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

BEATRIZ GONÇALVES NEVES

IDENTIFICAÇÃO MOLECULAR DE BACTÉRIAS EM LESÕES CARIOSAS DENTINÁRIAS E EM BIOFILME DE CRIANÇAS COM DIFERENTES ESTÁGIOS DA CÁRIE PRECOCE DA INFÂNCIA

FORTALEZA
2014

BEATRIZ GONÇALVES NEVES

IDENTIFICAÇÃO MOLECULAR DE BACTÉRIAS EM LESÕES CARIOSAS DENTINÁRIAS E EM BIOFILME DE CRIANÇAS COM DIFERENTES ESTÁGIOS DA CÁRIE PRECOCE DA INFÂNCIA

Tese apresentada ao Programa de Pós-Graduação em Odontologia da Faculdade de Farmácia, Odontologia e Enfermagem da Universidade Federal do Ceará como um dos requisitos para a obtenção do Título de Doutor em Odontologia.

Área de concentração: Clínica Odontológica

Orientadora: Profa. Dra. Lidiany Karla Azevedo Rodrigues
Co-orientador: Prof. Dr. Rafael Nóbrega Stipp

FORTALEZA

2014

Dados Internacionais de Catalogação na Publicação
Universidade Federal do Ceará
Biblioteca de Ciências da Saúde

N422i Neves, Beatriz Gonçalves.
Identificação molecular de bactérias em lesões cariosas dentinárias e em biofilme de crianças com diferentes estágios da cárie precoce da infância. / Beatriz Gonçalves Neves. – 2014.
79 f. : il. color., enc. ; 30 cm.

Tese (doutorado) – Universidade Federal do Ceará; Centro de Ciências da Saúde; Faculdade de Farmácia, Odontologia e Enfermagem; Departamento de Odontologia; Programa de Pós-Graduação em Odontologia; Doutorado em Odontologia, Fortaleza, 2014.
Área de Concentração: Clínica Odontológica.
Orientação: Profa. Dra. Lidiany Karla Azevedo Rodrigues.
Co-Orientação: Prof. Dr. Rafael Nóbrega Stipp.

1. Cárie Dentária. 2. Dente Decíduo. 3. Placa Dentária. 4. Dentina. 5. Reação em Cadeia da Polimerase em Tempo Real. I. Título.

CDD 617.67

BEATRIZ GONÇALVES NEVES

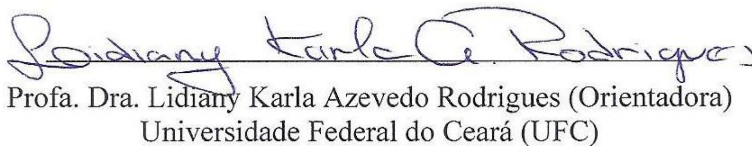
IDENTIFICAÇÃO MOLECULAR DE BACTÉRIAS EM LESÕES CARIOSAS DENTINÁRIAS E EM BIOFILME DE CRIANÇAS COM DIFERENTES ESTÁGIOS DA CÁRIE PRECOCE DA INFÂNCIA

Tese apresentada à Coordenação do Programa de Pós-Graduação em Odontologia da Universidade Federal do Ceará como requisito parcial para obtenção do Título de Doutor em Odontologia. Área de concentração: Clínica Odontológica.

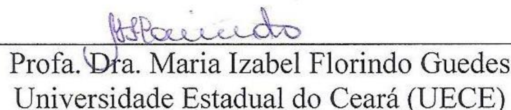
Aprovada em: 25/11/14

BANCA EXAMINADORA

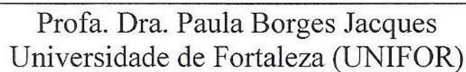
Identificação molecular de bactérias em lesões cariosas dentinárias e em biofilme de crianças com diferentes estágios da cárie precoce da infância



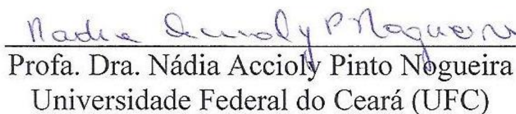
Profa. Dra. Lidiany Karla Azevedo Rodrigues (Orientadora)
Universidade Federal do Ceará (UFC)



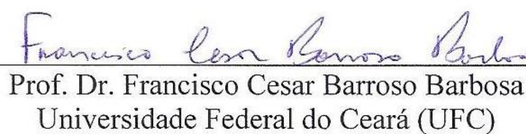
Profa. Dra. Maria Izabel Florindo Guedes
Universidade Estadual do Ceará (UECE)



Profa. Dra. Paula Borges Jacques
Universidade de Fortaleza (UNIFOR)



Profa. Dra. Nádia Accioly Pinto Nogueira
Universidade Federal do Ceará (UFC)



Prof. Dr. Francisco Cesar Barroso Barbosa
Universidade Federal do Ceará (UFC)

A **Deus,** em primeiro lugar, por toda a força e coragem que me concedeu, durante essa trajetória, imprescindíveis para realização deste projeto, tão importante na minha vida e da minha família.

Aos meus queridos pais, **Gilberto e Olivete**, pelo exemplo de vida, incentivo e confiança, sem os quais não seria possível a realização deste trabalho. Obrigada por todo carinho e apoio ! Amo vocês !

Ao meu irmão, **Bruno,** e minha cunhada, **Roberta,** pelo carinho, amizade, respeito e pelas palavras de apoio, tão importantes nesta caminhada. Peço desculpas por não ter sido tão presente, como gostaria, durante os preparativos para a chegada do nosso querido **Pedro,** mas o meu coração estava sempre com vocês.

Ao meu sobrinho **Pedro,** que está chegando no mesmo mês em que estou realizando a minha defesa. A expectativa do seu nascimento foi um dos fatores que me encorajaram a seguir com força e determinação para a conquista deste grande desafio.

AGRADECIMENTOS ESPECIAIS

À minha orientadora, **Profa. Dra. Lidiany Karla Azevedo Rodrigues**, pelo exemplo de profissional e pesquisadora que não mediu esforços na colaboração para o desenvolvimento desta pesquisa. Agradeço por ter acreditado neste projeto. Obrigada pelo incentivo constante e por contribuir para o meu crescimento profissional.

Ao meu co-orientador, **Prof. Dr. Rafael Nóbrega Stipp,** agradeço por ter me acolhido em um momento tão difícil e especial desta tese. Agradeço pelas orientações na área de Microbiologia e Biologia Molecular e pela contribuição ímpar para o desenvolvimento deste trabalho.

À equipe maravilhosa, **Daniela da Silva Bezerra e Sarah Florindo de Figueiredo Guedes,** os desafios e as dificuldades de cada momento contribuíram para que nos tornássemos amigas e companheiras até o final. Agradeço pela amizade e companheirismo fortalecidos durante a saudável convivência.

AGRADECIMENTOS

À Universidade Federal do Ceará (UFC), por meio do seu Magnífico Reitor **Prof. Dr. Jesualdo Pereira Farias**.

À Faculdade de Farmácia, Odontologia e Enfermagem da Universidade Federal do Ceará, na pessoa de sua diretora, **Profa. Maria Goretti Rodrigues de Queiroz** e vice-diretor **Prof. Sérgio Lima Santiago.**

Ao Coordenador do curso de Odontologia da Universidade Federal do Ceará, **Prof. Dr. Fabrício Bitu Sousa.**

À coordenação do Programa de Pós-Graduação em Odontologia da UFC, por meio da Coordenadora **Profa. Dra. Lidiany Azevedo Rodrigue**s e da vice-coordenadora **Profa. Dra. Cristiane Sá Roriz Fonteles.**

Ao Programa de Pós-Graduação de Odontologia (PPGO) da Universidade Federal do Ceará (UFC) e a todos os professores envolvidos, pela generosidade, bem como pelo estímulo, apoio e ensinamentos.

À Faculdade de Odontologia da Universidade de Campinas (UNICAMP) – Piracicaba e Laboratório de Imunologia e Biologia Molecular, na pessoa do **Prof. Dr. Rafael Nóbrega Stipp**, pela acolhida, disponibilidade e estrutura oferecidas para realização dos experimentos desta tese.

À Coordenação do Curso de Odontologia da Universidade Federal do Ceará (UFC) – Campus Sobral, em especial aos professores **Mario Áureo Gomes Moreira** e **Rodrigo Citó Rego.**

Ao Núcleo de Biotecnologia de Sobral (Nubis), em especial ao Prof. **Dr. José Roberto Viana Silva**, por ter permitido a realização de uma parte dos experimentos em Sobral.

Aos funcionários do PPGO da UFC, **Lúcia Ribeiro Marques Lustosa e Janaine Marques Leal,** pela constante colaboração e disponibilidade.

Ao **Davi Queiroz**, técnico do Laboratório do PPGO da UFC, pelo apoio, auxílio e disponibilidade sempre presentes.

Aos funcionários do Curso de Odontologia sempre dispostos a ajudar e solucionar os problemas surgidos.

Às colegas do Curso de Odontologia da UFC – Campus Sobral, **Alrieta Henrique Teixeira, Ana Karine Macedo, Celiane Mary Carneiro Tapety, Hellíada Vasconcelos Chaves**, **Iriana Carla Junqueira Zanin, Karuza Maria Alves Pereira e Mariana Ramalho de Farias,** pelo apoio e compreensão.

A **Cristiane Tomaz Rocha e Patricia Leal Dantas Lobo**, amigas e colegas de disciplina da Odontopediatria, pela compreensão, incentivo, apoio, ajuda e disponibilidade, imprescindíveis para que eu conseguisse concluir esta tese.

Às amigas **Denise Sá Maia Casseli e Virgínia Régia Souza da Silveira,** pela força, estímulo e incentivo constantes, bem como pela amizade desenvolvida ao longo de nossa convivência. Obrigada pelo carinho de vocês.

À **Juliana Nunes Botelho**, por toda a ajuda, companhia e carinho para comigo em vários momentos durante a minha estada em Piracicaba.

Aos colegas pós-graduandos da Faculdade de Odontologia da Unicamp – Piracicaba, **Erika Harth, Marcelle Buso, Natalia Vizoto, Tarsila Camargo, Lívia Alves, Felipe Joia, Ítalo Sarto e Thais Oliveira**, pela ajuda e atenção nos momentos em que precisei, nas minhas várias temporadas em Piracicaba. Meu agradecimento.

Aos colegas de doutorado da UFC**, Ana Patrícia Souza de Lima, Denise Lins de Sousa, Fabianni Apolônio, Ramille Araújo Lima, Juliana Marques Paiva, Mary Anne Sampaio de Mello, Vanara Passos Florêncio e Patrícia Maria Costa de Oliveira,** pela convivência harmoniosa e troca de experiências, bem como por todos os momentos em que nos apoiamos mutuamente.

À **Profa. Dra. Maria Izabel Florindo Guedes**, pela disponibilidade em ajudar quando precisei.

À amiga **Patrícia Maria Costa de Oliveira**, pela ajuda em vários momentos desta pesquisa. Muito obrigada!

Aos alunos de iniciação científica, especialmente **Edyr Pereira** e **Caroline Salema**, que contribuíram para o bom andamento deste trabalho.

Ao colega **Paulo Goberlânio de Barros Silva**, pela colaboração para a intepretação dos resultados. Obrigada pela paciência!

A todos os meus familiares, Vovó Alzirinha, meus avós que já partiram, "*in memoriam*" Boanerges, Cazuza e Joanila, tios, primos e primas, presentes em todos os momentos da minha vida. Em especial, minhas primas-irmãs, **Clarissa Lima de Oliveira, Mariana Lima de Oliveira e Marília de Oliveira Araújo**, que, apesar da distância física, estão sempre ao meu lado.

Aos meus padrinhos **Júlio** e **Lusenir Timbó**, pelo incentivo e apoio de sempre.

A minha sempre amiga **Fernanda Campos Machado,** companheira do Mestrado e, hoje, mesmo distante, fisicamente, continua presente me apoiando em todos os momentos da minha vida.

À **Maria Elena Evangelista**, muito querida, por toda a ajuda e apoio em casa, minimizando o meu cansaço. Obrigada!

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

Às escolas e creches municipais de José Batista de Oliveira, Padre Félice Pistoni, Aprisco, Vicente Fialho, São Raimundo, pela atenção dispensada, viabilizando a triagem de pacientes para este estudo.

Aos meus amigos, que aproveito para me desculpar pela ausência em muitos momentos importantes na vida de vocês, quando não foi possível estar presente para me dedicar a esta tese. Sei que me compreenderam, pela importância da causa.

Ao Conselho Nacional de Desenvolvimento Científico e Tecnológico **(CNPq),** pelo apoio financeiro para esta pesquisa, por meio do Edital Universal.

À Coordenação de Aperfeiçoamento de Pessoal de Nível Superior **(Capes),** pela concessão de bolsa em determinado período deste Doutorado.

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

MUITO OBRIGADA !

“Tudo posso naquele que me fortalece.”

(Filipenses 4:13)

RESUMO

A cárie precoce da infância (CPI) é considerada um grave problema de saúde pública em crianças pré-escolares em todo mundo. No entanto, muitos aspectos ainda devem ser explorados acerca da microbiota oral relacionada com a progressão da CPI e como a comunidade bacteriana se modifica de acordo com a atividade da lesão dentinária. Esta tese, constituída de dois capítulos, teve como objetivo identificar e quantificar através da técnica de reação em cadeia da polimerase quantitativa (qPCR) as bactérias *Actinomyces naeslundii, Bifidobacterium* spp*., Lactobacillus acidophilus, Streptococcus gordonii, Streptococcus mutans,* bem como espécies dos grupos *Lactobacillus casei* e *Mitis* em biofilme de crianças pré-escolares com diferentes estágios de progressão da cárie precoce da infância (Capítulo 1) e em lesões cariosas dentinárias ativas e inativas (Capítulo 2) e ainda verificar a associação destes microrganismos ao processo de saúde ou de doença. A amostra consistiu em pré-escolares com idade entre 2 e 5 anos de idade que frequentavam creches e escolas públicas de Fortaleza-CE. As crianças foram examinadas com o uso de índices visuais ICDAS II (*International Caries Detection Assessment System)* e Nyvad, a fim de avaliar a prevalência e atividade de cárie. A coleta de biofilme supragengival foi realizada em 75 crianças, as quais foram agrupadas de acordo com índice ICDAS II em três grupos: CF (livres de cárie) (n=20), ECL (presença de lesões de cárie em esmalte) (n=17) e DCL (presença de lesões de cárie em dentina) (n=38). Amostras de dentina cariada foram coletadas sob isolamento absoluto de 56 lesões cariosas dentinárias, sendo 17 inativas e 39 ativas. O DNA de todas as amostras coletadas foi extraído e purificado e, em seguida, testado para a presença das espécies/grupos bacterianos acima citados através de qPCR. A quantidade das bactérias foi comparada pelos testes Kruskal-Wallis e Mann-Whitney. Além disso, a associação da presença de bactérias e CPI foi analisada através do teste Qui-quadrado, com nível de significância de 5% e aplicado a regressão logística múltipla. Bactérias *L. acidophilus* e do grupo *L. casei* apresentaram baixa detecção no biofilme de todos os grupos avaliados. A presença de *S. mutans* e *Bifidobacterium* spp. mostrou forte associação com a progressão da doença no biofilme de crianças com lesões dentinárias com “odds ratio” de 21,5 e 5,9, respectivamente. Em lesões dentinárias ativas, concentrações de *Bifidobacterium* spp. e bactérias do grupo *L. casei* foram significativamente maiores quando comparadas às lesões inativas ($p<0.05$). Os níveis de *A. naeslundii,* bactérias do grupo *Mitis* e *S. gordonni* não apresentaram diferença significativa entre os grupos de biofilme, assim como nas lesões dentinárias. Conclui-se que as amostras de biofilme apresentaram alteração na proporção de bactérias acidogênicas e acidúricas com a

progressão da doença cárie. A presença de *Bifidobacterium* spp. e *S. mutans* apresentou forte associação com os estágios mais avançados da CPI. Em relação às lesões dentinárias, o aumento da concentração de bactérias *Bifidobacterium* spp. e do grupo *L. casei* evidenciou um papel importante destas bactérias na atividade de lesões dentinárias.

Palavras-chave: *Actinomyces*. Bactérias. *Bifidobacterium*. Cárie Dentária. Criança. Dente Decíduo. Dentina. *Lactobacillus*. Placa Dentária. Reação em Cadeia da Polimerase em Tempo Real. *Streptococcus*.

ABSTRACT

Early childhood caries (ECC) is considered a serious public health issue among children all over the world. However, many aspects should be explored about the oral microbiota related to the ECC progression and how the bacterial community modifies according to the dentine lesion activity. This thesis, composed by two chapters, aimed to investigate and quantify with quantitative polymerase chain reaction (qPCR) the following bacteria *Actinomyces naeslundii, Bifidobacterium* spp*., Lactobacillus acidophilus, Streptococcus gordonii*, *Streptococcus mutans,* as well as members of the groups *Lactobacillus casei* and *Mitis* on biofilm from pre-school children with different stages of early childhood caries progression (Chapter 1) and on active and inactive dentine carious lesions (Chapter 2), and also to verify the association of these microorganisms on the process of health or disease. The sample consisted on preschool children aged between 2 and 5 years from nurseries and public preeschools in Fortaleza-CE. The children were examined for caries diagnosis with ICDAS II index (International Caries Detection Assessment System), and the Nyvad criteria, in order to evaluate prevalence and caries activity. The supragingival biofilm collection was taken from 75 children, who were divided in three groups according to the ICDAS II: CF (caries free) (n=20), ECL (presence of enamel caries lesion) (n=17) and DCL (presence of dentine caries lesion) (n=38). Samples of carious dentine were collected under rubber dam isolation of 56 lesions of dentine affected by caries, being 17 inactive and 39 active. The DNA of all the collected samples was extracted and purified, then tested for the presence of the formerly mentioned bacterial species/groups through qPCR. The quantity of bacteria was compared through the Kruskal-Wallis and Mann-Whitney tests. Besides, the association between the presence of bacteria and ECC was analyzed through the Chi-square test, with a 5% significance level and the multiple logistic regression was applied. Bacteria from the group *L. casei* and *L. acidophilus* presented low detection on biofilm of all evaluated groups. The presence of *S. mutans* and *Bifidobacterium* spp. showed a strong association with dental caries progression on the biofilm from children with dentine lesions with odds ratio of 21,5 and 5,9; respectively. On active dentine lesions, concentrations of *Bifidobacterium* spp. and species from the *Lactobacillus casei* group were significantly higher when compared to the inactive lesions ($p<0.05$). The levels of *Actinomyces naeslundii, Streptococcus gordonni* and species from *Mitis* group were not significantly different among biofilm groups as well as comparing dentine lesions. In conclusion, the microbial profile from biofilm samples presented differences on the proportion of acidogenic and aciduric bacteria with dental caries progression. The presence of

Bifidobacterium spp. and *S. mutans* presented a strong association with the development of the more advanced stages of ECC. Regarding the activity of dentine lesions, higher detection levels of the group *L. casei* and *Bifidobacterium* spp. showed an important role of these bacteria in the dentine caries activity.

Key-words: *Actinomyces*. Bacteria. *Bifidobacterium.* Child. Dental caries. Dental plaque. Dentine. *Lactobacillus*. Primary teeth. Real-time polymerase chain reaction. *Streptococcus*.

SUMÁRIO

RESUMO

ABSTRACT

1 INTRODUÇÃO GERAL.....17

2 PROPOSIÇÃO.....22

3 CAPÍTULOS.....23

3.1 CAPÍTULO 1.....24

Molecular analysis of biofilm bacteria associated to different stages of early childhood caries

3.1.1 Introduction.....27

3.1.2 Methods.....28

3.1.3 Results.....31

3.1.4 Discussion.....32

3.1.5 References.....36

3.2 CAPÍTULO 2 46

Molecular detection of bacteria associated to caries activity in dentinal lesions

3.2.1 Introduction.....49

3.2.2 Methods.....50

3.2.3 Results 54

3.2.4 Discussion.....55

3.2.5 References.....60

4 CONCLUSÃO GERAL.....70

REFERÊNCIAS GERAIS.....71

APÊNDICES.....75

ANEXO.....79

1 INTRODUÇÃO GERAL

A cárie dentária é uma doença biofilme-açúcar-dependente, uma vez que é necessário que as bactérias bucais se organizem sobre os dentes na forma de biofilme e que haja ingestão frequente de açúcar para que ocorra desmineralização dos tecidos duros dentais (FEJERSKOV, 2004; LOESCHE, 1986). Dentre os fatores etiológicos, destacam-se o acúmulo de biofilme na superfície do dente, o frequente consumo de carboidratos fermentáveis e a susceptibilidade do indivíduo, após um período de tempo (MARSH, 1994). Esta desordem constitui uma das mais comuns doenças causadas por microrganismos em humanos e ainda é considerada como um significante problema de saúde pública em vários países (RAMOS-GOMEZ *et al*., 2002; PETERSEN *et al*., 2005), inclusive no Brasil. Em crianças, apresenta-se como a doença crônica mais prevalente da infância (de SILVA-SANIGORSKI *et al.*, 2010).

Os últimos levantamentos epidemiológicos de saúde bucal (BRASIL, 2012) apontam desigualdades entre grupos populacionais (COSTA *et al*., 2013), sendo que no relatório de saúde bucal de 2004, (BRASIL, 2004), o Brasil não atingiu a meta estabelecida pela Organização Mundial de Saúde (OMS), a qual preconizava que 50% das crianças de zero a cinco anos deveriam estar livres de cárie. Este levantamento verificou uma prevalência de quase 27% na experiência de cárie em crianças entre 18 e 36 meses. Em 2010, o levantamento em saúde bucal SB Brasil não incluiu nos exames o grupo da faixa etária de 18-36 meses, no entanto observou-se que aos 5 anos de idade, uma criança brasileira possui, em média, o índice de 2,43 dentes com experiência de cárie, com predomínio do componente cariado, sendo responsável por mais de 80% do índice (BRASIL, 2012).

A cárie precoce da infância (CPI), anteriormente conhecida como “cárie de mamadeira” e aceita mundialmente pelo termo “Early Childhood Caries’’ (ECC), é definida como a presença de uma ou mais superfícies cariadas (lesões cavitadas ou não-cavitadas), perdidas (devido à cárie) ou restauradas em qualquer dente decíduo em uma criança com menos de seis anos de idade (AAPD, 2014). O termo na língua inglesa Early Childhood Caries” foi traduzida para a língua portuguesa como “cárie de estabelecimento precoce”, “cárie de primeira infância”, ou com maior aceitação “cárie precoce da infância” (FELDENS , 2013). A presença de padrões atípicos, progressivos, agudos ou rampantes desta doença é designada cárie precoce da infância severa. Em crianças em idade pré-escolar, a grande quantidade de bactérias cariogênicas como estreptococos mutans e lactobacilos, a presença de biofilme dental, práticas inapropriadas de alimentação (AAPD, 2014; BERKOWITZ, 2003) e condição

sócio-econômica (SCHWENDICKE *et al.*, 2014) têm sido identificados como fatores predisponentes ao desenvolvimento de cárie.

A CPI é normalmente iniciada com lesões de mancha branca nos incisivos maxilares decíduos ao longo da margem gengival, onde o biofilme dentário normalmente acumula. Esta doença apresenta uma evolução rápida e progressiva, podendo ser dolorosa e causar sérias consequências para a criança (BERKOWITZ, 2003). Dentre as sequelas iniciais da doença não tratada pode-se citar dor, infecção e abscessos. Ainda pode acarretar várias consequências na saúde e desenvolvimento da criança, incluindo atraso no crescimento, problemas nutricionais e de sono, baixa auto-estima, além de prejuízo no rendimento escolar (RAMOS-GOMEZ *et al.*, 2002). Além disso, crianças acometidas por esta desordem podem apresentar um maior risco para o desenvolvimento de novas lesões de cárie em ambas dentições decídua e permanente (TINANOFF & O´SULLIVAN, 1997). Inicialmente, acreditava-se que a CPI estava associada ao uso prolongado de mamadeira (ISMAIL & SOHN, 1999), no entanto, somente o fator etiológico dieta não é capaz de causar a severidade característica desta doença (CLEATON-JONES *et al.*, 2000), apesar de exercer um papel imprescindível na manifestação clínica desta infecção (BERKOWITZ, 2003).

Há evidências consideráveis de que micro-organismos Gram-positivos acidogênicos, acidúricos e com capacidade de aderência às superfícies dentárias, pertencentes a um grupo heterogêneo denominado estreptococos do grupo mutans (EGM), são especialmente envolvidos na dinâmica do processo carioso (LOESCHE, 1986; PARISOTTO *et al.*, 2010). Estas bactérias são fortemente associadas com cárie dentária em virtude dos seus atributos metabólicos, ecológicos e epidemiológicos (LOESCHE, 1986). Dentre os EGM, *Streptococcus mutans* constitui uma espécie bacteriana predominante na microbiota de crianças pré-escolares com cárie de estabelecimento precoce (BECKER *et al.*, 2002; BERKOWITZ, 2003; SAXENA *et al.*, 2008). Apesar de que a associação entre *S. mutans* e CPI esteja estabelecida na literatura (BERKOWITZ, 2003; KOHLER *et al.*, 1988; VAN HOUTE *et al.*, 1982), tem sido demonstrado que nem todos os indivíduos que são colonizados por EGM apresentam a doença (CARLSSON, OLSSON & BRATTHALL, 1985; MATTOS-GRANER *et al.*, 2001) e que também a cárie pode ocorrer em sua ausência (AAS *et al.*, 2008; GROSS *et al.*, 2010; TANNER *et al.*, 2011).

A virulência de *S. mutans* reside em três atributos essenciais: a habilidade para formar biofilme, a produção de grandes quantidades de ácidos orgânicos (acidogenicidade), e a tolerância ao estresse ambiental, particularmente pH baixo (aciduricidade) (LAW *et al.*, 2007; SHAW, 1987; SHEN *et al.*, 2004). Embora o *S. mutans* seja um dos microrganismos

cariogênicos mais pesquisados, estudos têm sugerido que a etiologia da cárie dentária pode envolver comunidades mais complexas de espécies bacterianas (BECKER *et al*., 2002; VAN HOUTE, 1994).

Dessa forma, análises quantitativas e qualitativas de ecossistemas polimicrobianos como biofilme dentário e dentina cariada são desafiadoras, uma vez que as comunidades microbianas podem consistir em centenas de diferentes espécies bacterianas. É relatado na literatura existir mais de 700 espécies de bactérias na cavidade oral, sendo espécies cultiváveis apenas 50-60% (DEWHIRST et al , 2010). O advento de novos métodos moleculares através de identificação e quantificação bacteriana tem tornado possível reavaliar a patogênese de infecções orais de forma mais precisa. Em pesquisas utilizando identificação molecular de bactérias, estudos têm relatado que diversas comunidades bacterianas, incluindo novas espécies, estão associadas com a cárie dentária (BECKER *et al*., 2002; MUNSON *et al*.; 2004; AAS *et al*., 2008; TANNER *et al*., 2011; BELDA-FERRE *et al*., 2012; GROSS *et al*., 2012; JIANG *et al*., 2014; PETERSON *et al*. 2014). Além do *S. mutans*, espécies acidúricas e acidogênicas do grupo estreptococos não-mutans e algumas espécies dos gêneros *Veillonella*, *Lactobacillus*, *Bifidobacterium*, *Propionibacterium*, *Actinomyces*, e *Atopobium* podem também exercer uma importante função na progressão da cárie (BECKER *et al*., 2002; MUNSON et al., 2004; CHHOUR *et al*., 2005; AAS *et al*., 2008; BELDA-FERRE *et al*., 2012; GROSS *et al*., 2012). Estes achados apoiam a hipótese da placa ecológica, que propõe que o *S. mutans* é apenas um dos muitos micro-organismos envolvidos com a patogênese da cárie (MARSH, 2003; AAS *et al*., 2008; TAKAHASHI & NYVAD, 2011).

No estudo de AAS *et al*. (2008), foi observado que em 10 a 20% dos pacientes que apresentavam cárie severa não foram identificados níveis detectáveis de *S. mutans*, mas apresentavam outras espécies produtoras de ácidos. Além disso, em algumas lesões cariosas *S. mutans* pode ser um componente bacteriano minoritário do biofilme dental.

Em um estudo com cárie, BECKER *et al.* (2002) identificaram 10 novos filotipos na microbiota associada com lesões de cárie em um único indivíduo com cárie de estabelecimento precoce. Nesta mesma pesquisa, espécies bacterianas encontradas em crianças com cárie de estabelecimento precoce foram comparadas com aquelas identificadas em crianças livres de cárie. Algumas espécies como *Streptococcus sanguinis* foram associadas com saúde bucal, enquanto outras como *S. mutans*, *Lactobacillus fermentum* e algumas espécies dos gêneros *Streptococcus* spp., *Veillonella* spp., *Actinomyces* spp., *Bifidobacterium* spp. foram relacionadas com cárie.

Corby *et al.* (2005), através de métodos moleculares de identificação como *checkerboard hybridization*, verificaram bactérias associadas com cárie dentária e saúde em um grupo de 204 gêmeos de idade entre 1,5 e 7 anos. As bactérias *Actinomyces* spp., *S. mutans*, e *Lactobacillus* spp. foram detectadas em abundância no grupo cárie-ativo. Em contraste, espécies bacterianas, incluindo *Streptococcus parasanguinis*, *Abiotrophia defectiva*, *Streptococcus mitis*, *Streptococcus oralis*, e *S. sanguinis* predominaram na microbiota indígena bacteriana de indivíduos livres de cárie.

Estudos têm demonstrado que espécies consideradas como colonizadores iniciais da placa dentária, como estreptococos do grupo mitis, podem apresentar a capacidade de produzir álcali, gerando um impacto no equilíbrio do pH do biofilme (NASCIMENTO *et al.*, 2009). No entanto, em situações de acidúria e acidogenicidade, tais bactérias, comumente associadas à saúde dental, podem apresentar relação com atividade de cárie dentária (MARCHANT *et al.,* 2001; AAS *et al*., 2008 GROSS *et al.,* 2012).

Com o advento de métodos e sistemas de diagnóstico ao longo dos últimos anos, houve a inserção do ICDAS II (*International Caries Detection & Assessment System*), que é um sistema de detecção de cárie baseado na inspeção visual e inclui avaliação de lesões cariosas cavitadas e não-cavitadas. Esse sistema foi desenvolvido para uso na prática clínica, bem como em pesquisas clínicas e epidemiológicas (PITTS, 2004). O método provou ser bem aceito e prático, baseado na sua padronização de critérios e possibilidade de comparação com outros índices (SHOAIB *et al.*, 2009).

Com as mudanças no perfil de cárie nas últimas décadas (redução na prevalência e na progressão das lesões em uma parcela da população), a avaliação da atividade de cárie também se faz necessária juntamente com o diagnóstico, pois as lesões podem ter sido ou vir a ser inativadas por meio de medidas preventivas e mudanças comportamentais, tornando a atividade um fator importante na escolha do tratamento. Porém, pouco se tem pesquisado sobre a atividade das lesões em estudos de cárie em pré-escolares nos últimos anos (PIOSEVAN *et al.*, 2013), embora possa ser determinada pelo critério de Nyvad, um dos principais índices descritos e validados, o qual é baseado no diagnostico táctil e visual da lesão (NYVAD *et al.*, 1999; MACHIULSKIENE *et al.*, 1998).

Assim, uma vez que a microbiota envolvida com a doença cárie é considerada extremamente complexa e variável (MARTIN *et al.*, 2002), o estudo do papel etiológico das espécies relacionadas com a cárie e sua progressão revela-se essencial, assim como entender possíveis mudanças na comunidade bacteriana de acordo com as condições ambientais como

a atividade da lesão (KIANOUSH *et al*., 2014; TAKAHASHI & NYVAD, 2011).

2 PROPOSIÇÃO

Esta tese de doutorado será apresentada em capítulos, tendo como objetivo:

Detectar a presença e quantificar através da técnica de reação em cadeia da polimerase quantitativa (qPCR) as espécies/grupos de bactérias *Actinomyces naeslundii, Bifidobacterium* spp.*, Lactobacillus acidophilus,* grupo *Lactobacillus casei,* grupo *Mitis*, *Streptococcus gordonii* e *Streptococcus mutans* em biofilme de crianças pré-escolares com diferentes estágios de progressão de cárie precoce da infância e em lesões cariosas dentinárias ativas e inativas bem como verificar a associação destes micro-organismos ao processo de saúde ou de doença.

3 CAPÍTULOS

Esta tese está baseada no Artigo 46 do Regimento Interno do Programa de Pós-Graduação em Odontologia da Universidade Federal do Ceará, que regulamenta o formato alternativo para dissertações de Mestrado e teses de Doutorado e permite a inserção de artigos científicos de autoria e co-autoria do candidato. Por se tratarem de pesquisas envolvendo seres humanos, ou parte deles, o projeto de pesquisa referente a este trabalho foi submetido à apreciação do Comitê de Ética em Pesquisa da Faculdade de Medicina da Universidade Federal do Ceará, tendo sido aprovado sob protocolo nº 158/2011 (Anexo). Assim sendo, esta tese de Doutorado é composta por dois capítulos que contém artigos que serão submetidos para publicação em periódicos científicos, conforme descrito abaixo:

CAPÍTULO 1

"*Molecular analysis of biofilm bacteria associated to different stages of early childhood caries*"

B.G. Neves, R.N. Stipp, D.S. Bezerra, S.F.F. Guedes, L.K.A. Rodrigues

Este artigo será submetido à publicação no periódico "Caries Research".

CAPÍTULO 2

"*Molecular detection of bacteria associated to caries activity in dentinal lesions*"

B.G. Neves, R.N. Stipp, D.S. Bezerra, S.F.F. Guedes, L.K.A. Rodrigues

Este artigo será submetido à publicação no periódico "Caries Research".

CAPÍTULO 1

Molecular analysis of biofilm bacteria associated to different stages of early childhood caries

B.G. Neves[a], R.N. Stipp[b], D.S. Bezerra[a] , S.F.F. Guedes[a], L.K.A. Rodrigues[a]

[a]*Post-graduation Program, Faculty of Pharmacy, Dentistry and Nursing, Federal University of Ceará, Fortaleza, Ceará, Brazil;* [b]*Department of Microbiology and Immunology, Piracicaba Dental School, State University of Campinas, Piracicaba, SP, Brazil*

Short title: Bacteria detection in stages of early childhood caries

Key words: Dental Plaque, Bacteria, Early Childhood Caries, Quantitative Polymerase Chain Reaction, Preschoolers

Address all correspondence to:
Lidiany K. A. Rodrigues, DDS, MSc, PhD, Associate Professor
Postgraduate Program in Dentistry, Faculty of Pharmacy, Dentistry and Nursing
Federal University of Ceará, Fortaleza, Ceará, Brazil.
Rua Monsenhor Furtado, 1273 - Zip Code: 60430-355
Phone- #+558533668410/ Fax- #+558533668232
Email: lidianykarla@yahoo.com

Este artigo está escrito de acordo com as normas de publicação do periódico Caries Research.
http://www.karger.com/Journal/Guidelines/224219

Conflict of interests

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

ABSTRACT

Several bacterial species have been associated to early childhood caries (ECC), a prevalent public health problem among preschool children. The aim of this study was to quantify *Actinomyces naeslundii, Bifidobacterium* spp.*, Lactobacillus acidophilus, Lactobacillus casei* group*, Streptococcus gordonii, Mitis* group and *Streptococcus mutans* using quantitative polymerase chain reaction (qPCR) in dental biofilm from children with different stages of ECC. A total of 75 preschool children aged 2 to 5 years from public preeschools were clinically examined according to ICDAS criteria and were divided into three groups: caries-free (CF; n=20), enamel caries lesions (ECL; n=17) and dentine caries lesions (DCL; n=38). Pooled dental plaque sample of each child was collected and analyzed to detect the presence and quantity of the cited bacteria by qPCR. The bacterial presence and abundance data were analyzed by Kruskal-Wallis and chi-squared test followed by multiple logistic regressions. *Lactobacillus acidophilus* and *L. casei* group were absent in almost all plaque samples. No differences in relative proportions were observed for any stage of caries for *Actinomyces naeslundii, Mitis* group and *Streptococcus gordonii.* However, *Streptococcus mutans* and *Bifidobacterium* spp. were present in higher concentrations in the biofilm of children with DCL when compared to CF and ECL groups ($p<0.01$). Furthermore, multivariate analysis showed that *Streptococcus mutans* and *Bifidobacterium* spp. were strongly associated with biofilm in children with cavitated dentin lesions with an odds ratio of 21.5 and 5.9, respectively. The data indicated that *S. mutans* and *Bifidobacterium* spp. might be associated with the progression of early childhood caries.

INTRODUCTION

Dental caries is a progressive disorder associated with a complex etiology, including genetic, microbial, environmental and behavioral factors [Peterson et al., 2013] and remains as a major public health problem, being considered pandemic worldwide [Jiang et al., 2014; Peterson et al., 2014]. Early childhood caries (ECC) may cause pain and infection in preschool children, reduce quality of life and compromise primary dentition soon after eruption [AAPD, 2014; Gross et al., 2012]. Frequent sugared diet causes a growing acidification of dental biofilm (*i.e*., dental plaque), subsequently leading to an increase of the proportion of acidogenic/aciduric bacteria with the rapid progression of caries lesions [Takahashi and Nyvad, 2011; Burne et al., 2012].

The oral biofilm is composed by a structurally organized microbial community, being considered as a dynamic and extremely complex ecosystem [Marsh, 2006] and its role in the onset and progression of dental caries is unquestionable [Dige et al., 2014]. The human oral microbiome contains a diverse polymicrobial population, consisting of over 600 species [Dewhirst et al., 2010]. Efforts have been made to determine the bacterial species associated to oral health and disease [Dige et al., 2014]. However, researches of biofilms are particularly challenging and relevant, due to their dynamic changes in response to the constant exposure to complex diet-host-microbial interactions occurring in the oral cavity [Klein et al., 2011].

Streptococcus mutans (MS) has been identified as a major microbial pathogen in the development of early childhood caries [Koehler et al., 1988; Berkowitz, 2003; Parisotto et al., 2010a], however not all children with ECC harbor this bacteria [Becker et al., 2002; Tanner et al., 2011; Gross et al., 2012]. Thus, it is accepted that this association is not absolute, since dental caries-associated microbiota is highly complex and multiple members of the community can be implicated in the caries development [McLean et al, 2012; Obata et al, 2014]. ECC studies have detected other pathogens involved in dental caries such as *Bifidobacterium* [Becker et al., 2002; Aas et al., 2008; Mantzourani et al., 2009; Palmer et al., 2010], *Lactobacillus* [Becker et al., 2002; Aas et al., 2008] and *Actinomyces* [Marchant et al., 2001]. All these microorganisms are potential acid producers and able to survive in acid environment [Takahashi & Nyvad, 2011].

On the other hand, "pioneer colonizers" mainly comprised of *Mitis* group present in oral biofilm frequently are related to oral health, due to their capacity of producing alkaly, which can help to neutralize acids in pathogenic biofilms and favors pH increase [Nascimento et al.,

2009]. In addition, studies indicate that microorganisms present in dental plaque that have been frequently related with oral health [Li et al., 2004; Corby et al., 2005; Takahashi and Nyvad, 2008], have now been also associated with caries, then being considered as alternative pathogens [Gross et al., 2012] such as *Streptococcus oralis*, *Actinomyces naeslundii* [Marchant et al., 2001; Aas et al., 2008] and *Streptococcus parasanguinis* [Gross et al., 2012].

Considering that relatively few molecular studies have been performed using qPCR for studying specific bacteria associated with early childhood caries or oral health, the aim of the present study was to quantify *Actinomyces naeslundii, Bifidobacterium* spp*., Lactobacillus acidophilus, Lactobacillus casei* group*, Mitis* group, *Streptococcus gordonii* and *Streptococcus mutans* in dental biofilm from children with different stages of early childhood caries.

METHODS

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). Signed informed consents were obtained from the parents or guardians of all subjects. Samples were collected only after getting the approval from the children and their parents.

Study population

The study sample consisted of 75 children aged 2 to 5 years from public nurseries and preeschools from a suburban area in Fortaleza, Ceará, Brazil. Inclusion criteria were: the child was under 71 months of age; child had not be treated with antibiotics in the 3 months prior to the study and subjects had no salivary gland disorders or systemic diseases. Children, who did not cooperate with the dental exams, were excluded from the study.

The criteria used for early childhood caries (ECC) diagnosis was the International Caries Detection and Assessment System II (ICDAS II) [Pitts, 2004; Ismail et al., 2007]. Two calibrated dentists (B.G.N and D.S.B) conducted the examinations after children had their teeth brushed under the direct supervision of dentists. The examiners used a WHO periodontal probe, mirror, gauze and adequate illumination during the clinical evaluation. The intraoral examinations were performed at the children's schools from March 2012 to July 2013. The caries status was recorded on 0-6 scale by surface. Using the ICDAS criteria, caries status was assessed by recording all surfaces of primary teeth as 0 = sound; 1 = visual changes in enamel

(seen only when the tooth is dry); 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 lesions with visible dentine in more than 50% of the surface.

Screening of 420 children was performed in the current study. After meeting inclusion and exclusion criteria, 75 children were included in the final sample size. The distribution of the groups were defined by the different degrees of dental caries status, according to ICDAS coding:

- CF: caries-free children (n=20): code 0. Children who presented no caries lesions.
- ECL: children with the most severe score classified as enamel carious lesions (n=17): dmft ≥ 1 (including surfaces with ICDAS codes 2 and 3)
- DCL: children with the most severe score classified as dentine carious lesions (n=38): dmft ≥ 1 (including surfaces with ICDAS codes 5 and 6)

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.

Biofilm collection

Dental biofilm samples were collected at children's schools. Pooled supragengival plaque sample from each child was taken with autoclaved spoon excavators from vestibular and lingual surfaces of teeth, including anterior and posterior primary teeth. In children with dentin lesions, plaque was not collected from cavities. The participants were asked not to brush their teeth 24 h before sampling.

Plaque 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 of the 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.

Laboratory methods

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 as recommended by Bergey's Manual of Determinative Bacteriology [Holt *et al*. 1994]. 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.

DNA extraction

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 0.16g of 0.1mm diameter zirconia beads on a Mini-Bead Beater homogenizer (Biospec Products, Bartlesville, OK, USA) and subject to beating for 1 min at maximun 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_{260nm}) and purity (A_{260nm}/A_{280nm}) 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.

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

Quantitative real-time assays

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 appropriate forward/reverse primer, 2 μL of DNA sample and 2.4 μL of water free of nucleases. 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 (*C*t) 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 et a., 2002].

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%.

After examining the pattern of sample distribution (normality test Kolmogorov-Smirnov test) data were expressed as mean and standard deviation and compared between groups using Kruskal-Wallis test. Data were dichotomized according to the presence or absence of bacteria in the different groups and as the number of samples with higher or lower values and equal to its median assessment for bivariate (chi-square test) and multivariate (multinomial logistic regression).

RESULTS

In a cross-sectional design, bacterial species were detected and compared to different stages of caries. The mean $d_{1\text{-}6}$ mfs for the ECL group was 3.58 and for DCL group was 15.55. Levels of each species were calculated as a percent of total bacteria for each sample.

Table 2 shows mean and median proportions of oral bacteria in plaque oral samples. *S. mutans* displayed higher concentration in biofilm of children with dentine carious lesions. There was a statistically significant difference in median *S. mutans* and *Bifidobacterium* spp. between dentine caries lesions group and ECL and CF groups ($p < 0.001$). *Lactobacillus*

acidophilus and *Casei* group were almost absent in oral plaque samples from all groups (Median=0.00%). *Bifidobacterium* spp. concentrations were augmented significantly in the DCL group compared to the others ($p < 0.001$). No differences in relative proportions were observed for any stage of caries for any of the following species *S. gordonii, Mitis group* and *A. naeslundii.*

Table 3 shows the association between ECC stages and bacterial composition. After bivariate analysis, the variable that showed statistical significant association with ECL and CF groups was *Bifidobacterium* spp. (odds ratio =4.5). Considering CF versus DCL group, the bivariate analysis also revealed strong association with the presence of *S. mutans* (odds ratio=34) and *Bifidobacterium* spp (odds ratio =11.2). These variables were submitted to a multiple logistic regression analysis in order to obtain the best model possibility. The multivariate modeling indicated that *Streptococcus mutans* was strongly associated to the progressive stages of early childhood caries, with an odds ratio of 21.5. Moreover, *Bifidobacterium* was strongly associated with biofilm of children with dentine caries lesions (odds ratio=5.9) (Table 4 and 5).

DISCUSSION

A detailed investigation of the composition of oral biofilm microbiota is essential for a better understanding of the etiology of early childhood caries. Analysis of the data presented in this study demonstrates considerable differences in bacterial diversity and biofilm composition at different stages of ECC progression, as described from previous studies [Becker et al., 2002; Corby et al., 2005; Kanasi et al., 2010a; Gross et al., 2012; Jiang et al., 2014].

In the current study, the presence of *S. mutans* in dental plaque was strongly associated with biofilm in children with cavitated dentin lesions (odds ratio=21) in multivariate analysis, which is consistent with previous literature reports [Becker et al., 2002; Kanasi et al., 2010a; Parisotto et al., 2010b; Gross et al., 2012; Jiang et al., 2014]. *Streptococcus mutans* is highly acidogenic and aciduric with great capacity to adhere to enamel. It is considered the chief pathogen associated with caries initiation [Loesche, 1986; Tanzer et al., 2001] and also identified as a candidate risk factor for caries progression [Gross et al., 2012]. Moreover, we observed low levels of *S. mutans* in dental plaque samples with higher concentration in the biofilm of the DCL group ($p<0.05$) (Table 2), when compared to CF and ECL groups, making the role of this microorganism in caries progression evident. Despite, being present at low

concentrations, this can be explained because *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 [Xiao et al., 2012; Mattos-Graner et al., 2014]. This study has not found association with the presence of *S. mutans* and biofilm of children with enamel caries lesions. However, it is important to emphasize that patients from this study with advanced stage of caries also presented enamel lesions. Therefore, in this study, *S. mutans* is associated with the progression of the disease and not with individual caries lesions, considering that the sampling method was not site-specific.

Interestingly, members of the *L. casei* group and *L. acidophilus* were rarely identified or absent in most dental plaque samples even in DCL group, showing no association of these bacteria in biofilm with caries, which corroborates previous investigations [Marchant et al., 2001; Gross et al., 2012; Dige et al., 2014; Simón-Soro et al., 2014]. In contrast, some species of Lactobacilli have been found in biofilm in earlier studies [Parisotto et al., 2010b; Kanasi et al., 2010b] and also identified as metabolically active species in pH 4.5 [McLean et al., 2012]. Nevertheless, *Lactobacillus* are normally associated with the more advanced caries, being frequently detected in carious dentine [Byun et al., 2004; Chhour et al., 2005]. These conflicting results may be due to high heterogeneity between individuals [Gross et al., 2010] and also to variations in evaluation methods being molecular methods more accurate than culture-based techniques [Nyvad et al., 2013]. Besides, as long as no biofilm was collected from over dentinal lesions and considering that Lactobacilli are weakly adherent to smooth surfaces, they are more present in retentive sites such as cavities [van Houte, 1996; Gross et al., 2010], therefore it was not found in the biofilm samples of the current study.

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 dentine carious lesions compared to the other groups. Children with detected *Bifidobacterium* on dental biofilm were more likely to develop dentine carious lesions in the multilogistic regression model (odds ratio= 5.9). This result confirms that Bifidobacteria are involved with early childhood caries as previous studies have suggested [Becker et al., 2002; Aas et al., 2008; Palmer et al., 2010]. Bifidobacteria are considered aciduric and acidogenic [Mantzourani et al., 2009] similar to lactobacilli and more than MS [van Houte et al; 1996], indicating that the acidic environment provides a suitable habitat for the proliferation of these microorganisms

[Takahashi and Nyvad, 2011].

Most dental plaque samples from the current study presented among their composition *Actinomyces naeslundii*, *Mitis group* and *S. gordonii*. Nevertheless, no significantly differences in the relative levels of these species 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 also initial colonizers of tooth surfaces [Li et al., 2004; Takahashi and Nyvad, 2008; Dige et al., 2009]. Some metabolic activities of these bacteria may modulate dynamic caries processes, such as the use of lactate by *Actinomyces naeslundii* as a carbon source for growth converting it in weaker acids [Takahashi and Yamada, 1996] and the capacity of non-mutans streptococci to induce the arginine deiminase system producing alkalis [Nascimento et al., 2009], which can culminate in an increased pH in biofilms.

On the other hand, in certain circumstances, such as an acidic environment, non-MS may enhance their acidogenicity and acidurance adaptively [Takahashi and Yamada, 1999] playing a critical role for destabilizing the plaque homeostasis by facilitating a shift of the demineralization/remineralization balance [Takahashi and Nyvad, 2008]. Thus, these microorganisms were associated with dental caries in numerous studies and considered as alternative pathogens contributing to the disease process [Marchant et al., 2001; Becker et al., 2002; Aas et al., 2008; Belda-Ferre et al., 2012; Gross et al., 2012; Jiang et al., 2013; Peterson et al., 2013].

The discrepancies findings regarding the association of these species with health or disease might have been modulated by local conditions, since *Actinomyces* are as versatile to adjust oneself to different conditions in dental biofilm environment as are the non-MS [Takahashi and Nyvad, 2008]. This way, the molecular mechanisms through which non-MS bacteria participates in caries initiation remain unclear and further studies are needed to identify the ecological shifts leading to cariogenic biofilms [Mattos-Graner et al., 2014]. Despite all these factors, MS, lactobacilli and *Bifidobacterium* are more acidogenic, aciduric and competitive than non-mutans streptococci under severely acidic conditions [Takahashi, and Nyvad, 2011].

Several researchers have evaluated microbial composition of dental biofilms on pooled plaque samples in molecular studies [Becker et al., 2002; Aas et al., 2008; Parisotto et al., 2010b; Belda-Ferre et al., 2012; Peterson et al., 2013; Peterson et al., 2014]. 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. Therefore, the bacterial composition observed on this study could have varied if specific sites were included.

Molecular techniques are currently available to explore the bacterial microbiota, since some pathogens are not routinely cultivable [Paster et al., 2001]. Quantitative PCR represents an approach highly specific and considered as the gold standard for quantitative accuracy, however it has been underutilized [Peterson et al., 2011]. Its main limitation consists in not differentiate between live and dead cells [Alvarez et al., 2013], however a recent technique using propidium monoazide have made possible to assess cell viability with qPCR [Yasunaga et al., 2013]. While informative, this study does not provide data about the metabolic activity of the biofilm community. It seems essential to know what bacteria are doing [Takahashi and Nyvad, 2008]; since describing the identity of an organism does not necessary mean that the functional feature is apparent [Nyvad et al., 2013].

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, being considered as potentially key pathogens for ECC. Besides, it seems important to identify the metabolically active species present within these complex multi-species communities at the onset and progression of early childhood caries and also better understand how the bacterial population changes from healthy to diseased states in this dynamic/bacterial process.

Acknowledgements

This study was supported by CNPq - National Counsel of Technological and Scientific Development (Process # 475346/2011-4 MCT/CNPq 14/2011). We kindly thank the researcher Karina Maria Olbrich dos Santos (EMBRAPA) for providing *Bifidobacterium* strain and also Juliana Nunes Botelho for the technical assistance.

REFERENCES

American Academy of Pediatric Dentistry: Policy on Early Childhood Caries (ECC): classifications, consequences and preventive strategies. Reference Manual. Pediatric Dentistry 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.

Becker MR, Paster BJ, Leys EJ, Moeschberger ML, Kenyon SG, Galvin JL, Boches SK, Dewhirst FE, Griffen AL: Molecular analysis of bacterial species associated with childhood caries. J Clin Microbiol 2002;40:1001–1009.

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.

Berkowitz RJ: Acquisition and transmission of mutans streptococci. J Calif Dent Assoc 2003;31:135–138.

Byun R, Nadkarni MA, Chhour KL, Martin FE, Jacques NA, Hunter N: Quantitative analysis of diverse *Lactobacillus* species present in advanced dental caries. J Clin Microbiol. 2004;42:3128-3136.

Burne RA, Zeng L, Ahn SJ, Palmer SR, Liu Y, Lefebure T, Stanhope MJ, Nascimento MM: Progress dissecting the oral microbiome in caries and health. Adv Dent Res 2012;24:77–80.

Chhour K, Nadkarni MA, Byun R, Martin FE, Jacques NA, Hunter N: Molecular analysis of microbial diversity in advanced caries. J Clin Microbiol 2005;43:843–849.

Corby PM, Lyons-Weiler J, Bretz WA, Hart TC, Aas JA, Boumenna T, Goss J, Corby AL, Junior HM, Weyant RJ, Paster BJ: Microbial risk indicators of early childhood caries. J Clin Microbiol 2005;43:5753–5759.

Dewhirst FE, Chen T, Izard J, Paster BJ, Tanner AC, Yu WH, Lakshmanan A, Wade WG. The human oral microbiome. J Bacteriol 2010;192:5002-5017.

Dige I, Raarup MK, Nyengaard JR, Kilian M, Nyvad B. *Actinomyces naeslundii* in initial dental biofilm formation. Microbiology. 2009;155:2116-26.

Dige I, Grønkjær L, Nyvad B: Molecular studies of the structural ecology of natural occlusal caries. Caries Res 2014;48(5):451-60.

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

Gross EL, Leys EJ, Gasparovich SR, Firestone ND, Schwartzbaum JA, Janies DA, Asnani K, Griffen AL: Bacterial 16S sequence analysis of severe caries in young permanent teeth. J Clin Microbiol 2010;48:4121–4128.

Gross EL, Beall CJ, Kutsch SR, Firestone ND, Leys EJ, Griffen AL: Beyond Streptococcus

mutans: dental caries onset linked to multiple species by 16S rRNA community analysis. PLoS One 2012; 7:e47722.

Holt JG,Krieg NR, Sneath PHA, Staley JT,Williams ST: Bergey's Manual of Determinative Bacteriology 1994: 9 Edition. Williams & Wilkins. Baltimore.

Ismail AI, Sohn W, Tellez M, Amaya A, Sen A, Hasson H, Pitts NB: The International Caries Detection and Assessment System (ICDAS): an integrated system for measuring dental caries. Community Dent Oral Epidemiol 2007; 35:170–178.

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.

Li J, Helmerhorst, Leone CW, Troxler RF, Yaskell T, Haffajee AD, Socrasnsky SS, Oppenheim FG: Identification of early microbial coloniz- ers in human dental biofilm. J Appl Microbiol 2004;97:1311–1318.

Loesche WJ. Role of Streptococcus mutans in human dental decay. Microbiol Rev 1986; 50:353-380

Kanasi E, Dewhirst FE, Chalmers NI, Kent R, Moore A: Clonal analysis of the microbiota of severe early childhood caries. Caries Res 2010a; 44:485–497.

Kanasi E, Johansson I, Lu SC, Kressin NR, Nunn ME, Kent R Jr, Tanner AC.Microbial risk markers for childhood caries in pediatricians' offices. J Dent Res 2010b; 89:378-383.

Klein MI, Xiao J, Heydorn A, Koo H:An analytical tool-box for comprehensive biochemical, structural and transcriptome evaluation of oral biofilms mediated by mutans streptococci. J Vis Exp 2011; 25: pii: 2512. doi: 10.3791/2512.

Koehler B, Andreen I, Jonsson B: The earlier the colonization by mutans streptococci, the higher the caries prevalence at 4 years of age**.** Oral Microbiol Immunol 1988;3:14-17.

Mantzourani M, Gilbert SC, Sulong HNH, Sheehy EC, Tank S, Fenlon M, et al.: The isolation of bifidobacteria from occlusal carious lesions in children and adults. Caries Res 2009;43:308–313.

Marsh PD:Dental plaque as a biofilm and a microbial community - implications for health and disease. BMC Oral Health. 2006;15: Suppl 1:S14.

Mattos-Graner, RO, Klein MI, Smith DJ. Lessons learned from clinical studies: roles of mutans streptococci in the pathogenesis of dental caries. Curr Oral Health Rep 2014; 1:70–78.

McLean JS, Fansler SJ, Majors PD, McAteer K, Allen LZ, Shirtliff ME, Lux R, Shi W: Identifying low pH active and lactate-utilizing taxa within oral microbiome communities from healthy children using stable isotope probing techniques.PLoS One 2012;7:e32219.

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.

Nascimento MM, Burne RA: Caries prevention by arginine metabolism in oral biofilms: translating science into clinical success. Curr Oral Heal Reports 2014;1:79–85.

Nascimento MM, Gordan VV, Garvan CW, Browngardt CM, Burne RA. Correlations of oral bacterial arginine and urea catabolism with caries experience.Oral Microbiol Immunol 2009; 24:89-95.

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.

Nyvad B, Machiulskiene V, Baelum V: Construct and predictive validity of clinical caries diagnostic criteria assessing lesion activity. J Dent Res 2003;82:117–122.

Nyvad B, Crielaard, Mira A, Takahashi N, Beighton D: Dental caries in a molecular microbiological perspective. Caries Res 2013; 47: 89– 102.

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.

Park S, Kim YK, Kook J: Development of quantitative real-time PCR primers for detecting 42 bacterial species. Arch Microbiol 2013;195:473–482.

Parisotto TM, Steiner-Oliveira C, Silva CM, Rodrigues LK, Nobre-dos-Santos M: Early childhood caries and mutans streptococci: a systematic review. Oral Health Prev Dent. 2010a;8:59-70.

Parisotto TM, Steiner-Oliveira C, Duque C, Peres RCR, Rodrigues LKA, Nobre-dos-Santos M: Relationship among microbiological composition and presence of dental plaque, sugar exposure, social factors and different stages of early childhood caries. Arch Oral Biol 2010b;55:365–73.

Paster BJ, Boches SK, Galvin JL, Ericson RE, Lau CN, Levanos VA, Sahasrabudhe A, Dewhirst FE: Bacterial diversity in human subgingival plaque. J. Bacteriol 2001;183:3770–3783.

Peterson SN, Snesrud E, Schork NJ, Bretz WA: Dental caries pathogenicity: a genomic and metagenomic perspective. Int Dent J 2011;61:11–22.

Peterson SN, Snesrud E, Liu J, Ong AC, Kilian M, Schork NJ, Bretz W.: The dental plaque microbiome in health and disease. PLoS One 2013;8:e58487.

Peterson SN, Meissner T, Su AI, Snesrud E, Ong AC, Schork NJ, Bretz WA: Functional expression of dental plaque microbiota. Front Cell Infect Microbiol 2014;4:108.

Pitts NB: Modern concepts of caries measurement. J Dent Res 2004;83:43–47.

Rintillä T, Kassinen A, Malinen E, Krogius L, Palva A: 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 2004;97:1166–1177.

Simón-Soro A, Guillen-Navarro M, Mira A: Metatranscriptomics reveals overall active bacterial composition in caries lesions. Journal of Oral Microbiology 2014;6,25443

Tanner AC, Mathney JM, Kent RL, Chalmers NI, Hughes CV, Loo CY, Pradhan N, Kanasi E, Hwang J, Dahlan MA, Papadopolou E, Dewhirst FE: Cultivable anaerobic microbiota of severe early childhood caries. J Clin Microbiol 2011;49:1464-1474.

Takahashi N, Nyvad B: Caries ecology revisited: microbial dynamics and the caries process. Caries Res. 2008;42:409-18.

Takahashi N, Nyvad B: The role of bacteria in the caries process: ecological perspectives. J Dent Res 2011;90:294–303.

Takahashi, N. & Yamada, T: Catabolic pathway for aerobic degradation of lactate by *Actinomyces naeslundii*. Oral Microbiol Immunol 1996;11:193–198.

Takahashi N, Yamada T: Acid-induced acidogenicity and acid tolerance of non-mutans streptococci. Oral Microbiol Immunol 1999;14:43–48.

Tanzer JM, Livingston J, Thompson a M: The microbiology of primary dental caries in humans. J Dent Educ 2001;65:1028–1037.

van Houte J, Lopman J, Kent R: The final pH of bacteria comprising the predominant f lora on sound and carious human root and enamel surfaces. J Dent Res 1996;75:1008–1014.

Wilson K: Preparation of genomic DNA from bacteria. Curr Protoc Mol Biol 2001;Chapter 2:Unit 2.4.

Wolff, D, Freese C, Maier-Kraus T, Krueger T, Wolff B: Bacterial biofilm composition in caries and caries-free subjects. Caries Res 2013;47:69–77.

Xiao J, Klein MI, Falsetta ML, Lu B, Delahunty CM, Yates JR 3rd, Heydorn A, Koo H: The exopolysaccharide matrix modulates the interaction between 3D architecture and virulence of a mixed-species oral biofilm. PLoS Pathog 2012;8:e1002623.

Yano A, Kaneko N, Ida H, Yamaguchi T, Hanada N: Real-time PCR for quantification of *Streptococcus mutans*. FEMS Microbiology Letters 2002;217:23-30.

Yasunaga A, Yoshida A, Morikawa K, Maki K, Nakamura S, Soh I, Awano S, Ansai T: Monitoring the prevalence of viable and dead cariogenic bacteria in oral specimens and in vitro biofilms by qPCR combined with propidium monoazide. BMC Microbiol 2013; 13: doi: 10.1186/1471-2180-13-157.

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 et al., 2013
Bifidobacterium spp.[1]	F: TCGCGTC(C/T)GGTGTGAAAG R: CCACATCCAGC(A/G)TCCAC	58	243	Rintilla et al., 2004
Lactobacillus acidophilus	F: GATCGCATGATCAGCTTATA R: AGTCTCTCAACTCGGCTATG	60	124	Furet et al., 2004
L. casei group[2]	F: GCGGACGGGTGAGTAACACG R: GCTTACGCCATCTTTCAGCCAA	60	121	Furet et al., 2004
Mitis group[3]	F:TAGAACGCTGAAGGAAGGAGC R: GCAACATCTACTGTTATGCGG	60	133	Wolff et al., 2013
Streptococcus gordonii	F: CAGGAAGGGATGTTGGTGTT R: GACTCTCTTGGCGACGAATC	60	136	Wolff et al., 2013
Streptococcus mutans	F: AGCCATGCGCAATCAACAGGTT R: CGCAACGCGAACATCTTGATCAG	64	415	Yano et al., 2002

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. *Mitis* group *: Streptococcus mitis, S. oralis, S. pneumoniae, S. parasanguinis, S. australis*

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

Oral bacteria	Groups	Mean (%)	±SD	Median (%)	p-value
S. mutans	CF	0.190	0.680	0.000	<0.001
	ECL	0.052	0.118	0.001	
	DCL	1.191	2.068	0.250*	
L. acidophilus	CF	0.000	0.000	0.000	0.373
	ECL	0.000	0.000	0.000	
	DCL	0.000	0.001	0.000	
***Mitis* group**	CF	1.052	0.884	0.876	0.580
	ECL	1.573	1.453	1.178	
	DCL	3.361	6.123	0.892	
***L. casei* group**	CF	0.000	0.000	0.000	0.132
	ECL	0.000	0.000	0.000	
	DCL	0.001	0.002	0.000	
***Bifidobacterium* spp.**	CF	0.000	0.000	0.000	<0.001
	ECL	0.000	0.001	0.000	
	DCL	0.088	0.213	0.002*	
A. naeslundii	CF	18.062	17.021	15.053	0.092
	ECL	14.239	14.904	9.620	
	DCL	20.942	15.034	16.419	
S. gordonii	CF	0.513	0.777	0.294	0.075
	ECL	0.811	0.948	0.541	
	DCL	2.821	5.518	0.570	

SD= standard deviation; 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.

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 X ECL	CF X DCL	ECL X DCL
Bifidobacterium				<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

CAPÍTULO 2

Molecular detection of bacteria associated to caries activity in dentinal lesions

B.G. Neves[a], R.N. Stipp[b], D.S. Bezerra[a] , S.F.F. Guedes[a], L.K.A. Rodrigues[a]

[a]*Post-graduation Program, Faculty of Pharmacy, Dentistry and Nursing, Federal University of Ceará, Fortaleza, Ceará, Brazil;* [b]*Department of Microbiology and Immunology, Piracicaba Dental School, State University of Campinas, Piracicaba, SP, Brazil*

Short title: Dentine caries-associated bacteria in ECC-lesions

Key words: Early Childhood Caries, Quantitative Polymerase Chain Reaction, Bacteria, Dentin Caries, Preschoolers

Address all correspondence to:
Lidiany K. A. Rodrigues, DDS, MSc, PhD, Associate Professor
Postgraduate Program in Dentistry, Faculty of Pharmacy, Dentistry and Nursing
Federal University of Ceará, Fortaleza, Ceará, Brazil.
Rua Monsenhor Furtado, 1273 - Zip Code: 60430-355
Phone- #+558533668410/ Fax- #+558533668232
Email: lidianykarla@yahoo.com

Este artigo está escrito de acordo com as normas de publicação do periódico Caries Research.
http://www.karger.com/Journal/Guidelines/224219

Conflict of interests

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

ABSTRACT

Early childhood caries (ECC) is a microbial infection that severely compromises the dentition of young children. Few studies have focused on oral bacterial community changes within carious lesion activity. The aim of this study was to quantify *Actinomyces naeslundii, Bifidobacterium spp., Mitis* group, *Lactobacillus acidophilus, Lactobacillus casei* group*, Streptococcus gordonii* and *Streptococcus mutans* in active and inactive carious dentine lesions in ECC- children by using quantitative polymerase chain reaction (qPCR). Fifty-six dentine lesions samples, classified as active (n=39) or inactive (n=17), were collected from children aged 2 to 5 years. Relative quantification revealed that *Bifidobacterium* spp. and *L. casei* group were significantly more abundant in active dentine lesions ($p<0.05$). Concentrations of *Actinomyces naeslundii, Mitis* group and *Streptococcus gordonni* 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* (OR=15.1) and *S. gordonii* (OR=7.7) was significantly associated to the active lesions. The data indicate that higher detection levels of *Bifidobacterium* spp. and *L. casei* group may be linked to dentin lesion activity. Also, the presence of *L. acidophilus* and *S. gordonii* was associated to the lesion activity.

INTRODUCTION

Dental caries is one of the most prevalent diseases of childhood worldwide, especially in socially disadvantaged populations [Berkowitz, 2003]. Several factors, including microbial, genetic, immunological, behavioral and environmental, are involved and contribute to its development [Peterson et al., 2013]. In particular, in preschool children, this condition defined as early childhood caries (ECC), can devastate the primary teeth, affect child's self-esteem, impact general health and lead to nutritional deficiency [Ramos-Goméz et al., 2002]. Because of 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 [AAPD, 2014; Obata et al., 2014].

Cavitated dentine carious lesions are considered as the last stage of dental caries and also diverse ecosystems with high variability [Belda-Ferre et al., 2012]. Dentine provides a different environment for bacteria involved in caries progression, where only specialized bacteria are able to colonize and exploit [Simon-Sóro et al., 2013]. The bacterial profile in enamel and dentinal caries are significantly different [Simón-Soro et al., 2013; Obata et al., 2014] , since the microbiota in dentine is constantly submitted to changes, such as nutrient availability, oxygen concentration and pH [Lima et al., 2011; Takahashi and Nyvad, 2011]. Moreover, this tissue contains a higher proportion of organic matrix and lower inorganic component than enamel. Thus, the critical pH for dentine dissolution is higher compared to enamel, which allow colonization of bacteria that are not as acidogenic and aciduric as those required for initial enamel demineralization [Kinaoush et al., 2013], but also proteolytic bacteria are involved [Simón-Soro et al., 2013].

The advent of molecular researches to characterize the oral microbiota in health and disease is revealing the diversity of oral biofilms and dentinal caries and finding new candidates for disease-associated bacterial species [Becker et al., 2002; Aas et al., 2008; Corby et al., 2005; Gross et al., 2012] . Considering that the microbiota involved in dental caries are known to be highly diverse and variable [Martin et al., 2002; Takahashi and Nyvad, 2011], understanding the microbial etiology of caries and how environmental conditions in the oral cavity impact the disease process continues to change as technology advances [Kianoush et al. 2014]. Although the strong association of mutans streptococci and ECC is established in the literature [Kohler et al, 1988; Berkowitz, 2003; Kanasi et al., 2010; Parisotto et al., 2010], it seems that these bacteria are not present in all children with caries [Mattos-Graner et al., 2001; Aas et al., 2008]. Beyond *S. mutans*, molecular approaches have revealed a greater variability of the community of dentine caries-associated

microbiota including non-mutans streptococci and bacteria of the genera Actinomyces, *Bifidobacterium, Lactobacillus, Propionibacterium, Veillonella, Selenomonas and Atopobium* [Becker et al*.,* 2002; Martin et al*.,* 2002; Munson et al*.,* 2004; Chhour et al., 2005; Aas et al*.,* 2008; Mantzourani et al*.,* 2009; Belda-Ferre et al., 2012].

On the other hand, it can be suggested a possible contribution of other microorganisms to the inactivation of caries lesions, since several streptococcus, such as members of the *Mitis* group and *S. gordonii* are able to produce alkalis by the arginine deiminase system (ADS) [Nascimento and Burne, 2014]. These bacteria generate ammonia that neutralizes acids in cariogenic biofilms and favors pH increase, which are compatible with the pH of arrested lesions as previously demonstrated [Hojo et al., 1994; Kuribayashi et al., 2012]. However, the role of these bacteria in caries progression remains inconclusive.

Information about microbial communities present in dentinal caries lesions is not only important to understand the pathogenesis of dentinal caries, but also to contribute for developing novel approaches to dental caries treatment [Kianoush et al., 2014]. Early in the caries process, the pulp reflects changes within lesion activity [Bjordnal, 2014]. However, little is known about the dynamic characteristics of oral microbiota in caries progression [Jiang et al., 2014] and how the oral bacterial community changes within carious lesion activity [Takahashi, and Nyvad, 2011; Kianoush et al., 2014].

Molecular studies dealing with the microbiota in dentinal caries have mainly focused in comparing plaque samples within the same patient [Becker et al., 2002; Aas et al., 2008; Corby et al., 2005; Gross et al., 2010], rarely defining dentinal lesion activity, which may reveal different microbial patterns [Takahashi, and Nyvad, 2011]. Therefore, the aim of the present study was quantify *Actinomyces naeslundii, Bifidobacterium spp., Lactobacillus acidophilus, Lactobacillus casei* group*, 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 can 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 (COMEPE/UFC) (Protocol Number 158/2011). Verbal and written consents were

obtained from the parents of all subjects. Samples were taken only after getting the approval from the children and their parents.

Study population

Thirty-nine subjects aged from 2 to 5 years with severe early childhood caries were recruited for this study from public preeschools in Fortaleza, Ceará, Brazil. The schools were selected based on convenience and located in a suburban area of this city. A total of 420 children were examined at the first phase of this 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 previous to the study. Subjects had no salivary gland disorders or systemic diseases and also had no spontaneous symptoms associated with the caries lesions. Informed consent of the respective children and their families, their willingness to participate, and the presence of at least one cavitated dentine 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 [Pitts, 2004; Ismail et al., 2007]. 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 clinical evaluation.

Dentinal cavitated lesions were evaluated by Nyvad criteria [Nyvad et al., 1999; 2003] for the assessment of caries lesion activity, based on visual and tactile diagnoses. The teeth were examined after airdrying 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. A WHO periodontal probe was used to check the surface texture (rough or smooth). Dentine cavity classified as active were those easily visible with the naked eye, soft on gentle probing and with a whitish/yellowish appearance with loss of lustre. Inactive lesions were characterized by a whitish/brown/black shiny appearance and smooth/hard on gentle probing. Each site was ranked according to the scores proposed [Nyvad et al., 1999, 2003] for caries lesion activity.

All children involved in this study were enrolled in a dental care program that

included routine professional monitoring of their oral health status and their oral health is maintained until the present time.

Sampling and clinical data collection

All sample collections were performed by two calibrated dentists at the dental clinic of the Dental School, Federal University of Ceará, Fortaleza, Ceará, Brazil. Samples were collected from 39 children over a period of 15 months, between July 2012 and October 2013.

Dentine caries lesions selected were opened cavities scored with codes 5 (distinct cavity with visible dentine) and 6 (extensive distinct cavity with visible dentine), according to ICDAS II criteria [Ismail et al., 2007]. 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 dentine carious samples were collected from each child. Samples were not pooled across dentine 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 dentine lesions was swiped. Subsequently, all cavities were restored with resin-modified glass ionomer cement (Vitro Fil LC, DFL, Rio de Janeiro, Brasil). 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), immediately transported on ice to the laboratory, where they were frozen at - 20°C until analyses.

Laboratory methods

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 specificities of the primers 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* (UA 159).

Isolated bacteria were cultured in broth for 24 h as recommended by Bergey's Manual of Determinative Bacteriology [Holt *et al*. 1994]. 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.

DNA extraction

Extraction and purification of DNA from dentinal samples and bacterial cultures

All samples were transferred into a fresh 2 mL screw captube. 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 60s. 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}) and purity (A_{260}/A_{280}) 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 0.1µg/ ml ethidium bromide.

Quantification of target bacterial DNA in dentinal samples by 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 amplification curve and a melting-point product curve were obtained for each primer set. Amplifications of qPCR were performed using 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 appropriate forward/reverse primer, 2 µL of DNA sample and 2.4 µL water free of nucleases. 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 dentine samples were normalized relative to the total bacterial load estimated by the primer *Bacteria* 16S rDNA [Nadkarni et a., 2002].

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 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 mean and standard deviation and compared between groups using the Mann-Whitney test followed by post-test, Mann-Whitney test Bonferroni correction (nonparametric data).

A Spearman correlation (nonparametric data) was performed for evaluation of the interaction between different bacteria at different lesions activity (active or inactive). Data were dichotomized according to the presence or absence of bacteria in dentine carious lesions and as verified the number of samples with higher or lower values to its median proportion for bivariate analysis (chi-square test).

RESULTS

A total of 56 carious dentine samples were collected from cavitated dentine lesions (ICDAS 5, 6) Dentin samples were divided into two groups: active (n=39) and inactive (n=17) lesions, according to Nyvad criteria [Nyvad et al., 2013]. From one to three samples were collected from each patient and 5 children had lesions of both types.

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

Presence and absence of the bacteria in dentine lesions and also relative median of all groups are summarized in Table 3. *Lactobacillus acidophilus* was completely absent in all

carious inactive dentine samples. *S. mutans* was identified in all samples of the current study. *L. casei* group was absent in about 40% of inactive lesions. Considering presence in the carious dentine lesion in a bivariate analysis, *L. acidophilus* showed a statistical significant association to active dentine 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 dentine samples presented levels of *L. casei* group higher that the median value of both groups ($p<0.05$; OR=3.5) (Table 3).

Tables 4 and 5 show the results of Spearman's rank correlations, analysing bacterial relationships in the different groups. As shown in table 4, there was a significant inverse correlation between *Casei* group and *S. mutans* in active dentine lesions (p=0.039). *Bifidobacterium* spp and *Casei* group demonstrated a positive correlation (p=0.001). It was observed a significant direct correlation between *Mitis* group and *S. gordonni* both in active and inactive dentine lesions (Tables 4 and 5).

DISCUSSION

The microbiota in dental caries is highly complex and vary between individual lesions [Martin et al., 2002; Hahn and Liewehr, 2007]. Consequently, etiological studies must focus on site-specific analyses [Simón-Soro et al., 2013]. It has been suggested that the proportions and numbers of acid-base-producing bacteria are the core of dental caries activity [Kleinberg, 2002]. Different proportion of some bacterial populations regarding dentine lesion activity was observed in the current study.

The current results showed a higher concentration of *L. casei* group in active dentine lesions when compared to the arrested ones, which was previously demonstrated by a study that verified these bacteria as dominant in active dentine lesions in adults [Kuribayash et al., 2012]. Besides, although present in low quantity, presence of *L. acidophilus* was related with dentine lesion activity, since showed a statistical significant association to active dentine lesions (OR=15.1) since was completely absent in all carious inactive dentine samples. These findings demonstrated that dentine lesions, where these bacteria are present, showed 15.1 times more likely to be active. *Lactobacillus* spp. present great ability to produce organic acids, promoting low levels of pH and being responsible for decalcification of the dentinal matrix [Byun et al., 2004; Obata et al., 2014], which is a common situation in active lesions. The link *Lactobacillus* count and carious decay is unquestionable, however it

seems important to establish the relationship between the *Lactobacillus* count and the carious activity [Baded and Thebaud, 2008]. *Lactobacillus casei* and *Lactobacillus paracasei* (LCP) were frequently isolated from dentine sites in ECC [Nancy and Dorignac 1992; Marchant et al., 2001; Wen et al., 2010]. Moreover, Lactobacilli have shown robust association with more advanced stages of caries in many studies [Byun et al. 2004; Munson et al., 2004; Chhour et al., 2005; Aas et al. 2008; Lima et al., 2011; Simón-Soro et al., 2013; Jiang et al., 2014; Obata et al., 2014; Simón-Soro et al., 2014] and also have been implicated in the initial stages of pulp infection [Nadkarni et al., 2010], 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 with adult dentine lesions [Simón-Soro et al., 2013]. Interestingly, significant higher proportion was currently 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 [Mantzourani et al., 2009b]. Bifidobacteria have been detected from dentine carious lesions [Becker et al., 2002; Aas et al., 2008; Chour et al. 2005; Simón-Soro et al., 2013], suggesting that these bacteria may be implicated in dental caries progression [Mantzourani et al., 2009a; Nakajo et al., 2010] since these species are acidogenic and aciduric and also known to produce lactate [van Houte et al*.,* 1996; Mantzourani et al., 2009]. Besides, other 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 [Dige et al., 2014].

Several studies have associated *S. mutans* with progressive stages of caries and have detected these bacteria in cavitated lesions in dentine [Marchant et al., 2001; Aas et al., 2008; Mantzourani et al., 2009] as confirmed by the present results, since all dentine samples examined harbored *S. mutans*. Mutans streptococci comprise about 30% of the total microbiota according to microbial culture approaches [Loesche et al*.,* 1984; Milnes and Bowden, 1985]. These species may form biofilm on dentine, and incorporate and collaborate with various bacteria for induction of dentine acidulation [Maeda et al., 2006]. Surprisingly, *S. mutans* was identified with higher concentration in arrested lesions, which is not in agreement with Kuribayashi et al. [2012] that 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 the latter 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 those of permanent teeth [Aas et al., 2008]. Other factor to be considered is that arrested lesions have a restricted diffusion of carbohydrate [Hojo et al., 1994]; present a harder reactive dentine with irregularly and blocked dentinal tubules [Love and Jenkinson, 2002] and lower water and protein contents [Nanci, 2012]. In this way, proteolytic bacteria, generally higher in active dentinal caries [Simón-Soro et al., 2013], seem not to have adequate nutritional conditions for their survival, causing the proliferation of more acidogenic/aciduric organisms such as *S. mutans*.

However, further clinical studies are needed to evaluate viability and role of *S. mutans* in the activity of dentinal lesions. On the other hand, other studies have demonstrated low prevalence or even absence of *S. mutans* in dentinal caries lesions [Belda-Ferre et al., 2012; Simón-Soro et al., 2013] contradicting the current data. In fact, it must be emphasized that the earlier findings were based in 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 DNA extraction and bacterial lysis method and also PCR and primer bias [Munson et al., 2004; Kanasi et al., 2010]. In addition, it was observed that the oral microbiota is diverse in different ethnicities and races, which may explain heterogeneous composition of the microbiota in dentinal caries in different studies [Gross et al., 2012; Obata et al., 2014].

One particularly interesting finding of this study was the positive correlation between bacteria from *L. casei* group and *Bifidobacterium* spp. in active dentine lesions, since both bacterial genera are commonly detected in ECC lesions [Becker et al*.,* 2002; Aas et al., 2008] playing important roles in lowering the pH in active lesion environments and proliferating in acidic caries lesions [Mantzourani et al., 2009]. 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. [Mantzourani et al., 2009]. On the other hand, *S. mutans* and *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 [Takahashi and Nyvad, 2011]. This finding corroborates the results of a previous *in vitro* study where lactobacilli were capable of inhibiting the growth of mutans streptococci being *Lactobacillus paracasei, Lactobacillus plantarum*, and *Lactobacillus rhamnosus* the species with maximum interference capacity against mutans streptococci [Simark-Mattsson et al., 2007].

In this study, the presence of *S. gordonni* was significantly associated with active dentine lesions, which are data consistent with those reported by Peterson et al. [2013] in a dental plaque microbiome study that *S. gordonii* was associated with caries activity and also with a metagenomic study that detected abundance of this species in caries-active individuals [Belda-Ferre et al., 2012]. The role of *Streptococcus gordonii* in dental caries is yet undefined [Tanzer et al., 2001]. Despite being considered as a pioneer for dental plaque formation and associated with health [Gross et al., 2010], an *in vitro* study have shown that these bacteria were able to increase their acid tolerance and acidogenicity when exposed to an acidic environment [Takahashi and Yamada, 1999]. However, the contribution of *S. gordonii* on acidification of dentine remains unclear and deserves further investigation.

According to the present data, with regard to *Actinomyces naeslundii*, no statistical significant difference was found regardless dentine lesion activity, which is not a surprising result since *Actinomyces* spp. are prevalent in oral cavity and frequently found in association with both carious and sound surfaces [Marchant et al., 2001]. In addition, these species have been associated to dentine and root caries in adults [Brasilsford et al., 1999; Kianoush et al., 2014], and does not seem to play a relevant role in childhood caries [Becker et al., 2002]. In the same way, although members of the *Mitis* group were previously detected in active carious dentine lesions in adults and children [Marchant et al., 2001; Simón-Soro et al., 2013], they have been frequently associated to health [Corby et al., 2005; Gross et al., 2010], making the contribution of these bacteria on caries inactivation inconclusive.

A major limitation is that this study considered only the activity status of the lesions to compare different bacterial populations and also it must be emphasized that qPCR cannot distinguish between viable and non-viable bacterial cells, however a recent technique have been proposed to assess viability of live and dead cells with qPCR [Yasunaga et al., 2013]. Furthermore, the current study did not evaluate bacterial composition among different depths in the same lesion. However, no significant difference in the microbial composition in the different layers of dentinal caries lesions has been reported [Munson et al., 2004; Lima et al., 2011]. 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.

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. Besides, our study analysed an *in vivo* situation with severe ECC-children, differently from numerous studies with dentine caries that commonly evaluated extracted carious teeth [Marchant et al, 2001; Chour et al., 2005; Kianoush et al., 2013; Kianoush et al., 2014]. It is possible to infer if these teeth were necrotic already, which may alter the microbial composition. 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 [Kianoush et al., 2014]. Furthermore, dentine lesions present a different profile compared to other caries samples affecting different tissues [Simón-Soro et al., 2013].

The higher concentration of *Bifidobacterium* spp. and *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 state of dentin lesions.

Acknowledgements

This study was supported by CNPq - National Counsel of Technological and Scientific Development (Process # 475346/2011-4 MCT/CNPq 14/2011). We kindly thank the researcher Karina Maria Olbrich dos Santos (EMBRAPA) for providing *Bifidobacterium* strain and also Juliana Nunes Botelho for the technical assistance.

REFERENCES

American Academy of Pediatric Dentistry: Policy on Early Childhood Caries (ECC): classifications, consequences and preventive strategies. Reference Manual. Pediatric Dentistry 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.

Badet C, Thebaud NB. Ecology of lactobacilli in the oral cavity: a review of literature. Open Microbiol 2008; 2:38-48.

Becker MR, Paster BJ, Leys EJ, Moeschberger ML, Kenyon SG, Galvin JL, Boches SK, Dewhirst FE, Griffen AL: Molecular analysis of bacterial species associated with childhood caries. J Clin Microbiol 2002;40:1001–1009.

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.

Berkowitz RJ: Acquisition and transmission of mutans streptococci. J Calif Dent Assoc 2003;31:135–138.

Bjørndal L, Demant S, Dabelsteen S:Depth and activity of carious lesions as indicators for the regenrative potential of dental pulp after intervention. J Endod 2014: 40: :S76-81

Brasilsford SR, Tregaskis RB, Leftwich HS, Beighton D: The predominant *Actinomyces* spp. isolated from infected dentin of active root caries lesions. J Dent Res 1999;78:1525-1534.

Byun R, Nadkarni MA, Chhour KL, Martin FE, Jacques NA, Hunter N: Quantitative analysis of diverse *Lactobacillus* species present in advanced dental caries. J Clin Microbiol. 2004;42:3128-3136.

Chhour K, Nadkarni MA, Byun R, Martin FE, Jacques NA, Hunter N: Molecular analysis of microbial diversity in advanced caries. J Clin Microbiol 2005;43:843–849.

Corby PM, Lyons-Weiler J, Bretz WA, Hart TC, Aas JA, Boumenna T, Goss J, Corby AL, Junior HM, Weyant RJ, Paster BJ: Microbial risk indicators of early childhood caries. J Clin Microbiol 2005;43:5753–5759.

Dige I, Grønkjær L, Nyvad B: Molecular studies of the structural ecology of natural occlusal caries. Caries Res 2014;48(5):451-60.

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

Gross EL, Leys EJ, Gasparovich SR, Firestone ND, Schwartzbaum JA, Janies DA, Asnani K, Griffen AL: Bacterial 16S sequence analysis of severe caries in young permanent teeth. J Clin Microbiol 2010;48:4121–4128.

Gross EL, Beall CJ, Kutsch SR, Firestone ND, Leys EJ, Griffen AL: Beyond Streptococcus

mutans: dental caries onset linked to multiple species by 16S rRNA community analysis. PLoS One 2012; 7:e47722.

Hahn CL, Liewehr FR: Relationships between caries bacteria, host responses, and clinical signs and symptoms of pulpitis: J Endod 2007;33:213-219.

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

Holt JG,Krieg NR, Sneath PHA, Staley JT,Williams ST: Bergey's Manual of Determinative Bacteriology 1994: 9 Edition. Williams & Wilkins. Baltimore.

Ismail AI, Sohn W, Tellez M, Amaya A, Sen A, Hasson H, Pitts NB: The International Caries Detection and Assessment System (ICDAS): an integrated system for measuring dental caries. Community Dent Oral Epidemiol 2007; 35:170–178.

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.

Kanasi E, Dewhirst FE, Chalmers NI, Kent R, Moore A: Clonal analysis of the microbiota of severe early childhood caries. Caries Res 2010; 44:485–497.

Kianoush N, Nguyen K-AT, Browne G V, Simonian M, Hunter N: pH gradient and distribution of streptococci, lactobacilli, prevotellae, and fusobacteria in carious dentine. Clin Oral Investig 2013;16: DOI: 10.1007/s00784-013-1009-0.

Kianoush N, Adler CJ, Nguyen K-AT, Browne G V, Simonian M, Hunter N: Bacterial profile of dentine caries and the impact of pH on bacterial population diversity. PLoS One 2014;9:e92940.

Kleinberg I: 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 2002;13:108–125.

Koehler B, Andreen I, Jonsson B: The earlier the colonization by mutans streptococci, the higher the caries prevalence at 4 years of age**.** Oral Microbiol Immunol 1988;3:14-17.

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.

Lima KC, Coelho LT, Pinheiro IVA, Rocas IN, Siqueira JF Jr: Microbiota of dentinal caries as assessed by reverse-capture checkerboard analysis. Caries Res 2011;45:21–30.

Loesche WJ, Eklund S, Earnest R, Burt B: Longitudinal investigation of bacteriology of human fissure decay: epidemiological studies on molars shortly after eruption. Infect Immun 1984;46:765–772.

Love RM, Jenkinson HF: Invasion of dentinal tubules by oral bacteria. Crit Rev Oral Biol Med 2002;13:171–183.

Maeda T, Kitasako Y, Senpuku H, Burrow MF, Tagami J: Role of oral streptococci in the pH-dependent carious dentin. J Med Dent Sci 2006;53:159–166.

Mantzourani M, Fenlon M, Beighton D: Association between *Bifidobacteriaceae* and the clinical severity of root caries lesions. Oral Microbiol Immunol 2009;24:32–37.

Marchant S, Braislsford SR, Twomey AC, Roberts GJ, Beighton D: The predominant microflora of nursing caries lesions. Caries Res 2001;35:397-406.

Martin FE, Nadkarni MA, Nicholas A, Hunter N, Jacques NA: 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 2002;40:1698-1704.

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.

Milnes AR, Bowden GH: The microflora associated with developing lesions of nursing caries. Caries Res 1985;19:289–297.

Munson MA, Banerjee, Watson TF, Wade WG: Molecular analysis of the microflora associated with dental caries. J Clin Microbiol 2004;42:3023–3029.

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.

Nadkarni MA, Simonian MR, Harty DW, Zoellner H, Jacques NA, Hunter N**:** Lactobacilli are prominent in the initial stages of polymicrobial infection of dental pulp. J Clin Microbiol 2010: 48:1732-40.

Nakajo K, Takahashi N, Beighton D: Resistance to acidic environments of caries-associated bacteria: Bifidobacterium dentium and Bifidobacterium longum. Caries Res 2010;44(5):431-437.

Nanci A: ten Cate's Oral Histology; Development, Structure and Function. St Louis, Elsevier 2012;163–205.

Nascimento MM, Burne RA: Caries prevention by arginine metabolism in oral biofilms: translating science into clinical success. Curr Oral Heal Reports 2014;1:79–85.

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.

Nyvad B, Machiulskiene V, Baelum V: Construct and predictive validity of clinical caries diagnostic criteria assessing lesion activity. J Dent Res 2003;82:117–122.

Nyvad B, Crielaard, Mira A, Takahashi N, Beighton D: Dental caries in a molecular microbiological perspective. Caries Res 2013; 47: 89– 102.

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.

Park S, Kim YK, Kook J. Development of quantitative real-time PCR primers for detecting 42 bacterial species. Arch Microbiol 2013;195:473–482.

Parisotto TM, Steiner-Oliveira C, Silva CM, Rodrigues LK, Nobre-dos-Santos M: Early childhood caries and mutans streptococci: a systematic review. Oral Health Prev Dent. 2010;8:59-70.

Peterson SN, Snesrud E, Schork NJ, Bretz WA: Dental caries pathogenicity: a genomic and metagenomic perspective. Int Dent J 2011;61:11–22.

Peterson SN, Snesrud E, Liu J, Ong AC, Kilian M, Schork NJ, Bretz W.: The dental plaque microbiome in health and disease. PLoS One 2013;8:e58487.

Pitts NB: Modern concepts of caries measurement. J Dent Res 2004;83:43–47.

Ramos-Gomez FJ, Weintraub JA, Gansky SA, Hoover CI, Featherstone JD. Bacterial, behavioral and environmental factors associated with early childhood caries. J Clin Pediatr Dent 2002;26:165–73.

Rintillä T, Kassinen A, Malinen E, Krogius L, Palva A. 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 2004;97:1166–1177.

Simark-Mattsson C, Emilson CG, Håkansson EG, Jacobsson C, Roos K, Holm S: Lacto-bacillus-mediated interference of mutans streptococci in caries-free vs. caries-active subjects. Eur J Oral Sci 2007;115:308–314.

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. Journal of Oral Microbiology 2014;6,25443

Takahashi N, Nyvad B: The role of bacteria in the caries process: ecological perspectives. J Dent Res 2011;90:294–303.

Takahashi N, Yamada T : Acid-induced acidogenicity and acid tolerance of non-mutans streptococci. Oral Microbiol Immunol 1999; 14:43-48.

Tanzer JM, Livingston J, Thompson a M: The microbiology of primary dental caries in humans. J Dent Educ 2001;65:1028–1037.

van Houte J, Lopman J, Kent R: The final pH of bacteria comprising the predominant f lora on sound and carious human root and enamel surfaces. J Dent Res 1996;75:1008–1014.

Wilson K: Preparation of genomic DNA from bacteria. Curr Protoc Mol Biol 2001;Chapter 2:Unit 2.4.

Wolff, D, Freese C, Maier-Kraus T, Krueger T, Wolff B. Bacterial biofilm composition in caries and caries-free subjects. Caries Res 2013;47:69–77.

Yano A, Kaneko N, Ida H, Yamaguchi T, Hanada N: Real-time PCR for quantification of *Streptococcus mutans*. FEMS Microbiology Letters 2002;217:23-30.

Yasunaga A, Yoshida A, Morikawa K, Maki K, Nakamura S, Soh I, Awano S, Ansai T: Monitoring the prevalence of viable and dead cariogenic bacteria in oral specimens and in vitro biofilms by qPCR combined with propidium monoazide. BMC Microbiol 2013; 13: doi: 10.1186/1471-2180-13-157.

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 et al., 2013
Bifidobacterium spp.[1]	F: TCGCGTC(C/T)GGTGTGAAAG R: CCACATCCAGC(A/G)TCCAC	58	243	Rintilla et al., 2004
Lactobacillus acidophilus	F: GATCGCATGATCAGCTTATA R: AGTCTCTCAACTCGGCTATG	60	124	Furet et al., 2004
L. casei group[2]	F: GCGGACGGGTGAGTAACACG R: GCTTACGCCATCTTTCAGCCAA	60	121	Furet et al., 2004
Mitis group[3]	F:TAGAACGCTGAAGGAAGGAGC R: GCAACATCTACTGTTATGCGG	60	133	Wolff et al., 2013
Streptococcus gordonii	F: CAGGAAGGGATGTTGGTGTT R: GACTCTCTTGGCGACGAATC	60	136	Wolff et al., 2013
Streptococcus mutans	F: AGCCATGCGCAATCAACAGGTT R: CGCAACGCGAACATCTTGATCAG	64	415	Yano et al., 2002

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. *Mitis* group *: Streptococcus mitis, S. oralis, S. pneumoniae, S. parasanguinis, S. australis*

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	
***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	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. gordonni	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

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

Variables	Active lesions	Inactive lesions	p-value	Odds ratio (95% CI)
	n (%)	n (%)		
S. mutans				
Positive	39 (100%)	17 (100%)	1.000	-
Negative	0 (0%)	0 (0%)		
> 11.84%	16 (41%)	12 (70.6%)	0.080	0.3 (0.1 -1.0)
< 11.84%	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%)		
> 0.00%	7 (17.9%)	0 (0%)	0.088	-
< 0.00%	32 (82.1%)	17 (100%)		
***Mitis* group**				
Positive	37 (94.9%)	15 (88.2%)	0.577	2.5 (0.3 -19.1)
Negative	2 (5.1%)	2 (11.8%)		
>0.27%	17 (43.6%)	10 (58.8%)	0.294	0.5 (0.2 -1.7)
<0.27%	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				
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. * p<0.05. 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.

Table 4. Correlation among oral bacteria in active dentine carious lesions in severe ECC-children.

Oral bacteria		*S. mutans*	*L. acidophilus*	*Mitis* group	*L. casei* group	*Bifido spp.*	*A. naeslundii*	*S. gordoni*
S. mutans	r	-	-0.297	0.228	-0.332*	-0.062	0.165	0.140
	p-value	-	0.066	0.163	0.039	0.708	0.315	0.397
L. acidophilus	r	-0.297	-	-0.137	0.064	0.124	0.071	0.045
	p-value	0.066	-	0.405	0.697	0.452	0.667	0.784
***Mitis* group**	r	0.228	-0.137	-	0.208	0.055	0.288	0.542*
	p-value	0.163	0.405	-	0.205	0.740	0.075	0.000
***L. casei* group**	r	-0.332*	0.064	0.208	-	0.530*	0.212	0.333*
	p-value	0.039	0.697	0.205	-	0.001	0.195	0.038
Bifido spp.	r	-0.062	0.124	0.055	0.530*	-	0.075	0.192
	p-value	0.708	0.452	0.740	0.001	-	0.650	0.243
A. naeslundii	r	0.165	0.071	0.288	0.212	0.075	-	0.425*
	p-value	0.315	0.667	0.075	0.195	0.650	-	0.007
S. gordonii	r	0.140	0.045	0.542*	0.333*	0.192	0.425*	-
	p-value	0.397	0.784	0.000	0.038	0.243	0.007	-

*$p < 0.05$; Spearman's rank correlation.

Table 5. Correlation among oral bacteria in inactive dentine carious lesions in severe ECC-children.

Oral bacteria		***S. mutans***	***L. acidophilus***	***Mitis* group**	***L. casei* group**	***Bifido* spp.**	***A. naeslundii***	***S. gordonii***
S. mutans	**r**	-	-	0.118	0.025	0.071	-0.159	0.285
	p-value	-	-	0.653	0.923	0.786	0.541	0.267
L. acidophilus	**r**	-	-	-	-	-	-	-
	p-value	-	-	-	-	-	-	-
***Mitis* group**	**r**	0.118	-	-	-0.072	-0.341	0.377	0.738*
	p-value	0.653	-	-	0.784	0.180	0.136	0.001
***L. casei* group**	**r**	0.025	-	-0.072	-	0.296	-0.037	-0.255
	p-value	0.923	-	0.784	-	0.249	0.888	0.324
Bifido spp.	**r**	0.071	-	-0.341	0.296	-	0.010	-0.037
	p-value	0.786	-	0.180	0.249	-	0.970	0.887
A. naeslundii	**r**	-0.159	-	0.377	-0.037	0.010	-	0.251
	p-value	0.541	-	0.136	0.888	0.970	-	0.332
S. gordonii	**r**	0.285	-	0.738*	-0.255	-0.037	0.251	-
	p-value	0.267	-	0.001	0.324	0.887	0.332	-

*$p < 0.05$; Spearman's rank correlation.

4 CONCLUSÃO GERAL

Com base nos resultados desta tese, concluiu-se que:

1) As espécies de Lactobacilos (*L. acidophilus* e grupo *L. cas*ei) tiveram baixa prevalência em biofilme de crianças com cárie precoce da infância, não apresentando associação com a iniciação ou progressão da doença. Por outro lado, a presença de *S. mutans* e *Bifidobacterium* spp. no biofilme das crianças está fortemente associada à evolução da doença para lesões cavitadas dentinárias .

2) As bactérias do gênero *Bifidobacterium* e membros do grupo *L. casei* apresentaram uma forte associação e uma maior concentração em lesões ativas de cárie dentinária. Enquanto que *S. mutans* apresentou uma maior concentração em lesões inativas. Além disso, observou-se uma forte associação da presença de *L. acidophilus* e *S. gordonii* com atividade das em lesões dentinárias estudadas. Sendo assim, a presença e os níveis das bactérias dos grupos *Bifidobacterium* spp. e *L. casei* estão relacionadas com a atividade de lesões dentinárias de crianças com cárie precoce da infância.

REFERÊNCIAS GERAIS

AAS, J.A.; PASTER, B.J., STOKES, L.N.; OLSEN, I,; DEWHIRST, FE. Defining the normal bacterial flora of the oral cavity. **J Clin Microbiol**, v.43, p. 5721-32, 2005.

AAS, J.A; GRIFFEN, A.L; DARDIS, S.R.; LEE, A.M; OLSEN, I.; DEWHIRST, F.E.; LEYS, E.J.; PASTER, B.J. Bacteria of dental caries in primary and permanent teeth in children and young adults. **J Clin Microbiol**, v.46, n.4, p.1407-1417, 2008.

AMERICAN ACADEMY OF PEDIATRIC DENTISTRY. Policy on Early Childhood Caries (ECC): Classifications, Consequences and Preventive Strategies. Reference Manual. **Pediatric Dentistry**,v.36,p.50-52, 2014

BECKER, M. R.; PASTER B.J.; LEYS E.J.; MOESCHBERGER M.L.; KENYON S. G.; GALVIN, J.L.; BOCHES,S.K.; DEWHIRST, F.E.; GRIFFEN, A.L. Molecular analysis of bacterial species associated with childhood caries. **J. Clin. Microbiol**, v. 40, p. 1001–1009, 2002.

BELDA-FERRE P.; ALCARAZ L.D; CABRERA-RUBIO R.; ROMERO H.; SIMÓN-SORO A.; PIGNATELLI M.; MIRA A. The oral metagenome in health and disease. **ISME J,** v.g, p.46–56, 2012

BERKOWITZ, R.J. Causes, treatment and prevention of early childhood caries: a microbiologic perspective. **J Can Dent Assoc**, v.69, p. 304-307, 2003.

BRASIL. Ministério da Saúde. Secretaria de Atenção à Saúde. Departamento de Atenção Básica. Projeto SB Brasil 2003: condições de saúde bucal da população brasileira 2002-2003: resultados principais / Ministério da Saúde, Secretaria de Atenção à Saúde, Departamento de Atenção Básica. – Brasília: Ministério da Saúde, 2004.

BRASIL. 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, 2012.

CARLSSON, P.; OLSSON, B.; BRATTHALL, D. The relationship between the bacterium Streptococcus mutans in the saliva and dental caries in children in Mozambique. **Arch Oral Biol**., v. 30, n.3, p.265-268, 1985.

CHHOUR, K.; NADKARNI, M.A; BYUN, R.; MARTIN, F.E; JACQUES N.A; HUNTER N. Molecular analysis of microbial diversity in advanced caries. **J Clin Microbiol**, v.43,p.843–849, 2005.

CLEATON-JONES, P., RICHARDSON, B.D.; GRANATH, L.;, FATTI, L.; SINWELL, P.R.; WALKER, A. R. 2000. Nutritional status and dental caries in a large sample of 4- and 5-year-old South African children**. S. Afr. Med. J**., v. 90, p.631– 635, 2000.

CORBY, P.M.; LYONS-WEILER, J.; BRETZ, W.A.; HART, T.C.; AAS, J.A.; BOUMENNA, T.; GOSS, J.; CORBY, A.L.; JUNIOR, H.M.; WEYANT, R.J.; PASTER, B.J. Microbial risk indicators of early childhood caries. **J Clin Microbiol**, v.43, n.11, p.5753-5759, 2005.

COSTA, S.M; ABREU, M.H.N.G.; VASCONCELOS, M.; LIMA, R.C.G.S.; VERDI, M.;FERREIRA, E.F. Desigualdades na distribuição de cárie dentária no Brasil: uma abordagem bioética. **Ciênc. saúde coletiva,**v. 18, 2013.

DEWHIRST, F.E; CHEN, T.; IZARD, J.; PASTER, B.J; TANNER, A.C, YU, W.H; LAKSHMANAN, A.; WADE, W.G. The human oral microbiome. **J Bacteriol**, v.192,p.5002-5017, 2010.

FEJERSKOV, O. Changing paradigms in concepts on dental caries: consequences for oral health care. **Caries Res**, v. 38, n. 3, p. 182-191, 2004.

FELDENS, Carlos Alberto. **Cárie dentária na infância: abordagem contemporânea**. São Paulo: Santos, 2013. 312 p.

GROSS EL, LEYS EJ, GASPAROVICH SR, FIRESTONE ND, SCHWARTZBAUM JA, JANIES DA, ASNANI K, GRIFFEN AL: Bacterial 16S sequence analysis of severe caries in young permanent teeth. **J Clin Microbiol** v.48, p.4121–4128, 2010.

GROSS E.L; BEALL C.J; KUTSCH S.R; FIRESTONE N.D; LEYS E.J; GRIFFEN A.L; Beyond Streptococcus mutans: dental caries onset linked to multiple species by 16S rRNA community analysis. **PLoS One,v**.7, p.e47722, 2012.

ISMAIL, A.I.; SOHN W. A systematic review of clinical diagnostic criteria of early childhood caries. **J. Public Health Dent**, v.59, p.171–191, 1999.

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**, v.67,p.962–969,2014.

KANASI, E.; JOHANSSON, I.; LU, S.C.; KRESSIN, N.R.; NUNN, M.E.; KENT, R. J.R.; TANNER, A.C. Microbial risk markers for childhood caries in pediatricians' offices.**J Dent Res**., v.89, n.4, p.378-383, 2010.

KIANOUSH N, ADLER CJ, NGUYEN K-AT, BROWNE G V, SIMONIAN M, HUNTER N: Bacterial profile of dentine caries and the impact of pH on bacterial population diversity. **PLoS One**, v.9,e92940,2014.

KOHLER, B.; ANDREEN, I.; JONSSON, B. The earlier the colonization by mutans streptococci, the higher the caries prevalence at 4 years of age**. Oral Microbiol Immunol,** v.3, p.14-17, 1988.

LAW, V.; SEOW, WK.; TOWNSEND, G. Factors influencing oral colonization of *mutans streptococci* in young children**. Aust Dent J**, v.52, n. 2, p. 93-100, 2007.

LOESCHE WJ. Role of *Streptococcus mutans* in human dental decay. **Microbiol Rev,** v.50, n. 4, p. 353-380, 1986.

MACHIULSKIENE, V.; NYVAD, B.; BAELUM, V. Prevalence and severity of dental caries in 12-year-old children in Kaunas, Lithuania 1995. **Caries Res**, v. 32, n. 3, p. 175-180, 1998.

MARCHANT, S., BRAISLSFORD, S.R; TWOMEY, A.C; ROBERTS, G.J; BEIGHTON D: The predominant microflora of nursing caries lesions. Caries Res v. 35, p.397-406, 2001.

MARSH, PD. Are dental diseases examples of ecological catastrophes? **Microbiology**, v. 149, n. 2, p. 279-294, 2003.

MARSH, PD. Microbial ecology of dental plaque and its significance in health and disease. **Adv. Dent. Res**, v. 8, p.263–271, 1994.

MARSH PD. Sugar, fluoride, pH and microbial homeostasis in dental plaque**. Proc Finn Dent Soc**, v. 87, n. 4, p. 515-525, 1991.

MARTIN, F.E; NADKARNI, M.A; NICHOLAS, A; HUNTER, N.; JACQUES, N.A. 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 v.** 40, p.1698-1704,,2002.

MARTINS, C.; BUCZYNSKI, AK.; MAIA, LC.; SIQUEIRA, WL.; Castro, GF. Salivary proteins as a biomarker for dental caries--a systematic review. **J Dent**, v. 41, n. 1, p. 2-8, 2013.

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**, v. 61, n. 3, p. 161-167, 2001.

MUNSON, M.A.; BANERJEE A.; WATSON T.F.; WADE W.G.. Molecular analysis of the microflora associated with dental caries. **J. Clin. Microbiol**., v. 42, p.3023–3029, 2004.

NASCIMENTO, M.M.; GORDAN, V.V.; GARVAN, C.W.; BROWNGARDT, C.M.; BURNE, R.A. Correlations of oral bacterial arginine and urea catabolism with caries experience. **Oral Microbiol Immunol,** v.24, p:89-95, 2009.

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

PARISOTTO, T.M; STEINER-OLIVEIRA, C.; DUQUE, C; PERES, R.C.R.; RODRIGUES, L.K.A; NOBRE-DOS-SANTOS, M. Relationship among microbiological composition and presence of dental plaque, sugar exposure, social factors and different stages of early childhood caries. **Arch Oral Biol**., v. 55, n.2, p.365-373, 2010.

PASTER, B. J.; BOCHES. S.K.; GALVIN, J.K.; ERICSON, RE. *et al*. Bacterial diversity in human subgingival plaque. **J. Bacteriol**. v.183, p.3770–3783., 2001.

PETERSEN, P.E.; BOURGEOIS, D.; OGAWA, H.; ESTUPINAN-DAY, S.; NDIAYE C. The global burden of oral diseases and risks to oral health. Bulletin of the World Health Organization, v.83, p.661-669, 2005.

PETERSON, S.N; MEISSNER, T.; SU, A.I; SNESRUD, E.; ONG, A.C; SCHORK, N.J; BRETZ, W.A. Functional expression of dental plaque microbiota. **Front Cell Infect Microbiol,**v.4,p.108, 2014.

PITTS, N.B. Modern concepts of caries measurement. **J Dent Res,** v.83, p. 43–47, 2004.

RAMOS-GOMEZ, F.J.; WEINTRAUB, J.A.; GANSKY, S.A.;, HOOVER CI, FEATHERSTONE JD. Bacterial, behavioral and environmental factors associated with early childhood caries. **J Clin Pediatr Dent**.,v. 26, n.2, p.165-173, 2002.

SAXENA, D.; CAUFIELD, P.W.; LI, Y.; BROWN, S.; SONG, J.; NORMAN, R. Genetic classification of severe early childhood caries by use of subtracted DNA fragments from Streptococcus mutans. **J Clin Microbiol,** v. 46, n. 9, p**.** 2868-2873, 2008.

SHAW, JH. Causes and control of dental caries. **N Engl J Med**, v. 317, n. 16, p. 996-1004, 1987.

SHEN, S.; SAMARANAYAKE, LP.; YIP, HK. In vitro growth, acidogenicity and cariogenicity of predominant human root caries flora. **J Dent**, v. 32, v. 8, p. 667-678, 2004.

SHOAIB, L.; DEERY, C.; RICKETTS, DN.; NUGENT, ZJ. Validity and reproducibility of ICDAS II in primary teeth. **Caries Res**, v. 43, n. 6, p. 442-448, 2009.

SCHWENDICKE F.; DÖRFER, C.E., SCHLATTMANN P.; PAGE L.F; THOMSON W.M; PARIS S. Socioeconomic Inequality and Caries: A Systematic Review and Meta-Analysis. **J Dent Res** Nov 13. pii: 0022034514557546, 2014.

de SILVA-SANIGORSKI, A.M.; CALACHE, H.; GUSSY, M., DASHPER, S., GIBSON, J., WATERS, E. The VicGeneration study-a birth cohort to examine the environmental, behavioural and biological predictors of early childhood caries: background, aims and methods. **BMC Public Health** v.10, p.97, 2010.

TAKAHASHI N, NYVAD B: The role of bacteria in the caries process: ecological perspectives. **J Dent Res**, v. 90, p. 294–303, 2011.

TANNER A.C; MATHNEY J.M; KENT R.L; CHALMERS N.I; HUGHES C.V; LOO C.Y; PRADHAN N.; KANASI E.; HWANG J.; DAHLAN M.A; PAPADOPOLOU E.; DEWHIRST F.E. Cultivable anaerobic microbiota of severe early childhood caries. **J Clin Microbiol,**v.49,p.1464-1474. 2011

TINANOFF N.; O'SULLIVAN D. M. Early childhood caries: overview and recent findings. **Pediatr. Dent**, v.19, p. 12-6, 1997.

VAN HOUTE, J.; GIBBS, G.; BUTERA, C. 1982. Oral flora of children with "nursing bottle caries". **J. Dent. Res**., v. 61, p.382–385, 1982.

VAN HOUTE, J. Role of micro-organisms in caries etiology. **J. Dent. Res**, v.73, p.672–681, 1994.

APÊNDICE A

<u>Termo de consentimento livre e esclarecido</u>

Título do Projeto de pesquisa: Determinação de indicadores de risco relacionados a diferentes estágios da cárie precoce da infância

Você está sendo convidado pela pesquisadora Beatriz Gonçalves Neves a participar como voluntário de uma pesquisa juntamente com seu (sua) filho(a) a, e necessito que o Sr.(a) forneça informações a respeito de seu(sua) filho(a) em um questionário e diário alimentar devendo ocupá-lo(a) por 40 minutos para completar as respostas. Seu(sua) filho(a) será convidado a participar de um exame clínico odontológico e situação em que será realizada coleta de placa bacteriana dos seus dentes e de saliva para posterior avaliação em laboratório.

Após ser esclarecido(a) sobre as informações a seguir, no caso de aceitar fazer parte do estudo, assine ao final deste documento, que está em duas vias. Uma delas é sua e a outra é do pesquisador responsável.

Desde logo fica garantido o sigilo das informações. Em caso de recusa você não será penalizado(a) de forma alguma.

Este termo contém as informações sobre a pesquisa na qual você poderá participar. Por favor, leia-o atentamente. Qualquer dúvida que tiver, esclareça-a com a pesquisadora responsável, a qual estará a sua inteira disposição. Você deve decidir se deseja ou não participar desta pesquisa após entender todos os aspectos descritos a seguir, de modo que possa tomar uma decisão consciente.

INFORMAÇÕES SOBRE A PESQUISA:

Título do Projeto: Determinação de indicadores de risco relacionados a diferentes estágios da cárie precoce da infância

1) Objetivo:

Esta pesquisa tem como objetivo identificar de fatores de risco que possam estar relacionados com a cárie precoce da infância conhecida também como “cárie de mamadeira”. Assim, este estudo propõe avaliar a influência das bactérias presentes no biofilme dentário, hábitos de dieta, fatores socioeconômicos e hábitos de higiene bucal nos diferentes estágios de desenvolvimento da cárie precoce da infância dos pacientes deste estudo.

2) Justificativas:

A cárie dental é uma doença infecciosa, cujo tratamento sintomático restaurador é extremamente caro. A maior parte dos recursos públicos e privados ainda é aplicada em procedimentos curativo-restauradores e pouca ênfase é dada ao avanço de estratégias preventivas de controle desta doença. Busca-se com este estudo compreender a participação de vários fatores de risco na evolução da cárie precoce da infância. Assim, teremos maiores dados para o desenvolvimento de estratégias de controle de infecção que possam ser aplicados em programas de saúde pública.

3)Descrição da pesquisa:

Será realizado nas crianças participantes deste estudo o exame clínico (com espelho bucal e iluminação do refletor e secagem com seringa tríplice). Além disso, também serão coletados placa bacteriana de algumas superfícies dentárias e dentina cariada de alguns dentes com lesões. Esses procedimentos serão realizados em consultório odontológico. Além disso, os responsáveis pelas crianças serão convidados a responder um questionário com perguntas sobre hábitos de higiene da criança, aspectos socioeconômicos e também um diário de dieta alimentar, para descrever quais alimentos a criança costuma comer na sua rotina.

4) Desconfortos, riscos e benefícios esperados:

As crianças que participarem da pesquisa serão examinadas após limpeza profissional apenas com espelho intrabucal após a secagem dos dentes eventualmente presentes. O biofilme e a dentina cariada serão coletadas com a utilização de espátula estéril e oferecerão desconforto e riscos mínimos ao paciente. Não existem riscos adicionais. As mães serão entrevistadas nos dias que comparecerem para a primeira consulta com a pesquisadora. A realização da pesquisa não acarretará em nenhum prejuízo no andamento escolar do participante da pesquisa ou em seu tratamento odontológico. Os participantes da pesquisa, bem como seus responsáveis, participarão de atividade de educação em saúde bucal, na qual se tratará a respeito da cárie dentária e emprego de corretas técnicas para prevenção desta doença. Além disso, as crianças serão examinadas e, havendo necessidade de tratamento, serão encaminhadas para realização dos procedimentos e condutas necessárias.

5) Alternativas:

Não existem métodos alternativos para a obtenção das informações necessárias.

6) Compensação:

Não há previsão de indenização ou ressarcimento, pois não existirão gastos ou riscos relacionados exclusivamente com a pesquisa.

7) Confidencialidade dos registros:

Você terá direito à privacidade, visto que todas as informações obtidas dos fichas clínicas realizadas e das análises laboratoriais permanecerão confidenciais nos âmbitos possíveis da lei, assegurando a proteção da sua imagem. Serão respeitados seus valores culturais, sociais, morais, religiosos e éticos. Como participante desta pesquisa, você terá acesso aos resultados obtidos e permitirá o acesso dos mesmos aos pesquisadores envolvidos e aos membros da comissão de ética responsável. Os resultados deste trabalho poderão ser apresentados em congressos ou publicados em revistas científicas, porém sua identidade não será divulgada.

8) Direito de participar, recusar ou sair:

Sua participação é voluntária e você poderá recusar-se a participar ou mesmo interrromper sua participação a qualquer momento, sem penalidades ou perdas de seus benefícios aos quais de outra forma tenha direito. Os pesquisadores terão o direito de desligá-lo do estudo a qualquer momento, se julgarem necessário. Ao participar, você concorda em cooperar com a pesquisa, não abrindo mão de seus direitos legais ao assinar o termo de consentimento informado.

9) Contatos:

Informo que o Sr(a) tem a garantia de acesso, em qualquer etapa do estudo, sobre qualquer esclarecimento de eventuais dúvidas. Se tiver alguma consideração ou dúvida sobre a ética da pesquisa, entre em contato com o Comitê de Ética em Pesquisa da UFC na Rua Coronel Nunes de Melo, 1127 , Rodolfo Teófilo em Fortaleza-CE, fone: 3366.8344 ou comunique-se com a pesquisadora Beatriz Gonçalves Neves (85) 8827.0207 ou email: beatrizneves@hotmail.com.

Consentimento pós-informação

Eu ____, certifico que, tendo lido as informações prévias e tendo sido suficiente esclarecido pelos pesquisadores abaixo descritos, sobre todos os itens, estou plenamente de acordo com a realização do experimento, autorizando, inclusive que se execute a metodologia desta pesquisa no menor____, por qual sou responsável.

Ficou claro também que a participação do(a) meu(minha) filho(a) é isenta de despesas e que tenho garantia do acesso aos resultados e de esclarecer minhas dúvidas a qualquer tempo. Concordo voluntariamente em permitir a participação do(a) meu(minha) filho(a) deste estudo e poderei retirar o meu consentimento a qualquer momento, antes ou durante o mesmo, sem penalidade ou prejuízo.

Fortaleza, _______de ______________________de 20___.

Nome participante:____
Endereço:____
Nome responsável:___________________________RG:___________
Assinatura do pai (mãe) ou responsável: _________________________________

PESQUISADORA RESPONSÁVEL:

____

BEATRIZ GONÇALVES NEVES
CRO-CE 4677

APÊNDICE B

FICHA CLÍNICA

Nome da criança:____

Data de nascimento:_____________ Sexo:________________________

Endereço:________________________________TEL:_______________

Nome do responsável:____

Data do exame:__/__/__Escola/creche:____________________________

Upper Right | Upper Left

Surface				55	54	53	52	51	61	62	63	64	65			
	18	17	16	15	14	13	12	11	21	22	23	24	25	26	27	28
M																
O																
D																
B																
L																

Lower Right | Lower Left

Surface				85	84	83	82	81	71	72	73	74	75			
	48	47	46	45	44	43	42	41	31	32	33	34	35	36	37	38
M																
O																
D																
B																
L																

ANEXO

Universidade Federal do Ceará
Comitê de Ética em Pesquisa

Of. Nº 221/11 Fortaleza, 29 de agosto de 2011
Protocolo COMEPE nº 158/ 11
Pesquisador responsável: Beatriz Gonçalves Neves
Título do Projeto: "Determinação de indicadores de risco relacionados a diferentes estágios da cárie precoce na infância"

Levamos ao conhecimento de V.Sª. que o Comitê de Ética em Pesquisa da Universidade Federal do Ceará – COMEPE, dentro das normas que regulamentam a pesquisa em seres humanos, do Conselho Nacional de Saúde – Ministério da Saúde, Resolução nº 196 de 10 de outubro de 1996 e complementares, aprovou o protocolo e o TCLE do projeto supracitado na reunião do dia 18 de agosto de 2011.

Outrossim, informamos, que o pesquisador deverá se comprometer a enviar o relatório final do referido projeto.

Atenciosamente,

Dr. Fernando A. Frota Bezerra
Coordenador do Comitê
de Ética em Pesquisa