTCA	98	40	60
gtfC	F: CTCAACCAACCGCCACTGTT R: GGTTTAACGTCAAAATTAGCTGTATTAGC	93	40	60
aguD	F: TGGTGCTGCTCTTGCTAATG R: TAAAAGGACGCGGTGTATCC	188	45	60
nox	F: GGACAAGAATCTGGTGTTGA R: CAATATCAGTCTCTACCTTAGGC	115	45	58
fabM	F: ACTGATTAATGCCAATGGGAAAGTC R: TGCGAACAAGAGATTGTACATCATC	98	45	60
atpD	F: TGTTGATGGTCTGGGTGAAA R: TTTGACGGTCTCCGATAACC	176	45	60

F = *forward*; R = *reverse*. gtfB e gtfC: sequências dos *primers* retiradas de Fujiwara *et al*., (2002); aguD, nox, fabM e atpD: sequências dos *primers* retiradas de Bezerra *et al*., (2016).

Fonte: elaborado pelo autor.

4.8 Análise estatística

Os dados foram tabulados no *Microsoft Exce*l e exportados para o *software Statistical Package for the Social Sciences* (SPSS) versão 20,0 para *Windows,* no qual as análises foram realizadas adotando uma confiança de 95%. Foram expressas as médias e desvio padrão (DP) de cada medida, as quais foram submetidas ao teste de normalidade de Kolmogorov-Smirnov e comparadas por meio do teste de Kruskal-Wallis seguido do pós-teste de Dunn (dados não paramétricos).

5 RESULTADOS

S. mutans foi identificado em todas as amostras de biofilme testadas, sendo quase inexistente em amostras coletadas de superfícies dentárias saudáveis. A Tabela 6 mostra a presença de SM, ST oral e BT nos diferentes grupos, bem como a proporção de *S. mutans* em relação aos estreptococos totais (SM/ST) e em relação à carga bacteriana total (SM/BT). Não foram encontradas diferenças estatisticamente significantes na presença de BT (p = 0,222) e ST (p = 0,090) quando comparadas aos diferentes grupos, mas a presença de *S. mutans* foi significativamente maior no biofilme coletado nas lesões de cárie mais severas (grupo CDA) (p = 0,001). A proporção de SM relacionada à quantidade de ST (p <0,001) e BT (p <0,001) foi significativamente maior no biofilme de lesões dentinárias (grupos CDA e CDI).

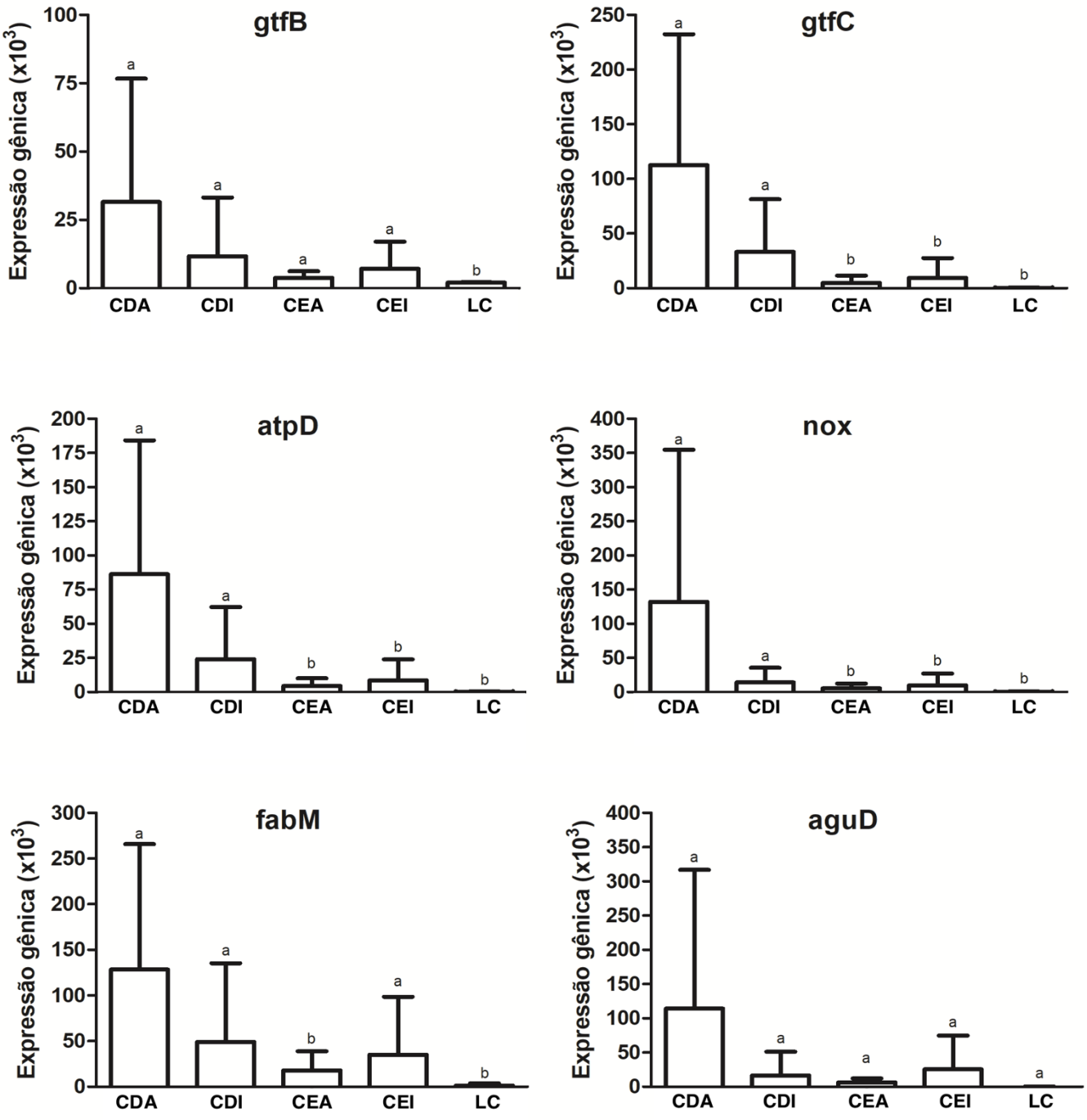
A Figura 1 mostra o perfil de expressão dos genes de *S. mutans* testados em amostras de biofilme de pacientes livres de cárie e com lesões cariosas ativas e inativas em esmalte e dentina. O gene gtfB foi mais expresso (p = 0,023) nos grupos cariados (grupos CDA, CDI, CEA e CEI) quando comparados ao grupo livre de cárie (grupo LC) enquanto os genes gtfC, atpD e nox foram expressos em níveis mais elevados (p = 0,001; p = 0,002 e p = 0,005) nos grupos de cárie dentinária (CDA e CDI) quando comparados aos grupos de cárie em esmalte (CEA e CEI) e livre de cárie (LC). O gene fabM foi mais expresso (p = 0,004) nos grupos CDA, CDI e CEI do que nos grupos CEA e LC. Não foram encontradas diferenças estatisticamente significativas na expressão do gene aguD entre os diferentes grupos (p = 0,209).

Tabela 6 – Quantificação de bactérias metabolicamente ativas (ng/μL) e a proporção de *S. mutans* em relação ao total de estreptococos e em relação à carga bacteriana total em amostras de biofilme dentário coletadas de pacientes livres de cárie e de lesões de cárie ativas e inativas de esmalte e dentina. Teste de Kruskal-Wallis/Dunn; letras diferentes = p<0,05 (média±DP).

Grupos	BT	ST	SM	SM/ST	SM/BT
CDA	484,31±438,39 [a]	40,02±64,70 [a]	7,30±7,48 [a]	62,03±128,31 [a]	1,98±2,39 [a]
CDI	116,03±160,40 [a]	3,86±4,76 [a]	1,93±3,14 [b]	108,43±140,70 [a]	16,42±37,40 [a]
CEA	273,38±266,28 [a]	20,81±13,05 [a]	0,28±0,36 [b]	1,85±2,11 [b]	0,15±0,18 [b]
CEI	271,69±258,40 [a]	22,61±23,86 [a]	0,82±1,58 [b]	4,58±7,08 [c]	0,45±0,77 [b]
LC	587,10±928,29 [a]	19,68±18,25 [a]	0,00±0,00 [b]	0,01±0,01 [c]	0,00±0,00 [c]
p-Valor	0,222	0,090	**0,001**	**<0,001**	**<0,001**

Fonte: elaborado pelo autor.

Figura 6 – Perfil de expressão dos genes testados de *S. mutans* em amostras de biofilme dentário coletadas de pacientes livres de cárie e de lesões de cárie ativas e inativas de esmalte e dentina.



Fonte: dados da pesquisa.

6 DISCUSSÃO

Os resultados indicam que *S. mutans* é parte da microbiota viável do biofilme sobre lesões de cárie de esmalte e dentina ativas e inativas de crianças. A análise da expressão gênica sugere que o perfil metabólico de *S. mutans* pode diferir de acordo com a severidade da lesão, mas não pelo *status* de atividade das lesões cariosas. Especificamente, os níveis de expressão do gene gtfB foram maiores em todos os grupos cariados, os dos genes gtfC, atpD e nox nas lesões de cárie dentinária, os do gene fabM nos grupos de cárie dentinária e no grupo de cárie de esmalte inativa quando comparados ao grupo livre de cárie; não houve diferença estatisticamente significativa para a expressão do gene aguD entre os grupos. Apesar da evidência da presença de *S. mutans* em todos os tipos de lesões cariosas, sua virulência pode ser afetada por mudanças ambientais (BEZERRA *et al*., 2016; MOYE *et al*., 2014; WEN *et al*., 2010).

S. mutans esteve presente em todas as amostras testadas neste estudo, tendo a sua quantificação maior no biofilme coletado nas lesões de cárie mais severas (grupo CDA) e quase nula em amostras coletadas de superfícies dentárias saudáveis (grupo LC). A proporção de SM relacionada à quantidade de ST e BT foi significativamente maior no biofilme quanto maior a severidade das lesões cariosas (grupos CDA e CDI). Resultado semelhante foi encontrado por Bachtiar & Bachtiar, (2018), no entanto, os autores quantificaram tanto as bactérias viáveis quanto as não viáveis, já que extraíram DNA para a realização da qPCR.

Um dos principais fatores de virulência do *S. mutans* é a sua capacidade de formar biofilme. Essa capacidade advém principalmente pela ação das suas GTFs. Existem vários fatores que podem influenciar a transcrição, a tradução e a secreção dos genes que codificam as GTFs, como a disponibilidade e a fonte de carboidrato, o pH do ambiente e a taxa ou fase de crescimento do *S. mutans* (BOWEN & KOO, 2011; FUJIWARA *et al*., 2002; KLEIN *et al*., 2009). A expressão dos genes gtfB e gtfC é induzida em resposta à acidificação do meio ou em resposta à presença de excesso de carboidrato metabolizável como a sacarose (LI & BURNE, 2001; BOWEN & KOO, 2011). Poucos estudos avaliaram e compararam a expressão dos genes gtfB e gtfC em isolados clínicos (FUJIWARA *et al*., 1998; STIPP *et al*., 2008), e este é o primeiro estudo que avaliou comparativamente a expressão desses dois genes em amostras clínicas de biofilme sobre lesões de cárie de esmalte e dentina ativas e inativas.

A síntese simultânea de glucanos pelas enzimas GtfB e GtfC é essencial para o estabelecimento de uma matriz extracelular que aumenta a coerência das células bacterianas e a aderência à superfície dentária, permitindo a formação de aglomerados celulares altamente

organizados e densos conhecidos como microcolônias (KOO *et al*., 2010; TAMESADA *et al*., 2004; XIAO & KOO, 2010). Usando um modelo *in vitro*, Koo *et al*., (2010) mostraram que uma cepa mutante de *S. mutans* UA159 que não expressa os genes gtfB e gtfC formou um biofilme deficiente com um acúmulo mínimo de polissacarídeos e células bacterianas na superfície de hidroxiapatita revestida com saliva quando comparada a uma cepa parental, e a deleção de cada um desses genes separadamente resultou em diferentes padrões de acumulação bacteriana nas superfícies dentárias. A cepa mutante sem expressão de gtfB formou um biofilme uniformemente ligado e acumulado na superfície, mas não formou microcolônias. Em contraste, a deleção do gtfC não diminuiu a capacidade da cepa mutante de formar microcolônias, embora houvesse menos microcolônias do que na cepa parental UA159. Imagens transversais dos biofilmes formados pela cepa parental e pela mutante sem expressão de gtfC mostraram que as microcolônias estavam envoltas por polissacarídeos, e que estes estavam preenchendo os espaços entre as microcolônias. Essas observações indicam que a presença do gene gtfB é crítica para o agrupamento de células bacterianas e para uma maior estabilização e desenvolvimento de microcolônias.

A primeira GTF detectada na película adquirida é a GtfC, por possuir uma maior afinidade com a hidroxiapatita que as outras GTFs (REN *et al*., 2016). Ela sintetiza glucanos solúveis e insolúveis: os insolúveis montam as camadas iniciais de PEC na superfície da hidroxiapatita que vão fornecer sítios de ligação aprimorados para colonização e acumulação de *S. mutans* (NGUYEN *et al*., 2014), e os solúveis, por serem facilmente digeridos, podem ser usados como uma fonte de reserva de energia e contribuir para a sobrevivência do microrganismo na placa cariogênica (BOWEN & KOO, 2011; PAES LEME *et al*., 2006). Esse fato pode explicar a maior expressão do gene gtfC nos grupos de cárie dentinária (CDA e CDI), no presente estudo, quando comparados aos grupos de cárie de esmalte (CEA e CEI) e LC.

A GtfB, codificada pelo gene gtfB, liga-se a muitos microrganismos orais, como *Actinomyces viscosus* e *Lactobacillus casei*, e, na presença de sacarose, forma uma matriz de polissacarídeos insolúvel, rígida e coesa. Além disso, é a principal responsável pelo desenvolvimento das microcolônias (BOWEN & KOO, 2011; KOO *et al*., 2010; NGUYEN *et al*., 2014). Neste estudo, o gene gtfB estava mais expresso nos grupos cariados (grupos CDA, CDI, CEA e CEI) quando comparados ao grupo LC, resultado semelhante ao encontrado por Bachtiar & Bachtiar, (2018) que compararam um grupo cariado com um livre de cárie sem levar o status da atividade de cárie em consideração. Essa maior expressão de gtfB sugere a relação desse gene com a presença de biofilme.

Além da capacidade de formar biofilme através da montagem de uma matriz insolúvel de PEC bem-estruturada com células bacterianas que gera microambientes acídicos e protetores dentro das microcolônias (KLEIN *et al*., 2012; XIAO *et al*., 2012), *S. mutans* produz ácido (acidogenicidade) e possui vários mecanismos para lidar com esses microambientes acídicos (aciduricidade) tanto no citoplasma quanto fora das células (KLEIN *et al*., 2012; LEMOS *et al*., 2008). A expressão de genes relacionados a esses fatores de virulência, como atpD, nox, fabM e aguD, também foi analisada neste estudo.

O gene atpD foi mais expresso no biofilme de lesões mais severas (cárie dentinária – grupos CDA e CDI) quando comparado ao biofilme de lesões iniciais (cárie de esmalte – grupos CEA e CEI) e ao biofilme de superfícies dentárias saudáveis (pacientes livres de cárie – grupo LC). Ele codifica a proteína transportadora de membrana denominada F_1F_0-ATPase, que é considerada um fator determinante para a tolerância ao ácido pelo *S. mutans*, já que, durante a glicólise, bombeia os prótons H^+ para fora da célula, prevenindo a acidificação do citoplasma que inibiria enzimas intracelulares. Além disso, em condições adversas, também gera ATP para o crescimento e a persistência do *S. mutans* (BEZERRA *et al*., 2016; LEMOS *et al*., 2005; NGUYEN *et al*., 2014). O estudo de Klein *et al*., (2012) mostrou que esse gene é altamente expresso pelo *S. mutans* em biofilme multiespécie quando o pH está baixo.

Além do sistema F_1F_0-ATPase, o sistema agmatina desiminase (AgDS), codificado por alguns genes, como o aguD, também é capaz de aumentar a competitividade e aptidão de *S. mutans*, contribuindo para a persistência e a patogênese desse organismo (XU *et al*., 2011). Esse sistema converte agmatina, um inibidor do crescimento de *S. mutans*, em putrescina, amônia e CO_2. A produção de amônia aumenta o pH citoplasmático e gera ATP que pode ser usado para o crescimento bacteriano ou para extrusão de prótons H^+ (GRISWOLD *et al*., 2006). No presente estudo, não foi verificada nenhuma diferença estatisticamente significativa da expressão do gene aguD nos diferentes grupos testados, que pode ser advinda pela mesma condição de exposição dos biofilmes à sacarose ou carboidratos fermentáveis, já que todas as crianças estavam sem comer há pelo menos duas horas.

O baixo pH desencadeia mudanças na composição de ácidos graxos da membrana plasmática e também afeta a permeabilidade da membrana aos prótons H^+. O gene fabM é responsável pela síntese de ácidos graxos monoinsaturados e a sua expressão é essencial para a sobrevivência de *S. mutans* em baixo pH (FOZO & QUIVEY, 2004b; KLEIN *et al*., 2012). O estudo de Fozo & Quivey (2004b) mostrou que uma cepa mutante de *S. mutans* sem a expressão de fabM apresentou uma membrana com composição alterada, gerando efeitos significativos sobre a glicólise, fosfotransferase, permeabilidade da membrana e atividade da F_1F_0-ATPase.

Assim, a alteração da composição dos ácidos graxos da membrana tem um impacto significativo na capacidade de sobrevivência ácida, bem como na atividade enzimática do *S. mutans*, o que foi comprovado também por Klein *et al*., (2012). Os resultados do presente estudo corroboram com essa implicação, já que o gene fabM foi mais expresso nos grupos de maior perda de tecido dentário (CDA e CDI).

O gene nox codifica a enzima NADH oxidase, que é uma das principais contribuintes para a resposta ao estresse oxidativo (BEZERRA *et al*., 2016). Em *S. mutans*, uma espécie anaeróbia facultativa, essa enzima age para reduzir o nível de O_2 convertendo-o em H_2O ou H_2O_2 através da oxidação de NADH em NAD^+. Essa reação auxilia na metabolização eficaz de substratos de açúcar para ácido lático, na via glicolítica, o que leva a uma acidificação da placa dentária (LEMOS *et al*., 2005). Um estudo realizado por Derr *et al*., (2012) mostrou que a perda da enzima NADH oxidase afetou a adaptação e a sobrevivência de *S. mutans* sob condições altamente ácidas, indicando uma forte associação entre as respostas de estresse oxidativo e ácido. No presente estudo, essa associação também foi verificada, já que os genes atpD, fabM e nox apresentaram níveis de expressão semelhantes, sendo mais expressos nos grupos de cárie dentinária (CDA e CDI).

Como o presente estudo foi realizado com amostras de biofilme *in vivo* coletadas de superfícies dentárias saudáveis ou de lesões de cárie, e o *S. mutans* sendo um dos microrganismos determinantes para a ocorrência das lesões, esperava-se que quanto maior fosse a severidade da doença maior seria a expressão dos genes de virulência, e que essa expressão seria diferente de acordo com a atividade da lesão. Em parte, isso foi mostrado no estudo, já que os genes gtfC, atpD, fabM e nox foram mais expressos nos grupos de dentina cariada, mas a não diferença de expressão dos genes entre os grupos de cárie ativa e inativa foi um fato inesperado. Com isso, os resultados sugerem que a atividade desses genes está relacionada com a severidade das lesões cariosas, mas que outros genes do próprio *S. mutans* ou de outros microrganismos podem estar relacionados com atividade das lesões cariosas.

7 CONCLUSÃO

Os achados desta pesquisa revelaram que o *S. mutans* faz parte da comunidade microbiana viável do biofilme de lesões ativas e inativas de esmalte e dentina. A expressão elevada dos genes gtfC, atpD, fabM e nox em grupos de cárie dentinária sugere a relação desses genes com a progressão de lesões cariosas, e a maior expressão do gene gtfB no biofilme de todos os grupos cariados sugere a relação desse gene com a presença de biofilme.

REFERÊNCIAS

AAS, J.A.; PASTER, B.J., STOKES, L.N.; OLSEN, I.; DEWHIRST, F.E. Defining the normal bacterial flora of the oral cavity. *Journal of Clinical Microbiology*, v. 43, n. 11, p. 5721-5732, 2005.

BACHTIAR, E.W.; BACHTIAR, B.M. Relationship between *Candida albicans* and *Streptococcus mutans* in early childhood caries, evaluated by quantitative PCR. *F1000Research*, v. 7, p. 1645-1657, 2018.

BAELUM, V.; HINTZE, H.; WENZEL, A.; DANIELSEN, B.; NYVAD, B. Implications of caries diagnostic strategies for clinical management decisions. *Community Dentistry and Oral Epidemiology*, v. 40, n. 3, p. 257-266, 2012.

BAKRY, A.S.; TAKAHASHI, H.; OTSUKI, M.; TAGAMI, J. Evaluation of new treatment for incipient enamel demineralization using 45S5 bioglass. *Dental Materials*, v. 30, n. 3, p. 314-320, 2014.

BANAS, J.A. Virulence properties of *Streptococcus mutans*. *Frontiers in Bioscience*, v. 9, p. 1267-1277, 2004.

BARATIERI, L.N. *Procedimentos preventivos e restauradores*. Rio de Janeiro: Editora Santos, 1989.

BECKER, M.R. *et al*. Molecular analysis of bacterial species associated with childhood caries. *Journal of Clinical Microbiology,* v. 40, n. 3, p.1001–1009, 2002.

BERKOWITZ, R.J. Acquisition and transmission of *mutans streptococci*. *Journal of the California Dental Association*, v. 31, n. 2, p. 135-138, 2003.

BEZERRA, D.S.; STIPP, R.N.; NEVES, B.G.; GUEDES, S.F.; NASCIMENTO, M.M.; RODRIGUES, L.K. Insights into the virulence traits of *Streptococcus mutans* in dentine carious lesions of children with early childhood caries. *Caries Research*, v. 50, n. 3, p. 279-287, 2016.

BOWEN, W.H.; KOO, H. Biology of *Streptococcus mutans*-derived glucosyltransferases: role in extracellular matrix formation of cariogenic biofilms. *Caries Research*, v. 45, n. 1, p. 69-86, 2011.

BOWEN, W.H. Dental caries – not just holes in teeth! A perspective. *Molecular Oral Microbiology*, v. 31, p. 228-233, 2016.

BURNE, R.A.; MARQUIS, R.E. Alkali production by oral bacteria and protection against dental caries. *FEMS Microbiology Letters*, v. 193, n. 1, p. 1–6, 2000.

CONRADS, G.; ABOUT, I. Pathophysiology of dental caries. *Monographs in Oral Science*, v. 27, p. 1-10, 2018.

CURY, J.A.; TENUTA, L.M. Enamel remineralization: controlling the caries disease or

treating early caries lesions? *Brazilian Oral Research*, v. 23, s. 1, p. 23-30, 2009.

DERR, A.M.; FAUSTOFERRI, R.C.;BETZENHAUSER, M.J.;GONZALEZ, K.; MARQUIS, R.E.; QUIVEY, R.G. Mutation of the NADH oxidase gene (nox) reveals an overlap of the oxygen- and acid-mediated stress responses in *Streptococcus mutans*. *Applied and Environmental Microbiology*, v. 78, n. 4, p. 1215-1227, 2012.

DIGE, I.; GRØNKJÆR, L.; NYVAD, B. Molecular studies of the structural ecology of natural occlusal caries. *Caries Research*, v. 48, n. 5, p. 451-460, 2014.

DO, T.; SHEEHY, E.C.; MULLI, T.; HUGHES, F.; BEIGHTON, D. Transcriptomic analysis of three Veillonella spp. present in carious dentine and in the saliva of caries-free individuals. *Frontiers in Cellular and Infection Microbiology*, p. 5-25, 2015.

FEJERSKOV, O.; KIDD, E.A.M. Dental caries: the disease and its clinical management. 2nd edition. Oxford: Blackwell Munksgaard, 2008, p. 209-230.

FITZGERALD, R.J.; ADAMS, B.O.; SANDHAM, H.J.; ABHYANKAR, S. cariogenicity of a lactate dehydrogenase deficient mutant of *Streptococcus mutans* serotype c in gnotobiotic rats. *Infection and Immunity*, v. 57, n. 3, p. 823-826, 1989.

FLEMMING, H.C; WINGENDER, J. The biofilm matrix. *Nature Reviews Microbiology*, v. 8, n. 9, p. 623-633, 2010.

FLOREZ SALAMANCA, E.J.; KLEIN, M.I. Extracellular matrix influence in *Streptococcus mutans* gene expression in a cariogenic biofilm. *Molecular Oral Microbiology*, v. 33, n. 2, p. 181-193, 2018.

FOZO, E.M.; QUIVEY, R.G. Shifts in the membrane fatty acid profile of *Streptococcus mutans* enhance survival in acidic environments. *Applied and Environmental Microbiology*, v. 70, n. 2, p. 929–936, 2004a.

FOZO, E.M.; QUIVEY, R.G. The fabM gene product of *Streptococcus mutans* is responsible for the synthesis of monounsaturated fatty acids and is necessary for survival at low pH. *Journal of Bacteriology*, v. 186, n. 13, p. 4152–4158, 2004b.

FUJIWARA, T. *et al*. Molecular analyses of glucosyltransferase genes among strains of *Streptococcus mutans*. *FEMS Microbiology Letters*, v. 161, n. 2, p. 331-336, 1998.

FUJIWARA, T.; HOSHINO, T.; OOSHIMA, T.; HAMADA, S. Differential and quantitative analyses of mRNA expression of glucosyltransferases from *Streptococcus mutans* MT8148. *Journal of Dental Research*, v. 81, n. 2, p. 109-113, 2002.

GARCIA, S.S.; DU, Q.; WU, H. *Streptococcus mutans* copper chaperone, CopZ, is critical for biofilm formation and competitiveness. *Molecular Oral Microbiology*, v. 31, n. 6, p. 515-525, 2016.

GRISWOLD, A.R.; CHEN, Y.Y.; BURNE, R.A. Analysis of an agmatine deiminase gene cluster in *Streptococcus mutans* UA159. *Journal of Bacteriology*, v. 186, n. 6, p. 1902–1904, 2004.

GRISWOLD, A.R.; JAMESON-LEE, M.; BURNE, R.A. Regulation and physiologic significance of the agmatine deiminase system of *Streptococcus mutans* UA159. *Journal of Bacteriology,* v. 188, n. 3, p. 834–841, 2006.

GUILBERT, J.J. The world health report 2002 - reducing risks, promoting healthy life. Education for health (Abingdon), v. 16, n. 2, p. 230-230, 2003.

HIGUCHI, M. *et al*. Functions of two types of NADH oxidases in energy metabolism and oxidative stress of *Streptococcus mutans*. *Journal of Bacteriology*, v. 181, n. 19, p. 5940-5947, 1999.

HILMAN, J.D.; CHEN, A.; SNOEP, J.L. Genetic and physiological analysis of the lethal effect of L-(+)-lactate dehydrogenase deficiency in *Streptococcus mutans*: complementation by alcohol dehydrogenase from *Zymomonas mobilis*. *Infection and Immunity*, v. 64, n. 10, p. 4319-4323, 1996.

HILMAN, J.D. Genetically modified *Streptococcus mutans* for the prevention of dental caries. *Antonie Van Leeuwenhoek*, v. 82, n. 1-4, p. 361-366, 2002.

ISMAIL, A.I. *et al*. The International Caries Detection and Assessment System (ICDAS): an integrated system for measuring dental caries. *Community Dentistry Oral Epidemiology*, v. 35, n. 3, p.170–178, 2007.

KLEIN, M.I., DUARTE, S.; XIAO, J.; MITRA, S.; FOSTER, T.H.; KOO, H. Structural and molecular basis of the role of starch and sucrose in *Streptococcus mutans* biofilms development. *Applied and Environmental Microbiology*, v. 75, n. 3, p. 837-841, 2009.

KLEIN, M.I.; XIAO, J.; LU, B.; DELAHUNTY, C.M.; YATES, J.R.; KOO, H. *Streptococcus mutans* protein synthesis during mixed-species biofilm development by high-throughput quantitative proteomics. *PLoS One*, v. 7, n. 9, e45796, 2012.

KÖHLER, B.; BIRKHED, D.; OLSSON, S. Acid production by human strains of *Streptococcus mutans* and *Streptococcus sobrinus*. *Caries Research*, v. 29, n. 5, p. 402-406, 1995.

KOLENBRANDER, P.E.; PALMER, R.J.; PERIASAMY, S.; JAKUBOVICS, N.S. Oral multispecies biofilm development and the key role of cell-cell distance. *Nature Reviews Microbiology*, v. 8, n. 7, p. 471-480, 2010.

KOO, H.; XIAO, J.; KLEIN, M.I.; JEON, J.G. Exopolysaccharides produced by *Streptococcus mutans* glucosyltransferases modulate the establishment of microcolonies within multispecies biofilms. *Journal of Bacteriology*, v. 192, n. 12, p. 3024-3032, 2010.

KRETH, J.; ZHU, L.; MERRITT, J.; SHI, W.; QI, F. Role of sucrose in the fitness of *Streptococcus mutans*. *Oral Microbiology Immunology*, v. 23, n. 3, p. 213–219, 2008.

LEMOS, J.A.; ABRANCHES, J.; BURNE, R.A. Responses of cariogenic *streptococci* to environmental stresses. *Current Issues in Molecular Biology*, v. 7, n. 1, p. 95-107, 2005.

LEMOS, J.A.; BURNE, R.A. A model of efficiency: stress tolerance by *Streptococcus mutans*. *Microbiology*, v. 154, n. 11, p. 3247–3255, 2008.

LI, Y.; BURNE, R.A. Regulation of the gtfBC and ftf genes of *Streptococcus mutans* in biofilms in response to pH and carbohydrate. *Microbiology*, v. 147, n. 10, p. 2841-2848, 2001.

LIU, Y.; ZHANG, X.; WANG, Y.; CHEN, F.; YU, Z.; CHEN, S.; GUO, M. Effect of citrus lemon oil on growth and adherence of *Streptococcus mutans*. *World Journal of Microbiology and Biotechnology*, v. 29, n. 7, p. 1161-1167, 2013.

LOESCHE, W. Role of *Streptococcus mutans* in human dental decay. *Microbiological Reviews*, v. 50, n. 4, p. 353-380, 1986.

LYNCH, D.J.; MICHALEK, S.M.; ZHU, M.; DRAKE, D.; QIAN, F.; BANAS, J.A. Cariogenicity of *Streptococcus mutans* glucan-binding protein deletion mutants. *Oral Health and Dental Management*, v. 12, n. 4, p. 191-199, 2013.

MARSH, P.D. Microbiology of dental plaque biofilms and their role in oral health and caries. *Dental Clinics of North America*, v. 54, n. 3, p. 441-454, 2010.

MARSH, P.D. Dental plaque as a biofilm and a microbial community - implications for health and disease. *BMC Oral Health*, v. 15, s. 14, 2006.

MATTOS-GRANER, R.O.; KLEIN, M.I.; SMITH, D.J. Lessons learned from clinical studies: roles of *Mutans Streptococci* in the pathogenesis of dental caries. *Current Oral Health Reports,* v. 1, n. 1, p. 70-78, 2014.

MATTOS-GRANER, R.O.; SMITH, D.J.; KING, W.F.; MAYER, M.P. Water-insoluble glucan synthesis by *mutans streptococcal* strains correlates with caries incidence in 12- to 30-month-old children. *Journal of Dental Research*, v. 79, n. 6, p. 1371-1377, 2000.

McNEILL, K.; HAMILTON, I.R. Acid tolerance response of biofilm cells of *Streptococcus mutans*. *FEMS Microbiology Letters*, v. 221, n. 1, p. 25-30, 2003.

MOYE, Z.D.; ZENG, L.; BURNE, R.A. Modification of gene expression and virulence traits in *Streptococcus mutans* in response to carbohydrate availability. *Applied and Environmental Microbiology*, v. 80, n. 3, p. 972-985, 2014.

MUNSON, M.A.; BANERJEE, A.; WATSON, T.F.; WADE, W.G. Molecular analysis of the microflora associated with dental caries. *Journal of Clinical Microbiology*, v. 42, n. 7, p. 3023–3029, 2004.

NADKARNI, M.A.; MARTIN, F.E.; JACQUES, N.A.; HUNTER, N. Determination of bacterial load by real-time PCR using a broad-range (universal) probe and primers set. *Microbiology*, v. 148, n. 1, p. 257-266, 2002.

NASCIMENTO, M.M.; BURNE, R.A. Caries prevention by arginine metabolism in oral biofilms: translating science into clinical success. *Current Oral Health Reports*, v. 1, p. 79–85, 2014.

NEUHAUS, K.W.; LUSSI, A. Carious lesion diagnosis: methods, problems, thresholds. *Monographs in Oral Science*, v. 27, p. 24-31, 2018.

NEVES, B.G.; STIPP, R.N.; BEZERRA, D.D.S.; GUEDES, S.F.F.; RODRIGUES, L.K.A. Quantitative analysis of biofilm bacteria according to different stages of early childhood caries. *Archives of Oral Biology*, v. 96, p. 155-161, 2018.

NGUYEN, P.T.M.; FALSETTA, M.L.; HWANG, G.; GONZALEZ-BEGNE, M.; KOO, H. α -Mangostin disrupts the development of *Streptococcus mutans* biofilms and facilitates its mechanical removal. *PLoS ONE*, v. 9, n. 10: e111312, 2014.

NYVAD, B.; MACHIULSKIENE, V.; BAELUM, V. Construct and predictive validity of clinical caries diagnostic criteria assessing lesion activity. *Journal of Dental Research*, v. 82, n. 2, p. 117-122, 2003.

NYVAD, B.; MACHIULSKIENE, V.; BAELUM, V. Reliability of a new caries diagnostic system differentiating between active and inactive caries lesions. *Caries Research*, v. 33, n. 4, p. 252-260, 1999.

NYVAD, B.; CRIELAARD, W.; MIRA, A.; TAKAHASHI, N.; BEIGHTON, D. Dental caries from a molecular microbiological perspective. *Caries Research*, v. 47, n. 2, p. 89-102, 2013.

PAES LEME, A.F.; KOO, H.; BELLATO, C.M.; BEDI, G.; CURY, J.A. The role of sucrose in cariogenic dental biofilm formation- new insight. *Journal of Dental Research*, v. 85, n. 10, p. 878-887, 2006.

PAPAIOANNU, W.; GIZANI, S.; HAFFAJEE, A.D.; QUIRYNEN, M.; MAMAI-HOMATA, E.; PAPAGIANNOULIS, L. The microbiota on different oral surfaces in healthy children. *Oral Microbiology and Immunology*, v. 24, n. 3, p. 183-189, 2009.

PASTER, B.J. *et al*. Bacterial diversity in human subgingival plaque. *Journal of Bacteriology*, v. 183, n. 12, p. 3770–3783, 2001.

PITTS, N.B. Modern concepts of caries measurement. *Journal of Dental Research*, v. 83, p. 43-47, 2004.

REN, Z. *et al*. Molecule targeting glucosyltransferase inhibits *Streptococcus mutans* biofilm formation and virulence. *Antimicrobial Agents and Chemotherapy*, v. 60, n. 1, p. 126-135, 2015.

SAKAGUCHI, S. *et al*. Bacterial rRNA-targeted reverse transcription-PCR used to identify pathogens responsible for fever with neutropenia. *Journal of Clinical Microbiology*, v. 48, n. 5, p. 1624-1628, 2010.

SOET, J.J.; NYVAD, B.; KILIAN, M. Strain-related acid production by oral *streptococci*. *Caries Research*, v. 34, n. 6, p. 486-490, 2000.

STIPP, R.N.; GONÇALVES, R.B.; HÖLFKING, J.F.; SMITH, D.J.; MATTOS-GRANER, R.O. Transcriptional analysis of gtfB, gtfC, and gbpB and their putative response regulators in several isolates of *Streptococcus mutans*. *Oral Microbiology Immunology*, v. 23, n. 6, p. 466-

473, 2008.

TAKAHASHI, N.; NYVAD, B. The role of bacteria in the caries process: ecological perspectives. *Journal of Dental Research*, v. 90, n. 3, p. 294-303, 2011.

TAMASADA, M.; KAWABATA, S.; FUJIWARA, T.; HAMADA, S. Synergistic effects of *streptococcal* glucosyltransferases on adhesive biofilm formation. Journal of Dental Research, v. 83, n. 11, p. 874-879, 2004.

TENUTA, L.M.A.; CURY, J.A. Fluoride: its role in dentistry. *Brazilian Oral Research*, v. 24, n. 1, p.9-17, 2010.

THOMAS, R.Z.; ZIJNGE, V.; CIÇEK, A.; DE SOET, J.J.; HARMSEN, H.J.; HUYSMANS, M.C. Shifts in the microbial population in relation to in situ caries progression. *Caries Research*, v. 46, n. 5, p. 427-432, 2012.

VISZWAPRIYA, D.; SUBRAMENIUM, G.A.; RADHIKA, S.; PANDIAN, S.K. Betulin inhibits cariogenic properties of *Streptococcus mutans* by targeting vicRK and gtf genes. *Antonie Van Leeuwenhoek*, v. 110, n. 1, p. 153-165, 2017.

WEN, Z.T.; YATES, D.; AHN, S.J.; BURNE, R.A. Biofilm formation and virulence expression by *Streptococcus mutans* are altered when grown in dual species model. *BMC Microbiology*, v. 10, n. 1, p. 111-119, 2010.

WILKINS, J.C.; HOMER, K.A.; BEIGHTON, D. Analysis of *Streptococcus mutans* proteins modulated by culture under acidic conditions. *Applied and Environmental Microbiology*, v. 68, n. 5, p. 2382-2390, 2002.

XIAO, J. *et al*. The exopolysaccharide matrix modulates the interaction between 3D architecture and virulence of a mixed-species oral biofilm. *PLoS Pathogens*, v. 8, n.4, e1002623, 2012.

XIAO, J.; KOO, H. Structural organization and dynamics of exopolysaccharide matrix and microcolonies formation by *Streptococcus mutans* in biofilms. *Journal of Applied Microbiology*, v. 108, n. 6, p. 2103-2113, 2010.

XU, X.; ZHOU, X.D.; WU, C.D. The tea catechin epigallocatechin gallate suppresses cariogenic virulence factors of *Streptococcus mutans*. *Antimicrobial Agents Chemotherapy*, v. 55, n. 3, p. 1229-1236, 2011.

YANO, A.; KANEKO, N.; IDA, H.; YAMAGUCHI, T.; HANADA, N. Real-time PCR for quantification of *Streptococcus mutans*. *FEMS Microbiology Letters*, v. 217, n. 1, p. 23–30, 2002.

YIN, W. *et al*. Oral health status in Sichuan Province: findings from the oral health survey of Sichuan, 2015–2016. *International Journal of Oral Science*, v. 9, n. 1, p. 10–15, 2017.

ANEXO A – PARECER DE APROVAÇÃO DO COMITÊ DE ÉTICA EM PESQUISA

UFC - UNIVERSIDADE FEDERAL DO CEARÁ /

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: EXPRESSÃO DE GENES DE VIRULÊNCIA DE Streptococcus mutans EM BIOFILME IN VIVO DE LESÕES CARIOSAS DE ESMALTE E DENTINA

Pesquisador: Lidiany Karla Azevedo Rodrigues

Área Temática:

Versão: 2

CAAE: 03019218.1.0000.5054

Instituição Proponente: UNIVERSIDADE FEDERAL DO CEARÁ

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 3.092.796

Apresentação do Projeto:

Uma melhor compreensão dos diversos mecanismos de virulência e do papel ecológico de S. mutans no biofilme de lesões cariosas ativas e inativas certamente ajudará no desenvolvimento de novas estratégias de prevenção da cárie. Portanto, o objetivo deste estudo será investigar o perfil de expressão de genes selecionados que são relacionados com as principais características de virulência do S. mutans, como gtfB e gtfC (adesão), atpD, nox, fabM, aguD (acidogenicidade e aciduricidade), em biofilme in vivo de lesões cariosas ativas e inativas de esmalte e dentina. Para isso amostras em cDNA armazenadas pertencentes a 5 grupos serão utilizadas: livres de cárie (LC), cárie de esmalte ativa (CEA), cárie de esmalte inativa (CEI), cárie dentinária ativa (CDA) e cárie dentinária inativa (CDI)

Objetivo da Pesquisa:

Investigar o perfil de expressão de genes selecionados que são relacionados com as principais características de virulência do S. mutans, como gtfB e gtfC (adesão), atpD, nox, fabM e aguD (acidogenicidade e aciduricidade), em biofilme in vivo de lesões cariosas ativas e inativas de esmalte e dentina.

Avaliação dos Riscos e Benefícios:

Riscos:

Riscos da pesquisa: as reações de qPCR não funcionarem devido ao tempo de armazenamento das

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amostras.

Riscos aos pacientes: Como se tratou de uma investigação com participação de seres humanos, haveria a possibilidade de trazer riscos, sobretudo a perda do sigilo das informações dos participantes. Apesar disso, o responsável pelo participante teve assegurado o direito a ressarcimento ou indenização no caso de quaisquer danos eventualmente produzidos pela pesquisa a criança sob sua responsabilidade. A realização da pesquisa não acarretou em nenhum prejuízo no andamento escolar dos participante da pesquisa ou em seu tratamento odontológico.

Benefícios:

Benefícios da pesquisa: como Streptococcus mutans é considerado o microrganismo mais cariogênico da microbiota bucal e está relacionado a diferentes formas de progressão da doença cárie uma melhor compreensão dos diversos mecanismos de virulência e do papel ecológico desse microrganismo, através da avaliação da expressão de genes relacionados a formação de biofilme (gtfB e gtfC), e relacionados a sua capacidade de produção de ácido e manutenção em ambientes ácidos (atpD, nox, fabM e aguD), no biofilme in vivo de lesões cariosas ativas e inativas de esmalte e dentina certamente ajudará no desenvolvimento de novas estratégias de prevenção da cárie.

Benefícios aos participantes: Os participantes da pesquisa, bem como seus responsáveis, participaram de atividade de educação em saúde bucal, na qual se tratou a respeito da cárie dentária e emprego de corretas técnicas para prevenção desta doença. As crianças foram examinadas e, havendo necessidade de tratamento, foram tratadas na Clínica de Dentística da Faculdade de Odontologia – Campus Fortaleza.

Comentários e Considerações sobre a Pesquisa:

Pesquisa laboratorial retrospectiva, na qual utilizará amostras em cDNA armazenadas pertencentes a 5 grupos serão utilizadas: livres de cárie (LC), cárie de esmalte ativa (CEA), cárie de esmalte inativa (CEI), cárie dentinária ativa (CDA) e cárie dentinária inativa (CDI). Reações em cadeia da polimerase quantitativa em tempo real da transcrição reversa (RT-qPCR) serão executadas para todas as amostras. Serão utilizadas amostras em cDNA que foram armazenadas no freezer -80 °C do Laboratório de Pesquisa do Programa de Pós-Graduação em Odontologia após a realização do projeto intitulado em “Determinação de indicadores de risco relacionados a diferentes estágios de cárie precoce da infância” aprovado sob protocolo COMEPE-UFC no. 158/2011. As amostras armazenadas são oriundas da extração de RNA bacteriano e conversão em cDNA de amostras biológicas (biofilme dentário), mas somente o cDNA bacteriano foi armazenado e será utilizado neste estudo.

Considerações sobre os Termos de apresentação obrigatória:

Todos os termos foram devidamente apresentados. Conforme solicitado a pesquisadora esclareceu os riscos e benefícios da pesquisa.

Recomendações:

Não se aplica.

Conclusões ou Pendências e Lista de Inadequações:

Não se aplica.

Considerações Finais a critério do CEP:

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_1230881.pdf	12/12/2018 11:35:37		Aceito
Projeto Detalhado / Brochura Investigador	Projeto_Biofilme_V2.pdf	12/12/2018 11:35:18	Lidiany Karla Azevedo Rodrigues	Aceito
Cronograma	Cronograma_biofilme.pdf	14/11/2018 16:34:15	Lidiany Karla Azevedo Rodrigues	Aceito
Folha de Rosto	Folha_de_rosto.pdf	14/11/2018 16:33:17	Lidiany Karla Azevedo Rodrigues	Aceito
Outros	Carta_solicitacao_apreciacao.pdf	01/10/2018 12:53:52	Lidiany Karla Azevedo Rodrigues	Aceito
Declaração de Manuseio Material Biológico / Biorepositório / Biobanco	Declaracao_de_fiel_depositario.pdf	01/10/2018 12:51:57	Lidiany Karla Azevedo Rodrigues	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	Isencao_TCLE.pdf	01/10/2018 12:50:46	Lidiany Karla Azevedo Rodrigues	Aceito
Orçamento	Orcamento.pdf	01/10/2018 12:50:21	Lidiany Karla Azevedo Rodrigues	Aceito
Declaração de Instituição e Infraestrutura	Autorizacao_institucional.pdf	01/10/2018 12:49:51	Lidiany Karla Azevedo Rodrigues	Aceito

Situação do Parecer:

UFC - UNIVERSIDADE FEDERAL DO CEARÁ /

Continuação do Parecer: 3.092.796

Aprovado

Necessita Apreciação da CONEP:
Não

FORTALEZA, 18 de Dezembro de 2018

Assinado por:
FERNANDO ANTONIO FROTA BEZERRA
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Archives of Oral Biology 96 (2018) 155–161

Contents lists available at ScienceDirect

Archives of Oral Biology

journal homepage: www.elsevier.com/locate/archoralbio

Quantitative analysis of biofilm bacteria according to different stages of early childhood caries

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ARTICLE INFO

Keywords:
Bacteria
Dental plaque
Early childhood caries
Preschoolers
Quantitative polymerase chain reaction

ABSTRACT

Objective: Monitoring selected key species related to health or disease may facilitate caries risk assessment and discovery of novel ecological preventive and therapeutic approaches. This study aimed at quantifying *Actinomyces naeslundii, Bifidobacterium* spp., *Lactobacillus acidophilus, Lactobacillus casei* group, *Streptococcus gordonii, Mitis* group and *Streptococcus mutans* by quantitative polymerase chain reaction (qPCR) in dental biofilm from Brazilian children with different stages of early childhood caries (ECC).

Design: Seventy-five preschool children were clinically evaluated by ICDAS criteria and divided into groups: caries-free (CF; n = 20), enamel caries lesions (ECL; n = 17) and dentine caries lesions (DCL; n = 38). Plaque samples from all children were collected for detection and quantification of the selected bacteria.

Results: *L. acidophilus* and *L. casei* group were absent in almost all plaque samples. No differences in relative proportions of *A. naeslundii, Mitis* group and *S. gordonii* were observed in any stage of caries. However, *S. mutans* and *Bifidobacterium* spp. were present at higher concentrations in the biofilm of children with DCL ($p < 0.001$). Multivariate analysis showed that *S. mutans* and *Bifidobacterium* spp. were strongly associated with biofilm in children with DCL.

Conclusion: Differences were observed in the proportion of acidogenic and aciduric bacteria with dental caries progression. The data indicate that *S. mutans* and *Bifidobacterium* spp. in dental biofilm may be involved in some progression processes for ECC.

1. Introduction

Dental caries is a progressive disorder characterized by demineralization of tooth tissues associated with dysbiosis of the colonizing microbiota (Tanner, Kressirer, Rothmiller, Johansson, & Chalmers, 2018). This disease presents a complex etiology, including genetic, microbial, environmental and behavioral factors (Rosier, Marsh, & Mira, 2018). Early childhood caries (ECC) can be defined as the presence of one or more carious (noncavitated or cavitated lesions), missing (due to caries), or restored dental surfaces in any deciduous tooth in a under 6-year-old child (American Academy of Pediatric Dentistry, 2018). This condition is considered as one of the most prevalent biofilm-dependent diseases in childhood worldwide (Hajishengallis, Parsaei, Klein, & Koo, 2017), may cause pain and infection in preschool children, result in extensive carious lesions and destruction of primary teeth and reduce quality of life (Gross et al., 2012). Frequent carbohydrate intake causes a prolonged acidification of dental biofilm, subsequently leading to an increase of saccharolytic, acidogenic and aciduric microbiota (Rosier et al., 2018).

The oral biofilm is composed by a structurally organized microbial community, being considered as a dynamic and extremely complex ecosystem (Sanz et al., 2017) and its role in the onset and progression of dental caries is unquestionable (Dige, Grønkjær, & Nyvad, 2014). The cariogenic activity of an oral biofilm is based on the ability of the community to produce acid under decreasing pH conditions (McLean et al., 2012; Sanz et al., 2017). Efforts have been made to understand the healthy and disease-associated oral microbiota (Dige et al., 2014).

Studies focusing on selected acidogenic and aciduric caries-associated species, which are considered important caries pathogens, may be crucial for caries risk assessment, prevention, and for the

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https://doi.org/10.1016/j.archoralbio.2018.09.007
Received 9 April 2018; Received in revised form 8 August 2018; Accepted 11 September 2018

improvement of anticaries approaches (Tanner et al., 2018). According to the extended caries ecological hypothesis, mutans streptococci and lactobacilli as well as aciduric strains of non-mutans streptococci, *Actinomyces*, and, bifidobacteria may become dominant in the aciduric stage of dental caries (Takahashi & Nyvad, 2011).

Streptococcus mutans has been identified as a major microbial pathogen in the development of early childhood caries (Parisotto, Steiner-Oliveira, Silva, Rodrigues, & Nobre-dos-Santos, 2010), however not all children with ECC harbor this bacteria (Becker et al., 2002; Gross et al., 2012). Thus, this association is not absolutely accepted since dental caries-associated microbiota is highly complex and multiple members of the community can be implicated in caries development (McLean et al., 2012). Beyond *S. mutans*, ECC studies have detected other pathogens involved in dental caries such as *Bifidobacterium* (Aas et al., 2008; Becker et al., 2002; Kanasi et al., 2010), *Lactobacillus* (Aas et al., 2008; Becker et al., 2002) and *Actinomyces* (Jiang et al., 2014). All these microorganisms are potential acid producers and able to survive in acid environment (Takahashi & Nyvad, 2011).

A health-promoting microbiota is related to an active equilibrium between acid production and alkali generation, resulting in an environment with a predominantly neutral pH. The identification of such species, involved in caries resilience, could promote disease control by benefiting the growth of health-associated communities and interfering with the mechanisms associated to the disease (Sanz et al., 2017). Abundant members of the normal oral flora, that colonize the teeth (e.g., *Streptococcus sanguinis*, *Streptococcus parasanguinis*, *Streptococcus oralis*, *Streptococcus mitis*, *Streptococcus gordonii* and *Actinomyces naeslundii)*, can generate alkali and ammonia by the catabolism of arginine or urea, increasing the local pH (Liu, Nascimento, & Burne, 2012; Nascimento, Gordan, Garvan, Browngardt, & Burne, 2009). Studies indicate that some of these species, frequently related to oral health, have been also involved with dental caries, then being considered as alternative pathogens (Gross et al., 2012) such as *Streptococcus oralis*, *Actinomyces naeslundii* (Aas et al., 2008; Marchant, Brailsford, Twomey, Roberts, & Beighton, 2001) and *Streptococcus parasanguinis* (Gross et al., 2012).

The ECC prevalence in Brazil, according to the National Epidemiologic Report, is as high as 48.2% for children under 5 years (Brasil, 2012), however, only few studies using molecular techniques were performed in biofilm samples of Brazilian-ECC population (Ximenes, Armas, Triches, Cardoso, & Vieira, 2018). The majority of molecular studies conducted in this population have identified bacteria associated to ECC in the saliva of ECC-children (Colombo et al., 2017; Neves et al., 2015), which may not represent the cariogenic microbiota (Simón-Soro & Mira, 2015) and not being the most appropriate sample for etiological studies or for bacterial caries-risk tests (Mira, 2018). Different bacterial species may play an acidogenic role in different individuals and populations, since caries microbiota is not always similar (Tanner et al., 2018). Therefore, the aim of the present study was to identify and quantify *Actinomyces naeslundii*, *Bifidobacterium* spp., *Lactobacillus acidophilus*, *Lactobacillus casei* group, *Mitis* group, *Streptococcus gordonii* and *Streptococcus mutans* in dental biofilm from Brazilian children with different stages of ECC.

2. Material and methods

2.1. Ethics statement

The study design and informed consent were approved by the Ethics Committee of the Federal University of Ceará (COMEPE/UFC) (Protocol Number 158/2011). A written and signed informed consent was obtained from the parents or legal guardians of each child enrolled in the study. Samples were collected only after getting the approval from the parents or guardians.

2.2. Study population

The study sample consisted of 75 children aged from 2 to 5 years from public preeschools in Fortaleza, Ceará, Brazil. The schools were selected based on convenience and located in a suburban area of the city. A total of 420 children was examined in the first phase of the study in order to select the study population.

The study excluded children with any health condition at the moment of examination, who had used antibiotics within the last 3 months, and those who were not cooperative during clinical examination. None of the subjects had salivary gland disorders or systemic diseases. Informed consent of the respective children and their families, their willingness to participate, and the child was under 71 months of age were used as inclusion conditions.

The criteria used for early childhood caries (ECC) diagnosis was the International Caries Detection and Assessment System II (ICDAS II) (Ismail et al., 2007). After children had their teeth brushed under the direct supervision of dentists, dental examinations were carried out by a previously calibrated examiner (intraexaminer weighted kappa = 0.72). The examiners used a WHO periodontal probe, mirror, gauze and adequate illumination for intraoral examinations. The caries status was recorded on 0 and 2–6 scale by surface. Using the ICDAS criteria, caries status was assessed by recording all surfaces of primary teeth as 0 = sound; 2 = visible non-cavitated lesion seen when wet and dry; 3 = cavitation in enamel; 4 = non-cavitated lesion extending into dentine seen as an undermining shadow; 5 = cavitated lesion with visible dentine: < 50% of surface; and 6 = extensive cavitated lesion with visible dentine in more than 50% of the surface. The score 1 was not recorded because this code is only detected after prolonged air drying, as clinical data were collected at public schools, there was no availability of compressed air.

After meeting inclusion and exclusion criteria, 75 children were included in the final sample size. All children had complete primary dentition and no child had permanent teeth. The distribution of the groups was defined by different rate of lesion progression, according to ICDAS coding:

- CF: caries-free children (no clinical or reported evidence of caries experience) (n = 20) - ICDAS score 0.
- ECL: children with the most severe score classified as enamel carious lesions (n = 17) – at least one ICDAS score 2 and/or 3.
- DCL: children with the most severe score classified as dentine carious lesions (n = 38) – at least one ICDAS score 5 and/or 6. Children from DCL group could also present other carious lesions with lower ICDAS scores (2, 3 and 4). From the DCL group, 5 children presented lesions classified with ICDAS score 4, but none of these subjects presented ICDAS score 4 as the most severe one, presenting also lesions classified with scores 5 and/or 6. Therefore, no child was excluded for presenting only ICDAS score 4.

The total mean decayed surfaces with enamel and/or dentine caries was calculated for the ECL and DCL groups. These values were calculated using data obtained by the ICDAS criteria, employing different thresholds: ECL group – enamel caries lesions (scores 2 and 3 for the ICDAS considered as enamel caries). In the analysis of the caries indices, ICDAS caries codes 2,3 were counted together as a measure of decayed enamel caries (d_{2-3}), resulting in a mean d_{2-3} surfaces = 3.58 for this group; DCL group - ICDAS codes 2–6 were considered for the calculation of the total decayed surfaces, resulting in a mean d_{2-6} surfaces = 15.55. The values for the measure of mean enamel caries surfaces for this group (d_{2-3}) was 7.39 and for the dentine caries lesions (d_{4-6}) was 8.16.

All children involved in this study were enrolled in a dental care program that included preventive counseling and dental treatment at the dental clinic of the Faculty of Dentistry, Federal University of Ceará, Fortaleza, Ceará, Brazil.

Table 1
Primers that were used for qPCR assays.

Target species	Sequence (5' 3')	Annealing temperature (°C)	Amplicon length (bp)	References
Bacteria 16S rDNA	F: TCCTACGGGAGGCAGCAGT R:GGACTACCAGGGTATCTAATCCTGTT	57	466	Nadkarni et al., 2002
Actinomyces naeslundii	F:CTGCTGCTGACATCGCCGCTCGTA R:TCCGCTCGCGCCACCTCTCGTTA	62	144	Park, Kim, & Kook, 2013
Bifidobacterium spp.[a]	F: TCGCGTC(C/T)GGTGTGAAAG R: CCACATCCAGC(A/G)TCCAC	58	243	Rintillä, Kassinen, Malinen, Krogius, & Palva, 2004
Lactobacillus acidophilus	F: GATCGCATGATCAGCTTATA R: AGTCTCTCAACTCGGCTATG	60	124	Furet, Quéneé, & Tailliez, 2004
L. casei group[b]	F: GCGGACGGGTGAGTAACACG R: GCTTACGCCATCTTTCAGCCAA	60	121	Furet et al., 2004
Mitis group[c]	F:TAGAACGCTGAAGGAAGGAGC R: GCAACATCTACTGTTATGCGG	60	133	Wolff, Freese, Maier-Kraus, Krueger, & Wolff, 2013
Streptococcus gordonii	F: CAGGAAGGGATGTTGGTGTT R: GACTCTCTTGGCGACGAATC	60	136	Wolff et al., 2013
Streptococcus mutans	F: AGCCATGCGCAATCAACAGGTT R: CGCAACGCGAACATCTTGATCAG	64	415	Yano, Kaneko, Ida, Yamaguchi, & Hanada, 2002

[a] *Bifidobacterium longum, B. minimum, B. angulatum, B. catenulatum, B. pseudocatenulatum, B. dentium, B. ruminantium, B. thermophilum, B. subtile, B. bifidum, B. boum, B. lactis, B. animalis, B. choerinum, B. gallicum, B. pseudolongum* subsp. *globosum, B. pseudolongum subsp. pseudolongum, B. magnum, B. infantis, B. indicum, B. gallinarum, B. pullorum, B. saeculare, B. suis*.
[b] *L. casei group: L. casei, L. paracasei, L. rhamnosus and L. zeae*.
[c] *Mitis group : Streptococcus mitis, S. oralis, S. pneumoniae, S. parasanguinis, S. australis*.

2.3. Biofilm collection

Pooled supragengival plaque sample was collected from each child with sterile curettes from vestibular and lingual surfaces of teeth, including anterior and posterior primary teeth. Samples were not pooled across patients. The participants were asked not to brush their teeth 24 h before sampling.

Biofilm samples were transferred into a sterile 1.5 mL microcentrifuge tube that contained 150 µL of TE (10 mM Tris − HCl, 1 mM EDTA pH 7.6). All samples were immediately transported on ice to the laboratory, not exceeding one hour after collection. Samples were stored and kept frozen at − 20°C until DNA extraction.

2.4. Bacterial strains and culture conditions

Quantitative PCR was performed to detect the presence/absence and to quantify targeted bacterial DNA in dental plaque samples. The presence of *Actinomyces naeslundii, Bifidobacterium* spp., *Lactobacillus acidophilus, Lactobacillus casei* group, *Mitis* group, *Streptococcus gordonii* and *Streptococcus mutans* was investigated using specific forward and reverse primers as listed in Table 1.

The bacteria strains used as positive controls to test the specificity of the primers and for qPCR standard curve preparations included *Actinomyces naeslundii* (ATCC 12104), *Bifidobacterium animalis subsp. lactis* BB-12®(Chr. Hansen), *Lactobacillus acidophilus* (ATCC 4356), *Lactobacillus paracasei subsp. paracasei* (ATCC 335), *Streptococcus gordonii* (ATCC 35105), *Streptococcus mitis* (ATCC 49456 – NTCC 12261) and *Streptococcus mutans* (UA159).

Isolated bacteria were cultured in Brain-Heart Infusion broth. The cells were centrifuged and washed in sterile saline solution (NaCl 0.9%). The quality and purity of bacterial cultures were checked by Gram staining.

2.5. Extraction and purification of DNA from dental plaque samples and bacterial cultures

All samples were transferred into a fresh 2 mL screw cap tube and the cells were mechanically lysed with 0.16 g of 0.1-mm diameter zirconia beads on a Mini-Bead Beater homogenizer (Biospec Products, Bartlesville, OK, USA) and subject to beating for 1 min at maximum power. DNA was recovered from all samples using an organic extraction protocol based on phenol/chloroform purification and alcohol precipitation (Wilson, 2001). The DNA concentration (A 260 nm) and purity (A260 nm/A280 nm) of the samples were evaluated by a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). Electrophoresis of the extracted DNA was performed on a 1.2% agarose gel in Tris/borate/EDTA buffer and stained with 0.1 µg/ml ethidium bromide.

2.6. Preparation of PCR standards and quantification of target bacterial DNA in plaque samples by quantitative PCR (qPCR)

Serial dilutions starting from 300 ng to 0.0003 ng (10-fold) of reference bacterial DNA concentrations were used as standards and positive controls for relative quantification of the targeted bacteria. A standard DNA amplification curve and a melting-point product curve were obtained for each primer set/run. SYBR Green real-time PCR amplifications were performed using MicroAmpFast Optical 48-Well Reaction Plate (Applied Biosystems, Foster City, CA, USA) in StepOne Real-Time PCR System (Applied Biosystems) covered with Optical Adhesive Film (Applied Biosystems). Each reaction mixture (10 µL) contained 5 µL of 2x Power SYBR Green Mastermix (Applied Biosystems), 0.3 µL of each forward/reverse primer 10 µM, 2 µL of DNA sample and 2.4 µL of nuclease-free water. Negative control included reactions without template to rule out primers dimers formation or presence of contaminating DNA. All samples were analyzed in duplicates. The final analyses were based on the mean of the two reactions. The standard curves were used to transform the cycle threshold (Ct) values to the mass of DNA and the results of the concentrations of bacteria in plaque samples were normalized relative as a percentage of the total bacterial load estimated by the primer *Bacteria* 16S rDNA (Nadkarni, Martin, Jacques, & Hunter, 2002).

2.7. Data treatment and statistical analysis

Data were tabulated in Microsoft Excel and exported to statistical software Statistical Package for Social Sciences (SPSS) version 17.0, on which all analyzes were performed considering a confidence level of 95%.

The pattern evaluation of the sample distribution (normality Kolmogorov-Smirnov test) showed that the data were nonparametric and, therefore, groups were compared using Kruskal-Wallis test followed by the Mann-Whitney/Bonferroni post-test and expressed as mean and standard deviation, median and quartile distance.

Table 2
Dental plaque bacteria of the groups caries-free, caries-active with enamel lesions and caries-active with dentine lesions determined by qPCR as a percentage of the total bacteria load.

Oral bacteria	Groups	Mean (%)	± SD	Median (%)	Q25 – Q75 (QD)	p-value
S. mutans	CF	0.190	0.680	0.000	0.000–0.000 (0.000)	< 0.001
	ECL	0.052	0.118	0.001	0.000–0.039 (0.039)	
	DCL	1.191	2.068	0.250*	0.020–1.542 (1.522)	
L. acidophilus	CF	0.000	0.000	0.000	0.000–0.000 (0.000)	0.373
	ECL	0.000	0.000	0.000	0.000–0.000 (0.000)	
	DCL	0.000	0.001	0.000	0.000–0.000 (0.000)	
***Mitis* group**	CF	1.052	0.884	0.876	0.242–1.619 (1.377)	0.580
	ECL	1.573	1.453	1.178	0.379–2.614 (2.235)	
	DCL	3.361	6.123	0.892	0.276–2.531 (2.255)	
***L. casei* group**	CF	0.000	0.000	0.000	0.000–0.000 (0.000)	0.132
	ECL	0.000	0.000	0.000	0.000–0.000 (0.000)	
	DCL	0.001	0.002	0.000	0.000–0.000 (0.000)	
***Bifidobacterium* spp.**	CF	0.000	0.000	0.000	0.000–0.000 (0.000)	< 0.001
	ECL	0.000	0.001	0.000	0.000–0.001 (0.001)	
	DCL	0.088	0.213	0.002*	0.000–0.033 (0.033)	
A. naeslundii	CF	18.062	17.021	15.053	6.873–24.680 (17.807)	0.092
	ECL	14.239	14.904	9.620	5.110 –18.085 (12.975)	
	DCL	20.942	15.034	16.419	10.553–29.856 (19.303)	
S. gordonii	CF	0.513	0.777	0.294	0.026–0.574 (0.548)	0.075
	ECL	0.811	0.948	0.541	0.017–1.213 (1.196)	
	DCL	2.821	5.518	0.570	0.148 –1.730 (1.583)	

SD = standard deviation; Q25 = 25% quartile; Q75 = 75% quartile; QD = quartile distance; CF = caries-free; ECL = enamel caries lesions; DCL = dentine caries lesions.
The data are mean ± standard deviation and median values of concentrations of bacterial species as a percent of total bacteria load.
Asterisks indicate that the values are significantly different from each other ($p < 0.05$); Kruskall-Wallis test followed by the Mann-Whitney/Bonferroni post-test.

Furthermore, data were dichotomized according to the presence or absence of bacteria in the different groups for bivariate (chi-square test) and multivariate analysis (multinomial logistic regression).

3. Results

In a cross-sectional design, bacterial species were detected and compared between children with different caries status. Table 2 shows mean and median proportions of selected bacteria in dental plaque samples. *S. mutans* and *Bifidobacterium* spp. were significantly higher in DCL group compared to CF and ECL groups ($p < 0.001$). *L. acidophilus* and *L. casei* group were absent in almost all dental plaque samples. No statistical differences were observed among the clinical groups for *S. gordonii, Mitis group* and *A. naeslundii* relative concentrations.

After bivariate analysis (Table 3), *Bifidobacterium* spp. showed statistical significant association with ECL compared with CF group (OR = 4.5). Considering CF versus DCL group, the bivariate analysis revealed strong association between the presence of *S. mutans* (OR = 34) and *Bifidobacterium* spp. (OR = 11.2) with DCL group. These variables were submitted to a multiple logistic regression analysis to obtain the best model possibility. According to Table 4, none of the studied bacteria showed association with biofilm of children with ECL in the multivariate analysis. The multivariate modeling indicated that *S. mutans* (OR = 21.5) and *Bifidobacterium* spp. (OR = 5.9) were strongly associated with biofilm of children with DCL (Table 5).

4. Discussion

This study reports qPCR analysis of supragengival plaque collected from Brazilian children with different caries status of ECC, contributing to a better understanding of the caries microbiota in this population. Since our goal was to detect and quantify target acidogenic caries-associated species, the method applied was quantitative PCR (qPCR), that has been shown to have a great sensitivity (Tanner et al., 2018). The data presented demonstrates shifts in the relative plaque composition in dental health and different stages of ECC.

In the current study, the *S. mutans* presence in dental plaque was strongly associated with biofilm from children with DCL, which is consistent with previous literature reports (Becker et al., 2002; Gross et al., 2012; Jiang et al., 2014; Parisotto, Steiner-Oliveira, Duque et al., 2010). *S. mutans* is considered the most common acid producer in caries initiation (Tanzer, Livingston, & Thompson, 2001) and identified as a candidate risk factor for caries progression (Gross et al., 2012). Mutans streptococci may comprise a relatively small percentage of the cariogenic microbiota (Mira, 2018). In this study, levels of *S. mutans* were present in low concentrations, but were significantly higher in biofilm from DCL group compared with CF and ECL groups. *S. mutans* functions in caries modulation seem not to be entirely dependent on bacterial levels, since virulence factors expressed by this species are shown to alter the biofilm structure and to promote ecological shifts leading to an acidogenic and acid-tolerant microbiota (Mattos-Graner, Klein, & Smith, 2014).

Interestingly, members of the *L. casei* group and *L. acidophilus* were rarely identified or absent in most dental plaque samples even in DCL group in the present study, showing no association of these bacteria in biofilm collected from non-cavitated surfaces with caries, which corroborates previous investigations (Dige et al., 2014; Gross et al., 2012; Marchant et al., 2001). In contrast, some species of *Lactobacilli* have been found in biofilm in earlier studies (Kanasi et al., 2010; Parisotto, Steiner-Oliveira, Duque et al., 2010) and identified as metabolically active species in pH 4.5 (McLean et al., 2012). These conflicting results in the distribution of prevalent species between studies may be due to interindividual variation, different sampling methods, and even differences in categorizing patients into groups (Carvalho et al., 2016) or also to variations in evaluation methods being molecular methods more accurate than culture-based techniques (Nyvad, Crielaard, Mira, Takahashi, & Beighton, 2013). In addition, as long as no biofilm was collected from over dentinal lesions and considering that *Lactobacilli* are weakly adherent to smooth surfaces, these species were rarely found in the biofilm samples of the current study, since they are more present in

Table 3
Bivariate analysis of the relationships between ECC status and related factors.

Variables	CF n (%)	ECL n (%)	DCL n (%)	p-value	Odds ratio (95% CI) CF v.s ECL	CF v.s DCL	ECL v.s DCL
***Bifidobacterium* spp.**				< 0.001	4.5 (1.1–19.2)	11.2 (3.0–41.6)	2.4 (0.7–8.2)
positive	4 (20)	9 (52.9)	28 (73.7)				
negative	16 (80)	8 (47.1)	10 (26.3)				
S. mutans				< 0.001	4.0 (0.9–17.4)	34.0 (7.5–153.6)	9.5 (2.3–39.1)
positive	4 (20)	9 (52.9)	34 (89.5)				
negative	16 (80)	8 (47.1)	4 (10.5)				
L. acidophilus				0.218			
positive	ND	ND	3 (7.9)				
negative	20 (100)	17 (100)	35 (92.1)				
***L. casei* group**				0.177			
positive	ND	1 (5.9)	ND				
negative	20 (100)	16 (94.1)	38 (100)				
***Mitis* group**				1.000			
positive	20 (100)	17 (100)	38 (100)				
negative	ND	ND	ND				
S. gordonii				0.343	0.8 (0.0–14.6)	5.9 (0.2–152.4)	7.0 (0.3–181.1)
positive	19 (95)	16 (94.1)	38 (100)				
negative	1 (5)	1 (5.9)	ND				
A. naeslundii				1.000			
positive	20 (100)	17 (100)	38 (100)				
negative	ND	ND	ND				

CF = caries-free; ECL = enamel caries lesions; DCL = dentine caries lesions; ND = not detected.
Variables were compared by the Chi-square test ($p < 0.05$). Presence and absence of the bacteria in dental plaque samples were described. Results presented are the non-adjusted odds ratios with their 95% confidence intervals.

Table 4
Multivariate modeling for enamel caries lesions.

Variables	p-value	Adjusted odds ratio (95% CI)
Bacteria detection		
S. mutans	0.063	4.439 (0.921–21.387)
L. acidophilus	1.000	–
Mitis group	1.000	–
L. casei group	1.000	–
Bifidobacterium spp.	0.170	3.128 (0.630–15.525)
A. naeslundii	1.000	–
S. gordonni	0.459	0.292 (0.015–5.741)

Table 5
Multivariate modeling for dentine caries lesions.

Variables	p-value	Adjusted odds ratio (95% CI)
Bacteria detection		
S. mutans	< 0.001*	21.501 (4.299–107.544)
L. acidophilus	1.000	–
Mitis group	1.000	–
L. casei group	1.000	–
Bifidobacterium	0.033*	5.903 (1.153–30.221)
A. naeslundii	1.000	–
S. gordonni	0.999	–

* $p < 0.05$ – statistically significant.

retentive sites such as cavities (Jiang et al., 2014). It is also important to highlight that recent high-throughput sequencing of the 16S gene has verified that these bacteria are only recovered from advanced, deep dentin cavities (Obata et al., 2014; Simón-Soro, Belda-Ferre, Cabrera-Rubio, Alcaraz, & Mira, 2013) and may not be fundamental in the initial stages of dental caries (Mira, 2018).

Presence of *Bifidobacterium* spp. was significantly associated with both stages of caries progression (ECL and DCL). Moreover, an important finding was that significantly higher levels of these bacteria were found in the biofilm composition of children with DCL compared to the other groups. A higher chance (odds ratio = 5.9) of detecting *Bifidobacterium* in dental biofilm of children with dentine carious lesions was demonstrated, confirming that Bifidobacteria are related to ECC in this population as previous studies have suggested (Aas et al., 2008; Becker et al., 2002). Bifidobacteria are considered aciduric and acidogenic microorganisms (Mantzourani et al., 2009), indicating that the acidic environment provides a suitable habitat for the proliferation of these bacteria (Takahashi & Nyvad, 2011). A previous study has suggested that the major secondary agent found in oral biofilm from children with severe ECC are not *Lactobacillus* spp., but rather *Bifidobacterium* spp. (Becker et al., 2002).

Most dental biofilm samples from the current study presented *A. naeslundii*, *Mitis group* and *S. gordonii*, nevertheless, no significantly differences in the relative levels of these microorganisms were observed for any stage of caries. Non-mutans bacteria (mainly non-mutans streptococci and *Actinomyces*) are considered common members of health-associated microbiota (Corby et al., 2005; Gross et al., 2012) and initial colonizers of tooth surfaces (Takahashi & Nyvad, 2008). However, these bacteria have been considered as alternative pathogens because some of their metabolic activities may modulate dynamic caries processes in a different way, such as the use of lactate by *Actinomyces naeslundii* as a carbon source for growth converting it in weaker acids (Takahashi & Yamada, 1996). In addition, the alkali production by these microorganisms directly impacts plaque pH, possibly interfering the initiation and/or progression of a caries lesion (Liu et al., 2012). Consequently, the association of these bacteria with health or disease can be modulated by local conditions, since *Actinomyces* are as versatile to adjust oneself to different conditions in dental biofilm environment as are the non-mutans streptococci (Takahashi & Nyvad, 2008). This way, the molecular mechanisms through which these microorganisms participate in caries initiation remain unclear and further studies are needed to identify the ecological shifts leading to cariogenic biofilms (Mattos-Graner et al., 2014).

A variety of methods are available for studies of dental biofilms

(Carvalho et al., 2016). Several researches have evaluated microbial composition of dental biofilms on pooled plaque samples in molecular studies (Aas et al., 2008; Becker et al., 2002; Johanson, Witkowska, Kaveh, Lif Holgerson, & Tanner, 2016; Parisotto, Steiner-Oliveira, Duque et al., 2010; Peterson et al., 2013). In this study, samples were not pooled across patients, being each sample corresponding to the collected biofilm of each child. A previous study supports this sampling method, since it was demonstrated that the dental microbiota associated with localized healthy tooth surfaces and caries lesions are similar within the same oral cavity (Corby et al., 2005). However, there are limitations to this procedure. According to Nyvad et al. (2013), individual site-specific sampling is more appropriate when trying to compare bacterial profiles with each stage of dental caries. On the other hand, the ideal situation for studying dental biofilms is collecting samples in their natural status, and sometimes the amount of biofilm found over carious sites may not be sufficient to perform accurate analyses providing relevance for pooled sample studies. According to Tanner et al. (2018), microbial monitoring in clinical practice would be important in detecting disease-associated microorganisms. Besides that, monitoring target putative caries pathogens may be more realistic to monitor and to evaluate reversal of the microbiome dysbiosis to that compatible with health (Tanner et al., 2018).

5. Conclusions

Differences were observed in the proportion of acidogenic and aciduric bacteria with dental caries progression. The presence and higher concentration of *Bifidobacterium* spp. and *Streptococcus mutans* in the biofilm of children with dentine caries lesions were strongly associated to the progression of early childhood caries, demonstrating that these bacteria may play an important role in caries evolution.

Conflict of interest

None.

Acknowledgements

This study was supported by CNPq - National Counsel of Technological and Scientific Development (Process # 475346/2011-4 MCT/CNPq 14/2011). The first author was supported by a CAPES (National Council for the Improvement of Higher Education) scholarship funded by the Brazilian government during her Ph.D course, when this research was conducted (Process # 1067129).

References

Aas, J. A., Griffen, A. L., Dardis, S. R., Lee, A. M., Olsen, I., Dewhirst, F. E., et al. (2008). Bacteria of dental caries in primary and permanent teeth in children and young adults. *Journal of Clinical Microbiology, 46*, 1407–1417.

American Academy of Pediatric Dentistry (2018). Policy on early childhood caries (ECC): Classifications, consequences, and preventive strategies. *Oral Health Policies, 39*, 59–61 (2017-18).

Becker, M. R., Paster, B. J., Leys, E. J., Moeschberger, M. L., Kenyon, S. G., Galvin, J. L., et al. (2002). Molecular analysis of bacterial species associated with childhood caries. *Journal of Clinical Microbiology, 40*, 1001–1009.

Brasil (2012). *Ministério da Saúde. Secretaria de Atenção à Saúde. Secretaria de Vigilância em Saúde. SB Brasil 2010: Pesquisa Nacional de Saúde Bucal: resultados principais/ Ministério da Saúde. Secretaria de Atenção à Saúde. Secretaria de Vigilância em Saúde.* Brasília: Ministério da Saúde.

Carvalho, J. C., Dige, I., Machiulskiene, V., Qvist, V., Bakhshandeh, A., Fatturi-Parolo, C., et al. (2016). Occlusal caries: Biological approach for its diagnosis and management. *Caries Research, 50*(6), 527–542.

Colombo, N. H., Kreling, P. F., Ribas, L. F. F., Pereira, J. A., Kressirer, C. A., Klein, M. I., et al. (2017). Quantitative assessment of salivar oral bacteria according to the severity of dental caries in childhood. *Archives of Oral Biology, 83*, 282–288.

Corby, P. M., Lyons-Weiler, J., Bretz, W. A., Hart, T. C., Aas, J. A., Boumenna, T., et al. (2005). Microbial risk indicators of early childhood caries. *Journal of Clinical Microbiology, 43*, 5753–5759.

Dige, I., Grønkjær, L., & Nyvad, B. (2014). Molecular studies of the structural ecology of natural occlusal caries. *Caries Research, 48*(5), 451–460.

Furet, J., Quéneé, P., & Tailliez, P. (2004). Molecular quantification of lactic acid bacteria in fermented milk products using real-time quantitative PCR. *International Journal of Food Microbiology, 97*, 197–207.

Gross, E. L., Beall, C. J., Kutsch, S. R., Firestone, N. D., Leys, E. J., & Griffen, A. L. (2012). Beyond streptococcus mutans: Dental caries onset linked to multiple species by 16S rRNA community analysis. *PLoS One, 7*, e47722.

Hajishengallis, E., Parsaei, Y., Klein, M. I., & Koo, H. (2017). Advances in the microbial etiology and pathogenesis of early childhood caries. *Molecular Oral Microbiology, 32*(1), 24–34.

Ismail, A. I., Sohn, W., Tellez, M., Amaya, A., Sen, A., Hasson, H., et al. (2007). The International Caries Detection and Assessment System (ICDAS): An integrated system for measuring dental caries. *Community Dentistry and Oral Epidemiology, 35*, 170–178.

Jiang, W., Ling, Z., Lin, X., Chen, Y., Zhang, J., Yu, J., et al. (2014). Pyrosequencing analysis of oral microbiota shifting in various caries states in childhood. *Microbial Ecology, 67*, 962–969.

Johanson, I., Witkowska, E., Kaveh, B., Lif Holgerson, P., & Tanner, A. C. R. (2016). The microbiome in populations with a low and high prevalence of caries. *Journal of Dental Research, 95*, 80–86.

Kanasi, E., Johansson, I., Lu, S. C., Kressin, N. R., Nunn, M. E., Kent, R., et al. (2010). Microbial risk markers for childhood caries in pediatricians' offices. *Journal of Dental Research, 89*, 378–383.

Liu, Y. L., Nascimento, M., & Burne, R. A. (2012). Progress toward understanding the contribution of alkali generation in dental biofilms to inhibition of dental caries. *International Journal of Oral Science, 4*(3), 135–140.

Mantzourani, M., Gilbert, S. C., Sulong, H. N., Sheehy, E. C., Tank, S., Fenlon, M., et al. (2009). The isolation of bifidobacteria from occlusal carious lesions in children and adults. *Caries Research, 43*, 308–313.

Marchant, S., Brailsford, S. R., Twomey, A. C., Roberts, G. J., & Beighton, D. (2001). The predominant microflora of nursing caries lesions. *Caries Research, 35*, 397–406.

Mattos-Graner, R. O., Klein, M. I., & Smith, D. J. (2014). Lessons learned from clinical studies: Roles of mutans streptococci in the pathogenesis of dental caries. *Current Oral Health Reports, 1*, 70–78.

McLean, J. S., Fansler, S. J., Majors, P. D., McAteer, K., Allen, L. Z., Shirtliff, M. E., et al. (2012). Identifying low pH active and lactate-utilizing taxa within oral microbiome communities from healthy children using stable isotope probing techniques. *PLoS One, 7*, e32219.

Mira, A. (2018). Oral microbiome studies: Potential diagnostic and therapeutic implications. *Advances in Dental Research, 29*(1), 71–77.

Nadkarni, M. A., Martin, F. E., Jacques, N. A., & Hunter, N. (2002). Determination of bacterial load by real-time PCR using a broad-range (universal) probe and primers set. *Microbiology, 148*(1), 257–266.

Nascimento, M. M., Gordan, V. V., Garvan, C. W., Browngardt, C. M., & Burne, R. A. (2009). Correlations of oral bacterial arginine and urea catabolism with caries experience. *Oral Microbiology and Immunology, 24*, 89–95.

Neves, A. B., Lobo, L. A., Pinto, K. C., Pires, E. S., Requejo, M., Maia, L. C., et al. (2015). Comparison between clinical aspects and salivary microbial profile of children with and without early childhood caries: A preliminary study. *Journal of Clinical Pediatric Dentistry, 39*(3), 209–214.

Nyvad, B., Crielaard, W., Mira, A., Takahashi, N., & Beighton, D. (2013). Dental caries in a molecular microbiological perspective. *Caries Research, 47*, 89–102.

Obata, J., Takeshita, T., Shibata, Y., Yamanaka, W., Unemori, M., Akamine, A., et al. (2014). Identification of the microbiota in carious dentin lesions using 16S rRNA gene sequencing. *PLoS One, 9*(8), e103712.

Parisotto, T. M., Steiner-Oliveira, C., Duque, C., Peres, R. C. R., Rodrigues, L. K., & Nobre-dos-Santos, M. (2010). Relationship among microbiological composition and presence of dental plaque, sugar exposure, social factors and different stages of early childhood caries. *Archives of Oral Biology, 55*, 365–373.

Parisotto, T. M., Steiner-Oliveira, C., Silva, C. M., Rodrigues, L. K., & Nobre-dos-Santos, M. (2010). Early childhood caries and mutans streptococci: A systematic review. *Oral Health Preventive Dentistry, 8*, 59–70.

Park, S., Kim, Y. K., & Kook, J. (2013). Development of quantitative real-time PCR primers for detecting 42 bacterial species. *Archives of Microbiology, 195*, 473–482.

Peterson, S. N., Snesrud, E., Liu, J., Ong, A. C., Kilian, M., Schork, N. J., et al. (2013). The dental plaque microbiome in health and disease. *PLoS One, 8*(3), e58487.

Rintillä, T., Kassinen, A., Malinen, E., Krogius, L., & Palva, A. (2004). Development of an extensive set of 16S rDNA-targeted primers for quantification of pathogenic and indigenous bacteria in faecal samples by real-time PCR. *Journal of Applied Microbiology, 97*, 1166–1177.

Rosier, B. T., Marsh, P. D., & Mira, A. (2018). Resilience of the oral microbiota in health: Mechanisms that prevent dysbiosis. *Journal of Dental Research, 97*(4), 371–380.

Sanz, M., Beighton, D., Curtis, M. A., Cury, J. A., Dige, I., Dommisch, H., et al. (2017). Role of microbial biofilms in the maintenance of oral health and in the development of dental caries and periodontal diseases. Consensus report of group 1 of the joint EFP/ORCA workshop on the boundaries between caries and periodontal disease. *Journal of Clinical Periodontology, 44*, S5–S11.

Simón-Soro, A., & Mira, A. (2015). Solving the etiology of dental caries. *Trends in Microbiology, 23*(2), 76–82.

Simón-Soro, A., Belda-Ferre, P., Cabrera-Rubio, R., Alcaraz, L. D., & Mira, A. (2013). A tissue-dependent hypothesis of dental caries. *Caries Research, 47*, 591–600.

Takahashi, N., & Nyvad, B. (2008). Caries ecology revisited: Microbial dynamics and the caries process. *Caries Research, 42*, 409–418.

Takahashi, N., & Nyvad, B. (2011). The role of bacteria in the caries process: Ecological perspectives. *Journal of Dental Research, 90*, 294–303.

Takahashi, N., & Yamada, T. (1996). Catabolic pathway for aerobic degradation of lactate by *Actinomyces naeslundii*. *Oral Microbiology and Immunology, 11*(3), 193–198.

Tanner, A. C. R., Kressirer, C. A., Rothmiller, S., Johansson, I., & Chalmers, N. I. (2018).

The caries microbiome: Implications for reversing dysbiosis. *Advances in Dental Research, 29*(1), 78–85.
Tanzer, J. M., Livingston, J., & Thompson, A. M. (2001). The microbiology of primary dental caries in humans. *Journal of Dental Education, 65*, 1028–1037.
Wilson, K. (2001). Preparation of genomic DNA from bacteria. *Current Protocols in Molecular Biology, 56*, 2.4.1–2.4.5 Chapter 2: Unit 2.4.
Wolff, D., Freese, C., Maier-Kraus, T., Krueger, T., & Wolff, B. (2013). Bacterial biofilm composition in caries and caries-free subjects. *Caries Research, 47*, 69–77.
Ximenes, M., Armas, R. D., Triches, T. C., Cardoso, M., & Vieira, R. S. (2018). Structure of the bacterial community in different stages of early childhood caries. *Oral Health & Preventive Dentistry, 15*, 1–5.
Yano, A., Kaneko, N., Ida, H., Yamaguchi, T., & Hanada, N. (2002). Real-time PCR for quantification of *Streptococcus mutans*. *FEMS Microbiology Letters, 217*, 23–30.

Received: 11 June 2018 | Revised: 18 October 2018 | Accepted: 22 October 2018

DOI: 10.1002/JPER.18-0366

JOURNAL OF Periodontology

ORIGINAL ARTICLE

Low-temperature plasma on peri-implant-related biofilm and gingival tissue

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Abstract

Background: Evaluate the effect of low-temperature plasma (LTP) on an anaerobic biofilm and on the biological response of an in vitro reconstituted gingival epithelium tissue.

Methods: *Porphyromonas gingivalis* W83 biofilm was cultured on titanium discs and reconstituted gingival tissues were submitted to similar treatment conditions. Treatments: LTP1—plasma treatment for 1 minute, LTP3—plasma treatment for 3 minute, CHX—0.2% chlorhexidine for 1 minute, GAS—gas only (no plasma) for 3 minute, and NEGATIVE—no treatment. TRITON group was included as a positive control for tissue analysis. Counting of viable colony forming units (CFU/mL) and confocal laser scanning microscopy were performed to evaluate LTP's antimicrobial effect. EpiGingival tissue was evaluated through cytotoxocity, viability, histology, and imunnohistochemistry (Ki67, vascular endothelial growth factor-A vascular endothelial growth factor A [VEGF-A], and terminal deoxynucleotidyl transferase dUTP nick end labeling terminal deoxynucleotidyl transferase dutp nick end labeling [TUNEL] expression).

Results: LTP1 and LTP3 presented significantly different reduced CFU/mL reduction in comparison to the negative control ($P < 0.001$), but it was not as effective as the positive control (CHX). Low cytotoxicity and high viability were observed in gingival epithelium of NEGATIVE, GAS, CHX, and both LTP groups. The morphologic analysis of gingival epithelium revealed minor cell damage in the plasma groups (score 1). LTP1, LTP3, GAS, and NEGATIVE groups exhibited less than 5% of basal layer positive cells. LTP1, LTP3, GAS, and CHX groups were not positive for TUNEL assay. LTP1 and LTP3 showed the most positivity for VEGF.

Conclusions: LTP treatment can be considered as an effective method for reducing *P. gingivalis* biofilm on implant surfaces, while being safe for the gingival epithelium. Furthermore, plasma treatment may be associated with cell repair.

KEYWORDS

dental implants, microbiology, periodontium, *Porphyromonas gingivalis*, therapeutics

1 | INTRODUCTION

Even though implant-based dental rehabilitation has now become a routine treatment to replace missing teeth with high survival rates, a "surviving" implant is certainly not a "successful" implant because many of those implants may present surrounding infected tissue.[1] If conservatively 10% of all implants have a complication, 100 000 implants per year will need treatment.[2] Among these complications, peri-implant mucositis has a prevalence of 43% and 22% for peri-implantitis.[3] Therefore, the implant biologic complications, which can potentially lead to implant failure, have attracted the attention of researchers and clinicians.

A significant debate exists regarding the properties of different antimicrobial agents and to what extent each contributes to inhibit microbes, and novel therapies using low-temperature plasma (LTP) have been considered.[4] Plasma reactive species are transiently generated and represent an especially good source of reactive oxygen and nitrogen species, including singlet oxygen (O), ozone (O_3), hydroxyl radicals (OH), NO, and NO_2.[5–7] Oxygen and nitrogen-based radicals are considered the most significant contributors to the sterilizing effects.[8] These reactive species have strong oxidative effects on the outer structures of bacterial cells, whether it is a spore coat or cell membrane.[8]

Another advantage of LTP application for biologic decontamination is their well-targeted local application.[9] Therefore, LTP could reach the site of infected implants during peri-implantitis surgery.[9] Furthermore, plasma can be considered supportive for the treatment of peri-implant diseases, because plasma treatment reduced contact angle and supported spreading of osteoblastic cells.[10]

Although previous study investigating the inactivation of peri-implant biofilms has shown that plasma could be a useful adjuvant treatment modality for peri-implant disease, this study was not conducted on *Porphyromonas gingivalis* biofilm, the major etiologic agent that contributes to chronic peri-implantitis.[11] In addition, studies regarding the biologic safety of plasma are still limited, particularly on gingival tissue. Therefore, the applicability of this approach requires more extensive studies.

Previously, our research group demonstrated that LTP is effective against *Candida albicans* and *Staphylococcus aureus* mature oral biofilms.[12] Furthermore, the effective dose against these microorganisms was tolerable for the reconstituted oral epithelium, because no significant alterations and cytotoxicity were found.[12] Our hypothesis is that LTP treatment is effective against *P. gingivalis* biofilm without causing any significant tissue damage to the in vitro reconstituted human gingival epithelium. The hypothesis was tested by determining the effect of LTP against *P. gingivalis* biofilm, and by characterizing the tissue response to LTP treatment using an in vitro reconstituted gingival epithelium.

2 | MATERIALS AND METHODS

2.1 | Substrate

Sterile, sandblasted, and acid-etched titanium grade 4 discs (Singular Implants—Dmr Ind. e Com. de Materiais Odontologicos Ltda, Parnamirim, Rio Grande do Norte, Brazil) with 6 mm diameter, 2 mm thickness, and 0.8505 ± 0.128 μm of mean roughness (*Ra*) Surface roughness profilometer Surftest SJ-401 (Mitutoyo Corporation, Kanagawa, Japan) were used.

2.2 | Biofilm

Porphyromonas gingivalis W83 biofilms were formed on brain heart infusion (BHI) supplemented with hemin (0.5 mg/mL) and menadione (5 mg/mL), and anaerobically incubated at 37°C for 5 days. The biofilms that started from a culture were adjusted to optical density (OD_{660}) of 1.2 in a spectrophotometer, which were equivalent to 1×10^5 colony forming units (CFU)/mL. Titanium discs were then incubated in 3 mL of the culture solution and after every 48 hours of incubation, 1.5 mL of the medium was removed, and an equal volume of fresh medium was added. After 5 days of biofilms development,[13] they were treated according to the following groups: LTP1—plasma for 1 minute, LTP3—plasma for 3 minutes, CHX—positive control with 0.2% chlorhexidine for 1 minute, GAS—negative control with argon gas only (no plasma) for 3 minutes, and NEGATIVE—negative control without treatment. The experiment was conducted in duplicates in three independent occasions.

2.3 | Plasma treatment

The LTP was generated through ionization of argon gas (Ar) at atmospheric pressure using the device Kinpen (Leibniz Institute for Plasma Science and Technology—INP, Greifswald, Germany). The device consists of a hand-held unit for generation of a plasma jet at atmospheric pressure, a DC power supply (system power: 8 W at 220 V, 50/60 Hz), and a gas supply unit.[12] The plasma tip-to-sample distance was set to 7 mm. The samples were moved horizontally during plasma application to allow scanning of the overall surface.[12] The experiment was conducted by a calibrated operator. The argon (Ar) gas flow was set to 5 slm and the flow rate was controlled by a flow controller.

2.4 | Positive control

The discs with *P. gingivalis* biofilm were immersed in 3 mL of 0.2% CHX solution for 1 minute. The samples were washed with phosphate buffered saline (PBS—10 mM PO_4^{3-}, 137 mM NaCl, 2.7 mM KCl).

2.5 | Analyses

2.5.1 | Viable colony forming units (CFU/mL)

The discs were removed from the culture plates and the bottom surface was rubbed with a sterilized swab to remove nontreated biofilm. Each disc was inserted in PBS and subjected to ultrasound bath 2 minutes for serial dilution. Serially diluted aliquots were plated in Anaerobe 5% Sheep Agar Blood and incubated anaerobically at 37°C for 5 days.

2.5.2 | Confocal laser scanning microscopy (CLSM)

The biofilms were stained using the Live/Dead BacLight Viability kit (Invitrogen-Molecular Probes, Waltham, MA) and incubated in anaerobiosis for 15 minutes at room temperature. Live cells are stained in green while dead cells are stained in red. A series of images of the biofilm was obtained using a Leica TCS SP5 II confocal microscope (Leica, Germany) with Leica HCX APO L 40x/0.8 W U-V-I water dipping lens (Leica, Germany).[12] Five random optical fields were examined for each specimen.

2.6 | Tissue culture

In vitro reconstituted gingival epithelium EpiGingival (GIN-100 MatTek Corporation, Promega, USA) model is based on normal human oral keratinocytes differentiated into tissues with a cornified, gingival phenotype, part number GIN-100. It was used to test the biologic response, as previously described.[12]

2.7 | Tissue analyses

CytoTox-ONE (Promega, USA) Homogeneous Integrity Assay kit was used as a fluorometric method to estimate cell viability based on the release of lactate dehydrogenase (LDH) from cells with damaged membrane. LDH release into the culture medium was measured by an enzymatic assay that results in the conversion of resazurin into resorufin. The MatTek MTT toxicology kit (MTT-100 MatTek Corporation, Fitchburg, Wisconsin) was used to check tissue viability.[12]

2.8 | Histology

Samples were immersed in 10% formalin, washed with PBS, dehydrated with 50% and 70% ethanol and prepared for paraffin embedding, cutting, and Hematoxylin/Eosin (H/E) staining. For tissue analysis, the slides were scanned with Panoramic MIDI 1.15 SPI 3D HISTECH® (Budapest, Hungary) and captured in Panoramic Viewer 1.115.2 3D HISTECH® (Budapest, Hungary). The histopathologic parameters (epithelium thickness and modification, hyperkeratosis, hyperplasia, and changes in cell morphology) were classified by semiquantitative analysis into scores from 0 to 3 (0—no modification, 1—minimum modification, 2—medium modification, and 3—severe modification).[14]

2.9 | Immunohistochemistry

To identify actively proliferating cells, angiogenic activity (tissue repair activity) and apoptosis, immunohistochemistry was performed on 4 μm formalin-fixed paraffin-embedded samples using the following markers: Ki67, vascular endothelial growth factor (VEGF-A), and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) (Thermo Scientific, Waltham, MA), respectively. The sections were deparaffinized in xylene, rehydrated through graded alcohols (100% and 95% ethanol), and rinsed in distilled water. Heat-induced epitoperetrieval was performed in a 1200 watt microwave oven at 100% power in 10 mM sodium citrate buffer, pH 6 for 20 and 10 minutes, respectively. Sections were allowed to cool for 30 minutes and then rinsed in distilled water. Antibody incubation and detection were carried out at 40°C on a Discovery instrument (Ventana Medical Systems, Tucson, AZ) using Ventana's reagent buffer and detection kits. Endogenous peroxidase activity was blocked with hydrogen peroxide. Antibody was diluted (1:400) in Dulbecco's PBS (Life Technologies, Waltham, MA). Samples were incubated overnight at room temperature. Antibody was detected with biotinylated goat anti-rabbit diluted at 1:200 (Vector Laboratories, Burlingame, CA) for 30 minute. This was followed by application of streptavidin-horseradish-peroxidase conjugate. The complex was visualized with 3,3-diaminobenzidene and enhanced with copper sulfate. Slides were washed in distilled water, counterstained with hematoxylin, dehydrated and mounted with permanent media. The experiment was conducted in duplicate in two independent occasions. Immunohistochemical reaction in epithelial cells for VEGF was also performed and analyzed.

2.10 | Statistical analyses

IBM SPSS v. 22 software (IBM Corp., Armonk, NY) was used for statistical analysis with a confidence level of 95%. CFU data were $\log_{10}$ transformed prior to analysis, and log CFU data were further rank transformed in some analyzes in order to homogenize within-group variances for analysis of variance (ANOVA). Group differences were compared using one-way ANOVA, and given a significant omnibus test, post hoc Tukey HSD tests were pursued. For tissue analyses, LDH data were analyzed again by one-way ANOVA and post hoc Tukey HSD tests were pursued using a pooled estimate of the standard error, and 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) (MTT) data were analyzed by percentile differences. Descriptive analyses were conducted for histologic and immunohistochemistry results.

TABLE 1 CFU mL (log10) and standard deviation obtained for each group

Treatments	CFU/mL (log_{10})	Standard deviation
LTP1	4.66[c]	2.03
LTP3	4.64[c]	0.76
GAS	5.92[a]	0.40
CHX	2.92[b]	1.49
NEGATIVE	6.02[a]	0.32

Means followed by the same letter in the column are not significantly different ($P > 0.05$).

3 | RESULTS

3.1 | Biofilm analysis

Table 1 shows means and standard deviations of CFU/mL (log_{10}) of all groups. The LTP groups showed a reduction in CFU/mL when compared to the NEGATIVE and GAS control groups ($P < 0.001$). Parallel analysis of a rank transformation of the log CFU data (which homogenized within cell variability) confirmed the results of the raw CFU/mL analysis. Figure 1 shows the CLSM biofilm images after treatment, in which live cells are shown in fluorescent green and dead cells are shown in fluorescent red. Plasma-treated samples visually showed less biofilm than the NEGATIVE and CHX groups, which could indicate a possible mechanical removal of dead bacteria, however, further studies would be necessary to prove this hypothesis.

3.2 | Tissue analysis

3.2.1 | Citotoxity and viability

Percent cytotoxicity of LDH release showed difference between TRITON and the others groups ($P < 0.05$). Post hoc *t*-tests showed that GAS ($5.09 \pm 2.56\%$), LTP1 ($3.83 \pm 1.42\%$), and LTP3 ($5.44 \pm 2.11\%$) were similar and different from control groups ($P < 0.05$). For MTT, except TRITON, all groups showed high viability (Figure 2).

3.3 | Histologic and immunohistochemistry analyses

Figure 3A–F shows the histologic analysis. Minor tissue alterations in plasma-treated samples (score 1) with slight keratinization in comparison to the NEGATIVE (score 0) group were seen. The immunohistochemistry analyzes are presented in Figure 4. VEGF was slightly more evident for LTP1 (A) and LTP3 (B) than for negative control (F) and was absent in the TRITON group (F); Ki-67 showed LTP1 (G), LTP3 (H), GAS (I), CHX (K), and control negative (L) groups with <5% of positive cells in basal layer and TRITON group with >15% of positive cells in superior stratum (J); TUNEL was negative for LTP1 (M), LTP3 (N), GAS (O), CHX (Q), and control negative (R) groups, however, TRITON group evidenced several positive cells (P).

4 | DISCUSSION

Porphyromonas gingivalis is considered the major etiologic agent involved in chronic peri-implantitis.[15,16] However, there is a lack of studies on the effect of LTP against *P. gingivalis* biofilm.

This study aimed to determine the effect of LTP on *P. gingivalis* biofilm cultured on titanium discs. Plasma-treated biofilms for 1 and 3 minute presented log_{10} CFU/mL reduction in comparison to negative control. The results confirm our hypothesis that LTP is effective against *P. gingivalis* biofilm. However, in terms of log_{10} CFU/mL reduction, 3 minute application did not provide any improvement when compared to 1 minute irradiation. These findings agree with previous study, where 60 and 120 s did not show different results when it was applied on *Streptococcus mitis* biofilm.[11] Considering biofilms have a strong extracellular matrix, the superficial layer of the dead biofilm could have blocked the plasma effect on the residual bacteria inside the biofilm. Tissue does not have as much protection as the biofilm extracellular matrix, so possibly this could be a reason why plasma was

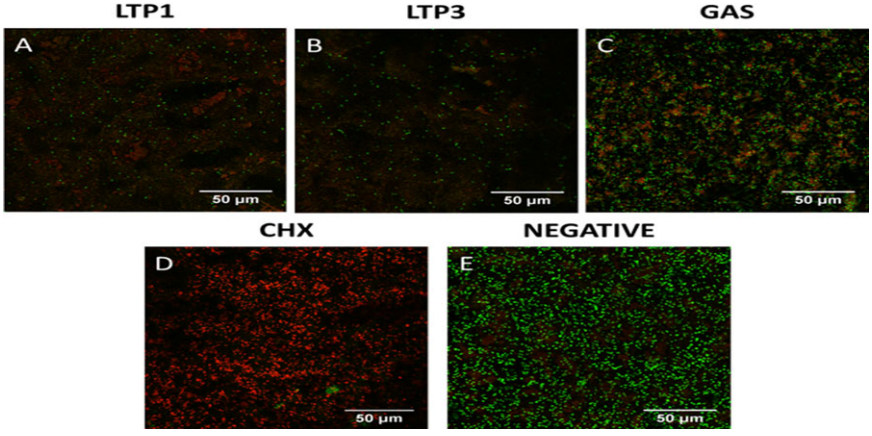

FIGURE 1 Confocal laser scanning microscopy of *P. gingivalis* biofilm after LTP treatment for 1 minute (A); LTP treatment for 3 minute(B); GAS for 3 minute; CHX-Positive Control for 1 minute (D); and NEGATIVE (E) 4 × zoom. Biofilm stained with Live/Dead BacLight Viability kit. Live cells in fluorescent green and dead cells in fluorescent red. Plasma-treated samples showed visually lower amount of biofilm than the NEGATIVE and CHX groups. After plasma treatment, images indicate a possible mechanical removal of death bacterium

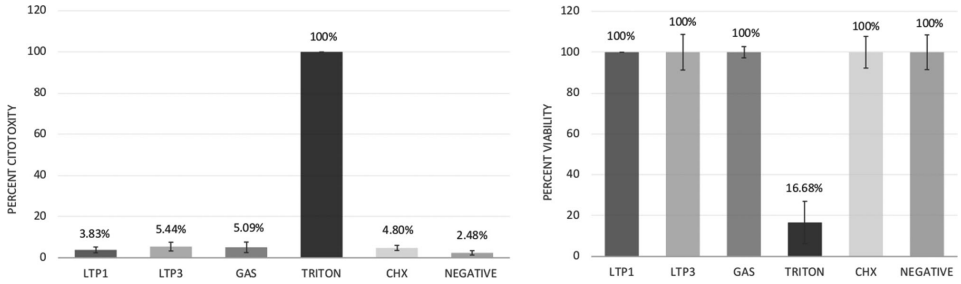

FIGURE 2 Mean (±SD) percent cytotoxicity and viability values of tissue. Considering the TRITON as a reference group for cytotoxicity calculation, the similarity between the results of the negative control and plasma groups indicates a minimum cytotoxic effect of plasma treatment on gingival epithelium within the dosage applied in this study. For MTT, considering the NEGATIVE control group as a reference for viability calculation, all groups showed 100% of percent viability, except TRITON

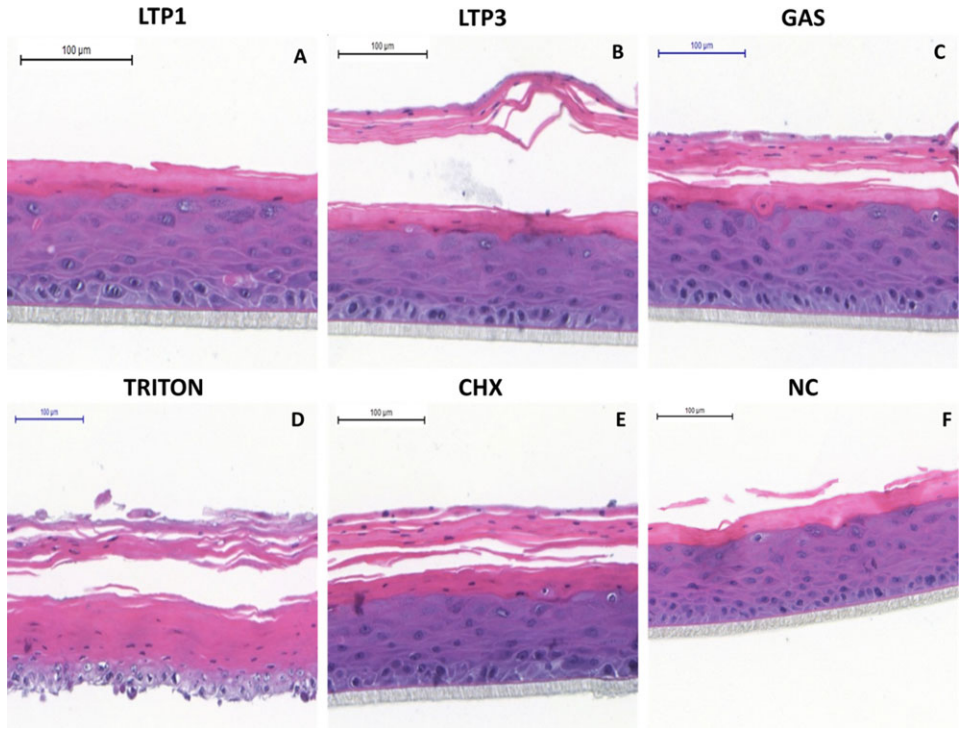

FIGURE 3 H/E: LTP1, score 1 (A); LTP3, score 1 (B); GAS Score 1(C); Triton, score 3 (D); CHX, score 0 (E); and negative control, score 0 (F). TRITON exhibited significant tissue damage with cell vacuolization and nuclear shrinkage (ie, pyknosis) (score 3). LTP1 and LTP3 showed similar morphology

more effective after a higher exposure time. Further studies are needed to understand these outcomes.

In our study, a control GAS group (without plasma) was included assuming that gas application could have any possible effect on removing and killing bacteria. The results did not show any difference between GAS and NEGATIVE treatments, meaning that biofilm inactivation was promoted by plasma and not by the gas or the mechanical influence. For the gold standard treatment for anti-plaque therapy, chlorhexidine digluconate (CHX 0.2%) was used as a positive control, considering it shows antibacterial effects against periodontopathogens.[17–20]

Previous studies have shown the antimicrobial effect of LTP application on *Streptococcus sanguinis*, *C. albicans,* and *S. aureus* biofilm, and *S. Mitis.*[11,12,21] A study conducted in beagles also showed a significant decrease in detection of bacteria (*P. gingivalis* and *Tannerella forsythia*) when nonequilibrium plasma treatment was applied as an adjunct to the conventional therapy.[22] It has been shown that reactive oxygen species and reactive nitrogen species are the central players in its actions of antimicrobial and cancer therapies.[23] Furthermore, plasma-induced apoptosis has been clearly demonstrated in a recent paper that investigated cellular signaling related to an apoptotic process.[24]

In addition to oral biofilm inactivation, the study in beagles observed a larger amount of new bone formation in the bone-to-implant contact surface area in the plasma group.[25] In another study, the ability to remove naturally grown biofilms

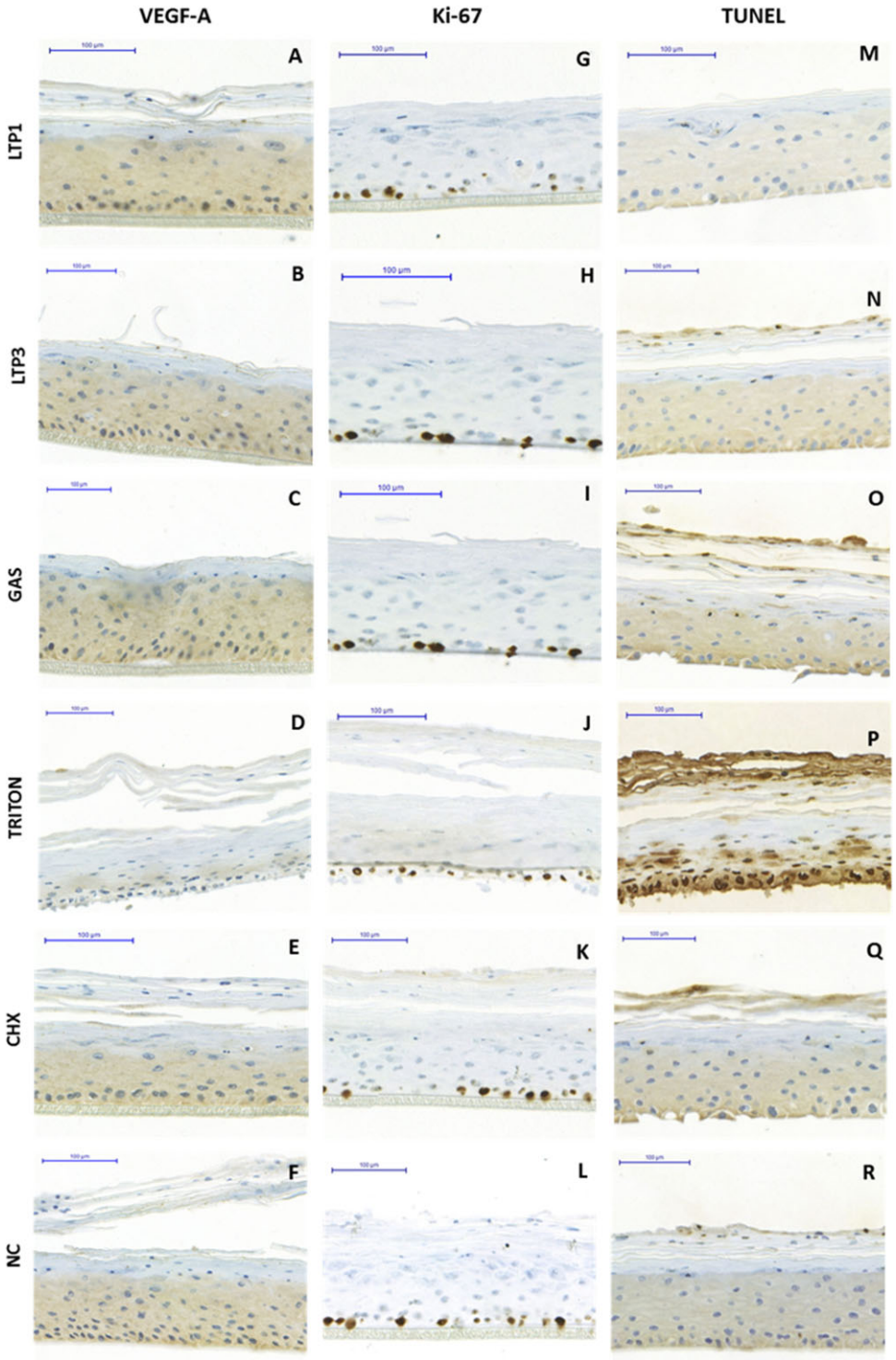

FIGURE 4 VEGF: Intense and diffuse immunostaining in LTP1, LTP3, GAS, CHX groups, and negative control (A, B, C, E, G) group. It shows discreetly more evident for LTP1 and LTP3. Absence of marking in the TRITON group (F); Ki-67: LTP1 (G), LTP3 (H), GAS (I), CHX (K), and control negative (L) groups with <5% of positive cells in basal layer; TRITON group with >15% of positive cells superior stratum (J); TUNEL: TP1 (M), TP3 (N), GAS (O), CHX (Q), and control negative (R) groups were negatives; TRITON group evidenced several positive cells (P)

on teeth with plasma treatment was compared to sonic brush, and biofilm removal was comparable for both treatments.[25]

However, it is important to note that killing the biofilm is not enough for its removal, considering there are other biofilm products that play a role in peri-implantitis, including cytokines.[26] It was demonstrated that removal of dead microbial residues was equally necessary to promote cell adhesion.[27] The visual analysis of the confocal images of the

present study showed fewer microorganisms in plasma-treated samples than in negative control groups. Although these findings indicate a possible mechanical removal of dead biofilm, the LTP potential to remove dead bacterium is still controversial. A recent study used 7-day-old biofilms to investigate the cleaning efficacy of air polishing with or without additional cold plasma treatment and concluded that concomitant use of air polishing and plasma treatment did not enhance osteoblast spreading.[28]

Despite the effective antimicrobial effect of plasma treatment, the fundamental nature of the interaction between plasma and human cells is still unknown to a large extent. So, a detailed knowledge of these interactions is essential for the evaluation of plasma effects in relation to cytotoxic and the establishment of new therapeutic plasma tools.

Various toxic responses or cellular and molecular functions are affected by LTP treatment in terms of up or down regulation of their associated proteins. Most of the toxicity responses are linked to oxidative stress response emphasizing oxidative stress as a possible key event in the regeneration process of epithelial cells, as well as in the adaptation to plasma exposure.[29] It is interesting to note that some recent studies have already revealed dose-dependent cellular effects in response to cold plasma treatment.[30,31] These studies indicate that increasing plasma doses could heighten the proportion of cells which are forced to undergo apoptosis, leading to a progressive negation of positive effects. Therefore, the biologic response should be tested for specific conditions.

To evaluate possible toxic responses by LTP application, plasma treatment was conducted on an in vitro reconstituted human gingival tissue containing normal and human-derived gingival cells. The cells have been cultured to form multilayered, highly differentiated models of gingival phenotypes. The tissues are cultured on specially prepared cell culture inserts using serum-free medium and attain levels of differentiation on the cutting edge of in vitro cell culture technology. The EpiGingival tissue models exhibit in vivo-like morphologic and growth characteristics, which are uniform and highly reproducible.[32] As gingival tissue has similar morphologic and physiologic characteristics of peri-implantitis soft tissue, testing plasma biologic response on gingival tissue is a distinction of this study. For our acknowledgment, no previous study with plasma was conducted on gingival tissue.

The results of the present study showed low cytotoxicity (Figure 2), high viability (Figure 3) levels after LTP application. It agrees with results of previous study that was conducted with the same plasma device, but in a different tissue, a reconstituted oral epithelium.[9] Regarding LDH, although negative control group showed statically significant difference in comparison to LTP groups, clinical relevance has to be further studied. Results for LTP1 were similar to GAS and CHX groups. However, LTP1 and LTP3 were different. Three minutes of plasma application was more cytotoxic than 1 minute. This data is in accordance to some recent studies that demonstrated dose-dependent cellular effects in response to cold plasma treatment.[33,34] Furthermore, LTP treatment is able to get selective killing effect between normal gingival cells and cancer cells due to sensitivity against reactive species.[30,35]

The histologic and immunohistochemical results also showed safety biologic response for LTP treatment. H/E stained sections showed minimal cell damage for plasma-treated groups, similar to the NEGATIVE and CHX groups. It means that the damage was repaired since the cellular changes were unobtrusive. Through labeling for Ki-67, it was possible to detect that LTP1 and LTP3 groups showed proliferative index similar to the NEGATIVE and CHX groups (<5%), similar to what was described previously, which illustrated that plasma was not able to act deep in the reconstituted oral epithelium tissue structure. Keratinized epithelium may also have a protective barrier against plasma reactive species.[12] TUNEL marker also showed that TRITON group showed diffuse positivity, different from all others groups. The epithelial cell analysis for anti-VEGF-A antibody demonstrated that LTP1 and LTP3 evidenced intense and diffuse cytoplasmic labeling in almost all epithelial layers. VEGF is a highly conserved, disulfide-bonded dimeric glycoprotein of 34-45 kDa and it is produced by several cell types, including fibroblasts, neutrophils, endothelial cells and peripheral blood mononuclear cells, macrophages, activated T-cells, and epidermal keratinocytes. VEGF binds to specific receptors expressed in endothelial cells.[36] VEGF plays a fundamental role in tissue repair, considering revascularization is a dynamic event necessary for the complete healing process. During angiogenesis, several growth factors are expressed and regulated. However, it is known that VEGF is the main factor in this process.[37,38] When the level of VEGF released in the culture media of human gingival epithelial cells from patients with generalized chronic periodontitis was analyzed, it was concluded that VEGF in periodontal disease may predict a greater regeneration capacity of gingival tissue.[39,40] Thus, the results of the present study indicate that VEGF-A immunoexpression identified in the epithelial cells of plasma-treated groups may be an induced repair factor.

Another possible advantage of LTP treatment is that it can potentially enhance peri-implant soft-tissue seal on titanium abutments, possibly preventing infection from the oral environment and decreasing implant failure. A recent study demonstrated that LTP treatment for 10 s improved the attachment of human gingival fibroblasts to titanium disks, perhaps due to oxygen present in functional groups on the surface, and/or decreased levels of carbon contamination.[4]

Even though significant reduction of viable biofilm was found in plasma-treated groups when compared to negative control, the magnitude of the reduction was less than 2 $\log_{10}$ CFU/mL. Further studies should be conducted in order to show whether this is a clinically relevant microbial reducing.

As we know, tip-to-sample distance, biofilm thickness, and treatment duration can influence plasma effect.[30,31] Furthermore, as CHX had the highest antimicrobial effect, further studies could consider additive or synergistic effects resulting from the combination of both treatments (CHX + LTP) or (LTP + CHX). This hypothesis would have to be further studied. In addition, biologic responses should be evaluated for each parameter. Despite the proven antimicrobial effect of CHX on titanium bound biofilms, CHX may compromise the biocompatibility of titanium surfaces, and its use is not recommended to detoxify implants. Then, LTP could be an alternative method for peri-implantitis treatment.[41] Further studies are also needed to determine the safety and efficacy of LTP application in vivo and in the clinical settings.

As a limitation, even if plasma treatment was effective in the studied *P. gingivalis* biofilm, in vivo peri-implantitis biofilms are much more structured than the one simulated here, which might affect the antimicrobial potential of LTP. So, future experiments should be conducted in more complex biofilm models aiming to understand the effect of the specific treatment on the biofilm structure, similar to the clinical peri-implant biofilm.

5 | CONCLUSION

Within the limitations of the present in vitro study, it can be concluded that LTP is a promising approach in peri-implant infection treatment. LTP treatment safely reduced *P. gingivalis* biofilm. In addition, plasma treatment may be associated with cellular report within a reconstituted gingival epithelium. Further studies are required to investigate the impact of LTP on clinical parameters, such as bleeding on probing and probing depth, and on the regeneration of gingival tissues surrounding titanium implants.

ACKNOWLEDGEMENTS

This study was performed in the Department of Basic Science & Craniofacial Biology at New York University College of Dentistry where the supervisor Dr. Simone Duarte was working as Clinical Associate Professor before moving to Indiana University School of Dentistry, where she is currently working as an Associate Professor. We would like to thank Singular Implants—Dmr Ind. e Com. de Materiais Odontologicos Ltda, Parnamirim, RN, Brazil for providing the titanium discs.

REFERENCES

1. Marcantonio C, Nicoli LG, Marcontonio Junior E, et al. Prevalence and possible risk factors of peri-implantitis: a concept review. *J Contemp Dent Pract*. 2012;16:750–757. https://doi.org/10.5005/jp-journals-10024-1752
2. Tarnow DP. Increasing prevalence of peri-implantitis: how will we manage. *J Dent Res*. 2016;95:7–8. https://doi.org/10.1177/0022034515616557
3. Derks J, Tomasi C. Peri-implant health and disease. A systematic review of current epidemiology. *J Clin Periodontol*. 2015;42(Suppl 16):S158–S171. https://doi.org/10.1111/jcpe.12334
4. Lee JH, Kim YH, Choi EH, et al. Air atmospheric-pressure plasma-jet treatment enhances the attachment of human gingival fibroblasts for early peri-implant soft tissue seals on titanium dental implant abutments. *Acta Odontol Scand*. 2015;73:67–75. https://doi.org/10.3109/00016357.2014.954265
5. Ermolaeva SA, Varfolomeev AF, Chernukha MY, et al. Bactericidal effects of non-thermal argon plasma *in vitro*, in biofilms and in the animal model of infected wounds. *J Med Microbiol*. 2011;60(Pt 1):75–83. https://doi.org/10.1099/jmm.0.020263-0
6. Joshi SG, Paff M, Friedman G, et al. Control of methicillin-resistant *Staphylococcus aureus* in planktonic form and biofilms: a biocidal efficacy study of nonthermal dielectric-barrier discharge plasma. *Am J Infect Control*. 2010;38:293–301. https://doi.org/10.1016/j.ajic.2009.11.002
7. Moisan M, Barbeau J, Moreau S, et al. Low-temperature sterilization using gas plasmas: a review of the experiments and an analysis of the inactivation mechanisms. *Int J Pharm*. 2001;226:1–21. https://doi.org/10.1016/S0378-5173(01)00752-9
8. Traba C, Liang JF. Susceptibility of *Staphylococcus aureus* biofilms to reactive discharge gases. *Biofouling*. 2011;27:763–772. https://doi.org/10.1080/08927014.2011.602188
9. Woedtke TV, Reuter S, Masur K, et al. Plasma for medicine. *Phys Reports*. 2013;530:291–320. https://doi.org/10.1016/j.physrep.2013.05.005
10. Duske K, Koban I, Kindel E, et al. Atmospheric plasma enhances wettability and cell spreading on dental implant metals. *J Clin Periodontol*. 2012;39:400–407. https://doi.org/10.1111/j.1600-051X.2012.01853.x
11. Preissner S, Wirtz HC, Tietz AK, et al. Bactericidal efficacy of tissue tolerable plasma on microrough titanium dental implants: an *in-vitro*-study. *J Biophotonics*. 2016;9:637–644. https://doi.org/10.1002/jbio.201500189
12. Delben JA, Zago CE, Tyhovych N, et al. Effect of atmospheric-pressure cold plasma on pathogenic oral biofilms and *in vitro* reconstituted oral epithelium. *PLoS One*. 2016;11:e0155427. https://doi.org/10.1371/journal.pone.0155427
13. Tavares LJ, Klein MI, Panariello BHD, et al. An in vitro model of *Fusobacterium nucleatum* and *Porphyromonas gingivalis in single*- and dual-species biofilms. *J Periodontal Implant Sci*. 2018;48:12–21. https://doi.org/10.5051/jpis.2018.48.1.12
14. Ebadian B, Razavi M, Soleimanpour S, et al. Evaluation of tissue reaction to some denture-base materials: an animal study. *J Contemp Dent Pract*. 2008;9:67–74.
15. Darveau RP, Hajishengallis G, Curtis MA. *Porphyromonas gingivalis* as a potential community activist for disease. *J Dent Res*. 201291:816–820. https://doi.org/10.1177/0022034512453589
16. Rutar A, Lang NP, Buser D, et al. Retrospective assessment of clinical and microbiological factors affecting periimplant tissue conditions. *Clin Oral Implants Res*. 2001;12:189–195. https://doi.org/10.1034/j.1600-0501.2001.012003189.x

17. Kadkhoda Z, Amarlu Z, Eshraghi S, et al. Antimicrobial effect of chlorhexidine on *Aggregatibacter actinomycetemcomitans* biofilms associated with peri-implantitis. *J Dent Res Dent Clin Dent Prospects*. 2016;10:176–180. https://doi.org/10.15171/joddd.2016.028

18. Jones CG. Chlorhexidine: I sit still the gold standard? *Periodontol 2000*. 1997;15:55–62

19. Koychev S, Dommisch H, Chen H, et al. Antimicrobial effects of mastic extract against oral periodontal pathogens. *J Periodontol*. 2017;88:511–517. https://doi.org/10.1902/jop.2017.150691

20. Lasserre J, Toma S, Dos Santos-Gonçalvez AM, et al. Evaluation of Emdogain antimicrobial effectiveness against biofilms containing the keystone pathogen *Porphyromonas gingivalis*. *New Microbiol*. 2018;41:73–76.

21. Gorynia S, Koban I, Matthes R, et al. In vitro efficacy of cold atmospheric pressure plasma on *S. sanguinis* biofilms in comparison of two test models. *GMS Hyg Infect Control*. 2013;8:Doc01. https://doi.org/10.3205/dgkh000201

22. Shi Q, Song K, Zhou X, et al. Effects of non-equilibrium plasma in the treatment of ligature-induced peri-implantitis. *J Clin Periodontol*. 2015;42:478–487. https://doi.org/10.1111/jcpe.12403

23. Conway GE, Casey A, Milosavljevic V, et al. Non-thermal atmospheric plasma induces ROS-independent cell death in U373MG glioma cells and augments the cytotoxicity of temozolomide. *Br J Cancer*. 2016;114:435–443. https://doi.org/10.1038/bjc.2016.12

24. Lee JH, Om JY, Kim YH, et al. Selective killing effects of cold atmospheric pressure plasma with no induced dysfunction of epidermal growth factor receptor in oral squamous cell carcinoma. *PLoS One*. 2016;11:e0150279. https://doi.org/10.1371/journal.pone.0150279

25. Jablonowski L, Fricke K, Matthes R, et al. Removal of naturally grown human biofilm with an atmospheric pressure plasma jet: an *in-vitro* study. *J Biophotonics*. 2017;10:718–726. https://doi.org/10.1002/jbio.201600166

26. Kato H, Taguchi Y, Tominaga K, et al. *Porphyromonas gingivalis* LPS inhibits osteoblastic differentiation and promotes pro-inflammatory cytokine production in human periodontal ligament stem cells. *Arch Oral Biol*. 2014;59:167–175. https://doi.org/10.1016/j.archoralbio.2013.11.008

27. Matthes R, Duske K, Kebede TG, et al. Osteoblast growth, after cleaning of biofilm-covered titanium discs with air-polishing and cold plasma. *J Clin Periodontol*. 2017b;44:672–680. https://doi.org/10.1111/jcpe.1272

28. Matthes R, Duske K, Kebede T, et al. Osteoblast growth after cleaning of biofilm covered titanium discs with air-polishing and cold plasma. *J Clin Periodontol*. 2017a;44:672–680. https://doi.org/10.1111/jcpe.12720.

29. Lendeckel D, Eymann C, Emicke P, et al. Proteomic changes of tissue-tolerable plasma treated airway epithelial cells and their relation to wound healing. *Biomed Res Int*. 2015; 2015:1–17. https://doi.org/10.1155/2015/506059

30. Blackert S, Haertel B, Wende K, et al. Influence of non-thermal atmospheric pressure plasma on cellular structures and processes in human keratinocytes (HaCaT). *J Dermatol Sci*. 2013;70:173–181. https://doi.org/10.1016/j.jdermsci.2013.01.012

31. Haertel B, Hähnel M, Blackert S, et al. Surface molecules on HaCaT keratinocytes after interaction with non-thermal atmospheric pressure plasma. *Cell Biol Int*. 2012;36:1217–1222. https://doi.org/10.1042/CBI20120139

32. Klausner M, Ayehunie S, Breyfogle BA, et al. Organotypic human oral tissue models for toxicological studies. *Toxicol In Vitro*. 2007;21:938–949. https://doi.org/10.1016/j.tiv.2007.01.024

33. Kim CH, Bahn JH, Lee SH, et al. Induction of cell growth arrest by atmospheric non-thermal plasma in colorectal cancer cells. *J Biotechnol*. 2010;150:530–538. https://doi.org/10.1016/j.jbiotec.2010

34. Lee JH, Kim KN. Effects of a nonthermal atmospheric pressure plasma jet on human gingival fibroblasts for biomedical application. *Biomed Res Int*. 2016; 2016:1–9. https://doi.org/10.1155/2016/2876916.

35. Weiss M, Gumbel D, Hanschmann EM, et al. Cold atmospheric plasma treatment induces anti-proliferative effects in prostate cancer cells by redox and apoptotic signaling pathways. *PLoS One*. 2015;10:e0130350. https://doi.org/10.1371/journal.pone.0130350

36. Scrieciu M, Mercu TV, Andrei OC, et al. Immunoexpression of vascular endothelial growth factor in gingival mucosa with *Papilloma* and *Condyloma acuminata*. *Rom J Morphol Embryol*. 2015;56:1077–1083.

37. Szpaderska AM, Walsh CG, Steinberg MJ, et al. Distinct patterns of angiogenesis in oral and skin wounds. *J Dent Res*. 2005;84:309–314. https://doi.org/10.1177/154405910508400403

38. Bao P, Kodra A, Tomic-Canic M, et al. The role of vascular endothelial growth factor in wound healing. *J Surg Res*. 2009;153:347–358. https://doi.org/10.1016/j.jss.2008.04.023

39. Lucarini G, Zizzi A, Aspriello SD, et al. Involvement of vascular endothelial growth factor, cd44 and cd133 in periodontal disease and diabetes: an immunohistochemical study. *J Clin Periodontol*. 2009;36:3–10. https://doi.org/10.1111/j.1600-051X.2008.01338.x

40. Momose M, Murata M, Kato Y, et al. Vascular endothelial growth factor and transforming growth factor-alpha and -beta1 are released from human cultured gingival epithelial sheets. *J Periodontol*. 2002;73:748–753. https://doi.org/10.1902/jop.2002.73.7.748

41. Kotsakis GA, Lan C, Barbosa J, et al. Antimicrobial agents used in the treatment of peri-implantitis alter the physicochemistry and cytocompatibility of titanium surfaces. *J Periodontol*. 2016;87:809–819. https://doi.org/10.1902/jop.2016.150684

How to cite this article: Carreiro AFP, Delben JA, Guedes S, et al. Low-temperature plasma on peri-implant-related biofilm and gingival tissue. *J Periodontol*. 2018;1–9. https://doi.org/10.1002/JPER.18-0366

Microbiology Research Journal International

22(6): 1-7, 2017; Article no.MRJI.39345
ISSN: 2456-7043
(Past name: British Microbiology Research Journal, Past ISSN: 2231-0886, NLM ID: 101608140)

Farnesol Anti-biofilm Activity against *Candida albicans* Reference and Mutant Strains

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Authors' contributions

This work was carried out in collaboration between all authors. Authors AFPC and SD designed the study. Author BHDP managed the literature search. Authors AFPC, SFFG and PVS performed the experiments and collected data. Author MNJ performed the statistical analysis. Results interpretation and discussion were performed by all authors. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/MRJI/2017/39345
Editor(s):
(1) Marcin Lukaszewicz, Department of Biotransformation, Faculty of Biotechnology, University of Wroclaw, Wroclaw, Poland and Division of Chemistry and Technology Fuels, Wroclaw University of Technology, Wroclaw, Poland.
Reviewers:
(1) İlknur Dag, Eskisehir Osmangazi University, Turkey.
(2) Mohammed Asaad Elmowafy, Mansoura University, Egypt.
(3) Carolina Pohl, University of the Free State, South Africa.
Complete Peer review History: http://www.sciencedomain.org/review-history/23227

Original Research Article

Received 2nd December 2017
Accepted 8th February 2018
Published 16th February 2018

ABSTRACT

Introduction: Farnesol is known as a quorum sensing (QS) molecule that has a role as an anti-biofilm agent. It is produced by *C. albicans* and blocks the morphological transition from yeasts to hyphae. The hyphal development is important for the formation of substantial biofilm biomass. Mutant strains lacking the filamentation genes EFG1 and TEC1 are less virulent than their reference strains.
Aims: To determine the role of the transcription factors EFG1 and TEC1 by using knockout strains

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(Δ/Δ efg1 and Δ/Δ tec 1) on farnesol's mechanism of action regarding dry weight and colony count of *Candida albicans* biofilms.
Study Design: *tt*-farnesol solutions at a concentration of 12.5 mM were prepared. Biofilms (n=6) of the strains *C. albicans* SN 425 (reference strain), *C. albicans* CJN 2330 (Δ/Δ tec1) and *C. albicans* CJN 2302 (Δ/Δ efg1) were treated twice daily with *tt*-farnesol or a vehicle control for 1 min during biofilm formation (48 h). Biofilms were also treated with 0.2% chlorhexidine (1 min) as a positive control and 0.89% NaCl (1 min) as a negative control. After treatments, biofilms were evaluated by dry weight (μg) and colony forming units per milliliters (CFU/mL).
Results: Data were analyzed by two-way ANOVA and *post-hoc* t-tests (α=0.05). Relative to the control, farnesol significantly reduced the CFU/mL of all the *C. albicans* strains and the biomass of the mutant strain CJN 2330.
Conclusion: Twice daily treatment with *tt*-farnesol at a concentration of 12.5 mM exhibited anti-biofilm activity against *C. albicans.* Analysis of strain differences suggests that the presence of the transcription factor TEC1 protects the biofilm against *tt*-farnesol mechanism of action.

Keywords: Candida; biofilms; farnesol; EFG 1; TEC 1; antimicrobials; candiadisis; oral.

1. INTRODUCTION

Denture stomatitis is a common inflammatory reaction in denture-wearing patients, characterized by an erythematous inflammation of mucosal areas covered by dentures. Although this clinical entity is multifactorial, *Candida albicans* is the major etiological agent [1] *Candida* infections are related mainly to the morphology transition of yeast to hyphae, because hyphae can adhere to and penetrate the host tissues [2]. This morphologic transition can be influenced by the quorum sensing system (QS), which is a complex cross- talking system where microorganisms communicate with each other in response to cell density via QS molecules [3].

Farnesol is a sesquiterpene alcohol (3,7,11-trimethyl-2,6,10-dodecatrien-1-ol) found in propolis and citrus fruits [4]. It has been found to be a natural anti-biofilm agent [5]. Topical applications of *tt*-farnesol for 1 min twice-daily reduced the exopolysaccharides amounts and development of single-species *Streptococcus mutans* biofilms on saliva-coated hydroxyapatite surfaces [5]. Additionally, *tt*-farnesol treatment in concentrations equal to or greater than 12.5 mM resulted in significant reductions in total biomass, CFUs and metabolic activity of a multispecies biofilm formed by *S. mutans* and *C. albicans*, and these reductions were also observed for the single species biofilms [6].

Farnesol has been recognized as a QS molecule involved in the coordination of activities among groups of many single-celled organisms [7]. It is produced by *C. albicans* and blocks the morphological transition from yeasts to hyphae [8] via inhibition of MAP kinase cascades [9]. The literature indicates farnesol has a role as an anti-biofilm agent [6]. The hyphal development pathway is critical for the establishment of significant biofilm mass [10]. Mutants defective in the enhanced filamentous growth transcriptional factor (EFG 1), a major activator of hyphal development, does not form a bulky and regular biofilm on polystyrene surfaces [10]. TEC 1 is a transcription factor required for hyphal development [11]. Mutant strains defective in these filamentation genes are less virulent than their reference strains and show lower levels of infectivity of endothelial cells and plasma-coated catheters [12-13].

The transcriptional network controlling *C. albicans* biofilm formation was previously investigated comparing the reference strain used in this study (SN425) to six gene knockout mutants of this diploid organism, including those with absence of the genes EFG 1 (Δ/Δ efg1) and TEC 1 (Δ/Δ tec 1) in both chromosomes [14]. In the same study, confocal images showed that the reference strain formed a biofilm with typical architecture and thickness of 250 μm in depth, containing both round budding yeast-form cells adjacent to the substrate and hyphal cells extending throughout the biofilm. But the mutants formed rudimentary biofilms of 20–80 mm in depth. Moreover, Δ/Δ efg1 mutant strain did not form hyphae [14].

Several chemical agents have been employed for cleaning dentures to prevent cross- infection. However, previous studies have demonstrated changes in characteristics of denture base resin due to immersion in chemical cleaners, such as sodium-hypochlorite (NaOCl) and alkaline

peroxides that may affect the lifetime of dentures [15]. These agents can compromise the mechanical properties and color stability of the dentures. Also, sodium- hypochlorite (NaOCl) has oxidative properties in metal framework [16]. Moreover, denture stomatitis is related to the growth of *Candida* biofilms on the prosthesis and not on the palate mucosa, so the ideal treatment to this disease should be directed to the prosthesis [17]. Thus, new disinfectants are necessary, considering an optimal denture disinfectant is not available. Farnesol is a potential anti-biofilm agent [5-6], however, its exact mechanism of action still unknown. In the present study we used knockout strains (Δ/Δ efg1 and Δ/Δ tec 1) to determine the role of the transcription factors EFG1 and TEC1 on farnesol's mechanism of action regarding dry weight and colony count (CFU/mL) of *C. albicans* biofilms.

2. METHODOLOGY

2.1 Inoculum and Biofilm Model

2.1.1 Biofilm inoculums

Three strains of *Candida- C. albicans* SN 425, CJN 2302 and CJN2330 [14]-, maintained as frozen stocks at – 80ºC, were reactivated on Sabouraud dextrose agar plates (SDA) plates. To prepare the inoculum, colonies of *Candida* were inoculated into 10 mL of Yeast Nitrogen Base (YNB) supplemented with 100 mM of glucose and incubated at 37ºC for 16 h. After 16 h of incubation, the inoculums were diluted into fresh YNB medium until the three strains reached the mid-log growth phase Then, the inoculums were adjusted to 10^7 cells/mL at the OD of 540 nm [18].

2.1.2 Biofilm formation

One (1) mL of the inoculums was transferred to the wells of a 24-well polystyrene plate. The biofilms were incubated for 90 minutes at 37ºC for the adhesion of cells to the bottom of the wells. After 90 min, the cells that were not adhered were removed by 2 washes with 1 mL of 0.89% NaCl. After washings, 1 mL of fresh RPMI 1640 medium buffered to pH 7.0 with 0.165 M morpholinepropanesulfonic acid (MOPS) was added to each sample. The biofilms were then incubated at 37ºC and treatments were applied twice-daily. After 48-hour incubation, the biofilms were processed analyzed.

2.2 Preparation of Treatment Solutions

The tested solutions were prepared at the time of use from a commercial solution of *tt*- farnesol (277541-1G Trans, trans-Farnesol, Sigma-Aldrich) using a solution containing 20% ethanol and 2% tween 80 as the vehicle. A solution of 12.5 mM of *tt*-farnesol was prepared for the assays [6]. A vehicle solution containing 20% ethanol and 2% tween 80 was also used as a control. As positive control, 0.2% chlorhexidine (CHX) was applied. As a negative control, 0.89% NaCl was used. The biofilms were treated with tt-farnesol or control solutions (1 min) twice-daily until complete 48 h of the experimental period.

2.3 Biofilm Analysis

After the last treatment, 1 mL of 0.89% NaCl was added to the biofilms and the biofilms were removed from the bottom of each well by scratching with a pipette tip. Then, the biofilms removed with 1 mL of NaCl were individually transferred to sterile centrifuge tubes. Additional 1 mL of 0.89% NaCl was added to these tubes, resulting in 2 mL of biofilm suspension. From this suspension, 100 µL was serially diluted and plated in SDA plates for the quantification of colony forming units (CFU/mL). For the dry weight (biomass), 100 µL of biofilm solution [18] was transferred to microcentrifuge tubes previously weighed. One (1) mL of ice-cold 99% ethanol was added to the tubes. Before, tubes were centrifuged (10,000 xg, 10 min, 4ºC) and the supernatant of each tube was discarded. The *pellet* was washed twice with ice-cold 75% ethanol and air-dried. The tubes were then weighed again, and the dry weight was determined in micrograms (µg).

2.4 Statistical Analyses

Tests were performed in three separate occasions in duplicate (n=6). IBM SPSS v. 22 software (IBM Corp., Armonk, NY, USA) was used for statistical analysis with a confidence level of 95%. CFU/mL data were Log_{10} transformed prior to analysis and were further rank transformed for analysis of variance (ANOVA). For biomass, ANOVA was performed with data in micrograms (µg). Group differences were compared using a two-way ANOVA with factors of treatment and strain, and given a significant omnibus test, *post-hoc* t-tests were pursued using a pooled estimate of the standard error.

3. RESULTS AND DISCUSSION

C. albicans is the major etiological agent of denture stomatitis [1] and the capacity to undergo a reversible yeast-hypha transformation is linked to its virulence [13]. When infecting humans and animals, *C. albicans* hyphae predominate at the primary site of infiltration of epithelial cell layers and tissues, while yeast cells are normally found either on the epithelial cell surface or emerging from penetrating hyphae that are infiltrating tissues [19]. Thus, reducing hyphae is important to reduce the penetration of *C. albicans* into the host tissues. Considering that the ideal treatment to denture stomatitis should be directed to the prosthesis [17], treating denture with farnesol might be a good alternative to the classic treatments. Farnesol effects on *C. albicans* biofilm were previously demonstrated [6, 20-22]; however, its exact mechanism of action is still unknown. The major question in this study was to investigate if the transcription factors EFG1 and TEC1 were involved in farnesol's mechanism of action in *C. albicans'* biofilm biomass and colony count.

In the present study, *tt*-farnesol was applied at a concentration of 12.5 mM, based on previous results [6]. Farnesol is known to act mainly in the morphological transition from yeasts to hyphae, which happens during the maturation of the biofilm [23]. Therefore, we performed the treatments during biofilm development, starting after the adhesion phase, twice a day, trying to simulate the denture cleaning that could be performed at home by the patient. Until we are aware, this is the first time *tt*-farnesol is tested against *C. albicans* mutant strains.

The results of CFU/mL are summarized in Fig. 1. Two-way ANOVA showed an interaction between "strain" and "treatment" factors ($p < .001$). Post-hoc testing then showed that *tt*-farnesol at 12.5 mM was associated with lesser colonies than the negative control group (NaCl) for all strains, but more colonies than the positive control, CHX.

Comparing strains for *tt*-farnesol 12.5 mM treatment, the reference strain (SN 425) exhibited the fewest colonies (Fig. 1). Moreover, the treatment with *tt*-farnesol caused a significant reduction in the CFU counts of all strains compared to the treatment with 0.89% NaCl. According to previous studies, in addition to the fact that farnesol blocks the conversion of yeast cells to hyphae in *C. albicans* [8],

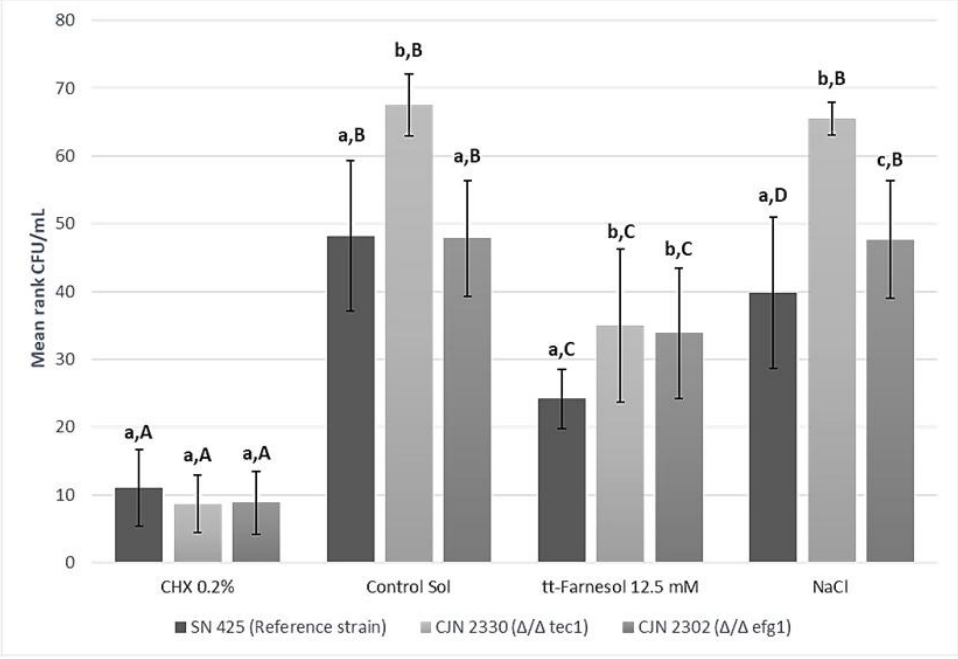

Fig. 1. Mean rank and standard deviations of rank CFU/mL. Means followed by the different letters are significantly different ($p < .05$)

Lowercase letters represent differences between strains within each treatment. Uppercase letters represent differences between treatments for each strain.

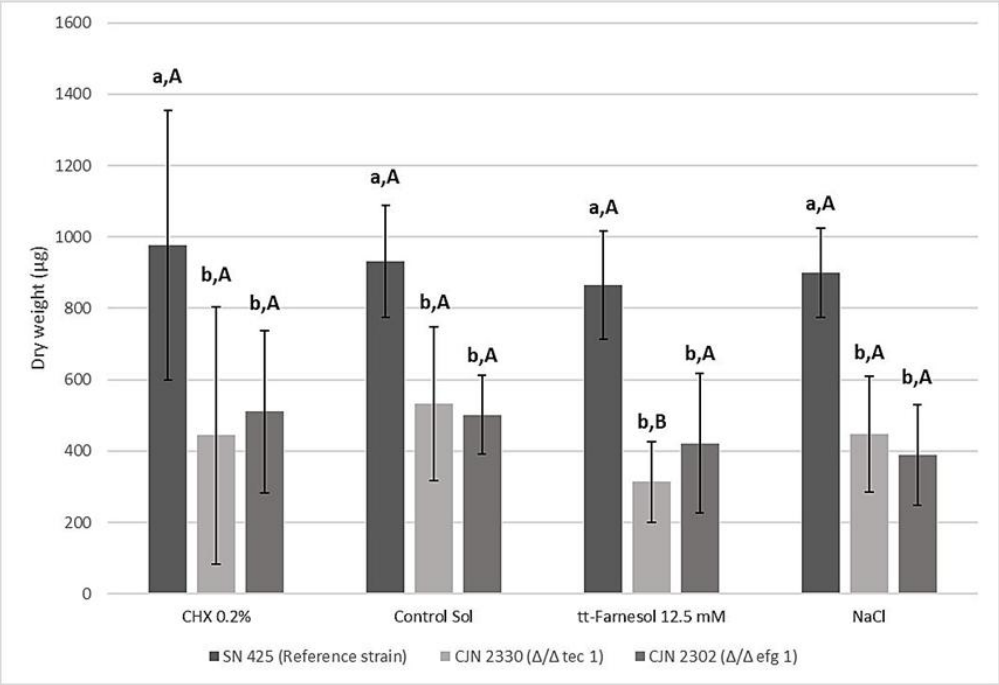

Fig. 2. Mean and standard deviations of dry weight (µg). Means followed by the different letters are significantly different (ρ < .05)

Lowercase letters represent differences between strains within each treatment. Uppercase letters represent differences between treatments for each strain.

it has also been shown to prevent cell adhesion and promote detachment of biofilms from different surfaces [24]. In contrast, *tt*-farnesol did not exhibit similar results of CFU/mL to the positive control (CHX). Although CHX is considered a gold standard antibacterial disinfectant [25], it has disadvantages including color changing in auto polymerizing reline resins for denture [26] and in acrylic denture teeth [27]. So, an ideal disinfectant is not available, and the positive results obtained with farnesol can open doors for new trials with this compound for denture disinfection.

For biomass (dry weight), results showed again an interaction between the factors "strain" and "treatment" (p < .001) (Fig. 2). While *tt*-farnesol treatment was associated with reduced biomass in the CJN 2330 strain, it had no effect on SN 425 or CJN 2302 strains.

The reference strain (SN 425) presented a higher biomass than the mutant strains for all treatments (Fig. 2), confirming the mutant strains have deficiency in biofilm formation. Interestingly, *tt*-farnesol significantly reduced the biomass only of the mutant strain CJN 2330 (Fig. 2), not only regarding Control and NaCl treatments, but also lower than CHX. The biomass comprises cells in the biofilm and the extracellular matrix produced by the biofilms, which is composed of exopolysaccharides, proteins and extracellular DNA [18]. Therefore, *tt*-farnesol acted reducing the colony counting of all strains but might not have reduced components of the extracellular matrix of CJN 2302 and SN 425. On the other hand, *tt*-farnesol might have reduced some component(s) of the extracellular matrix of CJN 2330 and might have acted to reduce the transition from yeast to hyphae, resulting in lower biomass. This outcome suggests that the presence of the transcription factor TEC1 protects the biofilm against *tt*-farnesol mechanism of action.

4. CONCLUSION

The aim of the study was to determine the role of the transcription factors EFG1 and TEC1 on farnesol's mechanism of action in *C. albica*ns' biofilm biomass and colony count. Within the limitations of this *in vitro* study, we conclude that the presence of the transcription factor TEC1 might protect the biofilm against *tt*-farnesol

mechanism of action and that twice-daily treatment with 12.5 mM *tt*-farnesol showed anti-biofilm effects against both *C. albicans.* mutant and reference strains. However, the biological significance of these findings would need further studies.

ACKNOWLEDGEMENT

We thank Dr. Alexander D. Johnson, Department of Microbiology and Immunology, UCSF, for his kind donation of the strains used in this study.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Gendreau L, Loewy ZG. Epidemiology and etiology of denture stomatitis. J Prosthodont. 2011;20(4):251-60.
DOI: 10.1111/j.1532-849X.2011.00698.x.
2. Rocha CR, Schroppel K, Harcus D, Marcil A, Dignard D, Taylor BN, Thomas DY, Whiteway M, Leberer E. Signaling through adenylyl cyclase is essential for hyphal growth and virulence in the pathogenic fungus *Candida albicans*. Mol Biol Cell. 2001;12(11):3631-43.
PMID: 11694594 PMCID: PMC60281
3. Wongsuk T, Pumeesat P, Luplertlop N. Fungal quorum sensing molecules: Role in fungal morphogenesis and pathogenicity. J Basic Microbiol. 2016;56(5):440-7.
DOI: 10.1002/jobm.201500759
4. Jeon JG, Pandit S, Xiao J, Gregoire S, Falsetta ML, Klein MI, Koo H. Influences of trans-trans farnesol, a membrane-targeting sesquiterpenoid, on Streptococcus mutans physiology and survival within mixed-species oral biofilms. Int J Oral Sci. 2011;3(2):98-106.
DOI:10.4248/IJOS11038
5. Koo H, Hayacibara MF, Schobel BD, Cury JA, Rosalen PL, Park YK, Vacca-Smith AM, Bowen WH. Inhibition of Streptococcus mutans biofilm accumulation and polysaccharide production by apigenin and tt-farnesol. J Antimicrob Chemother. 2003;52(5):782-9.
DOI: 10.1093/jac/dkg449
6. Fernandes RA, Monteiro DR, Arias LS, Fernandes GL, Delbem AC, Barbosa DB. Biofilm formation by *Candida albicans* and *Streptococcus mutans* in the presence of farnesol: a quantitative evaluation. Biofouling. 2016;32(3):329-38.
DOI:10.1080/08927014.2016.1144053
7. Alem MA, Oteef MD, Flowers TH, Douglas LJ. Production of tyrosol by *Candida albicans* biofilms and its role in quorum sensing and biofilm development. Eukaryot Cell. 2006;5(10):1770-9.
DOI:10.1128/EC.00219-06
8. Piispanen AE, Grahl N, Hollomon JM, Hogan DA. Regulated proteolysis of *Candida albicans* Ras1 is involved in morphogenesis and quorum sensing regulation. Mol Microbiol. 2013;89(1):166-78.
DOI:10.1111/mmi.12268
9. Sato T, Watanabe T, Mikami T, Matsumoto T. Farnesol, a morphogenetic autoregulatory substance in the dimorphic fungus *Candida albicans*, inhibits hyphae growth through suppression of a mitogen-activated protein kinase cascade. Biol Pharm Bull. 2004;27(5):751-2.
PMID: 15133261
10. Ramage G, VandeWalle K, López-Ribot JL, Wickes BL. The filamentation pathway controlled by the Efg1 regulator protein is required for normal biofilm formation and development in *Candida albicans*. FEMS Microbiol Lett. 2002;214(1):95-100.
PMID: 12204378
11. Schweizer A, Rupp S, Taylor BN, Rollinghoff M, Schroppel K. The TEA/ATTS transcription factor CaTec1p regulates hyphal development and virulence in *Candida albicans*. Mol Microbiol. 2000;38(3):435-45.
PMID: 11069668
12. Lewis RE, Lo HJ, Raad, II, Kontoyiannis DP. Lack of catheter infection by the efg1/efg1 cph1/cph1 double-null mutant, a *Candida albicans* strain that is defective in filamentous growth. Antimicrob Agents Chemother. 2002;46(4):1153-5.
PMID: 11897612 PMCID: PMC127119
13. Lo HJ, Kohler JR, DiDomenico B, Loebenberg D, Cacciapuoti A, Fink GR. Nonfilamentous C. albicans mutants are avirulent. Cell. 1997;90(5):939-49.
PMID: 9298905
14. Nobile CJ, Fox EP, Nett JE, Sorrells TR, Mitrovich QM, Hernday AD, Tuch BB, Andes DR, Johnson AD. A recently evolved transcriptional network controls biofilm development in *Candida albicans*. Cell. 2012;148(1-2):126-38.
DOI:10.1016/j.cell.2011.10.048.

15. Davi LR, Peracini A, Ribeiro Nde Q, Soares RB, da Silva CH, Paranhos Hde F, de Souza RF. Effect of the physical properties of acrylic resin of overnight immersion in sodium hypochlorite solution. Gerodontology. 2010;27(4):297-302.
16. Felipucci DN, Davi LR, Paranhos HF, Bezzon OL, Silva RF, Pagnano VO. Effect of different cleansers on the surface of removable partial denture. Braz Dent J. 2011;22(5):392-7.
PMID: 22011895
17. Webb BC, Thomas CJ, Harty DW, Willcox MD. Effectiveness of two methods of denture sterilization. J Oral Rehabil. 1998;25(6):416-23.
PMID: 9687113
18. Panariello BHD, Klein MI, Pavarina AC, Duarte S. Inactivation of genes TEC1 and EFG1 in *Candida albicans* influences extracellular matrix composition and biofilm morphology. J Oral Microbiol. 2017;9(1): 1385372.
DOI:10.1080/20002297.2017.1385372
19. Ray TL, Payne CD. Scanning electron microscopy of epidermal adherence and cavitation in murine candidiasis: a role for Candida acid proteinase. Infect Immun. 1988;56(8):1942-9.
PMID: 3294180 PMCID: PMC259505
20. Ramage G, Saville SP, Wickes BL, Lopez-Ribot JL. Inhibition of *Candida albicans* biofilm formation by farnesol, a quorum-sensing molecule. Appl Environ Microbiol. 2002;68(11):5459-63.
PMID: 12406738 PMCID: PMC129887
21. Cao YY, Cao YB, Xu Z, Ying K, Li Y, Xie Y, Zhu ZY, Chen WS, Jiang YY. cDNA microarray analysis of differential gene expression in *Candida albicans* biofilm exposed to farnesol. Antimicrob Agents Chemother. 2005;49(2):584-589.
DOI:10.1128/AAC.49.2.584-589.2005
22. Cordeiro RA, Teixeira CE, Brilhante RS, Castelo-Branco DS, Paiva MA, Giffoni Leite JJ, Lima DT, Monteiro AJ, Sidrim JJ, Rocha MF. Minimum inhibitory concentrations of amphotericin B, azoles and caspofungin against Candida species are reduced by farnesol. Med Mycol. 2013; 51(1):53-9.
DOI: 10.3109/13693786.2012.692489
23. Finkel JS, Mitchell AP. Genetic control of *Candida albicans* biofilm development. Nat Rev Microbiol. 2011;9(2):109-118.
DOI:10.1038/nrmicro2475
24. Nagy G, Hennig GW, Petrenyi K, Kovacs L, Pocsi I, Dombradi V, Banfalvi G. Time-lapse video microscopy and image analysis of adherence and growth patterns of *Candida albicans* strains. Appl Microbiol Biotechnol. 2014;98(11):5185-94.
DOI:10.1007/s00253-014-5696-5
25. Gendron R, Grenier D, Sorsa T, Mayrand D. Inhibition of the activities of matrix metalloproteinases 2, 8, and 9 by chlorhexidine. Clin Diagn Lab Immunol.1999;6(3):437-9.
PMID: 10225852
26. Panariello BH, Izumida FE, Moffa EB, Pavarina AC, Jorge JH, Giampaolo ET. Effects of short-term immersion and brushing with different denture cleansers on the roughness, hardness, and color of two types of acrylic resin. Am J Dent. 2015;28(3):150-6.
PMID: 26201226
27. Flotra L, Gjermo P, Rolla G, Waerhaug J. Side effects of chlorhexidine mouth washes. Scand J Dent Res. 1971;79(2): 119-125.

Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sciencedomain.org/review-history/23227

Clin Oral Invest (2017) 21:2053–2061
DOI 10.1007/s00784-016-1995-9

ORIGINAL ARTICLE

Molecular detection of bacteria associated to caries activity in dentinal lesions

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Received: 6 August 2015 / Accepted: 1 November 2016 / Published online: 12 November 2016

Abstract
Objectives This study aimed at identifying and quantifying *Actinomyces naeslundii*, *Bifidobacterium* spp., *Streptococcus mitis* group, *Lactobacillus acidophilus*, *Lactobacillus casei* group, *Streptococcus gordonii*, and *Streptococcus mutans* in active and inactive carious dentine lesions of children with early childhood caries by using quantitative polymerase chain reaction.
Material and methods Fifty-six dentin lesion samples, classified as active ($n = 39$) or inactive ($n = 17$), were collected from children aged from 2 to 5 years old. Dentinal-cavitated lesions were evaluated by Nyvad criteria for the assessment of caries lesion activity.
Results Relative quantification revealed that *Bifidobacterium* spp. and the *L. casei* group were significantly more abundant in active dentin lesions ($p < 0.05$). Concentrations of *A. naeslundii*, *S. mitis* group, and *S. gordonii* were not significantly different when comparing dentin lesion activity. The relative proportion of *S. mutans* was significantly greater in inactive than in active lesions ($p < 0.05$). *Bifidobacterium* spp. and *L. casei* group demonstrated a positive correlation ($p = 0.001$) in active lesions. The positive detection of *L. acidophilus* (odds ratio = 15.1) and *S. gordonii* (odds ratio = 7.7) was significantly associated to the active lesions.
Conclusions The data indicate that higher detection levels of *Bifidobacterium* spp. and the *L. casei* group may be linked to dentin lesion activity. Additionally, the presence of *L. acidophilus* and *S. gordonii* was associated with lesion activity.
Clinical relevance Considering that information about the oral microbiota related to dentin caries activity status is relevant, this study provides insights to better understand the differences in the microbiotas between active and arrested dentin cavities.

Keywords Bacteria · Child · Dentin caries · Early childhood caries · Quantitative polymerase chain reaction

Introduction

Dental caries is one of the most prevalent diseases of childhood worldwide, especially in socially disadvantaged populations [1]. Several factors, including microbial, genetic, immunological, behavioral, and environmental, are involved and contribute to its development [2]. In preschool children, in particular, this condition defined as early childhood caries (ECC), can devastate the primary teeth, affect the child's self-esteem, impact general health, and lead to nutritional deficiency [3]. Due to the aggressive pattern of ECC, areas of demineralization can rapidly progress, develop cavitation, and involve dental pulp tissues, causing serious consequences such as pain and pulp infection [4, 5].

Cavitated dentin carious lesions are considered as the last stage of dental caries and also diverse ecosystems with high variability in microbiota [6]. Dentin provides a different

Electronic supplementary material The online version of this article (doi:10.1007/s00784-016-1995-9) contains supplementary material, which is available to authorized users.

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environment for bacteria involved in caries progression, where only specialized bacteria are able to colonize and exploit [7]. The bacterial profile in enamel and dentinal caries are significantly different [5, 7], since the microbiota in dentin is constantly submitted to changes, such as nutrient availability, oxygen concentration, and pH [8, 9]. Moreover, this tissue contains a higher proportion of organic matrix and a lower inorganic component than enamel. Thus, the critical pH for dentin dissolution is higher when compared to enamel, which allows colonization of bacteria that may not be as acidogenic and aciduric as those required for initial enamel demineralization [10].

The advent of molecular research that characterizes the oral microbiota in health and disease revealed the diversity of oral biofilms and dentinal caries, introducing new candidates for disease-associated bacterial species [11–14]. Considering that the microbiota involved in dental caries are known to be highly diverse and variable [9, 15], understanding the microbial etiology of caries and how environmental conditions in the oral cavity impact the disease process continues to change as technology advances [16]. Even though the strong association of mutans streptococci and ECC is established in the literature [1, 17–19], it seems that these bacteria are not present in all children with caries [12, 20]. Beyond *S. mutans*, molecular approaches have revealed a greater variability of the community of dentine caries-associated microbiota including *Streptococcus* spp. and bacteria of the genera *Actinomyces*, *Bifidobacterium*, *Lactobacillus*, *Propionibacterium*, *Veillonella*, *Selenomonas*, and *Atopobium* [6, 11, 12, 15, 21–23].

On the other hand, it is possible that other microorganisms contribute to the inactivation of caries lesions, since several streptococci, such as members of the *S. mitis* group and *S. gordonii* are able to produce alkalis by the arginine deiminase system (ADS) [24]. These bacteria generate ammonia that neutralizes acids in cariogenic biofilms and favors pH increase, which could explain the pH of arrested lesions as previously demonstrated [25, 26]. However, the role of these bacteria in caries progression remains inconclusive.

Research into the microbial communities present in dentinal caries lesions is not only important in understanding the pathogenesis of dentinal caries, but also in developing novel approaches to dental caries treatment [16]. Early in the caries process, the pulp reflects changes within lesion activity [27]. However, little is known about the dynamic characteristics of oral microbiota in caries progression [28] and how the oral bacterial community changes within carious lesion activity [9, 16].

Molecular studies dealing with the microbiota in dentinal caries have mainly focused on comparing plaque samples within the same patient [11–14, 29], rarely defining dentinal lesion activity, which may reveal different microbial patterns [9]. Therefore, the aim of the present study was to quantify *Actinomyces naeslundii*, *Bifidobacterium* spp., *Lactobacillus acidophilus*, *Lactobacillus casei* group, *Streptococcus mitis* group, *Streptococcus gordonii*, and *Streptococcus mutans* in active and inactive dentine carious lesions from severe ECC children and also to evaluate whether caries lesion activity could be linked to a certain microbial composition.

Methods

Ethics statement

The study protocol was approved by the ethics committee of the Federal University of Ceará, Brazil (COMEPE/UFC) (Protocol Number 158/2011). Verbal and written consents were obtained from the parents of all subjects. Samples were taken only after obtaining the approval from the children and their parents.

Study population

Thirty-nine subjects aged from 2 to 5 years old with severe ECC were recruited for this study from public preschools in Fortaleza, Ceará, Brazil. The schools were selected based on convenience and located in a suburban area of the city. A total of 420 children was examined in the first phase of the study in order to select the study population. Patients were excluded from the study if history of significant medical disease or antimicrobial therapy was reported by their parents within the last 3 months prior to the study. None of the subjects had salivary gland disorders or systemic diseases, and none showed spontaneous symptoms associated with the carious lesions. Informed consent of the respective children and their families, their willingness to participate, and the presence of at least one cavitated dentinal carious lesion with no pulp exposure in primary teeth were used as inclusion conditions.

The presence of dental caries was assessed using the International Caries Detection and Assessment System (ICDAS) II [30, 31]. Children were examined under standardized conditions by two calibrated examiners (BGN and DSB). Professional oral cleaning was performed prior to clinical examination. A WHO periodontal probe, a mirror, an air syringe, and adequate illumination were used by the examiners during the clinical evaluation.

Dentinal cavitated lesions were evaluated by the Nyvad criteria [32] for the assessment of caries lesion activity, based on visual and tactile diagnoses. The teeth were examined after air-drying for 5 s. The examiners reviewed the clinical appearance of the selected sites based on color, opacity, and the presence of surface discontinuities or cavities. Gentle probing was used to assess the lesion surface integrity or texture (rough or smooth) and to remove dental plaque, if needed or not removed by professional oral cleaning. Dentine cavities

classified as active were those easily visible with the naked eye, soft on gentle probing, and with a whitish/yellowish appearance. Inactive lesions were characterized by a whitish/brown/black shiny appearance and were smooth/hard on gentle probing. Each site was ranked according to the scores proposed for caries lesion activity [32].

All children involved in this study were enrolled in a dental care program that included preventive counseling and dental treatment at the dental clinic of the Faculty of Dentistry at the Federal University of Ceará in Fortaleza, Ceará, Brazil.

Sampling and clinical data collection

All sample collections were performed by two calibrated dentists at the dental clinic of the Dental School at the Federal University of Ceará located at Fortaleza, Ceará, Brazil. Samples were collected from 39 children over a period of 15 months.

The dentinal caries lesions selected were opened cavities scored with codes 5 (distinct cavity with visible dentin) and 6 (extensive distinct cavity with visible dentin), according to the ICDAS II criteria [30]. Selected teeth had no clinical signs or symptoms of irreversible pulpitis. Absence of pulp exposure and radiolucent areas were evaluated radiographically.

After being classified as active or inactive, as previously reported, one to three dentin carious samples were collected from each child. Samples were not pooled across dentin lesions or patients. Carious samples were collected from cavitated dentine lesions from primary teeth with local anesthesia under rubber dam isolation with a sterile spoon excavator in order to reduce the risk of saliva contamination. Prior to sampling procedure, dental plaque on the surfaces of cavitated dentin lesions was swiped. Subsequently, all cavities were restored with resin-modified glass ionomer cement (Vitro Fil LC, DFL, Rio de Janeiro, Brazil). When necessary, an indirect pulp capping with calcium hydroxide liner was placed. Dentine carious samples were placed in a sterile 1.5-mL microcentrifuge tube containing 150 μL of TE (10 mM Tris-HCl, 1 mM EDTA pH 7.6) and immediately transported on ice to the laboratory, where they were frozen at a temperature of −20 °C until analysis.

Laboratory methods

Bacterial strains and culture conditions

Quantitative PCR was performed to detect the presence/absence and to quantify targeted bacterial DNA in carious dentin samples. The presence of *A. naeslundii*, *Bifidobacterium* spp., *L. acidophilus*, *L. casei* group, *S. mitis* group, *S. gordonii*, and *S. mutans* was investigated using specific forward and reverse primers as listed on Table 1.

The bacteria strains used as positive controls to test the specificities of the primers included *A. naeslundii* (ATCC 12104), *Bifidobacterium animalis subsp. lactis* BB-12 ® (Chr. Hansen), *L. acidophilus* (ATCC 4356), *Lactobacillus paracasei subsp. paracasei* (ATCC 335), *S. gordonii* (ATCC 35105), *Streptococcus mitis* (ATCC 49456—NTCC 12261), and *S. mutans* (UA 159).

Isolated bacteria were cultured in broth for 24 h as recommended by the Bergey's Manual of Determinative Bacteriology [39]. The cells were centrifuged and washed in sterile saline solution (sodium chloride 0.9%). The quality and purity of bacterial cultures were checked by Gram staining.

Extraction and purification of DNA from dentinal samples and bacterial cultures

All samples were transferred into a fresh 2-mL screw cap tube. Mechanical disruption of cells was carried out with 0.16 g of 0.1-mm diameter zirconia beads (Biospec Products, Bartlesville, OK, USA) on a Mini-beadbeater (Biospec Products) at maximum power for 60 s. DNA was recovered from all samples using an organic extraction protocol based on phenol/chloroform purification and alcohol precipitation [40]. The DNA concentration (A_{260}) and purity (A_{260}/A_{280}) of the samples were evaluated using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). Electrophoresis of the extracted DNA was performed on a 1.2% agarose gel in Tris/borate/EDTA buffer and stained with 0.1 μg/mL ethidium bromide.

Quantification of target bacterial DNA in dentinal samples by qPCR

Serial dilutions starting from 300 to 0.0003 ng (10-fold) of reference bacterial DNA concentrations were used as standards and positive controls for relative quantification of the targeted bacteria. A standard amplification curve and a melting-point product curve were obtained for each primer set. Amplifications of qPCR were performed using a MicroAmpFast Optical 48-Well Reaction Plate (Applied Biosystems, Foster City, CA, USA) covered with Optical Adhesive Film (Applied Biosystems) in a StepOne Real-Time PCR System (Applied Biosystems). Each reaction mixture (10 μL) contained 5 μL of 2x Power SYBR Green Mastermix (Applied Biosystems), 0.3 μL of each forward/reverse primer 10 μM, 2 μL of DNA sample, and 2.4 μL nuclease-free water. Assays were carried out in duplicate, and the final analyses were based on the mean of the two reactions. Negative control included reactions without template. The standard curves were used to transform the cycle threshold (*C*t) values to the mass of DNA, and the results of the concentrations of bacteria in carious dentin samples were normalized relative to the total bacterial load estimated by the primer *Bacteria* 16S rDNA [33].

Table 1 Primers that were used for qPCR assays

Target	Sequence (5′ 3′)	Annealing temperature (°C)	Amplicon length (bp)	References
Bacteria 16S rDNA	F: TCCTACGGGAGGCAGCAGT R:GGACTACCAGGGTATCTAATCCTGTT	57	466	[33]
Actinomyces naeslundii	F:CTGCTGCTGACATCGCCGCTCGTA R:TCCGCTCGCGCCACCTCTCGTTA	62	144	[34]
Bifidobacterium spp.[1]	F: TCGCGTC(C/T)GGTGTGAAAG R: CCACATCCAGC(A/G)TCCAC	58	243	[35]
Lactobacillus acidophilus	F: GATCGCATGATCAGCTTATA R: AGTCTCTCAACTCGGCTATG	60	124	[36]
L. casei group[2]	F: GCGGACGGGTGAGTAACACG R: GCTTACGCCATCTTTCAGCCAA	60	121	[36]
Streptococcus mitis group[3]	F:TAGAACGCTGAAGGAAGGAGC R: GCAACATCTACTGTTATGCGG	60	133	[37]
Streptococcus gordonii	F: CAGGAAGGGATGTTGGTGTT R: GACTCTCTTGGCGACGAATC	60	136	[37]
Streptococcus mutans	F: AGCCATGCGCAATCAACAGGTT R: CGCAACGCGAACATCTTGATCAG	64	415	[38]

[1] *Bifidobacterium longum*, *B. minimum*, *B. angulatum*, *B. catenulatum*, *B. pseudocatenulatum*, *B. dentium*, *B. ruminantium*, *B. thermophilum*, *B. subtile*, *B. bifidum*, *B. boum*, *B. lactis*, *B. animalis*, *B. choerinum*, *B. gallicum*, *B. pseudolongum subsp. globosum*, *B. pseudolongum subsp. pseudolongum*, *B. magnum*, *B. infantis*, *B. indicum*, *B. gallinarum*, *B. pullorum*, *B. saeculare*, *B. suis*

[2] *L. casei group: L. casei*, *L. paracasei*, *L. rhamnosus, and L. zeae*

[3] *Streptococcus mitis* group: *Streptococcus mitis*, *S. oralis*, *S. pneumoniae*, *S. parasanguinis*, *S. australis*

Statistical analysis

Data were tabulated in Microsoft Excel and exported to a statistical software Statistical Package for Social Sciences (SPSS) version 17.0, on which all analyses were performed considering a confidence level of 95%.

After examining the pattern of sample distribution (normality test Kolmogorov-Smirnov test), data were expressed as the mean and standard deviation and compared between groups using the Kruskal-Wallis test followed by post-test Mann-Whitney with Bonferroni correction (nonparametric data). For bacterial prevalence data, the values of the concentrations of the bacterial species were calculated as a percent of the total bacterial load.

Data were dichotomized according to the presence or absence of bacteria in active and inactive dentin carious lesions and stratified according to higher or lower values than the median proportion for bivariate analysis (chi-square test). Presence and absence of the bacteria in dentine lesions were described and also relative median of all groups was considered for this analysis. A Spearman correlation (nonparametric data) was performed for evaluation of the interaction between different bacteria and lesions activity (active or inactive).

Results

A total of 56 carious dentin samples were collected from cavitated dentin lesions (ICDAS 5, 6). Dentin samples were divided into two groups: active ($n = 39$) and inactive ($n = 17$) lesions, according to Nyvad criteria [32]. From one to three samples were collected from each patient, and five children had lesions of both types.

The concentrations of each strain were obtained by normalization to total bacteria present in the same dentine sample as determined using specific primers. Table 2 details the mean and median values of the prevalence of oral bacteria in active and inactive dentin carious lesions. Active lesions presented significantly higher concentrations of *Bifidobacterium* spp. and the *L. casei* group ($p < 0.05$) compared to inactive lesions. Concentrations of *A. naeslundii*, *S. gordonii*, and the *S. mitis* group were not significantly different when comparing active and inactive dentin lesions. The relative proportion of *S. mutans* was significantly greater in inactive than in active lesions ($p < 0.05$).

The presence and absence of the bacteria in dentine lesions and also relative median of all groups are summarized in Table 3. *L. acidophilus* was completely absent in all inactive carious dentin samples. *S. mutans* was identified in all samples of the current study. The *L. casei* group was absent in about 40% of inactive lesions. Considering presence in a carious dentin lesion in a bivariate analysis, *L. acidophilus* showed a statistically significant association with active dentin lesions (OR = 15.1) and also *S. gordonii* presented a greater chance to be present in active lesions (OR = 7.7). Only 29.4% of the inactive dentin samples presented levels of the *L. casei* group higher than the median value of both groups ($p < 0.05$; OR = 3.5) (Table 3).

According to the results of Spearman's rank correlations, there was a significant inverse correlation between *L. casei*

Table 2 Relative concentrations of oral bacteria in carious dentine samples as determined by qPCR and normalized by total bacteria as percentage

Oral bacteria	Lesion activity	Mean (%)	±SD	Median (%)	*p* value
S. mutans	Inactive	18.20	11.04	16.72*	0.015
	Active	11.72	12.58	8.71	
L. acidophillus	Inactive	0.00	0.00	0.00	0.065
	Active	0.07	0.42	0.00	
S. mitis group	Inactive	0.55	0.56	0.31	0.438
	Active	0.50	0.86	0.24	
L. casei group	Inactive	1.44	2.38	0.08	0.006
	Active	12.77	17.86	3.96*	
Bifidobacterium spp.	Inactive	2.14	5.15	0.35	0.012
	Active	3.39	4.48	1.67*	
A. naeslundii	Inactive	15.87	12.34	11.47	0.852
	Active	16.68	16.33	11.44	
S. gordonii	Inactive	0.19	0.52	0.02	0.052
	Active	0.24	0.34	0.07	

*The data are mean ± standard deviation and median values of concentrations of bacterial species as a percent of total bacteria load. *Asterisks* indicate that the values are significantly different (active vs. inactive) ($p < 0.05$); Mann-Whitney test

group and *S. mutans* in active dentine lesions ($p = 0.039$); when analyzing the bacterial relationships in the different groups, *Bifidobacterium* spp. and the *L. casei* group demonstrated a positive correlation ($p = 0.001$). A significant direct correlation was observed between the *S. mitis* group and *S. gordonii* in both active and inactive dentin lesions (Supplemental Tables).

Discussion

The microbiota in dental caries is highly complex and varies between individual lesions [15, 41]. Consequently, etiological studies must focus on site-specific analyses [7]. It has been suggested that the proportions and numbers of acid-base-producing bacteria are the core of dental caries activity [42]. Different proportions of some bacterial populations in active and inactive dentin lesions were observed in the current study.

The current results showed a higher concentration of the *L. casei* group in active dentin lesions when compared to the arrested lesions, which was previously demonstrated by a study that verified these bacteria as dominant in active dentine lesions in adults [26]. Although present in low quantity, the presence of *L. acidophilus* was related to dentin lesion activity and it showed a statistical significant association to active dentin lesions (OR = 15.1), since it was completely absent in all inactive carious dentin samples. These findings demonstrated that dentin lesions, where these bacteria are present, were 15.1 times more likely to be active. *Lactobacillus* spp. have the ability to produce organic acids, promoting low levels of pH and being responsible for the decalcification of the dentinal matrix [5, 43], which is a common situation in active lesions. The link between *Lactobacillus* count and carious decay is unquestionable. However, the relationship between the *Lactobacillus* count and the carious activity remains to be established [44]. *L. casei* and *L. paracasei* were frequently isolated from dentine sites in ECC [45]. Moreover, Lactobacilli have shown robust association with more advanced stages of caries in many studies [5, 7, 8, 12, 21, 23, 28, 43] and have also been implicated in the initial stages of pulp infection [46], indicating that they present a pathogenic potential and play a crucial role in caries progression.

Low proportions of *Bifidobacterium* spp. were detected in dentine lesions in the current study, which is in accordance with a previous study on adult dentine lesions [7]. Interestingly, a significantly higher proportion was verified in active ECC dentine lesions compared to inactive lesions, in agreement with an earlier study that isolated *Bifidobacterium* spp. from soft and active dentine lesions in primary teeth [22]. Bifidobacteria have been detected in dentine carious lesions [7, 11, 12, 21], suggesting that these bacteria may be implicated in dental caries progression [22], since these species are acidogenic and aciduric and also known to produce lactate [22, 47]. Additionally, another study has observed spatial distribution of bacterial taxa in vivo with confocal microscopy, showing a bacterial invasion into the dentinal tubules of *Bifidobacterium* inside cavitated caries lesions [48].

Several studies have associated *S. mutans* with progressive stages of caries and have detected these bacteria in cavitated lesions in dentin [12, 22, 45], and this was confirmed by the present results, since all dentine samples examined harbored

Table 3 Bivariate analysis comparing presence and median (%) of oral bacteria in different carious dentine lesions

Variables	Active lesions *n* (%)	Inactive lesions *n* (%)	*p* value	Odds ratio (95% CI)
S. mutans				
Positive	39 (100)	17 (100)	1.000	–
Negative	0 (0)	0 (0)		
>11.840 %	16 (41)	12 (70.6)	0.080	0.3 (0.1–1.0)
<11.840 %	23 (59)	5 (29.4)		
L. acidophilus				
Positive	12 (30.8)	0 (0)	0.011*	15.1 (1.0–286.3)
Negative	27 (69.2)	17 (100)		
>0.000 %	7 (17.9)	0 (0)	0.088	–
<0.000%	32 (82.1)	17 (100)		
S. mitis group				
Positive	37 (94.9)	15 (88.2)	0.577	2.5 (0.3–19.1)
Negative	2 (5.1)	2 (11.8)		
>0.270%	17 (43.6)	10 (58.8)	0.294	0.5 (0.2–1.7)
<0.270%	22 (56.4)	7 (41.2)		
L. casei group				
Positive	31 (79.5)	10 (58.8)	0.108	2.7 (0.8–9.4)
Negative	8 (20.5)	7 (41.2)		
>1.425%	23 (59)	5 (29.4)	0.042*	3.5 (1.0–11.7)
<1.425%	16 (41)	12 (70.6)		
Bifidobacterium spp.				
Positive	38 (97.4)	17 (100)	1.000	–
Negative	1 (2.6)	0 (0)		
>1.515%	23 (59)	5 (29.4)	0.042*	3.5 (1.0–11.7)
<1.515%	16 (41)	12 (70.6)		
A. naeslundii				
Positive	38 (97.4)	17 (100)	1.000	–
Negative	1 (2.6)	0 (0)		
>11.320%	18 (46.2)	9 (52.9)	0.640	0.8 (0.2–2.4)
<11.320%	21 (53.8)	8 (47.1)		
S. gordonii				
Positive	37 (94.9)	12 (70.6)	0.022*	7.7 (1.3–45.0)
Negative	2 (5.1)	5 (29.4)		
>0.059%	5 (12.8)	1 (5.9)	0.645	2.4 (0.3–21.8)
<0.059%	34 (87.2)	16 (94.1)		

*Variables were compared by the Chi-square test. Presence and absence of the bacteria in dentine lesions were described and also relative median of all groups was considered for this analysis. Results presented are the non-adjusted odds ratios with their 95% confidence intervals

*$p < 0.05$

S. mutans. Mutans streptococci comprise about 30% of the total microbiota according to microbial culture approaches [49, 50]. These species may form biofilm on dentine, and incorporate and collaborate with various bacteria for induction of dentine acidulation [51]. Surprisingly, *S. mutans* was identified with higher concentration in arrested lesions, which is not in agreement with Kuribayashi et al. [26], who demonstrated a high prevalence of *S. mutans* in carious dentine lesions regardless of the caries activity. These discrepant results may be due to the studied population, as their study evaluated adults. It has been suggested that *S. mutans* have a more dominating role in dentine and deep dentine caries of primary teeth that in permanent teeth [12].

Other studies have demonstrated a low prevalence or even an absence of *S. mutans* in dentinal caries lesions [6, 7], contradicting the findings of this study. However, it must be

emphasized that the earlier findings were based on smaller samples. Possible reasons for different detection levels of *S. mutans* in different studies are probably related to different approaches used for species detection, including the DNA extraction and the bacterial lysis method, as well as PCR and primer bias [18, 23]. In addition, it was observed that the oral microbiota is diverse in different ethnicities and races, which may explain discrepancies in the composition of the microbiota in dentinal caries in different studies [5, 14].

One particularly interesting finding of this study was the positive correlation between bacteria from the *L. casei* group and *Bifidobacterium* spp. in active dentine lesions. Both bacterial genera are commonly detected in ECC lesions [11, 12], where they play important roles in lowering the pH in active lesion environments and proliferating in acidic carious lesions [22]. A similar result was found in a study with active dentine lesions in deciduous teeth that verified a correlation between the proportion of *Bifidobacterium* spp. and *Lactobacillus* spp. [22]. *S. mutans* and *L. casei* group presented a negative correlation in active dentine lesions, which is supported by the idea that mutans streptococci and lactobacilli are more competitive under severely acidic conditions [9]. This finding corroborates with the results of a previous in vitro study in which the lactobacilli were capable of inhibiting the growth of mutans streptococci, being *L. paracasei*, *Lactobacillus plantarum*, and *Lactobacillus rhamnosus* the species with maximum interference capacity against mutans streptococci [52].

In this study, the presence of *S. gordonii* was significantly associated with active dentine lesions. The data is consistent with those reported by Peterson et al. [2] in a dental plaque microbiome study in which *S. gordonii* was associated with caries activity and also with a metagenomic study that detected abundance of this species in caries-active individuals [6]. The role of *S. gordonii* in dental caries is still undefined [53]. Despite being considered as a pioneer for dental plaque formation and associated with health [29], an in vitro study showed that these bacteria were able to increase their acid tolerance and acidogenicity when exposed to an acidic environment [54]. However, the contribution of *S. gordonii* on the acidification of dentin remains unclear and deserves further investigation.

With regard to *A. naeslundii*, no statistically significant difference was found between active and inactive lesions, which is not surprising as *Actinomyces* spp. are prevalent in oral cavity and frequently found in association with both carious and sound surfaces [45]. In addition, these species have been associated with dentin and root caries in adults [16, 55] and does not seem to play a relevant role in childhood caries [11]. Likewise, although members of the mitis group were previously detected in active carious dentine lesions in adults and children [7, 45], they have been frequently associated with health [13, 29], making the contribution of these bacteria to caries inactivation inconclusive.

Other microorganisms, like *Candida* spp., have been proposed to produce acid and contribute to dental caries. However, the role of *Candida* species in this disorder has not been clearly established. Studies have indicated a relationship between the presence of *Candida* species and dental caries progression [7], reflecting the polymicrobial etiology of dental caries although the invasion of these species into carious dentin has been questioned [56]. Since our goal was to mainly identify bacterial differences in active and inactive dentine caries lesions in primary teeth, fungal analysis was not accomplished in this study.

Real-time PCR was the chosen method to this study, since the use of qPCR is an accepted technology for the quantitative analysis of bacteria from mixed samples. Furthermore, this methodology allows the microorganisms to be assessed more accurately than they can be by cultural analysis. Quantitative PCR has the potential to account for the uncultivable portion of the oral microbial community, as well as, species which are more difficult to culture [57]. According to Chhour et al. [21], real-time PCR analysis of the total bacterial load in advanced carious lesions has shown that the total load exceeds the number of cultivable bacteria. A limitation of this methodology is that qPCR cannot distinguish between viable and non-viable bacterial cells, even though a recent technique has been proposed to assess the viability of live and dead cells with qPCR [58]. However, it seems that this study presents important findings, since differences in the microbiotas between active and arrested cavities have not yet been elucidated, according to Takahashi & Nyvad [59].

Moreover, the current study did not evaluate the bacterial composition among different depths in the same lesion. Nevertheless, no significant difference in the microbial composition in the different layers of dentinal caries lesions has been reported [8, 23]. In addition, stratified analysis of dentinal caries might be possible only with in vitro studies evaluating extracted teeth where sampling could be precisely taken from different zones, which may provide information about the dynamics of the disease process even in a cross-sectional study. In the present study, the dentine caries was well established and in an advanced stage of progression or arrest, and therefore was not representative of the initial stages of carious lesions.

Contamination during sampling technique must not be considered, since sampling of carious dentine was carried out carefully by rubber dam isolation and after removal of dental plaque and debris. Additionally, our study analyzed an in vivo situation with children with severe ECC, differently from numerous studies with dentine caries that commonly evaluated extracted carious teeth [10, 16, 21, 45]. It is possible to infer that if these teeth were already necrotic, their microbial composition might have been altered. It seems important to highlight that unlike plaque studies, there is no biologic control available for an established lesion where the bacterial DNA is

extracted directly from the caries mass [16]. Furthermore, dentine lesions present a different profile compared to other caries samples affecting different tissues [7].

The higher concentration of *Bifidobacterium* spp. and the *L. casei* group as well as the presence of *L. acidophilus* and *S. gordonii* in active ECC-dentine lesions show that these bacteria may be implicated in the activity status of dentin lesions.

Acknowledgements This study was supported by the CNPq—the National Counsel of Technological and Scientific Development (Process # 475346/2011-4 MCT/CNPq 14/2011).

Compliance with ethical standards The study protocol was approved by the ethics committee of the Federal University of Ceará, Brazil (COMEPE/UFC) (Protocol Number 158/2011). Verbal and written consents were obtained from the parents of all subjects. Samples were taken only after obtaining the approval from the children and their parents.

Conflict of interest The authors declare that they have no conflicts of interest.

Funding This study was supported by CNPq—National Counsel of Technological and Scientific Development (Process # 475346/2011-4 MCT/CNPq 14/2011).

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study.

References

1. Berkowitz RJ (2003) Acquisition and transmission of mutans streptococci. J Calif Dent Assoc 31:135–138
2. Peterson SN, Snesrud E, Liu J, Ong AC, Kilian M, Schork NJ, Bretz W (2013) The dental plaque microbiome in health and disease. PLoS One 8:e58487
3. Ramos-Gomez FJ, Weintraub JA, Gansky SA, Hoover CI, Featherstone JD (2002) Bacterial, behavioral and environmental factors associated with early childhood caries. J Clin Pediatr Dent 26:165–173
4. American Academy of Pediatric Dentistry (2014) Policy on early childhood caries (ECC): classifications, consequences and preventive strategies. Reference manual. Pediatr Dent 36:50–52
5. Obata J, Takeshita T, Shibata Y, Yamanaka W, Unemori M, Akamine A, Yamashita Y (2014) Identification of the microbiota in carious dentin lesions using 16S rRNA gene sequencing. PLoS One 9:e103712
6. Belda-Ferre P, Alcaraz LD, Cabrera-Rubio R, Romero H, Simón-Soro A, Pignatelli M, Mira A (2012) The oral metagenome in health and disease. ISME J 6:46–56
7. Simón-Soro A, Belda-Ferre P, Cabrera-Rubio R, Alcaraz LD, Mira A (2013) A tissue-dependent hypothesis of dental caries. Caries Res 47:591–600
8. Lima KC, Coelho LT, Pinheiro IVA, Rocas IN, Siqueira JF Jr (2011) Microbiota of dentinal caries as assessed by reverse-capture checkerboard analysis. Caries Res 45:21–30
9. Takahashi N, Nyvad B (2011) The role of bacteria in the caries process: ecological perspectives. J Dent Res 90:294–303
10. Kianoush N, Nguyen K-AT, Browne GV, Simonian M, Hunter N (2014) pH gradient and distribution of streptococci, lactobacilli, prevotellae, and fusobacteria in carious dentine. Clin Oral Investig 18:659–669
11. Becker MR, Paster BJ, Leys EJ, Moeschberger ML, Kenyon SG, Galvin JL, Boches SK, Dewhirst FE, Griffen AL (2002) Molecular analysis of bacterial species associated with childhood caries. J Clin Microbiol 40:1001–1009
12. Aas JA, Griffen AL, Dardis SR, Lee AM, Olsen I, Dewhirst FE, Leys EJ, Paster BJ (2008) Bacteria of dental caries in primary and permanent teeth in children and young adults. J Clin Microbiol 46:1407–1417
13. Corby PM, Lyons-Weiler J, Bretz WA, Hart TC, Aas JA, Boumenna T, Goss J, Corby AL, Junior HM, Weyant RJ, Paster BJ (2005) Microbial risk indicators of early childhood caries. J Clin Microbiol 43:5753–5759
14. Gross EL, Beall CJ, Kutsch SR, Firestone ND, Leys EJ, Griffen AL (2012) Beyond *Streptococcus mutans*: dental caries onset linked to multiple species by 16S rRNA community analysis. PLoS One 7:e47722
15. Martin FE, Nadkarni MA, Nicholas A, Hunter N, Jacques NA (2002) Quantitative microbiological study of human carious dentine by culture and real-time PCR: association of anaerobes with histopathological changes in chronic pulpitis. J Clin Microbiol 40:1698–1704
16. Kianoush N, Adler CJ, Nguyen K-AT, Browne GV, Simonian M, Hunter N (2014) Bacterial profile of dentine caries and the impact of pH on bacterial population diversity. PLoS One 9:e92940
17. Koehler B, Andreen I, Jonsson B (1988) The earlier the colonization by mutans streptococci, the higher the caries prevalence at 4 years of age. Oral Microbiol Immunol 3:14–17
18. Kanasi E, Dewhirst FE, Chalmers NI, Kent R, Moore A (2010) Clonal analysis of the microbiota of severe early childhood caries. Caries Res 44:485–497
19. Parisotto TM, Steiner-Oliveira C, Silva CM, Rodrigues LK, Nobre-dos-Santos M (2010) Early childhood caries and mutans streptococci: a systematic review. Oral Health Prev Dent 8:59–70
20. Mattos-Graner RO, Corrêa MS, Latorre MR, Peres RC, Mayer MP (2001) Mutans streptococci oral colonization in 12-30-month-old Brazilian children over a one-year follow-up period. J Public Health Dent 61:161–167
21. Chhour K, Nadkarni MA, Byun R, Martin FE, Jacques NA, Hunter N (2005) Molecular analysis of microbial diversity in advanced caries. J Clin Microbiol 43:843–849
22. Mantzourani M, Fenlon M, Beighton D (2009) Association between *Bifidobacteriaceae* and the clinical severity of root caries lesions. Oral Microbiol Immunol 24:32–37
23. Munson MA, Banerjee A, Watson TF, Wade WG (2004) Molecular analysis of the microflora associated with dental caries. J Clin Microbiol 42:3023–3029
24. Nascimento MM, Burne RA (2014) Caries prevention by arginine metabolism in oral biofilms: translating science into clinical success. Curr Oral Heal Reports 1:79–85
25. Hojo S, Komatsu M, Okuda R, Takahashi N, Yamada Y (1994) Acid profiles and pH of carious dentin in active and arrested lesions. J Dent Res 73:1853–1857
26. Kuribayashi M, Kitasako Y, Matin K, Sadr A, Shida K, Tagami J (2012) Intraoral pH measurement of carious lesions with qPCR of cariogenic bacteria to differentiate caries activity. J Dent 40:222–228

27. Bjørndal L, Demant S, Dabelsteen S (2014) Depth and activity of carious lesions as indicators for the regenerative potential of dental pulp after intervention. J Endod 40:S76–S81
28. Jiang W, Ling Z, Lin X, Chen Y, Zhang J, Yu J, Xiang C, Chen H (2014) Pyrosequencing analysis of oral microbiota shifting in various caries states in childhood. Microb Ecol 67:962–969
29. Gross EL, Leys EJ, Gasparovich SR, Firestone ND, Schwartzbaum JA, Janies DA, Asnani K, Griffen AL (2010) Bacterial 16S sequence analysis of severe caries in young permanent teeth. J Clin Microbiol 48:4121–4128
30. Ismail AI, Sohn W, Tellez M, Amaya A, Sen A, Hasson H, Pitts NB (2007) The international caries detection and assessment system (ICDAS): an integrated system for measuring dental caries. Community Dent Oral Epidemiol 35:170–178
31. Pitts NB (2004) Modern concepts of caries measurement. J Dent Res 83:43–47
32. Nyvad B, Machiulskiene V, Baelum V (1999) Reliability of a new caries diagnostic system differentiating between active and inactive caries lesions. Caries Res 33:252–260
33. Nadkarni MA, Martin FE, Jacques NA, Hunter N (2002) Determination of bacterial load by real-time PCR using a broad-range (universal) probe and primers set. Microbiology 148:257–266
34. Park S, Kim YK, Kook J (2013) Development of quantitative real-time PCR primers for detecting 42 bacterial species. Arch Microbiol 195:473–482
35. Rintillä T, Kassinen A, Malinen E, Krogius L, Palva A (2004) Development of an extensive set of 16S rDNA-targeted primers for quantification of pathogenic and indigenous bacteria in faecal samples by real-time PCR. J Appl Microbiol 97:1166–1177
36. Furet J, Quéneé P, Tailliez P (2004) Molecular quantification of lactic acid bacteria in fermented milk products using real-time quantitative PCR. Int J Food Microbiol 97:197–207
37. Wolff D, Freese C, Maier-Kraus T, Krueger T, Wolff B (2013) Bacterial biofilm composition in caries and caries-free subjects. Caries Res 47:69–77
38. Yano A, Kaneko N, Ida H, Yamaguchi T, Hanada N (2002) Real-time PCR for quantification of *Streptococcus mutans*. FEMS Microbiol Lett 217:23–30
39. Holt JG, Krieg NR, Sneath PHA, Staley JT, Williams ST (1994) Bergey's manual of determinative bacteriology. Williams & Wilkins, Baltimore
40. Wilson K (2001) Preparation of genomic DNA from bacteria. Curr Protoc Mol Biol Chapter 2:Unit 2.4. doi: 10.1002/0471142727.mb0204s56
41. Hahn CL, Liewehr FR (2007) Relationships between caries bacteria, host responses, and clinical signs and symptoms of pulpitis. J Endod 33:213–219
42. Kleinberg I (2002) A mixed-bacteria ecological approach to understanding the role of the oral bacteria in dental caries causation: an alternative to *Streptococcus mutans* and the specific-plaque hypothesis. Crit Rev Oral Biol Med 13:108–125
43. Byun R, Nadkarni MA, Chhour KL, Martin FE, Jacques NA, Hunter N (2004) Quantitative analysis of diverse *Lactobacillus* species present in advanced dental caries. J Clin Microbiol 42: 3128–3136
44. Badet C, Thebaud NB (2008) Ecology of lactobacilli in the oral cavity: a review of literature. Open Microbiol 2:38–48
45. Marchant S, Braislsford SR, Twomey AC, Roberts GJ, Beighton D (2001) The predominant microflora of nursing caries lesions. Caries Res 35:397–406
46. Nadkarni MA, Simonian MR, Harty DW, Zoellner H, Jacques NA, Hunter N (2010) Lactobacilli are prominent in the initial stages of polymicrobial infection of dental pulp. J Clin Microbiol 48:1732–1740
47. van Houte J, Lopman J, Kent R (1996) The final pH of bacteria comprising the predominant flora on sound and carious human root and enamel surfaces. J Dent Res 75:1008–1014
48. Dige I, Grønkjær L, Nyvad B (2014) Molecular studies of the structural ecology of natural occlusal caries. Caries Res 48:451–460
49. Loesche WJ, Eklund S, Earnest R, Burt B (1984) Longitudinal investigation of bacteriology of human fissure decay: epidemiological studies on molars shortly after eruption. Infect Immun 46:765–772
50. Milnes AR, Bowden GH (1985) The microflora associated with developing lesions of nursing caries. Caries Res 19:289–297
51. Maeda T, Kitasako Y, Senpuku H, Burrow MF, Tagami J (2006) Role of oral streptococci in the pH-dependent carious dentin. J Med Dent Sci 53:159–166
52. Simark-Mattsson C, Emilson CG, Håkansson EG, Jacobsson C, Roos K, Holm S (2007) Lactobacillus-mediated interference of mutans streptococci in caries-free vs. caries-active subjects. Eur J Oral Sci 115:308–314
53. Tanzer JM, Livingston J, Thompson AM (2001) The microbiology of primary dental caries in humans. J Dent Educ 65:1028–1037
54. Takahashi N, Yamada T (1999) Acid-induced acidogenicity and acid tolerance of non-mutans streptococci. Oral Microbiol Immunol 14:43–48
55. Brasilsford SR, Tregaskis RB, Leftwich HS, Beighton D (1999) The predominant *Actinomyces* spp. isolated from infected dentin of active root caries lesions. J Dent Res 78:1525–1534
56. Maijala M, Rautemaa R, Järvensivu A, Richard- son M, Salo T, Tjäderhane L (2007) *Candida albicans* does not invade carious human dentine. Oral Dis 13:279–284
57. Dalwai F, Spratt DA, Pratten J (2007) Use of quantitative PCR and culture methods to characterize ecological flux in bacterial biofilms. J Clin Microbiol 45(9):3072–3076
58. Yasunaga A, Yoshida A, Morikawa K, Maki K, Nakamura S, Soh I, Awano S, Ansai T (2013) Monitoring the prevalence of viable and dead cariogenic bacteria in oral specimens and in vitro biofilms by qPCR combined with propidium monoazide. BMC Microbiol 13: 157. doi:10.1186/1471-2180-13-157
59. Takahashi N, Nyvad B (2016) Ecological hypothesis of dentin and root caries. Caries Res 50:422–431

Original Paper

Caries Research

Caries Res 2016;50:279–287
DOI: 10.1159/000445256

Received: January 12, 2016
Accepted after revision: March 2, 2016
Published online: May 3, 2016

Insights into the Virulence Traits of *Streptococcus mutans* in Dentine Carious Lesions of Children with Early Childhood Caries

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Key Words
Dentine caries · Early childhood caries · Gene expression · *Streptococcus mutans*

Abstract
Streptococcus mutans is an oral bacterium considered to play a major role in the development of dental caries. This study aimed to investigate the prevalence of *S. mutans* in active and arrested dentine carious lesions of children with early childhood caries and to examine the expression profile of selected *S. mutans* genes associated with survival and virulence, within the same carious lesions. Dentine samples were collected from 29 active and 16 arrested carious lesions that were diagnosed in preschool children aged 2–5 years. Total RNA was extracted from the dentine samples, and reverse transcription quantitative real-time PCR analyses were performed for the quantification of *S. mutans* and for analyses of the expression of *S. mutans* genes associated with bacterial survival *(atpD*, *nox, pdhA)* and virulence *(fabM* and *aguD)*. There was no statistically significant difference in the prevalence of *S. mutans* between active and arrested carious lesions. Expression of the tested genes was detected in both types of carious dentine. The *pdhA* ($p = 0.04$) and *aguD* ($p = 0.05$) genes were expressed at higher levels in arrested as compared to active lesions. Our findings revealed that *S. mutans* is part of the viable microbial community in active and arrested dentine carious lesions. The increase in expression of the *pdhA* and *aguD* genes in arrested lesions is likely due to the unfavourable environmental conditions for microbial growth, inherent to this type of lesions.

Dental caries is one of the most prevalent biofilm-dependent diseases in both developing and developed countries, and particularly among young children of underprivileged communities worldwide [King and Wong, 2006; Bagramian et al., 2009; Marcenes et al., 2013]. Early childhood caries (ECC) is defined as the presence of one or more non-cavitated or cavitated carious lesions, missing (due to caries), or filled tooth surfaces in any primary tooth of a child under the age of 6 years [American Academy of Pediatric Dentistry, 2014]. Caries at such a young age can cause severe pain, weight loss, developmental issues, and lead to poor scholar performance; therefore, caries can adversely affect the quality of life of children [Filstrup et al., 2003; Feitosa et al., 2005; Abanto et al., 2011].

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The polymicrobial nature of caries has been well established [Simón-Soro et al., 2013, 2014; Jiang et al., 2014], and *S. mutans* is an oral bacterium that can contribute to the onset and progression of caries [Mattos-Graner et al., 2001; Nobre-dos-Santos et al., 2002; Aas et al., 2008; Takahashi and Nyvad, 2008; Palmer et al., 2010; Bowen and Koo, 2011; Luo et al., 2012]. The cariogenic potential of *S. mutans* is mainly attributed to its capacity to: (i) form biofilms on the tooth surfaces, (ii) produce weak acids, mainly lactic acid, and (iii) adapt to environmental stress conditions such as fluctuations in pH, oxygen tension and nutrient availability [Lemos and Burne, 2008]. More specifically, *S. mutans* can sustain growth and carry out glycolysis at pH values below the critical value to demineralize tooth enamel [Hazlett et al., 1999; de Soet et al., 2000; Quivey et al., 2001; Lemos et al., 2005].

Several adaptive strategies used by *S. mutans* to survive acid and oxidative stress in oral biofilms have been well characterized, such as the upregulation of the membrane-associated F_1F_0-ATPase and other stress-specific proteins, induction of pathways that contribute to cytoplasm buffering and DNA repair, as well as changes in membrane fatty acid composition [Hahn et al., 1999; Hanna et al., 2001; Kremer et al., 2001; Lemos et al., 2001; Quivey et al., 2001; Cox, 2007; Fozo et al., 2007; Lemos and Burne, 2008; Lemos et al., 2013]. These mechanisms are regulated by an enzymatic complex codified by several genes and operons acting according to bacterial requirements, such as the F_1F_0-ATPase complex, agmatine-deiminase system (AgDS), pyruvate dehydrogenase complex (PDH), H_2O-forming NADH oxidase and FabM protein. *S. mutans* has been estimated to comprise anywhere from 0.02 to 0.73% of the total oral biofilm microbiota depending on the cariogenic state of the sample [Simón-Soro et al., 2014]. Previous microbiological and molecular-based studies have revealed the presence of mutans streptococci in dentine carious lesions [Aas et al., 2008; Lima et al., 2011; Belda-Ferre et al., 2012; Kuribayashi et al., 2012; Wolff et al., 2012; Simón-Soro et al., 2013; Obata et al., 2014], and more recently, mutans streptococci were shown to be part of the metabolically active bacterial community of dentine caries [Simón-Soro et al., 2014].

The dynamic of the de- and remineralization processes, though physiological, are changeable and subjected to constant modifications. Thus, the use of caries activity diagnosis permits analysis of the dynamic nature of the disease. Active lesions exhibit evidence of progression over time [Filstrup et al., 2003], while arrested (or inactive) lesions exhibit evidence of no or slow progression [Nyvad and Fejerskov, 1997; Chu and Lo, 2008]. In this context, bacterial viability and virulence gene expression can be influenced by environmental changes of the harbouring dental tissues [Quivey et al., 2001; Lemos et al., 2005; Wen et al., 2010; Moye et al., 2014]. A better understanding of the various virulence mechanisms and the ecological role of *S. mutans* in dentine caries activity will certainly assist in the development of novel caries-preventive strategies. Therefore, the objectives of this study were to investigate: (1) the prevalence of *S. mutans* in relation to total streptococci (TS) and total bacteria (TB) in active and arrested cavitated dentine carious lesions of children with ECC, and (2) the expression profile of selected *S. mutans* genes involved in survival and virulence features. To the best of our knowledge, the present study is the first to report expression of genes related to survival and virulence of *S. mutans* in human carious dentine.

Materials and Methods

Study Population

A group of 298 children aged 40–71 months from four public schools of Fortaleza (Ceará, Brazil) received a dental examination as part of a previously conducted study [Rodrigues et al., 2015]. The study included children presenting at least one carious tooth surface with distinct cavity and visible dentine without pulpal involvement, as confirmed by clinical and radiographic examinations. The study excluded children with any health condition at the moment of examination, who had used antibiotics within the last 3 months, and those who were not cooperative during clinical examination. The children were routinely exposed to fluoride-containing toothpaste and fluoride intake; however, no information about the frequency of fluoride exposure was recorded. A convenience sample was used, consisting of 32 of these children who were diagnosed with ECC and met the inclusion criteria. Informed consent was obtained from the parents or legal guardians of each child under a protocol approved by the Ethics Committee of the Federal University of Ceará (protocol No. 548.405, Ceará, Brazil).

Caries Diagnosis

All oral and radiographic examinations were performed by calibrated researchers D.S.B. and B.G.N. at the Dental School of the Federal University of Ceará (Ceará, Brazil). Carious lesions were detected and diagnosed by the International Caries Detection and Assessment System II (ICDAS II) [Pitts, 2004; Ismail et al., 2007] associated with the Nyvad System for determination of caries activity and differentiation between active and arrested carious lesions [Nyvad et al., 1999]. Active lesions were clinically characterized by the presence of wet (after air drying), soft or leathery superficial layers of demineralized dentine. Inactive lesions are often characterized by colour changes (mostly darkness), shiny and dry (after air drying), and hard consistency of the superficial layers of dentine [Nyvad et al., 1997; Chu and Lo, 2008]. The ICDAS criteria have a score range from 0 to 6 as follows: 0 = no or slight change in enamel after air drying; 1 = first visual change in enamel after

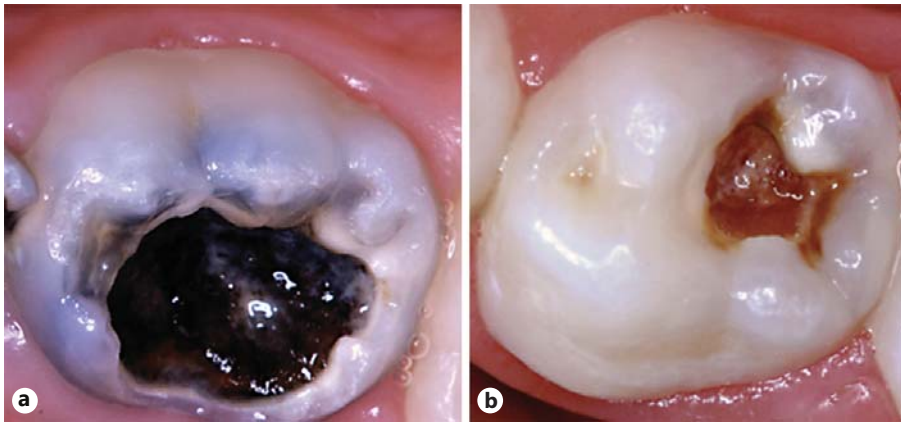

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Fig. 1. Dentine samples with ICDAS score 5 or 6. **a** Arrested dentine carious lesion (ICDAS score 6; Nyvad score 6). **b** Active dentine carious lesion (ICDAS score 5; Nyvad score 3).

air drying; 2 = distinct visual changes in enamel before air drying; 3 = localized enamel breakdown without visual signs of dentinal involvement; 4 = underlying dark shadow from dentine; 5 = distinct cavity with visible dentine; 6 = extensive and distinct cavity with visible dentine affecting more than half of the surface. Dentine samples were collected from 45 carious lesions with ICDAS score 5 or 6 (fig. 1), which were planned to receive restorative treatment. Out of these, 29 lesions were scored by the Nyvad System as 3 (active dentine caries with whitish/yellowish surface, feels soft or leathery upon gentle probing) and 16 lesions received score 6 (inactive dentine caries with brown/black and shiny surface, and feels hard upon gentle probing) [Nyvad et al., 1999].

Sample Collection

Prior to sample collection, the selected carious teeth were isolated with a rubber dam. After mechanical removal of the biofilm layer located over the lesions, carious dentine tissues were collected using sterilized and RNAse-free No. 5, 14 or 19 spoon excavators (S.S. White Duflex, Rio de Janeiro, RJ, Brazil), whose size was determined by lesion dimension. For active lesions, all soft and leathery carious tissue was collected until harder dentine was detected with a spoon excavator (S.S. White Duflex). For arrested lesions, a small amount of hard carious dentine tissue was collected by scrapping the superficial dentine off with a spoon excavator. Dentine was considered hard when the probe was not able to penetrate into the tissue under firm pressure. The samples were immediately transferred to sterile RNAse-free microtubes (Axygen, Union City, Calif., USA) containing RNA stabilizer solution (RNAlater™ – Ambion Inc., Austin, Tex., USA) and stored at 4°C for 18 h as recommended by the manufacturer. After this period, the tubes were stored at –80°C until RNA extraction. After the sample collection procedure, the carious lesions were lined and/or restored with a suitable material. Parents or guardians were instructed about caries prevention, and appointments were scheduled for continuation of dental treatment when needed.

RNA Extraction

The dentine samples were thawed and centrifuged (11,000 *g*/1 min/4°C). The RNAlater™ solution was removed using an automatic pipet without disturbing the pellets. The samples were carefully transferred to screw cap microtubes (Axygen, Union City, Calif., USA) containing 0.16 g of 0.1 mm diameter zirconium beads [Stipp et al., 2013]. The mechanical disruption of bacterial cells was made by Mini-Beadbeater (Biospec Products Inc., Bartlesville, Okla., USA) at maximum power (2 cycles of 60 s with 1 min rest on ice). A total of 850 µl of RLT buffer (Qiagen, Valencia, Calif., USA) with 1% of β-mercaptoethanol was added and the suspension homogenized by vortexing. After centrifugation (11,000 *g*/2 min/4°C), aliquots of 350 µl of the supernatant were subjected to RNA extraction using the RNeasy Mini Kit™ (Qiagen) according to the manufacturer's instructions. RNA concentration and purity (absorbance ratio A_{260}/A_{280} and A_{260}/A_{230}) were measured using a Nanodrop 2000c spectrophotometer (Thermo Scientific, Wilmington, Del., USA). The samples were then treated with Turbo™ DNAse (Applied Biosystems, Ambion) for removal of genomic DNA. The RNA solution was purified once again using an RNeasy Minielute™ CleanUp kit (Qiagen, Düsseldorf, Germany) according to the manufacturer's instructions. RNA integrity was verified by visualization of 16S and 23S ribosomal RNA bands on 1.2% formaldehyde-agarose gel stained with ethidium bromide, and the RNA samples were stored at –80°C.

Sanger Sequencing

Oligonucleotide primers used in this study for quantification of *S. mutans*, TS and TB are listed in table 1. The primers used for analysis of *S. mutans* gene expression are listed in table 2. The specificity of the primers in human samples was tested by previous studies [Nadkarni et al., 2002; Yano et al., 2002; Sakagushi et al., 2010], or here, via conventional PCR and subsequently Sanger sequencing. PCR assays were performed in a Veriti® Thermal Cycler (Applied Biosystems, Foster City, Calif., USA) and the reaction mix included 1 µl of 10× PCR buffer, 1 µl of dNTPs 10 mM, 2.5 µl of $MgCl_2$, 3 µl of each primer F/R 10 µM, 0.25 µl of Taq polymerase 5 U/µl, 50 mM 1 µl of template cDNA (10 ng/µl) and 37.25 µl of nuclease-free water. The thermal cycling consisted of 5 min at 95°C, 35 cycles of 15 s at 95°C, 60 s at the melting temperature of each primer (table 2), 20 s at 72°C, and 4 min at 60°C. The PCR products were purified using the QIAquick® PCR Purification Kit (Qiagen, USA), and the sequencing reactions were performed using a Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems) under the following thermal-cycling conditions: 20 s at 95°C; 40 cycles of 15 s at 96°C, 15 s at 50°C and 4 min at 60°C; 60 s at 60°C. The PCR products were then precipitated, dried, resuspended in 10 µl of formamide and then denatured for 5 min at

Table 1. Primers for bacterial identification used in RT-PCR

Gene	Bacteria	Primer sequence (5′ → 3′)	Product size, bp	Cycles, n	Annealing temperature, °C
Sm F5/R4	SM	F: AGCCATGCGCAATCAACAGG R: CGCAACGCGAACATCTTGATCAG	415	40	64
23S rRNA	TS	F: AGCTTAGAAGCAGCTATTCATTC R: GGATACACCTTTCGGTCTCTC	308	40	60
16S rDNA	TB	F: TCCTACGGGAGGCAGCAGT R: GGACTACCAGGGTATCTAATCCTGTT	466	40	57

SM = *S. mutans;* TS = total streptococci; TB = total bacteria; F = forward; R = reverse. *Sm F5/R4:* primer sequence from Yano et al. [2002]; *23S rRNA:* primer sequence from Sakaguchi et al. [2010]; *16S rDNA:* primer sequence from Nadkarni et al. [2002].

Table 2. Primers for analysis of *S. mutans* gene expression used in RT-PCR

Gene	Primer sequence (5′ → 3′)	Product size, bp	Cycles, n	Annealing temperature, °C
atpD	F: TGTTGATGGTCTGGGTGAAA R: TTTGACGGTCTCCGATAACC	176	45	60
fabM	F: ACTGATTAATGCCAATGGGAAAGTC R: TGCGAACAAGAGATTGTACATCATC	98	45	60
nox	F: GGACAAGAATCTGGTGTTGA R: CAATATCAGTCTCTACCTTAGGC	115	45	58
pdhA	F: ATGCCAAACTATAAAGATTTAC R: TCTTGGGCTTCAATATCT	113	40	56
aguD	F: TGGTGCTGCTCTTGCTAATG R: TAAAAGGACGCGGTGTATCC	188	45	60

F = Forward; R = reverse. *atpD, aguD:* primer sequences from Xu et al. [2011]; *fabM:* primer sequence from Jeon et al. [2011]; *nox:* primer sequence from Klein et al. [2012]; *pdhA:* primer sequence from Klein et al. [2010].

95°C. Sanger sequencing was performed in a 3500 Series Genetic Analyzer (8-capillary; Applied Biosystems). The sequencing data obtained was submitted to BioEdit software (version 7.2.5) to obtain *contigs* sequences. The BLAST (Basic Local Alignment Search Tool; http://estexplorer.biolinfo.org/hsd) program was used to analyse similarity among the sequences.

Reverse Transcription and Quantitative Real-Time PCR

To obtain cDNA, 1 µg of purified total RNA samples, 6 µl of 5× iScript reaction mix, 1 µl of iScript reverse transcriptase (iScript™ cDNA Synthesis Kit – Bio-Rad Laboratories Inc., Hercules, Calif., USA) and nuclease free-water were mixed to obtain a 30-µl solution. The reaction cycle included 5 min at 25°C, 120 min at 42°C, followed by 5 min at 85°C. Afterwards, the concentration of cDNA from all samples was adjusted to 10 ng/µl.

Standard curves used in every reverse transcription real-time quantitative PCR (RT-qPCR) run were prepared using purified DNA from *S. mutans* UA159 for testing of *S. mutans* genes, from *Streptococcus oralis* ATCC 35037 for TS and from *Streptococcus mitis* ATCC 49456 – NTCC 12261 for TB. Serial dilutions starting from 600 to 0.0006 ng (10-fold) of purified DNA were used as standards and positive controls for relative quantification of the targeted bacteria. A standard amplification curve and a melting-point product curve were obtained for each primer set. The RT-qPCR assays were carried out in duplicate in the StepOne™ Real Time PCR System (Applied Biosystems). The reactions were performed using a MicroAmpFast Optical 48-Well Reaction Plate (Applied Biosystems) covered with Optical Adhesive Film (Applied Biosystems). A mixture of 5 µl of Power SYBR® Green PCR Master Mix (Applied Biosystems), 2.4 µl of nuclease-free water, 0.3 µl of each primer F/R 10 µM and 2 µl of cDNA (10 ng/µl) was added in each spot of a 48-well plate. The final analyses were based on the mean of the two reactions. Negative control included reactions without template. The standard curves were used to transform the quantification cycle values to the mass of cDNA amplified.

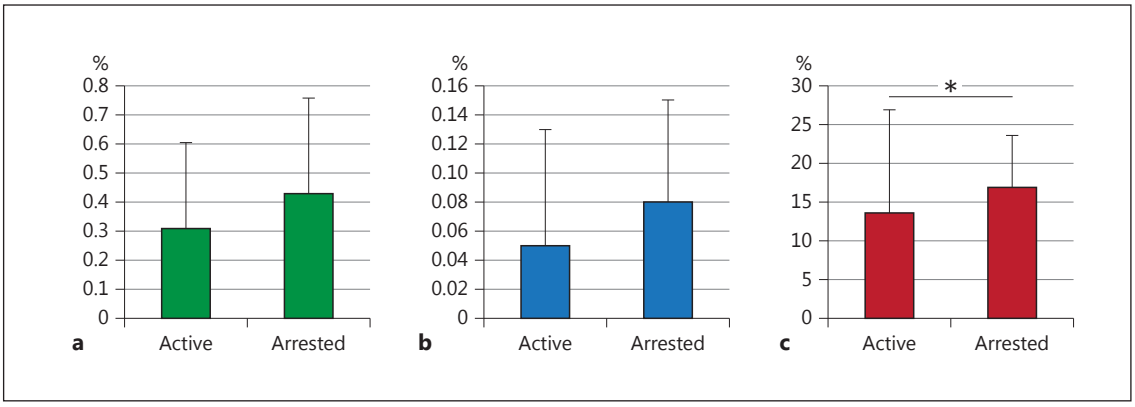

Fig. 2. *S. mutans* in proportion to both oral TS and oral TB. **a** *S. mutans*/TS. **b** *S. mutans*/TB. **c** TS/TB. * Statistically significant difference for TS/TB; p = 0.025 (Mann-Whitney test; α = 5%).

Statistical Analyses

An exploratory data analysis was performed to select the statistical tests. For bacterial prevalence data, the χ^2 test was used. The *S. mutans* quantification data were analysed by the Mann-Whitney test and TS and TB data by Student's t test. Data of *S. mutans* abundance in relation to TS were analysed by Student's t test, and in relation to TB were analysed by the Mann-Whitney test, as well as TS in relation to TB. Virulence gene expression data for the *atpD* and *aguD* genes were examined by the Mann-Whitney test, while *fabM, nox* and *pdhA* were transformed into logarithms to reach normality distribution and analysed by Student's t test. The statistical software SPSS 17.0 (Statistical Package for Windows Inc., Chicago, Ill., USA) was used to perform the analyses at a significance level of 5%.

Table 3. Quantification of metabolically active bacteria in carious dentine samples (ng/μl, mean ± SD)

Lesion activity	SM	TS	TB
Active	0.19±0.31	53.91±63.78	487.19±379.91
Arrested	0.27±0.30	55.52±53.83	462.54±368.87

SD = Standard deviation; SM = *S. mutans;* TS = total streptococci; TB = total bacteria. The Mann-Whitney test was applied for *S. mutans* and Student's t test for TS and TB (α = 5%).

Results

The mean age of the study population was 60.2 months and 48.4% were female. With regard to race, 25.8% of the participating children were Caucasian, 64.5% had a mixed race (Caucasian, Indian, African American), and 9.7% were African American. *S. mutans* was identified in 27 (93%) of the 29 active dentine lesions and in 15 (94%) of the 16 arrested dentine lesions tested. Table 3 shows the presence of *S. mutans*, oral TS, and oral TB in active and arrested dentine carious samples. No statistically significant differences in the presence of *S. mutans*, TS and TB were found when the two types of lesions were compared (p > 0.05).

The proportion of *S. mutans* in relation to total streptococci (*S. mutans*/TS) and in relation to the total bacterial load (*S. mutans*/TB), as well as the proportion of TS in relation to TB (TS/TB) are illustrated in figure 2. The TS proportion related to TB quantity was significantly higher in arrested dentine lesions (p = 0.025) compared to active lesions, while no significant correlation was found between *S. mutans*/TS or *S. mutans*/TB and the type of carious lesion (p > 0.05). Figure 3 shows the expression profile of the tested *S. mutans* genes in active and arrested dentine lesions. The two genes showing a statistically significant difference between active and arrested lesions were the *pdhA* (p = 0.04) and *aguD* (p = 0.05) genes; both genes were expressed at higher levels in arrested lesions as compared to active lesions.

Discussion

Our findings indicate that *S. mutans* is part of the viable microbiota of active and arrested dentine carious lesions of children with ECC. The analysis of gene expression suggests that the metabolic profile of *S. mutans* may differ according to the activity status of the carious

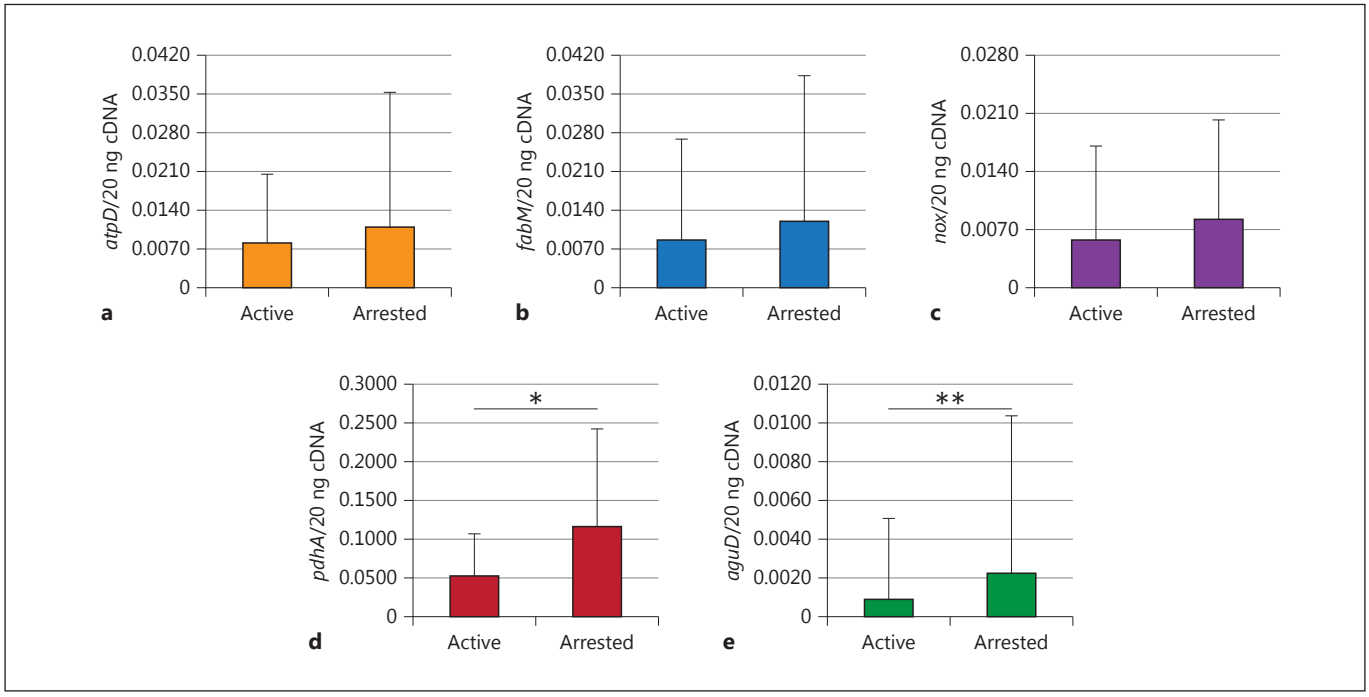

Fig. 3. Expression profile of the tested *S. mutans* genes in active and arrested dentine lesions. * $p < 0.04$; ** $p < 0.05$. **a** *atpD*. **b** *fabM*. **c** *nox*. **d** *pdhA*. **e** *aguD*.

lesions. Specifically, the expression levels of the *aguD* and *pdhA* genes were higher in arrested versus active lesions. Despite the evidence of the presence of *S. mutans* in carious dentine tissue, its virulence may be affected by environmental changes [Wen et al., 2010; Moye et al., 2014].

S. mutans was present in the majority of dentine carious samples tested in this study, however in low levels. Besides, *S. mutans* did neither show differences in its quantification between active and arrested lesions (table 3) nor in its proportion in relation to both oral TS and oral TB (fig. 2). Previous metagenomic and transcriptomic studies have also identified low levels of *S. mutans* in dentine caries [Aas et al., 2008; Lima et al., 2011; Wolff et al., 2012; Kianoush et al., 2014a; Simón-Soro et al., 2014]. On the other hand, recent metagenomic studies using pyrosequencing or fluorescence in situ hybridization did not observe the presence of *S. mutans* in the dentinal tubules at advanced stages of caries with cavity formation [Simón-Soro et al., 2013; Dige et al., 2014]. Other different previous findings [Kuribayashi et al., 2012] demonstrated *S. mutans* in the majority of active and arrested dentinal lesions, but in higher quantities. These divergences between the present and preceding results could be explained by factors inherent to each study methodology, such as the oral examination technique, caries criteria, number and age of the individuals, population ethnic differences, lesion depth and level of exposure to the oral environment of the collected dentinal tissues.

As previously cited, the microbiota of dental caries are highly complex and vary between individual lesions; however, it is notable that *S. mutans* is actually present in cavitated dentine carious lesions. The results of this study demonstrate that the role of *S. mutans* in caries development may not be strong enough when all the invading micro-organisms are taken into consideration. It should be emphasized that the critical pH for dentine dissolution is higher as compared to enamel, which allow colonization of bacteria that are not as acidogenic and aciduric as those required for initial enamel demineralization [Kianoush et al., 2014b]. Thus, dentine provides a different environment for bacteria involved in caries progression, where only certain types of bacteria may be able to colonize and exploit [Simón-Soro et al., 2013].

The current results showed a higher relative quantity of oral TS in arrested lesions, reaching a proportion of 17% from the oral TB. A similar result was found in previous studies involving dentine lesions found in children

[Jiang et al., 2014] and adults [Simón-Soro et al., 2013] without considering lesion activity status. A possible contribution of these micro-organisms to the inactivation of the lesions can be suggested, since several streptococcal species are able to produce alkali [Nascimento and Burne, 2014] that can neutralize acid in disease-associated biofilms and favour an increase in local pH, which is compatible with the high pH of arrested lesions [Hojo et al., 1994; Kuribayashi et al., 2012]. Another point to be considered is that arrested lesions present a harder reactive dentine with irregularly and blocked dentinal tubules [Love and Jenkinson, 2002] and lower water and protein contents [Nanci, 2012]. Hence, the high levels of proteolytic bacteria that are generally found in active dentine caries [Simón-Soro et al., 2013] may not have adequate nutritional conditions for their survival in arrested lesions, which potentially causes the overgrowth of streptococcal species.

S. mutans has been widely implicated in caries development for its capacity to form lactic acid from dietary carbohydrates that causes demineralization of tooth substrates. Among the virulence elements of this micro-organism are its capacity to grow at low pH (aciduricity) while producing acid (acidogenicity). The expression of genes related to these key properties (acidogenicity and aciduricity) and the metabolic activities of *S. mutans* were chosen to be analysed in this study in order to verify their relationship with caries activity in dentine tissues. Of note, we selected *S. mutans atpD, nox, pdhA*, *fabM* and *aguD* genes, which had been associated with bacterial survival, aciduricity and acidogenicity, and shown in this study by Sanger sequencing analysis to be specific for this organism when used in human dental samples.

The AgDS, encoded by the *aguBDAC* operon, is responsible for the catabolism of agmatine, an inhibitor of *S. mutans* growth, and consequently production of putrescine, ammonia, carbon dioxide and ATP. AgDS confers a competitive advantage to *S. mutans* by generating ATP, while removing the inhibitor agmatine from the environment and increasing acid tolerance via production of intracellular ammonia [Bardocz, 1993; Burne and Marquis, 2000; Sakakibara and Yanagisawa, 2003; Griswold et al., 2004, 2006, 2009]. The higher expression of the *aguD* gene in arrested lesions observed here may be explained by the bacterial need to produce ATP during survival in an antagonistic environment of poor nutrient availability and oxygen levels. No measurements of extracelluar agmatine capable of inducing the AgDs were made in this study. However, agmatine can be derived from human diet (maize, rice, soybeans, cucumbers) or produced by the metabolism of other bacteria [Griswold et al., 2006], such as *Prevotella* and *Neisseria*, which are abundant in dentine lesions [Simón-Soro et al., 2013, 2014].

The *pdhDABC* operon encodes the PDH complex involved in the glycolytic pathway, which is responsible for energy generation via metabolism of carbohydrates into pyruvate [Neijssel et al., 1997]. When the sugar concentration is high, lactate dehydrogenase enzyme converts pyruvate to lactic acid with oxidation of NADH to NAD^+ [Yamada and Carlsson, 1975]; however, when the sugar concentration is low, the metabolism of pyruvate in the presence of oxygen depends on the PDH complex to produce acetate and ATP with generation of NADH [Chary et al., 2005; Moye et al., 2014]. According to an in vitro study [Busuioc et al., 2010], a subset of *S. mutans* strains expressed the *pdh* operon in sugar-starved stationary phases, and seemed to be able to grow and divide even after several days. Thus, the higher expression of the *pdhA* gene in arrested lesions can also contribute to the survival of *S. mutans* in a potential sugar-deprived environment such as that found in arrested dentine lesions. A reduction in *S. mutans* levels has been shown when the *pdhDABC* operon was inactivated in vitro [Busuioc et al., 2010]. Moreover, the PDH complex promotes the production of acetate instead of lactate, which is an acid predominantly found in arrested lesions as previously demonstrated [Hojo et al., 1994].

No differences in expression of the *atpD, fabM* and *nox* genes between arrested and active dentinal caries were found. The *atpD* gene is a component of the *atpHAGDC* operon that encodes the F_0F_1-ATPase complex, which pumps protons from cells while maintaining the internal pH value [Kobayashi et al., 1986; Dashper and Reynolds, 1992; Kuhnert et al., 2004]. This gene seems to be expressed at higher levels during the early stages of caries, likely in the onset of cavitation. An in vitro experiment performed using multispecies biofilm showed a temporary overexpression of AtpD and FabM proteins during biofilm formation with declining levels of expression after 115 h [Klein et al., 2012]. The same interpretation may be made for the *fabM* gene, which encodes the FabM protein (trans-2-cis-3-decenoyl-ACP isomerase), responsible for the increased proportion of monounsaturated membrane fatty acids in response to environmental acidification and can affect the assembly of the ATPase system [Fozo and Quivey, 2004]. The *nox* gene encodes the Nox enzyme (H_2O-forming NADH oxidase), a major contributor to oxidative stress response by reducing oxygen levels in the glycolysis pathway through the

oxidation of NADH to NAD^+ [Derr et al., 2012]. In an in vitro study using a multispecies biofilm model, *nox* gene expression was reduced after 67 h of biofilm formation [Klein et al., 2012], which could indicate a more pronounced expression in the initial stages of caries lesions. In the present study, the dentine caries was well established and in an advanced stage of progression or arrest, and therefore was not representative of the initial stages of carious lesions.

Conclusion

Our findings revealed that *S. mutans* is part of the viable microbial community in active and arrested dentine carious lesions. The increased expression of the *pdhA* and *aguD* genes in arrested lesions is likely due to the unfavourable environmental conditions for microbial growth, inherent to this type of lesions. Future studies should continue to investigate the adaptive strategies used by *S. mutans* and other caries pathogens in order to identify novel ways to control caries.

Acknowledgements

This study was supported by the Brazilian National Council for Scientific and Technological Development (CNPq) (Process 475346/2011-4-MCT/CNPq14/2011-Universal).

Disclosure Statement

There are no potential conflicts of interest for any of the authors.

References

American Academy of Pediatric Dentistry: Policy on early childhood caries (ECC): classifications, consequences and preventive strategies. Reference manual. Pediatr Dent 2014;36:50–52.

Aas JA, Griffen AL, Dardis SR, Lee AM, Olsen I, Dewhirst FE, Leys EJ, Paster BJ: Bacteria of dental caries in primary and permanent teeth in children and young adults. J Clin Microbiol 2008;46:1407–1417.

Abanto J, Carvalho TS, Mendes FM, Wanderley MT, Bonecker M, Raggio DP: Impact of oral diseases and disorders on oral health-related quality of life of preschool children. Community Dent Oral Epidemiol 2011;39:105–114.

Bagramian RA, Garcia-Godoy F, Volpe AR: The global increase in dental caries. A pending public health crisis. Am J Dent 2009;22:3–8.

Bardocz S: The role of dietary polyamines. Eur J Clin Nutr 1993;47:683–690.

Belda-Ferre P, Alcaraz LD, Cabrera-Rubio R, Romero H, Simón-Soro A, Pignatelli M, Mira A: The oral metagenome in health and disease. ISME J 2012;6:46–56.

Bowen WH, Koo H: Biology of *Streptococcus mutans*-derived glucosyltransferases: role in extracellular matrix formation of cariogenic biofilms. Caries Res 2011;45:69–86.

Burne RA, Marquis RE: Alkali production by oral bacteria and protection against dental caries. FEMS Microbiol Lett 2000;193:1–6.

Busuioc M, Buttaro B, Piggot PJ: The *pdh* operon is expressed in a subpopulation of stationary-phase bacteria and is important for survival of sugar-starved *Streptococcus mutans*. J Bacteriol 2010;192:4395–4402.

Chary VK, Busuioc M, Renye JA Jr, Piggot PJ: Vectors that facilitate the replacement of transcriptional lacZ fusions in *Streptococcus mutans* and *Bacillus subtilis* with fusions to *gfp* or *gusA*. FEMS Microbiol Lett 2005;247:171–176.

Chu CH, Lo EC: Microhardness of dentine in primary teeth after topical fluoride applications. J Dent 2008;36:387–391.

Cox M: Regulation of bacterial RecA protein function. Crit Rev Biochem Mol Biol 2007;42:41–63.

Dashper SG, Reynolds EC: pH regulation by *Streptococcus mutans*. J Dent Res 1992;71:1159–1165.

Derr AM, Faustoferri RC, Betzenhauser MJ, Gonzalez K, Marquis RE, Quivey RG: Mutation of the NADH oxidase gene *(nox)* reveals an overlap of the oxygen- and acid-mediated stress responses in *Streptococcus mutans*. Appl Environ Microbiol 2012;78:1215–1227.

De Soet JJ, Nyvad B, Kilian M: Strain-related acid production by oral streptococci. Caries Res 2000;34:486–490.

Dige I, Grønkjær L, Nyvad B: Molecular studies of the structural ecology of natural occlusal caries. Caries Res 2014;48:451–460.

Feitosa S, Colares V, Pinkham J: The psychosocial effects of severe caries in 4-year-old children in Recife, Pernambuco, Brazil. Cad Saude Publica 2005;21:1550–1556.

Filstrup SL, Briskie D, da Fonseca M, Lawrence L, Wandera A, Inglehart MR: Early childhood caries and quality of life: child and parent perspectives. Pediatr Dent 2003;25:431–440.

Fozo EM, Quivey RG: Shifts in the membrane fatty acid profile of *Streptococcus mutans* enhance survival in acidic environments. Appl Environ Microbiol 2004;70:929–936.

Fozo EM, Scott-Anne K, Koo H, Quivey RG: Role of unsaturated fatty acid biosynthesis in virulence of *Streptococcus mutans*. Infect Immun 2007;75:1537–1539.

Griswold AR, Chen YY, Burne RA: Analysis of an agmatine deiminase gene cluster in *Streptococcus mutans* UA159. J Bacteriol 2004;186:1902–1904.

Griswold AR, Jameson-Lee M, Burne RA: Regulation and physiologic significance of the agmatine deiminase system of *Streptococcus mutans* UA159. J Bacteriol 2006;188:834–841.

Griswold AR, Nascimento MM, Burne RA: Distribution, regulation and role of the agmatine deiminase system in mutans streptococci. Oral Microbiol Immunol 2009;24:79–82.

Hahn K, Faustoferri RC, Quivey RG Jr: Induction of an AP endonuclease activity in *Streptococcus mutans* during growth at low pH. Mol Microbiol 1999;31:1489–1498.

Hanna MN, Ferguson RJ, Li YH, Cvitkovitch DG: *uvrA* is an acid-inducible gene involved in the adaptive response to low pH in *Streptococcus mutans*. J Bacteriol 2001;183:5964–5973.

Hazlett KR, Mazurkiewicz JE, Banas JA: Inactivation of the *gbpA* gene of *Streptococcus mutans* alters structural and functional aspects of plaque biofilm which are compensated by recombination of the *gtfB* and *gtfC*. Infect Immun 1999;67:3909–3914.

Hojo S, Komatsu M, Okuda R, Takahashi N, Yamada T: Acid profiles and pH of carious dentin in active and arrested lesions. J Dent Res 1994;73:1853–1857.

Ismail AI, Sohn W, Tellez Z, Amaya A, Sen A, Hasson H, Pitts NB: The International Caries Detection and Assessment System: an integrated system for measuring dental caries. Community Dent Oral Epidemiol 2007;35: 170–178.
Jeon JG, Pandit S, Xiao J, Gregoire S, Falsetta ML, Klein MI, Koo H: Influences of trans-trans farnesol, a membrane-targeting sesquiterpenoid, on *Streptococcus mutans* physiology and survival within mixed-species oral biofilms. Int J Oral Sci 2011;3:98–106.
Jiang W, Ling Z, Lin X, Chen Y, Zhang J, Yu J, Xiang C, Chen H: Pyrosequencing analysis of oral microbiota shifting in various caries states in childhood. Microb Ecol 2014;67: 962–969.
Kianoush N, Adler CJ , Nguyen KT, Browne GV, Simonian M, Hunter N: Bacterial profile of dentine caries and the impact of pH on bacterial population diversity. PLoS One 2014a;9:e92940.
Kianoush N, Nguyen K-AT, Browne GV, Simonian M, Hunter N: pH gradient and distribution of streptococci, lactobacilli, prevotellae, and fusobacteria in carious dentine. Clin Oral Investig 2014b;18:659–669.
King NM, Wong HM: More milk, stronger teeth? Part I. Dent Asia 2006, July/August, pp 17–21.
Klein MI, DeBaz L, Agidi S, Lee H, Xie G, Lin AH-M, Hamaker BR, Lemos JA, Koo H: Dynamics of *Streptococcus mutans* transcriptome in response to starch and sucrose during biofilm development. PLoS One 2010;5:e13478.
Klein MI, Xiao J, Lu B, Delahunty CM, Yates JR III, Koo H: *Streptococcus mutans* protein synthesis during mixed-species biofilm development by high-throughput quantitative proteomics. PLoS One 2012;7:e45795.
Kobayashi H, Suzuki T, Unemoto T: Streptococcal cytoplasmic pH is regulated by changes in amount and activity of a proton-translocating ATPase. J Biol Chem 1986;261:627–663.
Kremer BH, van der Kraan M, Crowley PJ, Hamilton IR, Brady LJ, Bleiweis AS: Characterization of the *sat* operon in *Streptococcus mutans*: evidence for a role of Ffh in acid tolerance. J. Bacteriol 2001;183:2543–2552.
Kuhnert WL, Zheng G, Roberta C, Faustoferri RC, Quivey RG Jr: The F-ATPase operon promoter of *Streptococcus mutans* is transcriptionally regulated in response to external pH. J Bacteriol 2004;186:8524–8528.
Kuribayashi M, Kitasako Y, Matin K, Sadr A, Shida K, Tagami J: Intraoral pH measurement of carious lesions with qPCR of cariogenic bacteria to differentiate caries activity. J Dent 2012;40:222–228.
Lemos JA, Chen YY, Burne RA: Genetic and physiologic analysis of the *groE* operon and role of the HrcA repressor in stress gene regulation and acid tolerance in *Streptococcus mutans.* J Bacteriol 2001;183:6074–6084.
Lemos JA, Burne RA: A model of efficiency: stress tolerance by *Streptococcus mutans*. Microbiology 2008;154:3247–3255.
Lemos JA, Abranches J, Burne RA: Responses of cariogenic streptococci to environmental stresses. Curr Issues Mol Biol 2005;7:95–107.
Lemos JA, Quivey RG, Koo H, Abranches J: *Streptococcus mutans*: a new Gram-positive paradigm? Microbiology 2013;159:436–445.
Lima KC, Coelho LT, Pinheiro IV, Rocas IN, Siqueira JF Jr: Microbiota of dentinal caries as assessed by reverse-capture checkerboard analysis. Caries Res 2011;45:21–30.
Love RM, Jenkinson HF: Invasion of dentinal tubules by oral bacteria. Crit Rev Oral Biol Med 2002;13:171–183.
Luo A, Yang D, Xin B, Paster B, Qin J: Microbial profiles in saliva from children with and without caries in mixed dentition. Oral Dis 2012; 6:595–601.
Marcenes W, Kassebaum NJ, Bernabé E, Flaxman A, Naghavi M, Lopez A, Murray JL: Global burden of oral conditions in 1990–2010: a systematic analysis. J Dent Res 2013;92:592–597.
Mattos-Graner RO, Corrêa MS, Latorre MR, Peres RC, Mayer MP: Mutans streptococci oral colonization in 12–30-month-old Brazilian children over a one-year follow-up period. J Public Health Dent 2001;61:161–167.
Moye ZD, Zeng L, Burne RA: Modification of gene expression and virulence traits in *Streptococcus mutans* in response to carbohydrate availability. Appl Environ Microbiol 2014;80: 972–985.
Nadkarni MA, Martin FE, Jacques NA, Hunter N: Determination of bacterial load by real-time PCR using a broad-range (universal) probe and primers set. Microbiology 2002;148:257–266.
Nanci A: ten Cate's Oral Histology: Development, Structure and Function. St Louis, Elsevier, 2012, pp 163–205.
Nascimento MM, Burne RA: Caries prevention by arginine metabolism in oral biofilms: translating science into clinical success. Curr Oral Heal Rep 2014;1:79–85.
Neijssel OM, Snoep JL, Teixeira de Mattos MJ: Regulation of energy source metabolism in streptococci. J Appl Microbiol 1997;83:12S–19S.
Nobre-dos-Santos M, Santos LM, Francisco SB, Cury JA: Relationship among dental plaque composition, daily sugar exposure and caries in the primary dentition. Caries Res 2002;36: 347–352.
Nyvad B, Fejerskov O: Assessing the stage of caries lesion activity on the basis of clinical and microbiological examination. Community Dent Oral Epidemiol 1997;25:69–75.
Nyvad B, Machiulskiene V, Baelum V: Reliability of a new caries diagnostic system differentiating between active and inactive caries lesions. Caries Res 1999;33:252–260.
Obata J, Takeshita T, Shibata Y, Yamanaka W, Unemori M, Akamine A, Yamashita Y: Identification of the microbiota in carious dentin lesions using 16S rRNA gene sequencing. PLoS One 2014;9:e103712.
Palmer CA, Kent R Jr, Loo CY, Hughes CV, Stutius E, Pradhan N, Dahlan M, Kanasi E, Arevalo Vasquez SS, Tanner AC: Diet and caries-associated bacteria in severe early childhood caries. J Dent Res 2010;89:1224–1229.
Pitts N: 'ICDAS' – an international system for caries detection and assessment being developed to facilitate caries epidemiology, research and appropriate clinical management. Community Dent Health 2004;21:193–198.
Quivey RG, Kuhnert WL, Hahn K: Genetics of acid adaptation in oral streptococci. Crit Rev Oral Biol Med 2001;12:301–314.
Rodrigues LKA, Guedes SF, Bezerra DS, Neves BG, Guedes MIF, Bassini A: Differential expression proteomics of saliva from children with caries at different stages of severity. ADR/AADR/CADR General Session and Exhibition, Boston, 2015. vol 1, p 1082.
Sakaguchi S, Saito M, Tsuji H, Asahara T, Takata O, Fujimura J, Nagata S, Nomoto K, Shimizu T: Bacterial rRNA-targeted reverse transcription-PCR used to identify pathogens responsible for fever with neutropenia. J Clin Microbiol 2010;48:1624–1628.
Sakakibara Y, Yanagisawa H: Agmatine deiminase from cucumber seedlings is a mono-specific enzyme: purification and characteristics. Protein Expr Purif 2003;30:88–93.
Simón-Soro A, Belda-Ferre P, Cabrera-Rubio R, Alcaraz LD, Mira A: A tissue-dependent hypothesis of dental caries. Caries Res 2013;47: 591–600.
Simón-Soro A, Guillen-Navarro M, Mira A: Metatranscriptomics reveals overall active bacterial composition in caries lesions. J Oral Microbiol 2014;6:25443.
Stipp RN, Boisvert H, Smith DJ, Höfling JF, Duncan MJ, Mattos-Graner RO: CovR and VicRK regulate cell surface biogenesis genes required for biofilm formation in *Streptococcus mutans*. PLoS One 2013;8:e58271.
Takahashi N, Nyvad B: Caries ecology revisited: microbial dynamics and the caries process. Caries Res 2008;42:409–418.
Wen ZT, Yates D, Ahn SJ, Burne RA: Biofilm formation and virulence expression by *Streptococcus mutans* are altered when grown in dual-species model. BMC Microbiol 2010;10:111.
Wolff D, Frese C, Maier-Kraus T, Krueger T, Wolff B: Bacterial biofilm composition in caries and caries-free subjects. Caries Res 2012; 47:69–77.
Xu X, Zhou XD, Wu CD: The tea catechin epigallocatechin gallate suppresses cariogenic virulence factors of *Streptococcus mutans.* Antimicrob Agents Chemother 2011;55:1229–1236.
Yamada T, Carlsson J: Regulation of lactate dehydrogenase and change of fermentation products in streptococci. J Bacteriol 1975;124:55–61.
Yano A, Kaneko N, Ida H, Yamaguchi T, Hanada N: Real-time PCR for quantification of *Streptococcus mutans.* FEMS Microbiol Lett 2002; 217:23–30.