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Tese de Doutorado

**AVALIAÇÃO DA RESISTÊNCIA À CLOREXIDINA EM
BACTÉRIAS PATOGÊNICAS ISOLADAS DA CAVIDADE ORAL
DE PACIENTES HOSPITALIZADOS**

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Brasília, 8 de novembro de 2023

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

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que sempre foi a base de tudo que sou.*

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*“Tudo posso naquele que me fortalece.”
Filipenses 4, 13*

RESUMO

A microbiota bucal é bastante diversificada e pode sofrer mudanças em sua composição em indivíduos hospitalizados, uma vez que ocorre a aquisição de microrganismos patogênicos que habitualmente não estariam presentes na boca. A clorexidina é um antisséptico frequentemente utilizado para higiene bucal, além de outros usos em ambientes hospitalares. Porém, o potencial desenvolvimento de resistência microbiana a desinfetantes e antissépticos, como a clorexidina, tem sido subestimado, a despeito do alerta de estudos recentes sobre o desenvolvimento de resistência à própria substância, a propagação de resistência cruzada a outros antimicrobianos, além do desenvolvimento de microrganismos multirresistentes. O objetivo deste estudo foi conhecer o estado de saúde e a microbiota patogênica presente na boca de pacientes hospitalizados, além de avaliar os isolados bacterianos quanto à susceptibilidade à clorexidina, à susceptibilidade aos fármacos antibacterianos e à possível mudança de perfil de resistência quando associados em biofilmes com uma linhagem probiótica de *Streptococcus salivarius*. Pacientes hospitalizados na enfermaria de Clínica Médica do Hospital Universitário de Brasília foram examinados por um cirurgião-dentista e swabs da mucosa bucal foram coletados. Os swabs foram processados para o isolamento de microrganismos patogênicos relacionados a infecções hospitalares. Foi realizado ensaio de concentração inibitória mínima (CIM) para determinação da sensibilidade dos isolados bacterianos à clorexidina. Os isolados que apresentaram resultados alterados foram submetidos a antibiograma. Os isolados multirresistentes foram avaliados quanto ao seu perfil de resistência à clorexidina em biofilmes simples ou em associação com *S. salivarius*. O estado de saúde bucal foi considerado mediano e o índice de saburra lingual foi classificado como elevado nos pacientes examinados. A prevalência de microrganismos patogênicos relacionados à resistência antimicrobiana foi alta nas amostras coletadas. Dos isolados analisados, 17.4% apresentaram redução da sensibilidade à clorexidina. Foi observada resistência de oito isolados a pelo menos um antibacteriano. Três isolados apresentaram resistência a mais de sete antibacterianos, sendo considerados multirresistentes. A associação com *S. salivarius* em biofilmes aumentou a susceptibilidade à clorexidina das bactérias patogênicas multirresistentes. Os resultados mostraram que a boca pode ser um reservatório de microrganismos patogênicos multirresistentes a antibacterianos e à clorexidina, e que a associação em biofilmes com bactérias da microbiota bucal como *S. salivarius* pode influenciar o processo de resistência aos antimicrobianos e seleção de microrganismos multirresistentes.

Palavras-chave: boca; microbiota; clorexidina; resistência microbiana a medicamentos; hospitalização; *Streptococcus salivarius*

ABSTRACT

Research has shown that oral health deteriorates in hospitalised patients. The oral microbiota may suffer a shift in its composition during a period of hospitalisation, acquiring pathogenic microorganisms that would not normally be in the mouth. Chlorhexidine is an antiseptic often used for oral hygiene, as well as other uses in hospital settings. However, the potential development of microbial resistance to disinfectants and antiseptics such as chlorhexidine has been underestimated, despite the warning of recent studies about the development of resistance to the substance itself, the spread of cross-resistance to other antimicrobials, and the development of multidrug-resistant microorganisms. The aim of this study was to characterize oral health and the pathogenic non-oral microbiota present in the mouth of hospitalized patients, as well as to assess bacterial isolates in terms of susceptibility to chlorhexidine, susceptibility to antibacterial drugs and the possible change in resistance profile when associated in biofilms with a probiotic strain of *Streptococcus salivarius*. Hospitalised patients admitted to the medical clinic ward of the University Hospital of Brasilia were examined by a dental surgeon and swabs of the oral mucosa were collected. The swabs were processed for the isolation of pathogenic microorganisms related to hospital-acquired infections. A minimum inhibitory concentration (MIC) test was carried out to determine the sensitivity of the bacterial isolates to chlorhexidine. Isolates with altered results were subjected to antibiograms in which various antibacterials were tested. Multidrug-resistant isolates were assessed for their chlorhexidine resistance profile in single biofilms or in association with *S. salivarius*. The prevalence of pathogenic microorganisms related to antimicrobial resistance was high in the patients examined. Of the isolates analysed, 17.4% had reduced sensitivity to chlorhexidine. It was observed that eight isolates exhibited resistance to at least one antibacterial agent. Three isolates demonstrated resistance to more than seven antibacterial agents, thereby classified as multidrug-resistant. The co-cultivation with *S. salivarius* in biofilms augmented the susceptibility of multidrug-resistant pathogenic bacteria to chlorhexidine. The findings underscore the oral cavity as a potential reservoir for multidrug-resistant pathogenic microorganisms, both to antibacterial agents and chlorhexidine. The association in biofilms with oral microbiota bacteria, such as *S. salivarius*, may significantly impact the antimicrobial resistance process and the selection of multidrug-resistant microorganisms.

Key words: chlorhexidine; microbial drug resistance; mouth; microbiota; hospitalization; *Streptococcus salivarius*

SUMÁRIO

1 INTRODUÇÃO, REVISÃO DA LITERATURA E OBJETIVOS	11
1.1 INTRODUÇÃO.....	11
1.2 REVISÃO DA LITERATURA	13
1.2.1 Microbiota bucal.....	13
1.2.2 Clorexidina.....	15
1.2.3 Resistência aos antimicrobianos.....	17
1.2.4 Resistência microbiana à clorexidina.....	19
1.3 OBJETIVOS	23
1.3.1 Objetivo geral.....	23
1.3.2 Objetivos específicos.....	23
1.4 REFERÊNCIAS.....	24
2 ORAL HEALTH AND THE PRESENCE OF INFECTIOUS MICROORGANISMS IN HOSPITALIZED PATIENTS: A PRELIMINARY OBSERVATIONAL STUDY.....	29
2.1 INTRODUCTION.....	31
2.2 METHODOLOGY.....	32
2.3 RESULTS.....	35
2.4 DISCUSSION.....	40
2.5 CONCLUSION.....	45
2.6 REFERENCES.....	48
3 CHLORHEXIDINE RESISTANCE IN PATHOGENIC BACTERIA ISOLATED FROM THE MOUTH OF HOSPITALIZED PATIENTS.....	52
3.1 INTRODUCTION.....	53
3.2 METHODOLOGY.....	54
3.3 RESULTS.....	62

3.4 DISCUSSION.....	69
3.5 CONCLUSION.....	76
3.6 REFERENCES.....	77
4 DISCUSSÃO GERAL E CONCLUSÕES DA TESE.....	82
4.1 DISCUSSÃO GERAL.....	82
4.2 CONCLUSÕES.....	85
4.3 REFERÊNCIAS.....	86
5 PRESS RELEASE.....	89
APÊNDICE A – TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO	90
APÊNDICE B – CASE REPORT FORM	91
APÊNDICE C – ARTIGO: Methotrexate induced erythema multiforme: a case report of accidental overdose	96
ANEXO A – PARECER DO COMITÊ DE ÉTICA	100
ANEXO B – ACEITE DO ARTIGO: Oral Health and the presence of infectious microorganisms in hospitalized patients: a preliminary observational study.....	106

1 INTRODUÇÃO, REVISÃO DA LITERATURA E OBJETIVOS

1.1 INTRODUÇÃO

A boca é colonizada por uma microbiota bastante diversificada que, quando mantida em equilíbrio, tem papel relevante na manutenção da saúde [1]. A composição da microbiota bucal pode ser alterada por condições do hospedeiro e do ambiente permitindo a proliferação de microrganismos potencialmente patogênicos [2]. Fatores como alimentação, presença ou ausência de dentes, higiene bucal e condições imunológicas do hospedeiro podem atuar no desequilíbrio da microbiota bucal, gerando disbiose [2]. Durante um período de hospitalização podem ocorrer modificações na composição da microbiota bucal, com aquisição de microrganismos gram-negativos com maior potencial patogênico, alguns deles resistentes a múltiplos fármacos [3].

O aumento de microrganismos resistentes aos antimicrobianos é um problema de saúde pública mundial. As infecções resistentes estão entre os maiores problemas enfrentados no ambiente hospitalar e a resistência aos antimicrobianos está entre as principais ameaças globais à saúde [4]. O problema tem gerado grande preocupação, uma vez que ameaça não somente a saúde das pessoas, mas também gera impactos nos meios de subsistência, na ordem econômica, social e política, comprometendo a segurança mundial e o cumprimento dos Objetivos de Desenvolvimento Sustentável [4].

A clorexidina é um antisséptico frequentemente utilizado em hospitais para higiene bucal de pacientes internados, principalmente dos que se encontram em unidades de terapia intensiva [3,5]. A substância é também utilizada como antisséptico e desinfetante para inúmeras outras finalidades nos dias atuais, como em sabonetes e outros produtos antissépticos [6,7]. Na Odontologia, é prescrita sob a forma de bochecho, gel, aerossol, verniz, líquidos de irrigação e chip subgingival para prevenção e tratamento de diversas condições como cárie, gengivite e periodontite [8]. A clorexidina também é encontrada em diversos produtos disponíveis comercialmente, sem

necessidade de prescrição, como enxaguatórios e cremes dentais [8]. É considerada uma substância segura com raros efeitos adversos sistêmicos a exemplo de alergias, e efeitos adversos locais mínimos ou transitórios, como a pigmentação dentária extrínseca, a xerostomia e a disgeusia [8].

No entanto, estudos recentes alertam para o desenvolvimento de resistência microbiana a desinfetantes e antissépticos, como a clorexidina e, ainda mais preocupante, o potencial desenvolvimento de resistência cruzada a outros antimicrobianos [9]. O surgimento de bactérias resistentes a antimicrobianos como possível resultado da multiplicidade de formas de utilização da clorexidina na Odontologia é um assunto que vem sendo discutido mas que ainda não foi avaliado de forma aprofundada [10].

Estudos sugeriram potencial de desenvolvimento de resistência microbiana à clorexidina e de desenvolvimento de resistência cruzada a determinados antibióticos, como a colistina (polimixina B), utilizada em infecções por bactérias multirresistentes [11]. Há ainda, a possibilidade da exposição à clorexidina estar relacionada ao desenvolvimento de microrganismos resistentes a múltiplos fármacos [12].

Não existe consenso sobre o papel do uso odontológico da clorexidina no desenvolvimento de microrganismos resistentes à própria substância e a outros antimicrobianos [9,10,13]. Também não está estabelecida na literatura científica a possibilidade da boca configurar um cenário importante para o desenvolvimento e a disseminação de microrganismos resistentes à clorexidina [9,10,13].

Portanto, mais estudos são necessários para investigar o papel do ambiente bucal como reservatório de microrganismos resistentes à clorexidina e a outros antimicrobianos. O desenvolvimento de resistência bacteriana e fúngica aos biocidas, como a clorexidina, e a possibilidade de resistência cruzada com antimicrobianos representa um importante problema de saúde pública. A confirmação dessas hipóteses poderá mostrar a necessidade de alterações nos protocolos de uso da clorexidina na Odontologia e de antisepsia e desinfecção em ambientes de assistência à saúde.

1.2 REVISÃO DA LITERATURA

1.2.1 Microbiota bucal

A boca é um complexo ecossistema que abriga mais de 600 espécies de microrganismos que interagem entre si e com o hospedeiro [14,15]. A colonização microbiana da boca é um processo contínuo que se inicia ao nascimento, e é influenciada por múltiplos fatores ao longo da vida, a exemplo de uso de antibiótico pela mãe no momento do parto, via de parto, condições ambientais, dieta, mudanças hormonais, higiene bucal, uso de medicamentos e hábitos como o tabagismo [16]. Portanto, ao longo do tempo, vai se estabelecendo uma microbiota extremamente diversificada cuja composição depende tanto de fatores do ambiente quanto do próprio hospedeiro [16].

Há duas grandes bases de dados com informações sobre a microbiota da cavidade bucal de indivíduos saudáveis: o projeto Microbioma Humano (<https://www.hmpdacc.org/hmp/>) que organizou dados referentes à identificação dos microrganismos encontrados em diversos sítios do corpo humano e o Banco de Dados do Microbioma Oral Humano (www.homd.org) [14,17]. Nestes bancos de dados foram disponibilizadas à comunidade científica informações sobre as mais de 600 espécies microbianas que formam o chamado microbioma bucal [14]. Há seis filos, *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Actinobacteria*, *Spirochaetes*, e *Fusobacteriota*, que compreendem 96% das espécies encontradas na boca de indivíduos saudáveis [14].

A microbiota bucal, quando em equilíbrio, contribui para um estado de simbiose entre os microrganismos e o hospedeiro, sendo a manutenção da homeostase fundamental para a saúde [18]. Porém, a cavidade oral é um ambiente aberto em que microrganismos do meio ambiente são continuamente introduzidos e ali permanecem de forma transitória [14]. Além disso, fatores como alterações imunológicas, mudanças na alimentação e nos hábitos de

higiene bucal e uso de medicamentos podem propiciar o rompimento do equilíbrio e contribuir para modificação da composição da microbiota, fenômeno que ao desestabilizar a relação de simbiose caracteriza a disbiose [18].

Estudo publicado em 2021 denominou como bactérias “não-orais” aquelas que comumente não fazem parte da composição da microbiota oral em indivíduos saudáveis [19]. Há no momento uma compreensão ainda limitada do papel destas bactérias “não-orais” na cavidade bucal [19]. Elas podem estar presentes de forma transitória na boca, mas podem se estabelecer como microbiota residente, principalmente em situações de desequilíbrio do sistema imunológico do hospedeiro [19]. Há muitas variáveis ecológicas e diferenças de metodologia entre os estudos, acarretando uma variabilidade nos resultados relacionados aos mecanismos biológicos das interações físicas ou metabólicas entre bactérias orais e não-orais [19]. Porém, está claro que as bactérias não-orais podem apresentar papel importante em doenças bucais e sistêmicas [19].

Durante um período de adoecimento e hospitalização pode haver colonização da boca por bactérias patogênicas que em condições normais não estariam na cavidade bucal [20,21]. Estudos mostraram aumento significativo de biofilme dentário e colonização bucal por microrganismos patogênicos relacionados a infecções nosocomiais, como *Staphylococcus* spp., *Enterobacter* spp. e *Candida* spp., atribuindo estas alterações à deficiência de higiene bucal durante a hospitalização [20,21]. Outro estudo mostrou que microrganismos relacionados à pneumonia nosocomial foram isolados da boca já no primeiro dia de hospitalização e aumentaram ao longo do período de permanência no hospital, com maior prevalência de colonização por microrganismos como *Staphylococcus* não produtor de coagulase, *Candida albicans*, *S. aureus* resistente à meticilina-MRSA, *Escherichia coli*-ESBL e *Moraxella catarrhalis* [22].

A higiene bucal tem sido uma das estratégias para reduzir a contaminação e prevenir infecções nosocomiais. Revisão sistemática publicada em 2020 pela Cochrane mostrou que, em pacientes sob ventilação mecânica, cuidados de higiene bucal estão associados a uma redução do risco de pneumonia associada à ventilação mecânica em pacientes de alto risco [5].

Concluiu ainda que, apesar de haver estudos controversos sobre a efetividade da clorexidina como antisséptico, ela provavelmente reduz o risco de pneumonia nosocomial, sendo mais efetiva quando associada à escovação dentária [5].

Outra possibilidade levantada para a recuperação da homeostase da microbiota bucal com consequente redução de microrganismos patogênicos na boca é o uso de probióticos [23]. Probióticos são microrganismos específicos que auxiliam a restauração da microbiota saudável, beneficiando o hospedeiro, a exemplo da redução de bactérias cariogênicas [23]. Estudo *in vitro* mostrou a ação de células livres sobrenadantes da bactéria probiótica *Streptococcus salivarius* na inibição de *Pseudomonas aeruginosa* e *Klebsiella pneumoniae*, ambos patógenos gram-negativos relacionados a infecções diversas [24]. Revisão sistemática realizada em 2017 concluiu que probióticos estão associados a redução de patógenos cariogênicos e inibição de patógenos periodontais, podendo modular a resposta inflamatória e exercer competição por superfícies de adesão e nutrientes [25].

Estudos atuais apontam evidências de que vários fatores colaboram para a disbiose da microbiota bucal, e que esta se associa tanto a doenças bucais quanto sistêmicas [17]. Portanto, considerando a diversidade microbiana existente na boca e sua susceptibilidade a diversos fatores, mais atenção deve ser dada ao estudo das interações entre os microrganismos dentro do ambiente bucal e também destes com o hospedeiro, pois esta compreensão poderá apontar caminhos que auxiliarão no tratamento de várias doenças [17].

1.2.2 Clorexidina

A clorexidina é um agente antimicrobiano de uso tópico em pele e mucosas, descrito pela primeira vez em 1954 [26]. Trata-se de um detergente catiônico da classe das biguanidas que afeta principalmente as membranas celulares microbianas, causando danos à sua estrutura, com consequente extravasamento de material citoplasmático [27]. Possui efeito bacteriostático em baixas concentrações e bactericida em concentrações mais altas [28].

Apresenta-se como molécula básica insolúvel, que combinada a ácidos gera sal solúvel em água. Pode ser encontrada sob as formas de acetato, hidrocloreto e digluconato, sendo este último, o sal de clorexidina mais comumente empregado [28].

A clorexidina apresenta amplo espectro de ação, agindo principalmente sobre bactérias Gram-positivas, mas também com menor atividade sobre bactérias Gram-negativas, fungos e vírus lipofílicos ou envelopados [27]. Não apresenta efeitos contra endósporos e apenas um efeito limitado contra micobactérias [27]. Tem seu efeito reduzido na presença de biofilmes, atuando melhor nos microrganismos em sua forma planctônica [29].

Dentre as propriedades que justificaram sua utilização como antisséptico, cita-se estabilidade, alta substantividade e baixa toxicidade [30]. A substantividade diferencia a clorexidina de outros antissépticos, pois trata-se da capacidade da substância de apresentar efeito por tempo prolongado devido à adsorção decorrente da sua interação química com as superfícies. Esta propriedade confere ação bacteriostática prolongada em pele e mucosa após sua utilização como antisséptico [28].

A clorexidina tem sido largamente usada em ambiente hospitalar, e também tem crescido sua utilização doméstica em sabonetes, xampus, antissépticos para ferimentos, soluções para limpeza de lentes de contato, enxaguatórios bucais, cremes dentais e até mesmo produtos cosméticos [6].

A clorexidina se mostrou eficaz na redução da quantidade de bactérias presente no ambiente bucal, sendo largamente utilizada na Odontologia [8,31,32]. Tem sido usada como antisséptico nos períodos pré e pós-operatório de cirurgias odontológicas, no controle do biofilme dentário para pacientes com dificuldade de realização de escovação, no tratamento de gengivite e doença periodontal e para irrigação e limpeza de canais radiculares [8]. Também é largamente utilizada para higiene bucal de pacientes hospitalizados, sob ventilação mecânica [3,33].

Porém, foi sugerido que o uso de enxaguatório bucal com clorexidina pode provocar alterações da microbiota bucal que parecem ser prejudiciais ao paciente. Estudo publicado em 2020 mostrou que bochechos com clorexidina 0,2% durante sete dias provocaram alteração do microbioma oral e redução do

pH salivar em pacientes saudáveis, o que pode criar um ambiente favorável para disbiose bucal [34]. Também a saúde sistêmica pode sofrer impactos pois a clorexidina alterou a diversidade bacteriana com diminuição das bactérias redutoras de nitrato, que contribuem para manutenção da saúde cardiovascular [34,35].

Portanto, é necessária a utilização criteriosa da clorexidina como antisséptico e desinfetante em ambiente hospitalar, assim como é questionável a disseminação de produtos odontológicos contendo clorexidina e o uso da substância para diversas outras finalidades. Há fortes indícios de que, além da mudança de diversidade microbiana na boca quando usada em forma de enxaguatório, a pressão seletiva exercida por diferentes vias de uso da clorexidina e de outros biocidas pode contribuir para o aumento de microrganismos resistentes a outros antimicrobianos [9].

1.2.3 Resistência aos antimicrobianos

O primeiro relato de resistência aos antimicrobianos foi escrito em 1924, antes mesmo da descoberta da penicilina, considerada o primeiro antimicrobiano natural. Tratava-se de um medicamento chamado Arsfenamina, mais conhecido à época como Salvarsan, usado no tratamento da sífilis [36]. O surgimento dos antimicrobianos foi um dos maiores avanços da Medicina, que possibilitou o tratamento de muitas doenças infecciosas e a preservação de muitas vidas. Porém, ao longo dos anos, os microrganismos têm manifestado uma capacidade de adaptação que tem sido demonstrada por diversos mecanismos de resistência aos antimicrobianos [37].

Hoje, a resistência aos antimicrobianos representa um grave problema de saúde pública que afeta praticamente todos os países do mundo e representa uma verdadeira ameaça para a humanidade [4]. Novos mecanismos de resistência vêm surgindo em velocidade maior que a descoberta de novos medicamentos e os microrganismos resistentes têm o potencial de se espalhar entre diferentes países [38]. Apesar de bactérias serem colocadas no centro da discussão, o problema é extensivo a todos os microrganismos que possuem

capacidade de adaptação [38]. Várias infecções bacterianas, fúngicas, virais e parasitárias estão se tornando de difícil controle, quando não impossíveis de tratar, devido à resistência de seus agentes etiológicos aos fármacos habitualmente empregados [38].

As infecções hospitalares ou nosocomiais estão entre os maiores problemas enfrentados em hospitais do mundo inteiro [38]. Com o rápido aumento da resistência microbiana aos antimicrobianos, as infecções vêm se tornando cada vez mais resistentes aos tratamentos disponíveis, gerando altos custos financeiros e sociais, com aumento do número de óbitos e riscos cada vez maiores aos pacientes [38]. As infecções resistentes a antimicrobianos são responsáveis por pelo menos 700.000 óbitos por ano em todo o mundo e existem projeções indicando que esse número poderá chegar a 10 milhões de mortes anuais no ano 2050 [38].

A pandemia de COVID-19, doença que surgiu em dezembro de 2019 e deflagrou uma crise de saúde repentina, exigindo uma rápida mobilização dos sistemas de saúde do mundo todo, trouxe, além de sua ameaça intrínseca, a exacerbação de uma ameaça que já existia e que agora foi amplificada, apesar de parecer ter ficado oculta por trás da pandemia [39]. O uso generalizado e desnecessário de antimicrobianos durante a pandemia e o aumento do número de hospitalizações e de pacientes graves facilitaram a evolução de microrganismos cada vez mais resistentes, aumentando o risco de infecções impossíveis de tratar com os recursos atuais [39].

Relatório sobre resistência antimicrobiana da Organização Mundial de Saúde (OMS) publicado em 2020 apresentou dados de 78 países, coletados entre maio e julho de 2019. Foram observadas altas taxas de resistência de vários microrganismos em relação a antimicrobianos usados para tratar infecções bacterianas comuns. A taxa de resistência da bactéria *Klebsiella pneumoniae* à ciprofloxacina, antibiótico habitualmente usado no tratamento de infecções urinárias, variou de 4,1% a 79,4% e da bactéria *Escherichia coli* foi de 8,4% a 92,9% para o mesmo antibiótico em pelo menos 33 países. A taxa média de infecções por cepas de *Staphylococcus aureus* resistente à metilina

(MRSA) foi de 12,11% e por *E. coli* resistente à 3ª geração de cefalosporinas foi de 36% [4].

O novo relatório da OMS publicado em 2022 mostrou aumento de resistência em bactérias causadoras de infecções comuns, porém acredita-se que esse aumento seja subestimado pela ausência de dados de vários países [4]. Um dos objetivos almejados no relatório é o compromisso de governos em ações transversais, como o desenvolvimento de sistemas de vigilância nos diversos países, que possam fornecer os dados com qualidade para o monitoramento da resistência antimicrobiana [4].

Nos últimos anos, a abordagem designada Saúde Única vem ganhando destaque nas discussões que envolvem a resistência aos antimicrobianos. Dentre inúmeros outros exemplos, a pandemia de COVID-19, cujo vírus parece ter se originado em animais [40], impulsionou o entendimento da relação direta entre a saúde humana, o equilíbrio dos ecossistemas e a saúde dos animais [41]. Alguns organismos internacionais como a OMS, a Organização Mundial de Saúde Animal (OIE), o Programa das Nações Unidas para o Meio Ambiente (UNEP) e a Organização das Nações Unidas para Agricultura e Alimentação (FAO) passaram a trabalhar em conjunto dentro da estratégia Saúde Única. Esta abordagem privilegia a transdisciplinaridade, onde pesquisas e outras iniciativas devem ter uma abordagem ampla, englobando todos os níveis, considerando seres humanos, animais, plantas e meio ambiente [42]. Busca-se ampliar as fronteiras disciplinares para propiciar a participação de vários segmentos da sociedade num trabalho colaborativo. O audacioso objetivo é encontrar o equilíbrio entre saúde, bem estar, preservação do meio ambiente, desenvolvimento sustentável, reconhecendo que seres vivos e meio ambiente são indissociáveis [41].

1.2.4 Resistência microbiana à clorexidina

A questão da resistência aos antimicrobianos é uma preocupação mundial amplamente estudada e debatida, porém, o potencial de resistência microbiana aos biocidas como a clorexidina tem sido deixado em segundo

plano [9]. Este fato pode ser comprovado pela falta de parâmetros ou diretrizes para a realização de estudos. Para a maioria dos antimicrobianos, são bem estabelecidos valores de referência de concentração inibitória mínima (CIM) que possibilitam determinar a susceptibilidade dos diversos microrganismos a cada fármaco. Porém, para os biocidas como a clorexidina, não há definição de pontos de corte de CIM para estabelecer susceptibilidade ou resistência de microrganismos [7,43]. Também não existe relatado na literatura um método padronizado para o teste de susceptibilidade à clorexidina, o que dificulta a comparação entre diferentes estudos [7]. Além disso, as concentrações disponíveis comercialmente são bem mais altas que as definidas nos testes de CIM para clorexidina [7,43].

Além das dificuldades relatadas em relação ao estudo da susceptibilidade microbiana à clorexidina, é importante também mencionar três conceitos que têm sido usados na literatura: resistência, tolerância e persistência. A resistência trata de mecanismos ativos de defesa associados a mutações que são transmitidas de forma hereditária, fazendo com que o microrganismo tenha a capacidade de crescer na presença de altas concentrações de um antimicrobiano e independente do tempo de exposição [9,44]. Tolerância pode ser conceituada como a capacidade, hereditária ou não, de sobreviver à exposição transitória a altas concentrações de um antimicrobiano, sem mudanças nos valores de concentração inibitória mínima (CIM), devido a desaceleração de processos metabólicos [9,44]. Já persistência indica uma habilidade não hereditária de sobreviver a um antimicrobiano pela paralisação de seu metabolismo e total inatividade enquanto durar a exposição ao fármaco [9,44]. De forma geral, a resistência aos antimicrobianos confere ao microrganismo a capacidade de continuar sua replicação e divisão celular mesmo na presença do antimicrobiano, enquanto na tolerância e na persistência microbiana os microrganismos não se replicam na presença do antimicrobiano, mas também não são mortos; uma vez removida a presença do antimicrobiano, a replicação das células microbianas é retomada [9,44]. Pode-se ainda fazer uma analogia dos fenômenos de tolerância e persistência microbianas aos efeitos microbiostáticos dos

antimicrobianos que inibem a divisão celular e replicação dos microrganismos, mas não os matam.

Portanto, a resistência aos antimicrobianos e aos biocidas pode ser intrínseca, envolvendo uma capacidade inata do microrganismo, ou adquirida. Esporos e micobactérias possuem resistência intrínseca à clorexidina devido à constituição de sua parede celular, que apresenta uma barreira à ação da substância [43].

Os mecanismos de resistência adquirida podem se desenvolver através de adaptações fenotípicas ou mudanças genéticas que podem envolver mutações ou aquisição horizontal de material genético como plasmídeos, por exemplo [9]. Adaptações fenotípicas podem ser exemplificadas por redução do metabolismo para possibilitar a sobrevivência ou por condições transitórias como a presença de um biofilme que protege o microrganismo do biocida [43]. Alterações da membrana celular ou da bomba de efluxo de fármacos exemplificam mecanismos de resistência adquiridos geneticamente. Nestes casos, ocorre aquisição de genes comumente encontrados em elementos genéticos móveis como os plasmídeos e que codificam essas funções da célula microbiana [43].

Foram encontradas modificações genéticas nos genes *smvA/R* que codificam o funcionamento da bomba de efluxo em cepas de *K. pneumoniae* que se tornaram resistentes à clorexidina depois de expostas a altas concentrações [11]. No mesmo estudo, foi verificado que este mecanismo confere à bactéria resistência aumentada à colistina, antibiótico utilizado no tratamento de infecções por bactérias multirresistentes [11].

Outro estudo publicado em 2019 fez análise proteômica de microrganismos Gram-negativos expostos à clorexidina. Após sucessivas exposições à clorexidina, valores de CIM aumentados de 4 a 8 vezes foram encontrados e os microrganismos demonstraram susceptibilidade reduzida ao antibiótico colistina, apesar de não terem sofrido exposição ao mesmo [29]. Foram observadas modificações proteômicas nas cepas que se tornaram resistentes em relação às cepas originais utilizadas no estudo. Várias proteínas tiveram sua expressão reduzida e houve aumento na expressão de outras que possuem conexão com mecanismos de resistência a antimicrobianos, como

aquelas relacionadas com metabolismo celular, bombas de efluxo, chaperonas moleculares e movimento de flagelos, dentre outras funções biológicas [29].

Estudo publicado em 2020 também descreveu modificações em nível molecular em isolados de *K. pneumoniae* produtoras de carbapenemase expostas à clorexidina [44]. Em uma das linhagens estudadas, foi identificado um novo fragmento de plasmídeo, chamado PemI/PemK, responsável pelo desenvolvimento de resistência e pela formação de biofilmes [44].

Revisão sistemática publicada em 2020 relatou que, dentre onze estudos clínicos analisados, dois mostraram seleção de microrganismos tolerantes à clorexidina após exposição à substância. A resistência à clorexidina foi atribuída à aquisição de genes e à expressão de proteínas que acionam bombas de efluxo [45]. O estudo concluiu que é necessário manter a vigilância, apesar do desenvolvimento de novos mecanismos de resistência à clorexidina não parecer comum [45].

A associação entre resistência microbiana à clorexidina e a outros fármacos vem sendo relatada também em microrganismos bucais. Saleem e colaboradores detectaram a presença de espécies bacterianas resistentes à clorexidina e a vários antimicrobianos no ambiente bucal, caracterizando resistência a múltiplos fármacos [12]. Na boca, a exposição das camadas mais profundas do biofilme a concentrações mais baixas de clorexidina pode contribuir para o desenvolvimento de resistência à própria substância e a outros antimicrobianos [12].

Portanto, o uso odontológico da clorexidina e a identificação de resistência aumentada à substância em cepas de bactérias consideradas patogênicas, que não são encontradas normalmente na boca, é preocupante [44,46,47]. As principais questões são a possibilidade de resistência cruzada com antibióticos [11,29,48] e o risco da boca atuar como reservatório e propagação desta microbiota patogênica em condições específicas [9].

1.3 OBJETIVOS

1.3.1 Objetivo Geral

Avaliar o estado de saúde bucal e a presença de bactérias patogênicas “não-orais” resistentes à clorexidina na boca de pacientes hospitalizados

1.3.2 Objetivos específicos

Artigo 1: Avaliar o status de saúde bucal e descrever espécies bacterianas patogênicas potencialmente associadas à resistência antimicrobiana encontradas na boca de pacientes hospitalizados.

Artigo 2: Caracterizar espécies bacterianas patogênicas provenientes da boca de pacientes hospitalizados quanto a valores de concentração inibitória mínima para clorexidina, susceptibilidade a antibióticos e susceptibilidade à clorexidina quando cultivadas em biofilme com espécie bacteriana probiótica.

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2 ORAL HEALTH AND THE PRESENCE OF INFECTIOUS MICROORGANISMS IN HOSPITALIZED PATIENTS: A PRELIMINARY OBSERVATIONAL STUDY

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Abstract

Objective: Characterize oral health, and the presence in the oral cavity of pathogenic non-oral microorganisms potentially associated with nosocomial infections and antimicrobial resistance in non-intubated patients admitted to a Brazilian university hospital. **Materials and methods:** An intraoral examination and oral swab were performed in hospitalized individuals at three different times, T1 (within 48 hours of hospitalization), T2 (48 hours after T1) and T3 (7 days after hospitalization). The oral health status was defined by the Oral Health Assessment Tool (OHAT) and Tongue Coating Status (TCS). The swabs containing microbiological samples were processed and microorganisms potentially related to nosocomial infections were phenotypically identified through colony morphology, staining and microscopy. **Results:** A quarter of the patients presented oral lesions; the majority were women (62,1%), no smokers (88%), were under oral feeding (98,1%), and did not need assistance for oral hygiene (95.1%) at T1. The prevalence of potentially pathogenic microorganisms was high and constant from the first 48 hours to the seventh day of hospitalization. The most prevalent microorganisms were *E. coli*, *Enterococcus* spp., *Enterobacter* spp., *Pseudomonas* spp., *Candida albicans*, and *S. aureus*. The oral health status was considered median, and the tongue coating index was considered high throughout the study period. The prevalence of potentially pathogenic non-oral microorganisms was high and constant from the first 48 hours to the seventh day of hospitalization. **Conclusions:** The results point out that the mouth can act as a reservoir of epidemiologically important pathogens within hospital settings, even in patients without mechanical ventilation, thus increasing the risk of nosocomial infections in susceptible individuals.

Keywords: Hospitalization. Mouth. Oral health. Microbiota.

2.1 INTRODUCTION

The human mouth is naturally colonized by a diversified microbiota, composed of about 700 species of bacteria, in addition to fungi, archaea, viruses and protozoa [1], which exhibits commensalism, symbiosis and pathogenic relations with the host. [2] Several factors alter both the homeostasis and the composition of the oral microbiome, such as chemical interactions with enzymes or microorganisms, decreased salivary flow, reduced production of immunoglobulins, presence of proteases and neuraminidase associated with gingivitis and periodontal disease. [3] These conditions are associated with the occurrence of early colonization of the oral cavity by Gram-negative microorganisms and strains resistant to multiple antimicrobials, which can occur during a period of hospitalization. [3]

The study conducted by Cecon *et al.* in 2010 showed time-dependent colonization of the mouth by *Enterobacteriaceae*, *Staphylococcus aureus* and *Candida* spp. in comatose patients who did not receive oral hygiene. [4] When self-care with oral hygiene is compromised, such as hospitalizations in which the patient is restricted to bed or is unconscious, oral health deterioration occurs due to the accumulation of oral biofilm, deposited on the teeth and dental prostheses. [5,6]

Considering that the mouth has a direct relationship with both respiratory and digestive tracts, and that it gathers all the ideal conditions of temperature, humidity and nutrition, there may be microorganisms' translocation between such means, especially in individuals with poor oral hygiene, intubated and systemically weakened. [2] Studies have shown that the mouth acts as a reservoir of mandatory anaerobic respiratory pathogens (belonging to the *Prevotella* and *Fusobacterium* genera) in institutionalized elderly and hospitalized individuals who have poor oral hygiene, which seems to be a risk factor for the development of diseases, such as aspiration pneumonia. [7-9] Colonization of endotracheal tubes by pathogenic microorganisms, related to pneumonia from the mouth, has been reported in patients on mechanical ventilation.[10]

A study demonstrated that the saliva and oral biofilm of non-intubated patients were highly colonized by respiratory pathogens in approximately 14 days of hospitalization that preceded elective myocardial revascularization surgery. [11] These data show that the mouth can act as a reservoir of mandatory anaerobic respiratory pathogens even in patients without mechanical ventilation, increasing the risk of nosocomial pneumonia in

susceptible individuals. [11]

Considering the scarcity of studies on the pathogenic non-oral microorganisms in non-intubated hospitalized individuals, the objective of our study was to evaluate the oral health status and the presence of aerobic microorganisms potentially associated with hospital infections and resistance to antimicrobials in the oral cavity of individuals admitted to a Brazilian university hospital.

2.2 Methodology

2.2.1 Study design

This is an observational, longitudinal study with before-after analysis, developed at the University Hospital of Brasília (Brazil). The study was conducted in accordance with the Declaration of Helsinki and was approved by the Human Research Ethics Committee of the Medicine School of the University of Brasília (Certificate of Presentations of Ethical Appreciation number 87378818.7.0000.5558, technical opinion number 2.628.620).

2.2.2 Study Population

The study was carried out between the months of July and December 2018, with patients hospitalized in the Medical Clinic Ward of the University Hospital of Brasília, Brasília, Brazil. Participants received clarifications about the research and signed the free and informed consent form.

Participants were included if aged 18 years or over, hospitalized for less than 48 hours and without cognitive impairment. They were excluded due to immunosuppression (under chemotherapy, post-transplant, HIV) and if they had a history of recent hospitalizations that occurred in the last 30 days.

2.2.3 Initial interview and clinical record data

Participants were interviewed about their oral hygiene routine performed at the hospital, food route used during hospitalization, alcoholism, smoking and complaints related to oral health.

By consulting the medical record, data were collected on the reasons for hospitalization, presence of comorbidities and medication prescribed during hospitalization and of continuous use.

2.2.4 Oral Health Assessment

Oral health assessment was performed by a single calibrated dental surgeon, in hospital bed, under artificial lighting and in accordance with all biosafety standards. The intraoral examination was performed in three moments, the first happened within 48 hours after hospital admission (T1); the second, performed 48 hours after T1; and the third, performed 7 days after hospital admission (T3).

The OHAT (Oral Health Assessment Tool) and the TCS (Tongue Coating Status) were used to determine the oral health status. The intra-examiner Kappa index obtained for OHAT was 0.83 and for TCI was 0.74.

OHAT assesses soft tissues, saliva quality, presence and appearance of natural teeth and prostheses, oral hygiene pattern and presence of pain. [12] Each item is scored from 0 to 2, with the lowest score representing no change and gradually increasing the score according to the presence of changes. The sum of the scores obtained in each of the items defines the final score, which varies between 0 (very healthy) and 16 (very sick). [12]

The TCS classifies the tongue coating according to its length. [13] A coated tongue can be associated with a range of conditions and occurs when there is accumulation of a layer composed by bacteria, food matter and dead cells on the tongue surface. For this purpose, the division of the tongue body into three thirds is considered and the presence or absence of tongue coating is evaluated, without considering its thickness. Scores vary from 0 to 3, with 0 indicating no visible coating, 1 indicating less than $\frac{1}{3}$ of the tongue body, 2 indicating less than $\frac{2}{3}$ and 3 greater than $\frac{2}{3}$ of the tongue body covered by coating. [13]

2.2.5 Collection of microbiological samples and identification of the pathogenic non-oral microorganisms

In order to identify the presence, in the oral cavity, of non-oral microorganisms potentially related to the occurrence of antimicrobial resistance and hospital infections,

microbiological samples were collected by rubbing a sterile swab on the unilateral vestibular surface of lower molars when present, unilateral vestibular mucosa in the region of lower molars and tongue body. Collections were always performed in the morning, approximately one hour after feeding. The swabs, contained in individual closed packages without culture medium, were transported in a refrigerated container to the Laboratory of Molecular Analysis of Pathogens (LAMP) of the Institute of Biological Sciences at the University of Brasília, where they were processed on the same day of collection.

The samples contained in the swabs were individually homogenized in 1 mL of sterile saline and centrifuged at 10,000 x *g* for 1 minute and 30 seconds at room temperature. The 800-microliter volume of the supernatant was discarded, and the precipitate, contained in the remaining volume, was homogenized again by pipetting in order to perform seeding by exhaustion in plates with chromogenic culture media CHROmagar™*Candida* (BD, Germany) and CHROmagar™ Orientation (BD, Germany).

CHROmagar™ Orientation is used for isolation and differentiation of urinary tract pathogens, but can also be used to differentiate various microorganisms in other infected areas. [14] Its composition has chromogenic substrates that reveal metabolic enzymes of the microorganisms. It allows full differentiation of the pathogens by different color and typical appearance of each microorganism on the plate. [14] CHROmagar™*Candida* is used for isolation and differentiation of major clinical-significant *Candida* species. It provides intense colony coloration helping to differentiate species with high specificity and sensitivity for major *Candida* species. [15]

The microorganisms of the study were identified by means of colony morphology, staining and microscopy. The microorganisms found were isolated and a new inoculation on the same chromogenic culture media was performed to confirm their identification.

2.2.6 Statistical analysis

The data were presented in the form of descriptive statistics such as mean, standard deviation, absolute distribution and percentage of the variables studied. In order to evaluate the effect of the length of hospitalization on oral health indexes (OHAT and TCS) the Friedman test was used, followed by the Dunn post-test, and, for that purpose, the statistical software GraphPad Prism 5.0a Software was used (GraphPad Software Inc., San Diego, CA, USA).

In order to assess differences in the proportion of microorganisms found in each

collection time, the Cochran's Q test was used for related groups and to perform an analysis of the association between the presence of microorganisms and the oral health condition, the Pearson's Chi-square test was performed with continuity correction when necessary. Both analyzes were performed using the statistical software IBM SPSS (Statistical Package for the Social Sciences) 23, 2015.

2.3 RESULTS

Initially, 403 patients and their medical records were evaluated and 300 were excluded. Out of the 103 patients assessed at T1, 63 remained hospitalized at T2 and 46 at T3. (Figure 1).

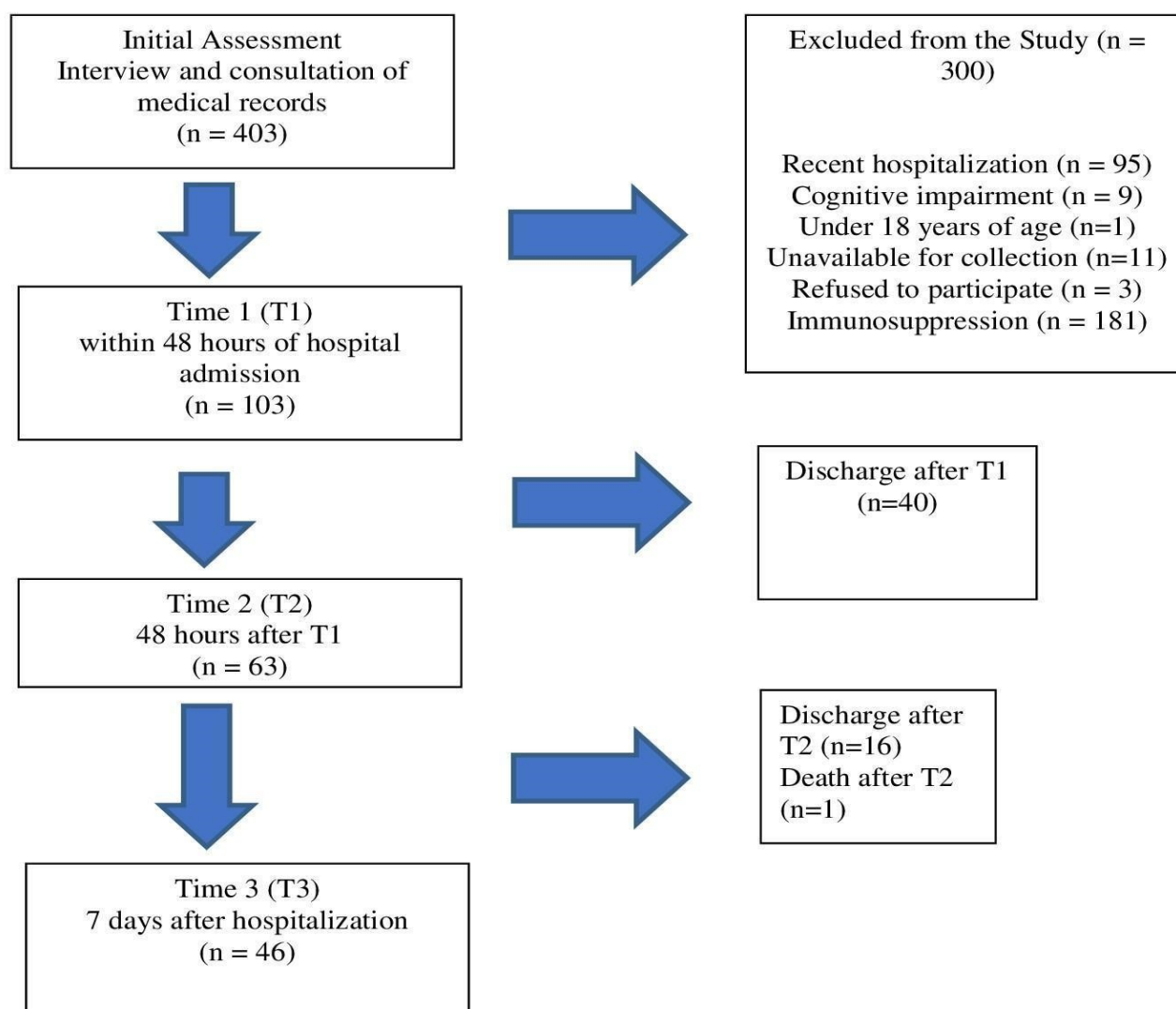


Figure 1 – Flowchart of the clinical phase of the study

Table 1 describes the characterization of the population assessed in the study at T1. At T1, the majority of the patients were women (62.1%), no smokers (88%), were under physiological oral feeding (98.1%), and did not need assistance for oral hygiene (95.1%). About 40% of the patients performed oral hygiene three times per day. The average number of teeth was 13 (± 12), and one-third of the patients were edentulous. About 48.5% of the patients were users of a removable dental prosthesis, and 50% of them did not remove it during sleep. Anti-hypertensive and anticoagulant drugs, besides diuretics, and antimicrobials were the main drugs utilized during hospitalization. Xerostomia was the principal oral complaint. The prevalence of oral lesions was 25.2% at T1 (ulcers, leukoplakia, candidiasis, fibroma, and hyperplasia); 26.9% in T2 and 19.5% in T3. Five patients, who did not present oral lesions at the first examination (T1), developed oral ulcers throughout the seven-days follow-up.

Table 1 - Characterization of the sample composed of participants hospitalized in the Medical Clinic Unit of University Hospital of Brasília from July to December 2018, in T1. Mean and standard deviation (SD) or absolute distribution (n) and percentage (%) of responses (n = 103)

		N	%	Mean(SD)
Sex	Male	39	37,9	-
	Female	64	62,1	-
Age (years)				53 (± 17)
Smoking	Yes	12	11,6	-
	No	91	88,3	-
Route of feeding	Physiological oral route	101	98,1	-
	Other	2	1,9	-
Number of teeth present				13 (± 12)
Total edentulism		34	33	-
Removable prosthesis user	Yes	50	48,5	-
	No	53	51,5	-
Remove the prosthesis to sleep (n = 50)	Yes	9	18	-
	No	41	82	-
Need for oral hygiene assistance	Yes	5	4,8	-
	No	98	95,1	-

Daily frequency of oral hygiene	No	8	7,8	-
	1 x	15	14,6	-
	2 x	39	37,9	-
	3 x or more	41	39,8	-
Main medications used	Anti-hypertensive	58	56,3	-
	Anticoagulants	51	49,5	-
	Diuretics	40	38,8	-
	Antibiotics	33	32,0	-
	Hypoglycemic	26	25,2	-
	Corticosteroids	20	19,4	-
	Statins	20	19,4	-
Main oral health complaints	Xerostomia	55	53,4	-
	Chewing difficulty	28	27,2	-
	Halitosis	22	21,4	-
	Decayed or fractured tooth	18	17,5	-
	Decreased taste	17	16,5	-
	Gingival bleeding	15	14,6	-
Most common oral lesions	Ulcers	6	5,8	-
	Leukoplakia	5	4,8	-
	Candidosis	3	2,9	-
	Fibroma	3	2,9	-
	Hyperplasia	3	2,9	-

Table 2 shows the OHAT and TCS indexes of patients who remained hospitalized in the three assessment periods. The median of the OHAT index was 6.0, and the mean and standard deviation was 6.2 at T1. The OHAT index improved throughout hospitalization. The TCS index was high and remained constant during the evaluation period, as well as oral hygiene.

Table 2 - Oral Health Assessment Tool (OHAT), Tongue Coating Status (TCS) and Oral Hygiene (isolated from OHAT) of the participants hospitalized at the Medical Clinic Unit of University Hospital of Brasilia in the three times of the study, from July to December 2018. Data presented as mean and standard deviation. Friedman's Analysis of Variance; * p <0.05.

	T1 (n = 46)	T2 (n = 46)	T3 (n = 46)	P-value
OHAT	6.2 (± 1.8)	5.9 (± 2.1)	5.8 (± 2.3)	0.036
TCS	2.4 (± 0.9)	2.3 (± 0.8)	2.5 (± 0.7)	0.441
ORAL HYGIENE	1.3 (± 0.8)	1.3 (± 0.6)	1.2 (± 0.6)	0.417

In T1, 103 biological samples were collected, in T2, 63 samples and in T3, 46 samples, totaling 212 samples. Each sample was inoculated on two plates with culture media CHROMagar™ Candida and CHROMagar™ Orientation. A total of 882 microbial colonies were identified in the 212 samples collected.

Table 3 shows the sample frequencies with positive results for each microorganism identified at T1, T2 and T3. The number of microorganisms identified over the three hospitalization times remained stable, with no significant differences. The most prevalent microorganisms were *E. coli*, *Enterococcus* spp., *Enterobacter* spp., *Pseudomonas* spp., *Candida albicans* and *S. aureus*.

Table 3 - Absolute and percentage distribution of microorganisms present in patients admitted to the Medical Clinic Ward at University Hospital of Brasilia in the three collection times (2018). * Cochran's Q test

	T1	T2	T3	P*
	n (%)	n (%)	n (%)	
<i>E. coli</i>	37 (80,4)	36 (78,6)	34 (73,9)	0,584
<i>Enterococcus</i> spp.	32 (69,6)	32 (69,6)	34 (73,9)	0,641
<i>Enterobacter</i> spp.	28 (60,9)	24 (52,2)	21 (45,7)	0,214
<i>Pseudomonas</i> spp.	24 (52,2)	25 (54,3)	25 (54,3)	0,951
<i>C. albicans</i>	24 (52,2)	23 (50,0)	21 (45,7)	0,627
<i>S. aureus</i>	18 (39,1)	19 (41,3)	15 (32,6)	0,444
<i>Candida tropicalis</i>	11 (23,9)	7 (15,2)	9 (19,6)	0,368
<i>Klebsiella</i> spp.	8 (17,4)	6 (13,0)	3 (6,5)	0,232
<i>C. glabrata</i>	5 (10,9)	9 (17,4)	9 (17,4)	0,472
<i>C. krusei</i>	5 (10,9)	7 (15,2)	5 (10,9)	0,695
<i>Proteus</i> spp.	1 (2,2)	1 (2,2)	3 (6,5)	0,135
<i>Streptococcus</i> spp.	0 (0,0)	1 (2,2)	2 (4,3)	0,223
Total	46	46	46	

Table 5 shows the frequency of microorganisms according to the oral health status, using the median of OHAT value. At T1, patients with poorer oral health (OHAT > 6) had a higher frequency of *S. aureus*, *Candida krusei* and *Candida glabrata*, while patients with OHAT ≤ 6 were 5.4 times more likely to have *Escherichia coli* in relation to patients with higher OHAT values (Table 4). At T2, *S. aureus* and *C. krusei* were more frequent in patients with OHAT > 6, while *Enterococcus* spp. was more frequent in patients with OHAT ≤ 6 (Table 5). In T3, there was no statistically significant difference in the frequency of the microorganisms found, according to the division of the OHAT index.

Table 4 - Analysis of cross-sectional association of microorganisms in relation to the OHAT index less than or equal to 6 and greater than 6 of patients admitted to T1 at the Medical Clinic Ward of the University Hospital of Brasília (HUB) (2018).

	OHAT		P*	RC	IC (95%)
	≤ 6	> 6			
	n (%)	n (%)			
<i>E. coli</i>	61 (93,8)	28 (73,7)	0,004	5,446	1,572 - 18,874
<i>Enterobacter</i> spp.	34 (52,3)	17 (44,7)	0,458	1,355	0,607 - 3,026
<i>S. aureus</i>	14 (21,5)	15 (39,5)	0,049	0,421	0,175 - 0,998
<i>Streptococcus</i> spp.	1 (1,5)	0 (0,0)	1,000	-	-
<i>S. saprophyticus</i>	0 (0,0)	0 (0,0)	-	-	-
<i>Enterococcus</i> spp.	55 (84,6)	27 (71,1)	0,099	2,241	0,847 - 5,925
<i>Pseudomonas</i> ssp.	35 (53,8)	14 (36,8)	0,095	2,000	0,881 - 4,541
<i>Proteus</i> spp.	3 (4,6)	4 (10,5)	0,457	0,411	0,087 - 1,946
<i>Klebsiella</i> spp.	10 (15,4)	3 (7,9)	0,425	2,121	0,546 - 8,248
<i>Citrobacter</i> spp.	0 (0,0)	0 (0,0)	-	-	-
<i>C. albicans</i>	23 (35,4)	19 (50,0)	0,145	0,548	0,243 - 1,236
<i>C. tropicalis</i>	10 (15,4)	10 (26,3)	0,176	0,509	0,190 - 1,367
<i>C. krusei</i>	1 (1,5)	4 (10,5)	0,041	0,133	0,014 - 0,926
<i>C. glabrata</i>	5 (7,7)	8 (21,1)	0,049	0,313	0,094 - 0,979
Total	65 (100,0)	38 (100,0)			

* Pearson's Chi Squared Test.

Table 5 - Analysis of the cross-sectional association of microorganisms in relation to the OHAT index less than or equal to 6 and greater than 6 of patients admitted to T2 at the Medical Clinic Ward of the University Hospital of Brasília (2018).

	OHAT		P*	RC	IC (95%)
	≤ 6	> 6			
	n (%)	n (%)			
<i>E. coli</i>	35 (87,5)	16 (69,6)	0,158	3,063	0,842 - 11,138
<i>Enterobacter</i> spp.	19 (47,5)	12 (52,2)	0,721	0,829	0,297 - 2,316
<i>S. aureus</i>	12 (30,0)	13 (56,5)	0,038	0,330	0,114 - 0,958
<i>Streptococcus</i> spp	1 (2,5)	0 (0,0)	1,000	-	-
<i>S. saprophyticus</i>	0 (0,0)	0 (0,0)	-	-	-
<i>Enterococcus</i> spp.	34 (85,0)	14 (60,9)	0,030	3,643	1,091 - 12,168
<i>Pseudomonas</i> spp.	22 (55,0)	10 (43,5)	0,378	1,589	0,565 - 4,465
<i>Proteus</i> spp.	2 (5,0)	2 (8,7)	0,966	0,553	0,072 - 4,213
<i>Klebsiella</i> spp.	3 (7,5)	3 (13,0)	0,783	0,541	0,100 - 2,930
<i>Citrobacter</i> spp.	0 (0,0)	0 (0,0)	-	-	-
<i>C. albicans</i>	18 (45,0)	10 (43,5)	0,907	1,064	0,378 - 2,989
<i>C. tropicalis</i>	8 (20,0)	3 (13,0)	0,722	1,667	0,395 - 7,033
<i>C. krusei</i>	2 (5,0)	5 (21,7)	0,042	0,189	0,033 - 0,972
<i>C. glabrata</i>	5 (12,5)	7 (30,4)	0,158	0,327	0,090 - 1,188
Total	40 (100,0)	23 (100,0)			

* Pearson's Chi Squared Test.

4- DISCUSSION

The present study investigated the oral health status and the presence of pathogenic non-oral microorganisms in the oral cavity of patients hospitalized in the ward, non-intubated and mostly independent for self-care. The oral health status presented mean values that remained from the second to the seventh day of hospitalization. The presence of microorganisms related to the epidemiology of nosocomial infections and resistance to antimicrobials was high and constant in the three times of the study.

In general, the most prevalent microorganisms in our study were *E. coli*, *Enterococcus* spp., *Enterobacter* spp., *Pseudomonas* spp., *Candida albicans* and *S. aureus*. They are all microorganisms that have strains resistant to antimicrobials associated with difficult-to-control nosocomial infections and are, therefore, important from an epidemiological point of view in the worrying scenario of growing prevalence of antimicrobial resistance worldwide. [16] Corroborating these results, findings similar to ours were presented in a study conducted in a hospital in Curitiba (Brazil), with samples collected from the tongue body of patients hospitalized in the intensive care unit (ICU), in which a variety of gram-negative microorganisms, related to nosocomial pneumonia and other hospital infections as well, were identified in three collection times (within 24 hours, 72 hours and 120 hours of admission to the ICU). [17]

Some microorganisms identified in our study, such as *E. coli* and *S. aureus*, had strains with broad antimicrobial resistance described in the latest WHO report published in 2020. This report presented data collected from 78 countries between May and July 2019 and high rates of resistance of various microorganisms to antimicrobials used to treat common bacterial infections were observed. For *E. coli*, the rate of resistance to ciprofloxacin, an antibiotic commonly used to treat urinary infections, ranged from 8.4% to 92.9% in at least 33 countries. [16] The average rate of infections by methicillin-resistant *S. aureus* (MRSA) was 12.11% and by *E. coli* resistant to the 3rd generation of cephalosporins was 36%. [16]

E. coli, *Enterobacter* spp., *Pseudomonas* spp. and *S. aureus* are pathogens known for their involvement in respiratory tract infections acquired within hospital settings. [18] *Pseudomonas* spp. are Gram-negative bacilli that rarely infect healthy patients [19], however, in addition to respiratory infections, they can also cause urinary infections or opportunistic bacteremia acquired mainly by burn victims or those under mechanical ventilation. [20]

C. albicans are commensal organisms that inhabit the oral cavity, gastrointestinal tract and, sometimes, the skin. Under normal conditions, they are non-pathogenic microorganisms, but in immunocompromised patients they are responsible for a large part of systemic fungal infections that are associated with high mortality rate. [21,22]

Some of these microorganisms, identified in our study, are part of a group of bacteria collectively identified by the acronym ESKAPE. [23] This acronym represents six pathogens that have shown increasing virulence and are related to nosocomial infections and resistance to multiple antibacterials. They are *Enterococcus faecium*, *S. aureus*,

Klebsiella pneumoniae, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* spp. [23,24] This group of pathogens is responsible for most nosocomial infections and has been listed by the World Health Organization as a priority in research for the development of new antibiotics. [25]

The microbial profile found in our study may come from community colonization prior to contact with the hospital environment and early colonization from the hospital environment in less than 48 hours of hospitalization. Microorganisms were identified in the first moment of our study, and their presence remained constant until the seventh day of hospitalization. In Tuon et al. (2017) study, the occurrence of MRSA and carbapenem-resistant *Enterobacteria* were also recorded in the initial sample, considering that colonization may have occurred on the patient's admission to emergency care or during recent medical care. [3]

There are studies demonstrating that *Staphylococcus* spp. can be isolated in the oral cavity of healthy individuals. [26] In immunocompetent adult individuals with gingival-periodontal disease, *Staphylococcus* spp. and *Candida* spp were isolated in high number and the prevalence was even higher in patients who used dental appliances.[27] These studies suggest that these microorganisms can colonize the oral cavity regularly, but it is not well established if it is a transient presence.[26,27]

In our study, the microbiological results found may indicate the existence of a transient microbiota that may reach a greater or lesser degree of permanence in the mouth, depending on the conditions of the host. Ewan et al. (2018) study indicated that, in frail patients, hospital pathogens may be present at the time of hospital admission [18], which may have happened to the participants in our study. In addition, we found a higher frequency of *S. aureus* and some *Candida* species in patients whose oral health was considered worse, possibly showing a tendency of associations between these microorganisms. There are studies showing the interaction between *Candida* species and bacteria, such as *S. aureus* and *P. aeruginosa*, with the formation of polymicrobial biofilms in which the relationship between microorganisms occurs synergistically, contributing to greater virulence, mutations and greater resistance to antimicrobials from the microorganisms involved. [28-30]

Although potentially pathogenic microorganisms were detected in our study in the first 48 hours after hospital admission, there were no significant changes in their prevalence at the three observation times. A study with non-intubated elderly hospitalized for 14 days concluded that factors related to the host, such as age, degree of fragility and

comorbidities, had a greater impact on the composition of the oropharynx microbiota than the length of hospitalization. [18] The same study also showed that there was relative stability of this microbiota during hospitalization period. [18] Another study compared a group of unconscious patients and another group with preserved ability to perform their oral hygiene and found a progressive increase in the count of microorganisms in the first group and not in the second over up to seven days. [4] These factors may explain the absence of important variations in the microbial load in the three times of our study, for the sample was mostly composed of independent participants to perform self-care.

The microbiological findings of our study support the idea that, in non-intubated patients, the microorganisms present in the oral cavity can represent a risk for nosocomial infections. A study carried out with non-intubated patients, hospitalized due to lower limb fractures, demonstrated the relationship between nosocomial pneumonia and oral microbiota. [31] The occurrence of pneumonia was associated with the presence of two or more positive oral samples for *S. aureus*, MRSA, *E. coli* or *P. aeruginosa*, at any time of hospitalization, but it was not associated with oral health at certain aspects such as number of teeth or presence of a large amount of dental biofilm in teeth and dentures. [31]

In relation to oral health, the most reported complaint by the study participants was xerostomia, followed by chewing difficulty and halitosis. The use of a large number of medications during hospitalization may be related to reports of xerostomia and halitosis. [32] Although we did not measure the salivary flow, this complaint may point out its reduction. There are studies showing the relationship between hyposalivation and increased oral colonization by microorganisms, such as *Candida* spp. [33,34] In our study, a prevalence of *C. albicans* was found in approximately 50% of the samples at the three collection times, which may indicate some relationship with the xerostomia reported by the patients. The chewing difficulty presented is probably related to the large number of totally edentulous participants, of whom, a quarter did not have dental prosthesis. In our study, neither the worsening of oral health detectable by the indexes used was identified, nor the worsening of oral hygiene itself during the three assessment times. However, findings in the literature reveal worsening of oral hygiene with increased accumulation of bacterial biofilm and tongue coating during a 72-hour to a 14-day hospitalization period. [6,35-38] Available studies in the literature deal with patients hospitalized in ICUs, therefore, one of the factors suggested to explain our results was the autonomy presented by most patients to perform their own oral hygiene, for only five of them (4,85%) needed assistance to perform this task. In a study conducted with patients hospitalized for fractures of the lower

and non-ventilated limbs, there was a greater increase in the dental biofilm score among the most dependent, the ones with decreased mobility, the most fragile and the ones with dementia. [36]

In the present study, about 25% of the participants presented oral lesions in each of the three moments of the evaluation. A study carried out with patients hospitalized for infectious diseases reported a prevalence of oral lesions in 84,3% of them. [39] Although our study showed a lower prevalence of oral lesions than the one mentioned above, which probably occurred due to the different profile of the sample, it can still be considered a high prevalence, which demonstrates the need for a dental surgeon in multidisciplinary team within hospital settings, as most of these injuries cause considerable discomfort, reduced quality of life for the patient and the need of specific attention.

The oral health status remained median between the second and the seventh day of hospitalization. The tongue coating index was considered high throughout the follow-up period. The prevalence of potentially pathogenic microorganisms and related to the transmission of resistance to antimicrobials in the mouth was high from the first 48 hours to the seventh day of hospitalization. Although the composition of these microorganisms has not undergone significant changes during hospitalization, the identification of microorganisms related to nosocomial infections and resistance to antimicrobials, may suggest the occurrence of previous or early colonization of the oral cavity.

Among the limitations in our study, we can mention the loss of follow-up of participants due to hospital discharge or death. In addition, the index used to measure patients' oral health conditions evaluated categories that did not change for seven days, such as number of teeth and use of removable prosthesis. These factors may have affected the results of the study as we couldn't detect the worsening in oral health and hygiene during the three assessment times. Furthermore, although the chosen culture media are considered to have good sensitivity and specificity for isolation and identification of most microorganisms at species or genus level, there is a group of four Enterobacteriaceae (*Klebsiella* spp., *Citrobacter* spp., *Enterobacter* spp. and *Serratia* spp.) that cannot be differentiated among them [14,40,41], what may affect the accuracy of part of the results. However, considering these four microorganisms as a group, the accuracy in identifying pathogenic anaerobic microorganisms by the chromogenic culture medium is between 96% and 100% compared to other methods of phenotypic identification (colony morphology and biochemical tests) and molecular methods such as PCR, genetic and protein sequencing. [14,15,40-42]

The results of this study point out that the mouth can act as a reservoir of epidemiologically important pathogens within hospital settings even in patients without mechanical ventilation, increasing the risk of nosocomial infections in susceptible individuals.

The present study is the first part of a larger research that intends to perform the isolation and differentiation of all the found microorganisms using MALDI-TOF mass spectrometry or molecular methods such as polymerase chain reaction (PCR) and genetic sequencing. The aim is to evaluate the susceptibility of each species to chlorhexidine. The minimal inhibitory concentration for chlorhexidine will be determined for all the depicted isolates. Species with altered susceptibility to chlorhexidine will be submitted to antibiogram analysis to identify possible resistance to antimicrobials for clinical use in humans.

5 CONCLUSION

The oral health status was considered average, and the tongue coating index was considered high throughout the study period. The prevalence of potentially pathogenic non-oral microorganisms was high and constant from the first 48 hours to the seventh day of hospitalization. It may suggest colonization of the mouth before hospitalization or during the first hours within hospital settings.

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Declaration of interest statement

All authors declare that there are no conflicts of interest with the topic addressed.

Author contributions statement

All authors were involved in the conception and design of the work. ASCC, YPF and DMG were involved in the acquisition of data. ASCC, HSM, VPM and ENL were involved in the analysis and interpretation of the data. All authors were involved in drafting the manuscript. All authors reviewed and approved the final version and agree to be held accountable for all aspects of the work.

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Ethical approval

The study was approved by the Committee of Ethics in Research with Human Beings of the Medical School at the University of Brasília and all the patients signed the Free and Informed Consent Form.

Availability of Data and Materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request and were deposited at the Open Science Framework (OSF)https://osf.io/dp7qb/?view_only=aed524399f494421b12eb71876ce6934.

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3 CHLORHEXIDINE RESISTANCE IN PATHOGENIC BACTERIA ISOLATED FROM THE MOUTH OF HOSPITALIZED PATIENTS

ABSTRACT

Chlorhexidine is an antiseptic often used for oral hygiene, as well as other uses in hospital settings. Nevertheless, concerns have arisen regarding potential adverse effects of this substance, including the development of resistance to the substance itself, the spread of cross-resistance to other antimicrobials, and the development of multidrug-resistant microorganisms. The aim of this study was to characterize the pathogenic microbiota present in the mouth of hospitalized patients in terms of susceptibility to chlorhexidine, susceptibility to antibacterial drugs and the possible change in resistance profile when associated in biofilms with a probiotic strain of *Streptococcus salivarius*. The clinical strains were isolated from oral swabs of non-intubated patients hospitalized in the ward. A minimum inhibitory concentration (MIC) test was carried out to determine the sensitivity of the bacterial isolates to chlorhexidine. Isolates with altered results were subjected to antibiograms in which various antibacterials were tested. Multidrug-resistant isolates were assessed for their chlorhexidine resistance profile in single biofilms or in association with *S. salivarius*. Of the isolates analysed, 17.4% had reduced sensitivity to chlorhexidine. Resistance of certain isolates to both chlorhexidine and antibiotics was observed. The association with *S. salivarius* in biofilms increased the chlorhexidine susceptibility of pathogenic multidrug-resistant bacteria. The results highlight the oral cavity as a potential reservoir for pathogenic microorganisms that exhibit multidrug resistance to both antibacterials and chlorhexidine, and demonstrate that co-cultivation in biofilms with the probiotic bacteria *S. salivarius* can enhance the susceptibility of resistant microorganisms to chlorhexidine.

Key-words: chlorhexidine; microbial drug resistance; mouth; microbiota; hospitalization; *Streptococcus salivarius*

3.1 INTRODUCTION

Chlorhexidine is a disinfectant and antiseptic widely used in clinical and home settings. In the field of Dentistry, chlorhexidine is employed as an antiseptic for multiple indications [1] and remains a preferred choice for oral hygiene maintenance in intubated patients within Intensive Care Units (ICUs) [2]. Nevertheless, concerns have arisen regarding potential adverse effects of this substance, including the development of antimicrobial resistance and an increased risk of mortality among hospitalized patients [3,4]. Previous studies have shown that the use of chlorhexidine as mouthwash causes a shift in the composition of oral microbiota [5]. In hospitalized patients, it can lead to dysbiosis that can favor colonization by pathogenic microorganisms existing in the hospital environment [6].

The oral microbiome is critical to the balance of oral health, and there is increasing evidence that it is also related to systemic health [7]. The microbiota composition in hospitalized patients seems to play a critical role, since hospital environments harbor multidrug resistant microorganisms that can colonize the mouth and other sites in the human body [8]. In this scenario, the use of chlorhexidine can both contribute to the development of resistant microorganisms and to make the oral cavity more susceptible to their colonization [4].

There is no consensus on the role of the dental use of chlorhexidine in the development of resistant microorganisms to the substance itself and to other antimicrobials [9–11]. Nor, it is clear whether the oral cavity can be an important setting for the development and spread of chlorhexidine-resistant microorganisms [9–11]. It is important to establish whether the mouth plays a relevant role as a reservoir for chlorhexidine-resistant microorganisms.

Considering the potential role of chlorhexidine in the growing problem of microbial drug resistance, the aim of this study was to characterize pathogenic microorganisms isolated from the mouth of non-intubated hospitalized patients in terms of susceptibility to chlorhexidine, susceptibility to antimicrobial drugs, and the outcome of the probiotic strain of *S. salivarius* in the formation of mixed biofilms and susceptibility to chlorhexidine.

3.2 METHODOLOGY

3.2.1 Microorganisms

The clinical strains used in this study were isolated from oral swabs collected from patients hospitalized in the Medical Clinic Ward of the University Hospital of Brasília, Brazil, in 2018. The swab collection was conducted in three moments; the first one within 48 hours after hospital admission (T1); the second was performed 48 hours after T1; and the third was performed 7 days after hospital admission (T3). The strains isolated and clinical characteristics of patients were widely described in a study published in 2022 [12].

S. salivarius K12 was kindly donated from the Division of Oral Biology of the University of Leeds, United Kingdom. All isolates were stored at -80 °C in a glycerol stock solution to maintain the viability of microorganisms.

The study was conducted in accordance with the Declaration of Helsinki and was approved by the Ethics Committee of the Medicine School of the University of Brasília (Certificate of Presentations of Ethical Appreciation number 87378818.7.0000.5558, technical opinion number 2.628.620).

3.2.2 Determination of chlorhexidine minimal inhibitory concentration

The guideline “Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically” [13], published by the Clinical and Laboratory Standards Institute (CLSI) in 2012, was used as a reference to determine the minimum inhibitory concentration (MIC) for chlorhexidine.

The inoculum was prepared by transferring 10 µL of each isolate to tubes containing BHI broth (Brain-Heart-Infusion media). After 24 hours at 37 °C, the isolates were inoculated in plates of Chromagar Orientation™ media to confirm their isolation and identification. After growth, the microorganisms were inoculated in tubes containing Muller-Hinton broth. The final inocula were adjusted to the density of a 0.5 McFarland standard in order to achieve bacterial suspension of $1,5 \times 10^8$ CFU/mL.

The assay to determine the MIC for chlorhexidine was performed using the broth microdilution method in 96-well plates containing Muller-Hinton media,

as recommended by the CLSI manual for susceptibility tests involving aerobic or facultative anaerobic microorganisms. In each row of the plate, identified with the letters A to H, an isolate was analyzed. In the wells of column 1 only Muller-Hinton media (200 μL) was placed to obtain a negative control for contaminations (no bacterial growth was expected in this column). In the wells of column 2, the culture medium (100 μL) was placed with the bacterial inoculum (100 μL) without chlorhexidine addition for a positive control of bacterial growth. In columns 3 to 12, bacterial inoculum (100 μL) and increasing concentrations of chlorhexidine (100 μL) were placed, comprising the following final concentrations 0.18 $\mu\text{g/mL}$, 0.37 $\mu\text{g/mL}$, 0.75 $\mu\text{g/mL}$, 1.56 $\mu\text{g/mL}$, 3.12 $\mu\text{g/mL}$, 6.25 $\mu\text{g/mL}$, 12.5 $\mu\text{g/mL}$, 25 $\mu\text{g/mL}$, 50 $\mu\text{g/mL}$, 100 $\mu\text{g/mL}$ (Figure 1). The plates were incubated at 37 °C for 24 hours. After this period, the plates were analyzed to verify the microorganism's growth. The MIC was the lowest concentration of chlorhexidine that could prevent visible growth of each microorganism.

The MIC values were organized in a table to be compared with other values found in the literature for each identified microorganism. The cutoff values to define the susceptibility or resistance of each microorganism to chlorhexidine were defined based on the average values found in the literature. A comparison was also made between the MIC values of isolates from different collection moments, that is, at the beginning and after seven days of the patient's hospitalization, to detect whether there was a change in the pattern of susceptibility of microorganisms to chlorhexidine throughout the hospitalization period.

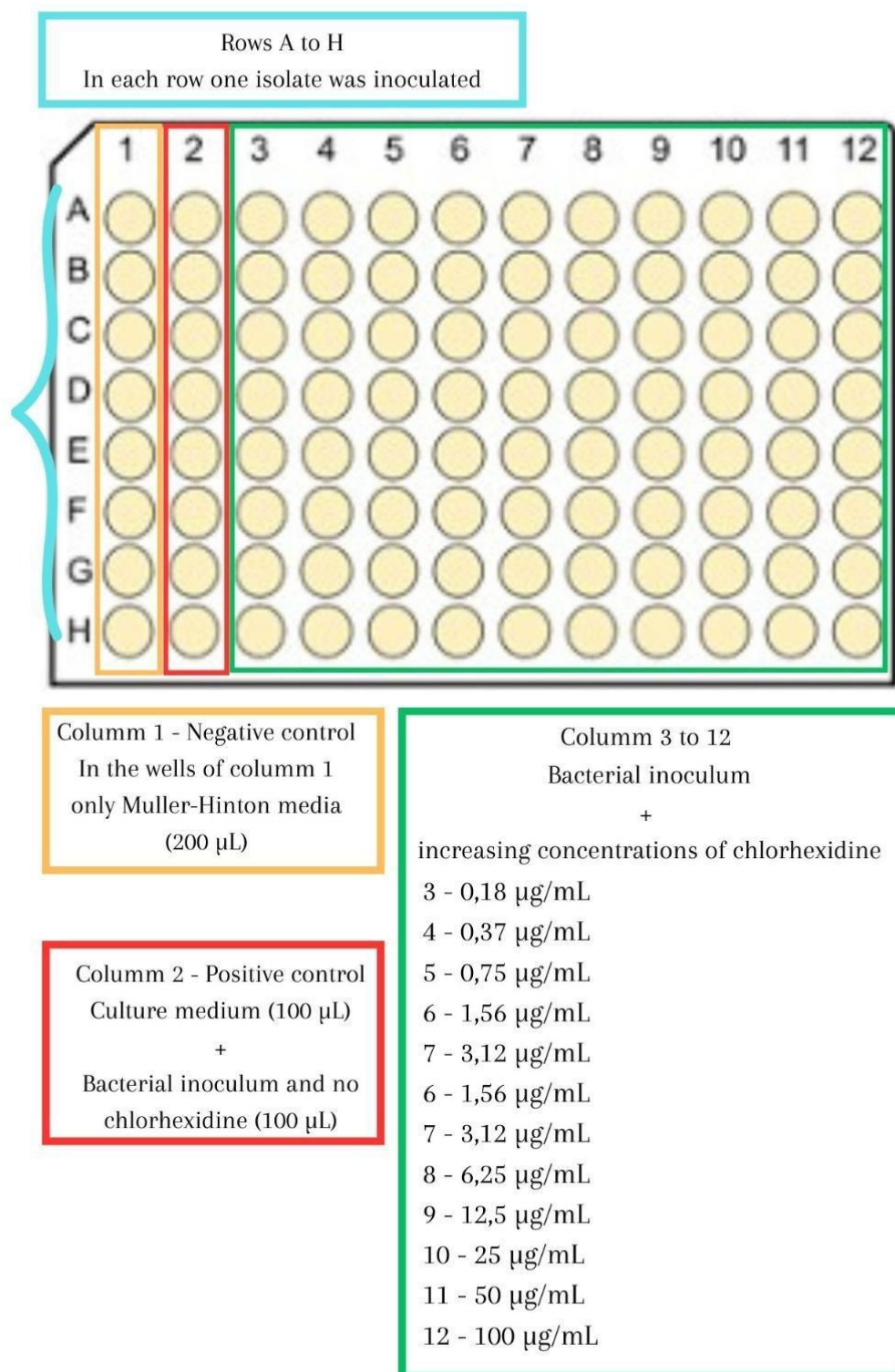


Figure 1. Schematic representation of chlorhexidine concentrations employed in the preparation of a 96-well plate for determination of the minimum inhibitory concentration.

3.2.3 Determination of susceptibility to antimicrobial drugs

Isolates that showed altered susceptibility values to chlorhexidine were subjected to susceptibility tests to other antimicrobials, mainly drugs used in the treatment of hospital infections. Laborclin (Solabia Group) antibiogram disks were used to perform the disk diffusion method as described by Kirby-Bauer [14]. The antibiotics tested were polymyxin (300 UI), tobramycin (10 µg), amikacin (30 µg), norfloxacin (10 µg), cefepime (30 µg), ciprofloxacin (5 µg), piperacillin/tazobactam (100 µg / 10 µg), levofloxacin (5 µg), lomefloxacin (10 µg), ofloxacin (5 µg), gentamicin (10 µg), ticarcillin/clavulanic acid (75 µg / 10 µg), aztreonam (30 µg), meropenem (10 µg) and imipenem (10 µg).

The microorganisms were inoculated in tubes containing Muller-Hinton broth. The final inocula were adjusted to the density of a 0.5 McFarland standard to achieve bacterial suspension of $1,5 \times 10^8$ CFU/mL. Each isolate was seeded uniformly covering the entire surface of Muller-Hinton agar placed on a plate. The paper disks saturated with each antimicrobial agent were placed on the surface of Muller-Hinton Agar. The plates were incubated overnight at 37 °C and on the next day it was measured the diameter of the zone of growth inhibition around the disks. The zone diameters were interpreted as resistant, intermediate, or sensitive values for each antimicrobial drug according to CLSI standards [15].

3.2.4 Biofilm formation

The isolates that were resistant to chlorhexidine in the MIC assays and also presented resistance to at least seven antimicrobial drugs in the antibiogram tests were assessed for susceptibility to chlorhexidine in single biofilms and in dual-species biofilms with *S. salivarius* K12, a probiotic strain. A total of three isolates meeting these criteria were identified: one of *Pseudomonas aeruginosa*, one of *Staphylococcus aureus*, and one of *Escherichia coli*.

Each isolate was grown overnight, aerobically at 37 °C, on Brain-heart-infusion (BHI) agar plates. The inoculum was prepared growing each isolate in

tubes with 5 mL of BHI broth that were incubated overnight, aerobically at 37°C. The optical density (OD) of each inoculum was adjusted to 1.0 of the McFarland standards.

The assay to grow the biofilms and test their susceptibility to chlorhexidine was performed on the Calgary biofilm devices (CBD). The CBD is a 96-well plate where the lid has 96 pegs that seat in the wells of the plate. It is a model system to mimic the *in vivo* situations using *in vitro* devices to study susceptibility to antimicrobials in microbial biofilms [16].

The pegs were coated with sterile saliva previously prepared and incubated overnight at 37 °C on a shaker at 65 rotations per minute (RPM). The saliva preparation was made adding 25 µL of DTT 2.5 molar to 5 mL of collected saliva. It was processed by centrifugation for 20 minutes at 7000 x g at 4 °C. The supernatant was diluted 50% on phosphate buffered saline (PBS) and filtered through 0.2 µm membrane for sterilization.

In each row of the plate, identified with the letters B to G, one biofilm with different composition was cultivated. The wells close to the edge of the plate were not used due to the risk of contamination. There were seven biofilms with different compositions: the three resistant isolates in single biofilms, the probiotic strain in single biofilm and dual-species biofilms combining each resistant isolate and the probiotic strain. After the first 24 hours of the pegs immersion in saliva, the wells were filled with the inoculum and the media was changed daily for a fresh BHI during seven days.

In the seventh day of biofilm cultivation, the media of the wells was exchanged for BHI with different concentrations of chlorhexidine. Three different concentrations of chlorhexidine were used in each plate: 0.007%, 0.03% and 0.12% in plate 1 and 0.003%, 0.015% and 0.12% in plate 2. The 0.12% concentration was chosen because it is the most used clinically in Dentistry. The other concentrations were chosen according to the microorganism's MIC for chlorhexidine. The pegs were submerged for 10 minutes in these chlorhexidine solutions at 37 °C on a shaker at 65 RPM. After the 10-minute treatment with chlorhexidine, the pegs were washed twice in plates with wells filled with BHI broth. The plates were kept under incubation at 37 °C on a

shaker at 65 RPM and the media were changed daily for fresh BHI during three days.

At the end of three days, the pegs that would be used for confocal were snipped off the CBD plates with sterile pliers. The plates with remaining pegs were stored at -80 °C until further experiments.

3.2.5 Confocal microscopy

The pegs appointed for confocal (0.12% chlorhexidine concentration) were washed in another plate with sterile PBS to remove loosely adherent cells and then incubated in the LIVE/DEAD staining (Molecular Probes, Inc.) for 30 minutes at room temperature, in the dark.

Samples were scanned using a Confocal Laser Scanning Microscope (Leica Microsystems) at 1- μm intervals. All samples were observed by an oil lens and all images were taken at the same magnification of 630x. Three-D images were generated and the images of biofilm layers were combined into a 2D image to calculate the area of red and green fluorescence (ImageJ software; RGB measurement tool).

The viable cells counting was performed using the Image J software (or maximum projection and RGB measurement). Rates of biofilm viability were calculated as proportion of green fluorescence areas.

3.2.6 DNA isolation

To analyze bacteria proportion by quantitative PCR, it was performed the DNA extraction from the biofilms. The pegs were snipped off the CBD plate and harvested by carefully scraping each peg with a sterile dental curette. Attached biofilm was scraped by the same person for 5 minutes, by going around each three times. The protocol for DNA extraction was performed as described for Ausubel et al. [17]. The scrapped material was placed in a microcentrifuge tube with 200 μL of Tris-EDTA buffer solution and vortexed for 3 minutes before centrifugation at 12.000 x g for 3 minutes. The supernatant was discarded, and 50 μL of lysozyme (10 mg/mL) and 1 μL of RNase A (5 mg/mL) were added.

The suspension should be thoroughly mixed on a vortex for 10 seconds and incubated at 37 °C for at least one hour.

Following incubation, 70 µL of 10% SDS (Sodium Dodecyl Sulfate) and 5 µL of proteinase K (10 mg/mL) were added. The solution was vortexed and incubated at 65 °C for 20 minutes. After this period, 100 µL of 5 M NaCl and 100 µL of the CTAB/NaCl solution preheated to 65 °C in a water bath were added. The solution was vortexed once more until a milky appearance was achieved, after which it was incubated at 65 °C for 10 minutes. After incubation, 750 µL of a chloroform and isoamyl alcohol solution (24:1) was added, followed by vortex homogenization for 10 seconds. The mixture was then centrifuged at 12,000 x *g* for 5 minutes, and the supernatant (approximately 200 µL) was carefully transferred to a clean microcentrifuge tube. Subsequently, 120 µL of isopropanol at -20 °C was added, and the mixture was placed in a freezer at -20°C for a minimum of 30 minutes.

The mixture was centrifuged at 12,000 x *g* for 5 minutes and subsequently washed twice with -20 °C 70% ethanol, followed by centrifugation at 12,000 x *g* for 3 minutes at the end of each wash. The precipitated DNA was air-dried at room temperature. Once dry, 50 µL of sterile deionized Milli-Q water was added to the DNA. The concentration and purity of the DNA were determined using a nanodrop spectrophotometer at 260 nm and 280 nm.

3.2.7 Quantitative real-time polymerase chain reaction (qRT-PCR)

The qPCR assays followed the protocol previously described in 2012 by Ammann et al. [18]. At first, the qRT-PCR was conducted for the *Pseudomonas* spp. isolate, which stood out due to its higher resistance to antibiotics and chlorhexidine. There were available specific primers to target the following genes: *oprL* for *Pseudomonas aeruginosa*, *nucA* for *Staphylococcus aureus*, and *uidA* for *Escherichia coli*. The DNA intercalating dye Sybr Green was employed as part of the method. The total reaction volume for each assay was 15 µL, comprising 7.5 µL of Sybr Green PCR Master Mix, 6 µL of DNA extract (diluted to contain 0.1 ng of DNA), and 1.5 µL of a primer solution (10 µM, consisting of a combination of forward and reverse primers). The amplification

steps were set as follows: 2 min at 50 °C, followed by 10 min at 95 °C, then 40 cycles of 15 sec at 95 °C and 1 min at 60 °C.

To generate standard curves, pure cultures of each microorganism were grown overnight, aerobically at 37 °C. Inoculum was prepared growing each isolate in tubes with 5 mL of BHI broth. DNA was isolated from these cultures as described above in section 3.2.6. The standard curves were generated with 5 different points using a 1:10 serial dilution of DNA samples, from each different bacteria, ranging from 10 ng to 0.001 ng per qPCR reaction. The standard curve for each pair of primers should have high linearity ($R^2 = 0.99$).

From the obtained crossing point (Cp) values, the DNA concentration was calculated as unto the theoretical genome weight of each microorganism. The number of copies of each microorganism was determined using the following formula: $n^\circ \text{ of copies} = (x \text{ ng} * 6.0221 * 10^{23}) / (N * 660 \text{g} / \text{mol} * 10^9 \text{ ng/g})$. Where, $x \text{ ng}$ = the amount of DNA from the samples that was extrapolated from the standard curve of each microorganism species; N = genome size of each microorganism species based on genome sequence published in NCBI gene bank.

3.2.8 Statistical analysis

The data was presented in the form of descriptive statistics using the absolute frequencies and percentages pertaining to the conditions of interest, namely: samples classified as resistant or susceptible to chlorhexidine, as well as the proportion of bacteria and their viability within biofilms after treatment with chlorhexidine.

To assess the effects of hospitalization on the proportion of samples resistant to chlorhexidine at T1 and T3, the Mann-Whitney test for unpaired groups was conducted. For the qRT-PCR data, a 2-way Anova test and Tukey procedure were used to analyze differences in bacterial viability after chlorhexidine treatment in the samples of single and dual-species biofilms. The statistical analyses were conducted in Jamovi (v. 2.3.28.0). For all analyses, $p < 0.05$ was considered statistically significant.

3.3 RESULTS

A total of 444 clinical isolates are stored, including *Candida* spp. and various genera and species of bacteria, derived from oral swabs collected from hospitalized patients (Figure 2). Among these, 151 bacterial isolates were selected for the determination of MIC for chlorhexidine, originating from patients who remained hospitalized for at least seven days, meaning they were swabbed within the first 48 hours (T1) and on the seventh day of hospitalization (T3). Since some microorganisms did not exhibit growth, MIC values were obtained for 69 isolates, of which 17.4% showed resistance to chlorhexidine, corresponding to 12 isolates (Table 1).

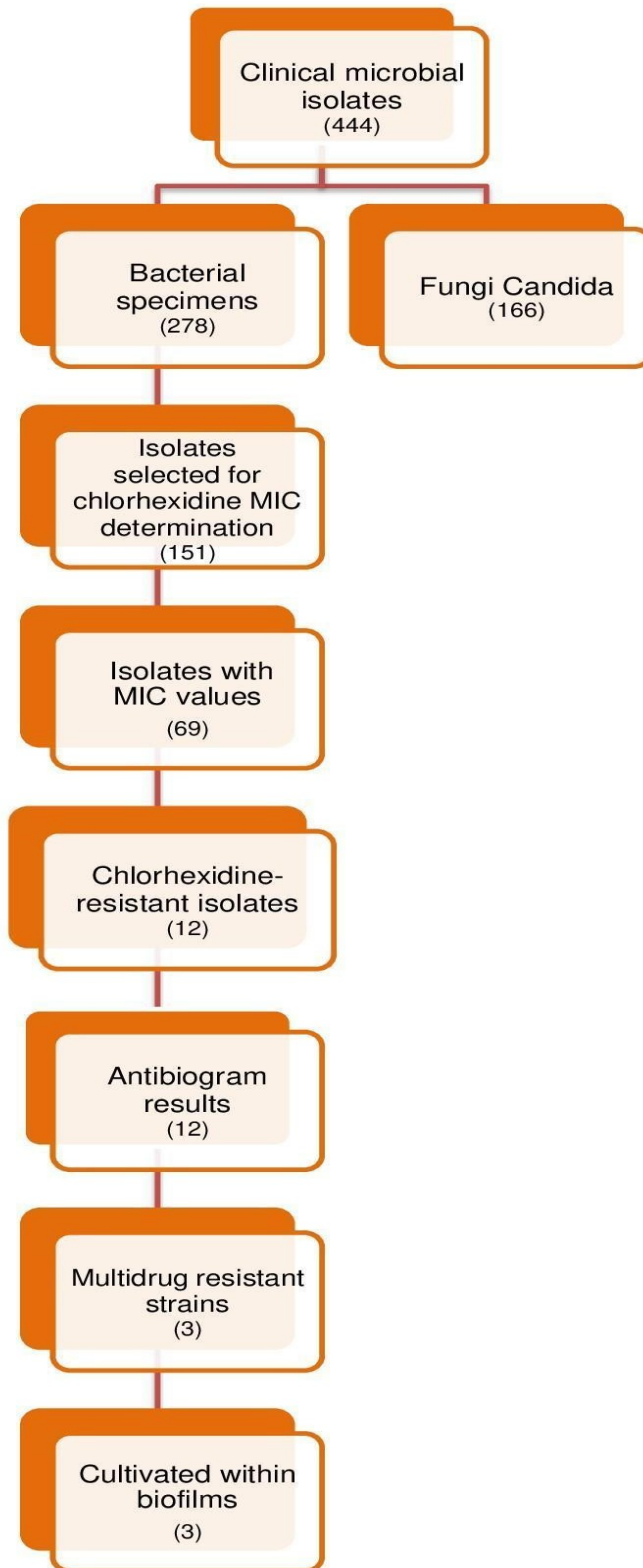


Figure 2. Flowchart of the selection process for microbial samples.

Table 1. Isolates from oral samples of patients admitted to the University Hospital of Brasilia between July and December 2018, classified according to chlorhexidine susceptibility. (S = susceptible, I = intermediate, R = resistant)

Phenotypic Identification	Chlorhexidine susceptibility									TOTAL
	Time 1			Time 2			Time 3			
	S	I	R	S	I	R	S	I	R	
<i>E. coli</i>	0	0	1	0	0	0	0	0	1	2
<i>Enterobacter</i> spp.	2	1	0	0	1	0	2	1	0	7
<i>Enterococcus</i> spp.	2	2	0	2	2	0	2	1	0	11
<i>Klebsiella</i> spp.	0	2	1	0	2	2	0	2	1	10
<i>Proteus</i> spp.	0	0	0	0	0	0	2	1	1	4
<i>Pseudomonas</i> spp.	1	1	0	2	0	1	3	1	2	11
<i>S. aureus</i>	0	7	0	0	8	0	0	7	2	24
TOTAL	5	13	2	4	13	3	9	13	7	69

The MIC values adopted for characterizing microorganisms in terms of susceptibility to chlorhexidine are detailed in Table 2. These values were determined by the authors based on averages derived from articles found in the literature [19–25] as there is no official standardization for chlorhexidine, as exists for other antimicrobials.

Despite the observed increase in the number of chlorhexidine-resistant isolates over the course of hospitalization, as depicted in Table 1, the statistical comparison of chlorhexidine resistance/susceptibility profile between isolates

collected at T1 and T3 did not reveal significant differences, as depicted in Figure 3 ($p = 0.745$).

Table 2. Minimum Inhibitory Concentration (MIC) values adopted for susceptibility classification to chlorhexidine of microorganisms isolated from oral samples of patients admitted to the University Hospital of Brasília between July and December 2018.

Genus/species	Susceptible (less than X $\mu\text{g/mL}$)	Intermediate (between X $\mu\text{g/mL}$ and Y $\mu\text{g/mL}$)	Resistant (greater than Y $\mu\text{g/mL}$)
<i>E. coli</i>	0,5	0,5 – 5	5
<i>Enterobacter</i> spp.	10	10 – 48	48
<i>Enterococcus</i> spp.	2,5	2,5 – 32	32
<i>Klebsiella</i> spp.	0,5	0,5 – 45	45
<i>Proteus</i> spp.	10	10 – 50	50
<i>Pseudomonas</i> spp.	10	10 – 50	50
<i>S. aureus</i>	0,5	0,5 – 8	8

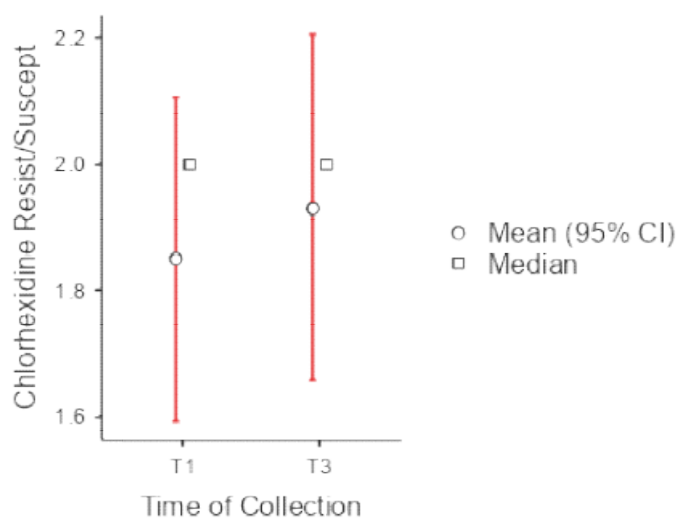


Figure 3. Comparison of the chlorhexidine resistance/susceptibility profile between isolates collected from the mouth of hospitalized patients at T1 and T3 (Mann-Whitney Test, $p = 0.745$).

Antibiograms were performed for the 12 bacterial isolates that were categorized as resistant in chlorhexidine susceptibility tests. Table 3 shows the results of antibiogram, with resistance-indicative values highlighted in red.

Table 3. Antibiogram values of 12 clinical isolates non-susceptible to chlorhexidine isolated from oral samples of patients admitted to the University Hospital of Brasília between July and December 2018.

N. sample	Identification	Polymyxin (POL300)	Tobramycin (TOB10)	Amikacin (AM130)	Norfloracin (NOR10)	Cefepime (CPM30)	Ciprofloxacin (CIP5)	Piperacillin/Tazobactam (PPT110)	Levofloxacin (LVX5)	Lomefloxacin (LMX10)	Ofloxacin (OFX5)	Gentamicin (GEN10)	Ticarcillin/Clavulanic acid (TAC85)	Aztreonam (ATM30)	Meropenem (MER10)	Imipenem (IPM10)
2012D	<i>Pseudomonas</i> spp.	13	22	24	>30	22	30	26	24	20	20	20	20	20	26	28
3012A	<i>Pseudomonas</i> spp.	12	20	22	>30	24	>30	24	22	20	18	18	18	18	24	22
2014F	<i>Klebsiella</i> spp.	12	14	20	22	18	24	20	26	22	22	20	14	16	22	20
3042E	<i>Pseudomonas</i> spp.	8	14	14	0	7	7	12	12	0	8	0	0	12	8	13
4049E	<i>Proteus</i> spp.	0	22	20	>30	>30	>30	24	>30	32	32	20	16	32	24	22
3056B	<i>S. aureus</i>	10	14	16	8	0	8	0	8	0	0	7	0	0	0	20
1074C	<i>E. coli</i>	12	18	20	>30	>30	>30	24	22	30	30	26	20	>30	30	30
1082D	<i>Klebsiella</i> spp.	10	18	18	30	30	32	22	30	28	28	16	22	22	22	20
3092D	<i>E. coli</i>	14	12	16	0	22	0	24	0	0	0	14	14	20	30	30
2094D	<i>Klebsiella</i> spp.	12	20	14	32	30	32	22	30	28	26	16	22	20	20	18
3094H	<i>S. aureus</i>	10	24	22	30	>30	>30	>30	30	26	24	20	>30	8	>30	>30
3094I	<i>Klebsiella</i> spp.	12	24	24	>30	>30	>30	22	>30	30	30	18	18	22	18	20

Within the antibiogram results, three multidrug-resistant isolates were identified: one sample of *Pseudomonas* spp., one of *S. aureus*, and one of *E. coli*. They were cultured in biofilms separately or in association with the probiotic bacterium *S. salivarius* K12.

Subsequently, one sample from each biofilm that had been treated with 0.12% chlorhexidine was scanned using a confocal laser scanning microscope. Rates of biofilm viability were calculated in ImageJ software (National Institute of Health, USA) as proportion of green fluorescence areas (Figure 4). It appears that there was a reduction in cellular viability within the biofilms where an association of *Pseudomonas* + *S. salivarius* and *S. aureus* + *S. salivarius* occurred. However, it is not possible to ascertain which of the species experienced diminished viability in this analysis.

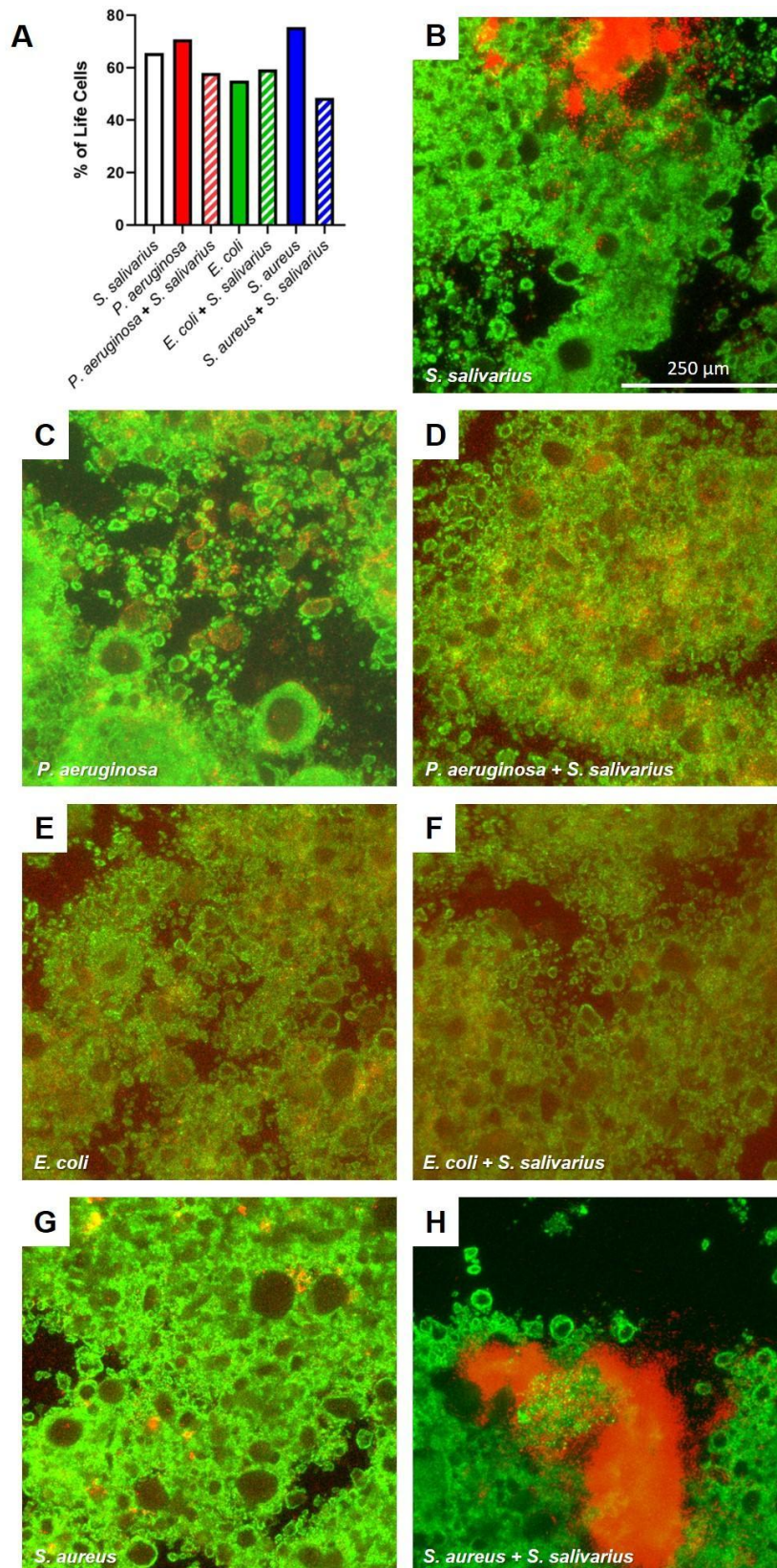


Figure 4. Rates of biofilm viability in single and dual biofilms. A), percentage of life cells in biofilm images (green fluorescence). B – H, images of biofilms used to generate the graph of life cells (A). Life cells are labeled by green fluorescence and dead cells are in red fluorescence. The scale bar is representative for images B to H. The microorganisms in each biofilm are: (B) *S. salivarius*; (C) *P. aeruginosa*; (D) *P. aeruginosa* + *S. salivarius*; (E) *E. coli*; (F) *E. coli* + *S. salivarius*; (G) *S. aureus*; (H) *S. aureus* + *S. salivarius*.

Quantitative real-time polymerase chain reaction (qRT-PCR) was performed on DNA extracted from single biofilms of *Pseudomonas* spp. and *S. salivarius*, as well as from dual biofilm in which the two microorganisms were co-associated. The results showed that in the dual biofilm, after treatment with 0.07% chlorhexidine, there was a statistically significant decrease in the viability of both microorganisms compared to the single biofilms, as observed in Figure 5 ($p < 0.0001$). This suggests that the association between *Pseudomonas* spp. and *S. salivarius* can increase the susceptibility of both to chlorhexidine. At the 0.03% chlorhexidine concentration, although no statistically significant difference was observed, there was a decrease in cell viability in the dual biofilm.

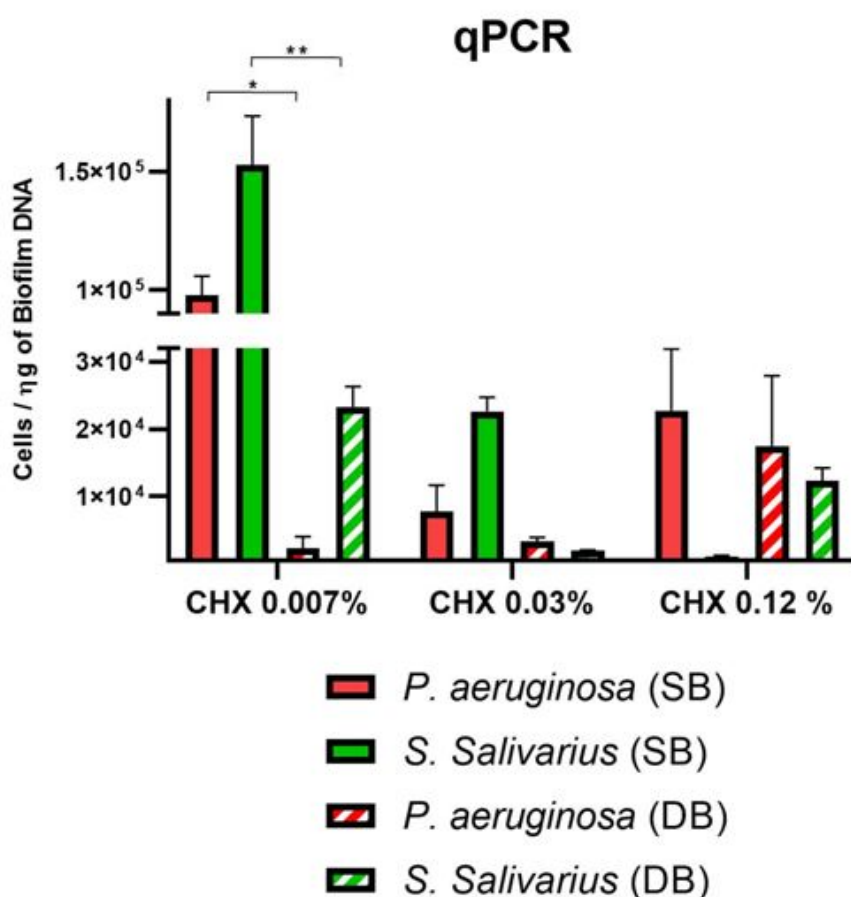


Figure 5. Comparison of the microorganisms viability in single biofilm (SB) and dual biofilm (DB) after treatment with chlorhexidine in different concentrations. * $p < 0.0001$, ** $p < 0.0001$ (2-way Anova test, Tukey procedure)

3.4 DISCUSSION

The bacterial isolates utilized in this investigation were sourced from oral samples of patients undergoing hospitalization in the ward, and the presence of pathogenic and resistant isolates was detected. Previous research efforts have already provided evidence of the colonization by pathogenic bacteria during the hospitalization process [8,26,27]. Cecon et al.'s study described a progressive temporal rise in the count of pulmonary pathogens in the mouth of comatose patients [8]. Nevertheless, most of the studies have primarily focused on critically ill patients admitted to ICU, which makes the comparisons challenging, as our investigation focuses on patients in the general ward, the majority of whom exhibit preserved autonomy for self-care in hygiene practices.

The findings of our investigation showed that the mouth can function as a reservoir for pathogenic bacteria, even in non-intubated patients, with pathogenic microorganisms being present within the initial 48 hours of hospitalization. Previous study demonstrated the presence of pathogenic bacteria in the mouth of patients upon their admission to the ICU, as early as the first day of hospitalization, followed by subsequent colonization by a variety of predominantly gram-negative microorganisms [28]. It is plausible to posit the presence of pathogenic microorganisms in the mouth even before the patient's admission to the hospital.

There were 69 isolates evaluated in our study, of which 17.4% showed resistance to chlorhexidine and no significant difference in the proportion of resistant samples was observed between T1 and T3. Chlorhexidine is extensively employed in Dentistry; however, it is noteworthy that the study participants under investigation were in a clinical ward and were not undergoing chlorhexidine mouth rinses on the days of data collection. In addition to its dental applications, chlorhexidine exhibits a wide array of alternative uses, both within hospital and domestic settings [1]. There are studies suggesting that the proliferation of chlorhexidine usage methods has contributed to the development of microorganisms resistant to the substance [11,29].

Previous research demonstrates that oral bacteria can adapt to chlorhexidine and by the same mechanism decrease their susceptibility to antimicrobial drugs [30]. In our research, a total of twelve chlorhexidine-resistant isolates were subjected to antibacterial susceptibility testing, with eight of them exhibiting resistance to at least one drug. The primary antibiotic-resistant bacteria isolated included *Pseudomonas* spp., *Klebsiella* spp., *S. aureus*, and *E. coli*. Similarly, a substantial proportion of antibiotic-resistant microorganisms was observed in non-hospitalized patients with odontogenic infections, with notable resistance levels identified in microorganisms such as *Staphylococcus* spp., *Streptococcus* spp., *Klebsiella pneumoniae*, *Enterococcus* spp., *Pseudomonas aeruginosa*, and *Proteus mirabilis* [31]. Another study examined the presence of microbial resistance genes and phenotypic resistance in microorganisms isolated from the oral cavity of both healthy patients and those with dental caries and periodontal disease [32]. It described a broad spectrum of resistance genes within the oral microbiota, revealing a substantial prevalence of phenotypically expressed resistance in the oral biofilm [32].

Notably, among the resistant isolates identified in our investigation, three exhibited resistance to chlorhexidine and to seven or more antibiotics, classifying them as multidrug-resistant. These isolates included one sample of *E. coli*, one of *S. aureus*, and one of *Pseudomonas* spp. Next, we will provide a brief description of each of them, relating each one to the clinical aspects of the patients from whose oral cavity they originated.

The *E. coli* isolate was derived from an oral cavity swab collected from a 63-year-old female patient who presented six teeth and was not utilizing dental prostheses. The patient had been hospitalized due to pneumonia and had a medical history of hypertension, arrhythmia, and chronic obstructive pulmonary disease, requiring oxygen therapy. She reported a smoking history of more than 20 years but had quit smoking. Levofloxacin antibiotic therapy was initiated on the first day of hospitalization. On the seventh day of admission, the patient developed anuria and abdominal pain, prompting the empirical use of cefepime as an antibiotic, and a urinalysis was requested. Unfortunately, we were unable to access the results of this urinalysis. Sputum culture collected on the third day of hospitalization revealed the presence of *E. coli* that exhibited resistance to

several antibacterial agents, including levofloxacin and cefepime. In the antibiogram conducted as part of our study, the *E. coli* isolated from the patient's mouth was resistant to levofloxacin but susceptible to cefepime. The patient was discharged from the hospital after a 17-day hospitalization period.

In our research analyses, the *E. coli* isolate exhibited a minimum inhibitory concentration (MIC) of 100 mg/L for chlorhexidine and displayed resistance to the antibiotics tobramycin, norfloxacin, ciprofloxacin, levofloxacin, lomefloxacin, ofloxacin, and ticarcillin/clavulanic acid. A study conducted in 1981 analyzed 148 *E. coli* isolates from clinical lesions, and 12.8% of them were classified as chlorhexidine-resistant [33]. However, a study that considered the chlorhexidine resistance breakpoint to be 64 mg/L reported rare cases of chlorhexidine-resistant *E. coli* isolates [25]. Another investigation that assessed genetic mechanisms of chlorhexidine resistance in *E. coli* did not confirm the possibility of cross-resistance to antibiotics and described alterations in proteins involved in lipid transport mechanisms and resistance to acids and antimicrobial stress in chlorhexidine-resistant strains [34].

The *S. aureus* isolate was derived from the oral cavity of a 65-year-old female patient who was completely edentulous and was wearing only the upper complete denture. The patient had been hospitalized due to pneumonia and had a medical history of hypertension and heart failure. Antibiotic therapy with azithromycin and piperacillin/tazobactam was prescribed. In the antibiogram conducted as part of our study, the *S. aureus* isolated from the patient's oral cavity exhibited resistance to piperacillin/tazobactam and was not tested for azithromycin or other macrolide-class antibiotics. We found no patient record in the medical chart documenting a culture or antibiotic susceptibility test. The patient was discharged from the hospital after 14 days of hospitalization.

In our investigation, the *S. aureus* isolate exhibited a Minimum Inhibitory Concentration (MIC) of 12.5 mg/L for chlorhexidine and demonstrated resistance to the antibiotics norfloxacin, cefepime, ciprofloxacin, piperacillin/tazobactam, levofloxacin, lomefloxacin, ofloxacin, gentamicin, ticarcillin/clavulanic acid, aztreonam, and meropenem. Previous studies have reported the prevalence of specific biocide resistance genes in *S. aureus* strains

[22,25]. An *in vitro* study demonstrated that exposure of *S. aureus* isolates to chlorhexidine resulted in the development of genotypic and phenotypic traits, leading to reduced susceptibility of the bacterium to both chlorhexidine and the antibiotic vancomycin [14]. Variations in susceptibility to chlorhexidine exist between methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-sensitive strains, with MRSA strains displaying higher MIC values [(19,22)]. Among 206 MRSA isolates, 35% exhibited chlorhexidine resistance, with nearly all carrying *qacA/B* genes responsible for their reduced susceptibility to the drug [22]. However, these genes may also be present in strains with unchanged susceptibility to chlorhexidine [35]. Several of these genes are associated with resistance to both chlorhexidine and antibacterials, suggesting a shared resistance mechanism between the two drugs [25].

The *Pseudomonas* spp. isolated was obtained from the mouth of a 69-year-old male patient, who had six remaining teeth and was wearing an upper removable partial denture and a lower complete denture. An ulcer was detected on the anterior mucosa of the lower alveolar ridge, and the patient reported discomfort upon admission. He had been hospitalized due to infected lower limb ulcers and had a medical history that included hypertension, cardiac disease, chronic obstructive pulmonary disease, diabetes, chronic nephropathy, with a renal transplant performed 13 years ago, which experienced rejection after 2 years, necessitating the resumption of hemodialysis. Prior to admission, the patient was undergoing treatment for paracoccidioidomycosis and had previously received itraconazole followed by sulfamethoxazole/trimethoprim therapy. In addition to these medications, the patient was on various other drugs, including opioid analgesics. In the hospital, ertapenem was initiated after culture results from foot ulcer material indicated the presence of *Klebsiella pneumoniae* ESBL, which was susceptible to ertapenem, meropenem, and tigecycline. Throughout the hospitalization period, the patient was administered the following antimicrobials: imipenem/cilastatin, linezolid, meropenem, polymyxin B, amikacin, and vancomycin.

On the seventh day of hospitalization, the patient exhibited delirium and mental decline, with suspected diagnosis of septic encephalopathy or opioid

intoxication, leading to transfer to the intensive care unit and intubation on the ninth day of hospitalization. On the twentieth day of hospitalization, the patient was transferred to another medical facility in critical condition, displaying hemodynamic instability and a diagnosis of refractory septic shock, beyond which no further patient records are available. Although several blood cultures and tracheal aspirate cultures were requested during the hospitalization period, we were unable to access the results of these examinations.

The *Pseudomonas* spp. isolate described in this study exhibited a Minimum Inhibitory Concentration (MIC) result of 50 mg/mL for chlorhexidine and demonstrated resistance to 14 antibiotics, including polymyxin, amikacin, norfloxacin, cefepime, ciprofloxacin, piperacillin/tazobactam, levofloxacin, lomefloxacin, ofloxacin, gentamicin, ticarcillin/clavulanic acid, aztreonam, meropenem, and imipenem. Previous studies have indicated that clinical isolates of *Pseudomonas* spp. undergo genotypic and phenotypic changes, resulting in resistance to various antibiotics when exposed to chlorhexidine, as well as resistance to the biocide itself [36,37]. Sub-inhibitory concentrations of chlorhexidine induced a significant reduction in susceptibility to the compound in both *P. aeruginosa* and *S. aureus* [24]. A systematic review conducted in 2021 demonstrated a decrease in susceptibility to chlorhexidine among Gram-negative bacteria. Moreover, an extremely pronounced reduction in susceptibility to chlorhexidine in *P. aeruginosa* over an 80-year period was observed, with clinical isolates of *P. aeruginosa* exhibiting lower susceptibility compared to non-clinical isolates [19].

The main mechanisms described for bacterial resistance to chlorhexidine include alterations in lipid synthesis and transport, changes in porin expression, and increased efflux pump activity [34]. Chlorhexidine resistance is less common in *E. coli*, *Salmonella* spp., and *S. aureus*, but more prevalent in *Enterobacter* spp., *Pseudomonas* spp., *Proteus* spp., *Providencia* spp., and *Enterococcus* spp. [25]. Therefore, resistance to chlorhexidine in Gram-negative bacteria poses a significant and substantial concern [34]. Studies have demonstrated cross-resistance between chlorhexidine and the antibiotic colistin in *Klebsiella* spp. and other Enterobacteriaceae [37,38].

Similarities between mechanisms of microbial resistance to biocides and antibiotics have been demonstrated *in vitro* [19]. Although the linkage between chlorhexidine resistance and resistance to clinically important antibiotics remains incompletely understood, this comprehension is indispensable for addressing antimicrobial resistance [19,39]. Antimicrobial resistance represents a significant contemporary concern. It is a global health crisis that requires immediate attention and action from governments and international organizations [40]. There are many challenges to find real solutions and, although there are some initiatives to tackle the issue, it seems that the goal is far from being achieved.

The One Health strategy poses a bold holistic approach to face the problem. It claims that human beings, animals, and the environment are considered all part of the same problem [41]. It emphasizes that there is no single solution and that collaborative efforts are needed from scientists, governments, and population, where all the actions must agree with some principles: equity, inclusivity, equal access, parity, socio ecological equilibrium, stewardship, and transdisciplinarity [41].

Analyzing the issue of microbial resistance to biocides from the perspective of the One Health strategy, it is necessary an examination of the organizational trait found in many microorganisms, which form communities known as biofilms. Within these biofilms, microorganisms can undergo adaptive interactions that render them more resilient, potentially resulting in additional effects, such as increased virulence and colonization of novel environments [39].

In our study, the viability of the three multidrug-resistant isolates was assessed in biofilms treated with chlorhexidine, either in single or dual-biofilms in association with a probiotic bacterium, *S. salivarius* K12. Biofilms represent sessile communities of bacteria or fungi, adhered to a surface or to each other, ensconced within a matrix primarily composed of self-produced extracellular materials [39]. The interest in understanding the interactions of microorganisms within biofilms is growing. It has been observed that cells within these communities exhibit altered phenotypes compared to their planktonic counterparts, contingent on the composition of the biofilm [39].

We chose to combine the resistant isolates with a probiotic strain because probiotics have been suggested as an alternative for restoring microbiota balance [42]. Probiotics consist of specific microorganisms believed to confer health benefits and are considered an alternative for preventing and treating various diseases, including drug-resistant infections [43]. *Streptococcus salivarius* K12 is a probiotic microorganism recognized as an early colonizer of the oral cavity. Previous research has shown its ability to inhibit the growth of *Streptococcus mutans* and prevent the formation of cariogenic biofilms [44].

In the confocal microscopy analysis of biofilms cultivated in our experiment, it appears that there was a reduction in cellular viability following treatment with 0.12% chlorhexidine within the biofilms where an association of *Pseudomonas* + *S. salivarius* and *S. aureus* + *S. salivarius* occurred. However, it is not possible to ascertain which of the species experienced diminished viability. In the analysis conducted using qRT-PCR, the microorganisms *Pseudomonas* spp. and *S. salivarius* exhibited a significant reduction in cellular viability following treatment with 0.007% chlorhexidine when co-associated in a dual biofilm. At the concentration of 0.03%, a similar outcome was observed, although without statistical significance. In the concentration of 0.12%, an unexpected outcome was observed, with a lower cellular viability of *S. salivarius* in the single biofilm as compared to the dual biofilm. Despite this, our findings suggest that the probiotic bacterium *S. salivarius* has the potential to enhance the susceptibility of bacteria resistant to chlorhexidine.

Literature reports have indicated an increased sensitivity to the antibiotic cefoperazone and a reduction in the growth of *P. aeruginosa* and *K. pneumoniae* when exposed to the *S. salivarius* M18 strain in its planktonic form in liquid media [45]. Another study demonstrated a reduction in the bacterial load of *P. aeruginosa* within the airways of rats when co-infected with *S. salivarius*, leading to a decreased host inflammatory response [46]. However, clinical isolates displayed an ability to survive at elevated concentrations of chlorhexidine within biofilms, well above the MIC [47]. This phenomenon, observed in this study, has been described as the "Eagle Effect", signifying enhanced isolate survival at chlorhexidine concentrations exceeding the MIC. An alert was raised regarding the potential confusion in microbial susceptibility

testing due to the Eagle Effect [47]. The Eagle Effect has previously been reported for antibiotics and is described as an incompletely understood phenomenon in which bacteria, when exposed to a much higher concentration than the usual bactericidal concentration of an antimicrobial agent, exhibit improved survival performance compared to exposure to the usual bactericidal concentration [47].

Significant advances have been made in understanding the internal dynamics of microorganisms within biofilms and their interactions with the surrounding environment [39]. Exposure of the inner layers of biofilms to sub-inhibitory concentrations of chlorhexidine appears to induce tolerance and the emergence of acquired resistance [39]. Studies that simulate *in vivo* conditions of biofilms are imperative to enhance the comprehension of the entire spectrum of characteristics and phenomena occurring within these microbial communities [39].

3.5 CONCLUSION

In clinical samples obtained from the mouth of hospitalized patients in the ward, the presence of pathogenic microorganisms resistant to chlorhexidine and various antimicrobial agents was detected. When these microorganisms were co-cultured in biofilms with the probiotic strain *S. salivarius* K12, alterations in cell viability were observed following treatment with chlorhexidine. The probiotic bacterium *S. salivarius* K12 showed the potential to enhance the susceptibility of chlorhexidine-resistant bacteria. This finding supports the alteration in bacterial behavior when co-associated in biofilms.

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4 DISCUSSÃO GERAL E CONCLUSÕES DA TESE

4.1 DISCUSSÃO GERAL

Nosso estudo refere-se à saúde e à microbiota patogênica presente na boca de pacientes hospitalizados em Enfermaria. O estudo mostrou que, mesmo em pacientes não entubados e que não estão em unidade de terapia intensiva (UTI), a boca pode ser um reservatório de microrganismos patogênicos epidemiologicamente importantes na disseminação de infecções resistentes a antimicrobianos. Outros estudos já mostraram colonização da boca por microrganismos patogênicos em pacientes hospitalizados, porém a maioria deles investigou pacientes internados em UTI [1–3].

Na primeira fase do estudo, em que foi realizado exame e coleta dos dados clínicos da pesquisa, foram detectadas lesões e outras condições orais cujo acompanhamento e tratamento foram necessários e por vezes fundamentais para a resolução do problema que levou à hospitalização do paciente. Algumas vezes a equipe de pesquisadores foi chamada a auxiliar no manejo de alguma condição presente na boca dos pacientes, como em casos de xerostomia, dificuldade de mastigação, próteses mal adaptadas e úlceras bucais. A partir de uma dessas situações, foi feito o acompanhamento de uma paciente cujo diagnóstico foi intoxicação por metotrexato e foi publicado relato de caso, que se encontra no Apêndice 3 deste trabalho.

A fase microbiológica da pesquisa detectou nas amostras bucais microrganismos que possuem linhagens resistentes a antimicrobianos e associação a infecções nosocomiais de difícil controle, como *E. coli*, *Enterococcus* spp., *Enterobacter* spp., *Pseudomonas* spp. e *S. aureus*. Alguns destes microrganismos integram um grupo de bactérias coletivamente identificadas pelo acrônimo ESKAPE [4]. Este acrônimo representa seis patógenos que têm apresentado crescente patogenicidade e estão relacionados a infecções nosocomiais e resistência a múltiplos antibacterianos. São eles *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* e *Enterobacter* spp. [5].

Este grupo de patógenos é responsável pela maioria das infecções nosocomiais e foi listado pela Organização Mundial de Saúde como prioritário nas pesquisas para o monitoramento de sua susceptibilidade aos antimicrobianos e para o desenvolvimento de novos fármacos [6].

Foram detectados microrganismos com susceptibilidade reduzida à clorexidina e a outros antimicrobianos de forma simultânea. Porém, esta associação entre resistência a antibióticos e a biocidas permanece controversa [7]. A clorexidina é um biocida amplamente utilizado sobre o qual paira forte suspeita de contribuição para o desenvolvimento e a seleção de microrganismos resistentes a diversos antimicrobianos. Há na literatura relato de amostras de *K. pneumoniae* que foram expostas à clorexidina e se tornaram resistentes à substância e também ao antibacteriano colistina [8]. Porém, os métodos para testar susceptibilidade de microrganismos à clorexidina não são padronizados, gerando uma dificuldade para o estudo da substância no cenário da resistência antimicrobiana. Há uma variedade de métodos laboratoriais para testar a susceptibilidade *in vitro* de microrganismos a agentes antimicrobianos [9]. Em nosso estudo foi escolhido o método de microdiluição em caldo por fornecer dados numéricos de concentração inibitória mínima, além de ser mais confiável e econômico. No manual “Métodos para testes de sensibilidade aos antimicrobianos por diluição em caldo para bactérias que crescem aerobicamente”, publicado pelo Clinical and Laboratory Standards Institute (CLSI) em 2012 [10], foram descritos os procedimentos padronizados para a metodologia de microdiluição em caldo. Essa descrição é voltada para os testes relacionados a antibióticos. Porém, na ausência de uma metodologia específica para antissépticos e desinfetantes, foi a referência utilizada para a realização dos testes de susceptibilidade à clorexidina neste estudo.

Diversos fatores contribuem para o grave problema da resistência antimicrobiana. A estratégia Saúde Única propõe ampliar o foco de ação no combate ao problema da resistência antimicrobiana. Há inúmeros mecanismos contribuintes que vão muito além do uso de medicamentos, como desequilíbrios ecológicos, mudanças climáticas, modos de produção e comercialização internacional de produtos e deslocamentos humanos e de

outras espécies [11]. Não basta buscar novos medicamentos capazes de combater os microrganismos resistentes. É necessário considerar outras questões como as interações entre seres vivos e meio ambiente tanto de um ponto de vista socioeconômico quanto nos biofilmes formados por diferentes espécies de microrganismos [12].

As interações entre os microrganismos em biofilmes parecem ser fundamentais para a sobrevivência deles aos antimicrobianos. Na boca há mais de 700 espécies de microrganismos que se organizam em biofilmes [13]. Em nosso estudo, a associação de cepas bacterianas multirresistentes à bactéria probiótica *S. salivarius* K12 em biofilmes mostrou redução da viabilidade celular de ambas as bactérias após tratamento com clorexidina quando comparada à viabilidade nos biofilmes unitários. Há estudos mostrando atuação positiva de probióticos na modulação do comportamento de bactérias patogênicas na boca [14–16].

A despeito do risco de resistência microbiana, a clorexidina ainda é um antimicrobiano importante, com uso difundido na Odontologia e em ambientes clínicos [17]. As evidências de que seu uso tenha como consequência direta a seleção de células resistentes em ambiente clínico ainda não são consideradas consistentes [18] e a resistência cruzada entre antimicrobianos e clorexidina ainda não é um consenso na literatura (7,19). Portanto, o uso de clorexidina como antisséptico deveria ser restrito às situações em que o benefício é claro.

4.2 CONCLUSÕES

Os achados clínicos do nosso estudo sugeriram a necessidade de maior atenção às condições bucais de pacientes hospitalizados. Os dados microbiológicos demonstraram que, em pacientes não entubados hospitalizados em Enfermaria, a boca pode abrigar microrganismos patogênicos simultaneamente resistentes à clorexidina e aos antimicrobianos, representando um risco para infecções nosocomiais resistentes. Houve redução da viabilidade celular, demonstrando aumento de susceptibilidade à clorexidina, quando os isolados bacterianos resistentes ao biocida foram associados à cepa probiótica *S. salivarius* K12 em biofilmes. Apesar de não haver consenso sobre o papel da clorexidina no desenvolvimento de resistência aos antimicrobianos, a substância deve ser usada com cautela somente nas situações em que o benefício clínico for evidente.

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5 PRESS RELEASE

Existem suspeitas de que a clorexidina, uma substância antisséptica utilizada como enxaguatório bucal, possa estar contribuindo para o desenvolvimento de bactérias resistentes aos antibióticos. A resistência aos antibióticos é um grave problema mundial e muitas pessoas morrem todos os anos por infecções que não respondem ao tratamento com os medicamentos disponíveis. Pacientes hospitalizados podem ter sua saúde bucal comprometida e sua boca colonizada por microorganismos que podem provocar doenças, sendo mais susceptíveis a contraírem infecções resistentes em ambiente hospitalar. O objetivo deste estudo foi conhecer o estado de saúde bucal e avaliar os microorganismos patogênicos presentes na boca de pacientes hospitalizados quanto à resistência à clorexidina e aos antibióticos. Foram identificados microorganismos resistentes à clorexidina e simultaneamente resistentes aos antibióticos. Foram feitos experimentos *in vitro* associando a bactéria probiótica *Streptococcus salivarius* a bactérias multirresistentes provenientes da boca dos pacientes para testar a susceptibilidade à clorexidina. Os resultados do estudo mostraram que a boca pode ser um reservatório de microorganismos patogênicos multirresistentes a antibacterianos e à clorexidina, e que a associação de bactérias probióticas como *S. salivarius* pode aumentar a susceptibilidade de microorganismos multirresistentes à clorexidina.

Apêndice A – Termo de Consentimento Livre e Esclarecido (TCLE)



Termo de Consentimento Livre e Esclarecido - TCLE

O (a) Senhor(a) está sendo convidado(a) a participar do projeto de pesquisa “Avaliação da resistência à clorexidina da microbiota bucal de pacientes internados no HUB”.

O objetivo desta pesquisa é conhecer a saúde da boca e comparar os tipos de bactérias e fungos que se encontram na boca de pessoas internadas no Hospital Universitário de Brasília. Também queremos saber se essas bactérias e fungos podem ser combatidos por um produto chamado clorexidina, utilizado em bochechos para a limpeza da boca no hospital. Com isso, poderemos ajudar as pessoas a encontrarem um melhor método de limpeza da boca quando estiverem hospitalizadas. O(a) senhor(a) receberá todas as informações necessárias antes e durante a pesquisa e lhe asseguramos que seu nome não aparecerá, sendo mantido o mais rigoroso sigilo(segredo) e nenhuma informação que poderá lhe identificar irá aparecer.

Para participar, o Senhor(a) deverá responder perguntas sobre sua saúde e seu prontuário será consultado. Além disso, sua boca será examinada e um cotonete será usado para coletar um material da sua boca, que será esfregado com cuidado na sua língua, alguns dentes e na bochecha. Isso não causa dor ou desconforto e será repetido em três dias diferentes, sendo o primeiro dia até 2 dias depois da sua internação no hospital, o segundo 2 dias após o primeiro dia e o terceiro 7 dias depois da sua internação. Há também a possibilidade de, após o exame no laboratório, encontrarmos alguma bactéria ou fungo mais agressivo. Neste caso, a Comissão de Controle de Infecção do Hospital será avisada para tomar as providências necessárias. Ainda, toda necessidade urgente em relação à saúde da boca que o Sr(a) apresentar, será atendida por nós. O Sr(a) pode desistir de participar em qualquer momento da pesquisa por qualquer motivo, sem nenhum prejuízo, ou seja, seu tratamento de saúde continuará da mesma forma. Sua participação é voluntária, isto é, não há pagamento por sua colaboração.

Os resultados da pesquisa serão divulgados na Universidade de Brasília podendo ser publicados depois. Os dados e materiais utilizados na pesquisa ficarão sob a guarda do pesquisador por um período de no mínimo cinco anos, após isso serão destruídos ou mantidos na instituição.

Se o(a) Senhor(a) tiver qualquer dúvida em relação à pesquisa, os pesquisadores estarão à sua disposição para quaisquer esclarecimentos nos telefones: Adriana Silva da Costa Cruz (99298.8124), Érica Negrini Lia (99116.7148). Este projeto foi Aprovado pelo Comitê de Ética em Pesquisa da Faculdade de Medicina da Universidade de Brasília. As dúvidas com relação à assinatura do TCLE ou os direitos do participante da pesquisa podem ser obtidos através do telefone: (61) 3107-1918 ou do e-mail cepfm@unb.br. Este documento foi elaborado em duas vias, uma ficará com o pesquisador responsável e a outra com o participante da pesquisa.

Nome / assinatura

Adriana Silva da Costa Cruz - Pesquisadora Responsável

Brasília, ____ de ____ de ____

Apêndice B – Ficha Clínica (CRF – Case ReportForm)

Data ____ / ____ / _____

Prontuário HUB:

CRF – Case Report Form

PROJETO AVALIAÇÃO DA RESISTÊNCIA À CLOREXIDINA DA
MICROBIOTA BUCAL DE PACIENTES INTERNADOS NO HUB

IDENTIFICAÇÃO DO PACIENTE	
Nome:	Idade:
Telefones de contato:	Raça / cor: <input type="checkbox"/> F <input type="checkbox"/> M
CRITÉRIOS DE INCLUSÃO	
Idade ≥ 18 anos	<input type="checkbox"/> S <input type="checkbox"/> N
Hospitalizado há menos de 48 horas?	<input type="checkbox"/> S <input type="checkbox"/> N
CRITÉRIOS DE EXCLUSÃO	
Condição de imunossupressão?	<input type="checkbox"/> S <input type="checkbox"/> N
Em caso afirmativo:	<input type="checkbox"/> Quimioterapia <input type="checkbox"/> Transplante <input type="checkbox"/> HIV <input type="checkbox"/> Outros: _____
Passou por hospitalização recente nos últimos 30 dias?	<input type="checkbox"/> S <input type="checkbox"/> N
Comprometimento cognitivo ou dificuldade de compreensão?	<input type="checkbox"/> S <input type="checkbox"/> N
Observação: excluir o paciente do estudo caso a(s) resposta(s) acima seja(m) afirmativa(s)	
COLETOU TCLE?	<input type="checkbox"/> S <input type="checkbox"/> N DATA DA COLETA ____ / ____ / _____
DADOS DE SAÚDE	
Clínica e motivo principal da internação	

Lista de problemas	

Medicamentos em uso	
Uso regular	

Uso hospitalar	

Prontuário HUB:

DADOS DE SAÚDE			
Alimentando-se por via oral?	<input type="checkbox"/> S	<input type="checkbox"/> N	
Em caso negativo:	<input type="checkbox"/> Sonda nasointestinal	<input type="checkbox"/> Gastrostomia	<input type="checkbox"/> Parenteral <input type="checkbox"/> Jejum
Alguma restrição alimentar?	<input type="checkbox"/> S	<input type="checkbox"/> N	
Quais?	_____		
Faz higiene bucal no hospital?	<input type="checkbox"/> Sozinho	<input type="checkbox"/> Com auxílio	<input type="checkbox"/> Não faz
Quantas vezes ao dia?	<input type="checkbox"/> 1X	<input type="checkbox"/> 2X	<input type="checkbox"/> 3X <input type="checkbox"/> Outros <input type="checkbox"/> Não se aplica
Usa:	<input type="checkbox"/> Escova dental	<input type="checkbox"/> Creme dental	<input type="checkbox"/> Fio dental <input type="checkbox"/> Não se aplica
	<input type="checkbox"/> Outros – Quais?	_____	
Fumante?	<input type="checkbox"/> S	<input type="checkbox"/> N	
Etilista?	<input type="checkbox"/> S	<input type="checkbox"/> N	
Alguma queixa bucal?	<input type="checkbox"/> S	<input type="checkbox"/> N	
<input type="checkbox"/> 1	Ardência bucal		
<input type="checkbox"/> 2	Dor dental		
<input type="checkbox"/> 3	Dificuldade mastigatória		
<input type="checkbox"/> 4	Dificuldade de deglutição		
<input type="checkbox"/> 5	Diminuição do paladar		
<input type="checkbox"/> 6	Halitose		
<input type="checkbox"/> 7	Boca seca		
<input type="checkbox"/> 8	Cárie		
<input type="checkbox"/> 9	Sangramento		
<input type="checkbox"/> 10	Dente amolecido		
<input type="checkbox"/> 11	Outras: _____		

Usa prótese dentária removível?	<input type="checkbox"/> S	<input type="checkbox"/> N	
Observações:	_____		

Dorme com a prótese?	<input type="checkbox"/> S	<input type="checkbox"/> N	<input type="checkbox"/> Não se aplica

Prontuário HUB:

V1 – 1ª Coleta: Data ____ / ____ / ____

Data da internação: ____ / ____ / ____ Horário da internação: ____ : ____

Coleta de swab? S N Código: _____

Número de dentes na boca: _____

Presença de lesões bucais? S N

1 Candidíase	2 Leucoplasia
3 Úlcera	4 Hiperplasia por prótese
5 Afta	6 Herpes
7 Fibroma	8 Queilite angular
9 Abscesso	10 Fístula
11 Outras: _____	

Observações: _____

INSTRUMENTO DE AVALIAÇÃO DA SAÚDE BUCAL PARA TRIAGEM ODONTOLÓGICA (IASBT, Gonçalves LHT, Mello ALSF e Zimemann K, 2010)

1. Categoria	0 = saudável	1 = presença de alterações	2 = não saudável	Pontuação por categoria
2. Lábios	* Lírios, rosados, úmidos	* Rachados * Avermelhados nas comissuras * Secos * Avermelhados nas comissuras * Avermelhados nas comissuras	* Inchaço ou caroço * Mancha branca ou avermelhada * Úlcera * Sangramento * Inflamação nas comissuras	
3. Língua	* Normal, úmida, rosada, rosada	* Presença de fissuras * Descolorido por substância * Avermelhada * Mancha	* Inflamação nas comissuras * Úlcera * Inchaço * Mancha avermelhada ou branca	
4. Gengivas e tecidos	* Rosados, úmidos, macios * Sem sangramento	* Avermelhados * Secos * Inchados * Diferentes * Apertadíssimos * Manchas ou úlceras ambas das dentaduras	* Manchas brancas ou avermelhadas * Vermelhidão generalizada * Gengivas inchadas * Sangramento * Úlceras	
5. Saliva	* Tecidos úmidos, salivagem adequada * Flúo livre despedaçado sem obstrução	* Tecidos secos e pegajosos * Presença de placa salivar	* Tecidos ressecados e avermelhados * Protrusão ou refluxo salivar * Saliva muito espessa	
6. Dentes naturais Sim ou Não	* Todos os dentes sãos	* 1 a 2 áreas ou dentes com cáries ou quebrados * Os dentes muito desgastados	* 1 ou mais áreas ou dentes com cáries ou quebrados * Ou presença de restos de 4 dentes * Ou ainda dentes muito desgastados	
7. Dentaduras Sim ou Não	* Nenhuma área ou dente quebrado * Dentaduras utilizadas em ambas arcadas continuamente durante o dia	* 1 área ou 1 dente danificado * Dentaduras usadas por apenas 1 a 2 horas ao dia * Dentaduras soltas/difíceis * Uma somente 1 dentadura (superior ou inferior)	* Mais de 1 área ou mais de 1 dente danificado * Falta de dentadura ou dentadura não utilizada * Prática de adesivo para dentadura	
8. Higiene bucal	* Boca limpa sem resíduos de alimento, sem tártaro na boca ou nas dentaduras	* Resíduos de alimento, tártaro ou placa em 1 a 2 áreas da boca ou em pequena área de dentadura * Mau hálito	* Resíduos de alimento ou tártaro ou placa bastante na maioria das áreas da boca ou das dentaduras * Mau hálito forte	
9. Dor de dente	* Sem sinais comportamentais, verbais ou físicos de dor de dente	* Sinais verbais ou comportamentais de dor de dente, como carúas, irritadas nos lábios, falta de apetite, agressividade	* Sinais físicos como inchaço facial, abscessos nas gengivas, dentes quebrados, grande ulceração, e sinais verbais ou comportamentais como carúas, mordidas nos lábios, falta de apetite, agressividade	

A pontuação final resulta da soma das oito categorias e varia entre 0 (muito saudável) e 10 (muito doente)

TOTAL _____

ÍNDICE DE SABURRA LINGUAL (Miyazaki et al. 1995)

0 nenhuma saburra visível

1 menos de 1/3 do dorso da língua coberto

2 menos de 2/3 do dorso da língua coberto

3 mais de 2/3 do dorso da língua coberto

Swab enviado para LAMP? S N

Resultados microbiológicos: _____

Microorganismos encontrados: _____

Resistência a CHX (anotar a concentração na qual não houve turvação): _____

Responsável pelo preenchimento: _____

Prontuário HUB:

V2 – 2ª Coleta: Data / /

Incluiu algum medicamento? S N Qual(is)? _____

Coleta de swab? S N Código: _____

Presença de lesões bucais? S N

1 Candidíase	2 Leucoplasia
3 Úlcera	4 Hiperplasia por prótese
5 Afta	6 Herpes
7 Fibroma	8 Queilite angular
9 Abscesso	10 Fístula
11 Outras: _____	

Observações: _____

INSTRUMENTO DE AVALIAÇÃO DA SAÚDE BUCAL PARA TRIAGEM ODONTOLÓGICA (IASBO, Gonçalves LHT, Mello ALB e Zimmermann K, 2010)

1. Categoria	0 = saudável	1 = presença de alterações	2 = não saudável	Pontuação por categoria
2. Lábios	* Lírios, rosados, úmidos	* Rachados * Avermelhados nas comissuras * Secos * Avermelhados nas comissuras * Presença de fissuras	* Inchaço ou caroço * Mancha branca ou avermelhada * Úlcera * Sangramento * Inflamação nas comissuras	
3. Língua	* Normal, úmida, rosada	* Presença de fissuras * Secante por salivaria * Avermelhada * Manchada	* Úlcera * Inchaço * Mancha avermelhada ou branca	
4. Gengivas e tecidos	* Rosados, úmidos, macios * Sem sangramento	* Avermelhados * Secos * Inchados * Bolhosos * Asperos/irregulares * Manchas ou úlceras embaixo das dentaduras	* Manchas brancas ou avermelhadas * Vermelhidão generalizada * Gengivas inchadas * Sangramento * Úlceras	
5. Saliva	* Tecidos úmidos, salivagem espessa * Fluzo livre desmontável sem obstrução	* Tecidos secos e pegajosos * Presença de pouca saliva	* Tecidos ressecados e avermelhados * Pausadas ou nenhuma saliva * Saliva muito espessa	
6. Dentes naturais Sim ou Não	* Todos os dentes íntegros	* 1 a 3 dentes ou dentes com cáries ou quebrados * Os dentes muito desgastados	* 4 ou mais dentes ou dentes com cáries ou quebrados * Ou presença de menos de 4 dentes * Ou dentes muito desgastados	
7. Dentaduras Sim ou Não	* Nenhuma área ou dente quebrado * Dentaduras utilizadas em ambos arcos continuamente durante o dia	* 1 área ou 1 dente danificado * Dentaduras usadas por apenas 1 a 2 horas ao dia * Dentaduras soltas/difíceis * Usa somente 1 dentadura (superior ou inferior)	* 2 área de dentadura ou dentadura não utilizada * Falta de adesivo para dentadura	
8. Higiene bucal	* Boca limpa, sem resíduos de alimento, sem tártaro na boca ou nas dentaduras	* Resíduos de alimento, tártaro ou placa em 1 a 2 áreas da boca ou em pequena área da dentadura * Mau hálito	* Restos de alimento ou tártaro ou placa bacteriana na maioria das áreas da boca ou das dentaduras * Mau hálito severo	
9. Dor de dente	* Sem sinais comportamentais, verbais ou físicos de dor de dente	* Sinais verbais ou comportamentais de dor de dente, como carêtas, mordidas nos lábios, falta de apatia, agressividade	* Sinais físicos como inchaço facial, abscessos nas gengivas, dentes quebrados, grande ulceração, e sinais verbais ou comportamentais como carêtas, mordidas nos lábios, falta de apatia, agressividade	
A pontuação final resulta da soma das oito categorias e varia entre 0 (muito saudável) e 16 (muito doente)				TOTAL _____

ÍNDICE DE SABURRA LINGUAL (Miyazaki et al. 1995)

0 nenhuma saburra visível

1 menos de 1/3 do dorso da língua coberto

2 menos de 2/3 do dorso da língua coberto

3 mais de 2/3 do dorso da língua coberto

Swab enviado para LAMP? S N

Resultados microbiológicos: _____

Microrganismos encontrados: _____

Resistência a CHX (anotar a concentração na qual não houve turvação): _____

Responsável pelo preenchimento: _____

Prontuário HUB:

V3 – 3ª Coleta: Data / /

Incluiu algum medicamento? S N Qual(is)?

Coleta de swab? S N Código:

Presença de lesões bucais? S N

1 Candidíase	2 Leucoplasia
3 Úlcera	4 Hiperplasia por prótese
5 Afta	6 Herpes
7 Fibroma	8 Queilite angular
9 Abscesso	10 Fístula
11 Outras: <input type="text"/>	

Observações:

INSTRUMENTO DE AVALIAÇÃO DA SAÚDE BUCAL PARA TRIAGEM ODONTOLÓGICA (IASBTO, Gonçalves LHT, Mallo ALSF e Zimmermann K, 2010)

1. Categoria	0 = saudável	1 = presença de alterações	2 = não saudável	Pontuação por categoria
2. Lábios	* Lírios, resados, úmidos	* Inchados * Acometidos nas comissuras * Secos * Acometidos nas comissuras * Acometidos nas comissuras	* Inchado ou caroço * Mancha branca ou acometida * Úlcera * Sangramento * Inflamação nas comissuras	
3. Língua	* Normal, úmida, rosada, rosada	* Presença de fissuras * Recoberto por saburra * Acometida * Manchas	* Ulcerada * Inchada * Mancha acometida ou branca	
4. Gengivas e tecidos	* Rosadas, úmidos, macios * Sem sangramento	* Acometidos * Secos * Inchados * Inflamados * Aparentemente * Manchas ou úlceras entre as dentaduras	* Manchas brancas ou acometidas * Vermelhidão generalizada * Gengivas inchadas * Sangramento * Úlceras	
5. Saliva	* Fácil de untar, salvação espessa * Flúo livre desprendido sem obstrução	* Fácil de secar e pegajoso * Presença de pouca saliva	* Fácil de ressecar e acometidos * Paucossaliva ou nenhuma saliva * Saliva muito espessa	
6. Dentes naturais sim ou não	* Todos os dentes íntegros	* 1 a 3 raios ou dentes com caries ou quadrados * Os dentes muito desgastados	* 4 ou mais raios ou dentes com caries ou quadrados * Os dentes muito desgastados	
7. Dentaduras sim ou não	* Nenhuma área ou dente quadrado * Dentaduras utilizadas em ambas arcadas continuamente durante o dia	* 1 área ou 1 dente quadrado * Dentaduras usadas por apenas 1 a 2 horas no dia * Dentaduras veladas/ruins * Usa somente 1 dentadura (superior ou inferior)	* De 2 a 3 áreas ou dentes quadrados * Mais de 1 área ou mais de 1 dente quadrado * Falta de dentadura ou dentadura não utilizada * Falta de adesivo para dentadura	
8. Higiene bucal	* Boca limpa, sem resíduos de alimento, sem tártaro na boca ou nas dentaduras	* Resíduos de alimento, tártaro ou placa em 1 a 2 áreas da boca ou em pequena área da dentadura * Mau hálito	* Resíduos de alimento ou tártaro ou placa localizada na maioria das áreas da boca ou das dentaduras * Mau hálito severo	
9. Dor de dente	* Sem sinais comportamentais, veritais ou físicos de dor de dente	* Sinais veritais ou comportamentais de dor de dente, como caries, mochadas nos lábios, falta de apêxito, agressividade	* Sinais físicos como inchaço facial, abscessos nas gengivas, dentes quadrados, grande ulcerações, e sinais veritais ou comportamentais como caries, mochadas nos lábios, falta de apêxito, agressividade	

A pontuação final resulta da soma das oito categorias e varia entre 0 (muito saudável) e 16 (muito doente)

TOTAL

(ÍNDICE DE SABURRA LINGUAL (Miyazaki et al, 1995))

0 nenhuma saburra visível

1 menos de 1/3 do dorso da língua coberto

2 menos de 1/2 do dorso da língua coberto

3 mais de 1/2 do dorso da língua coberto

Swab enviado para LAMP? S N

Resultados microbiológicos:

Microrganismos encontrados:

Resistência a CHX (anotar a concentração na qual não houve turvação):

Responsável pelo preenchimento:

Apêndice C

Methotrexate induced erythema multiforme: a case report of accidental overdose

❖ Artigo publicado durante coleta de dados da pesquisa de Doutorado

<https://doi.org/10.30968/rbfhss.2021.123.0642>

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Original Paper

Open Access

Methotrexate induced erythema multiforme: a case report of accidental overdose

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Abstract

Methotrexate is the first-line treatment for rheumatoid arthritis (RA) and can be used in patients with systemic lupus erythematosus (SLE) with poor symptom control. In addition to reducing disease symptoms, methotrexate can cause a series of side effects, including erythema multiforme, which can affect oral mucosa and cause painful ulcerations. This article presents a case of a 39-year-old patient with an unconfirmed diagnosis of SLE that received a prescription of 10 capsules of methotrexate (2.5 mg) to be used orally once a week. However, the patient misunderstood the prescription and ingested the prescribed monthly dose in one week. She was hospitalized with multiple intraoral and extraoral lesions, intense pain and dysphagia. In addition to that, she could not take oral antibiotics and had difficulty ingesting solid food. During hospitalization, the patient was treated with topical medications and laser therapy for pain and other symptoms and was released after 9 days to continue outpatient treatment. This case report shows the importance of good communication between health care professional and patient, especially regarding the understanding of the drug prescription.

Key words: drug-related side effects and adverse reactions; erythema multiforme; mouth disease; dental care; systemic lupus erythematosus, methotrexate.

Eritema multiforme induzido por metotrexato: relato de caso de overdose acidental

Resumo

O metotrexato é o medicamento de primeira linha no tratamento da artrite reumatoide e pode ser utilizado em casos de lúpus eritematoso sistêmico quando há dificuldade no controle dos sintomas. Apesar de seu efeito no controle dos sintomas de diversas doenças, o metotrexato pode causar uma série de efeitos adversos, incluindo eritema multiforme, que é uma reação de hipersensibilidade caracterizada por úlceras dolorosas que podem afetar a pele e a mucosa oral. Esse artigo apresenta o caso de uma paciente do sexo feminino, 39 anos de idade, com diagnóstico inconclusivo de lúpus eritematoso sistêmico, que recebeu uma prescrição de 10 cápsulas de metotrexato (2,5 mg) a serem usadas por via oral uma vez por semana, sendo duas pela manhã, duas no almoço, duas no jantar, duas na manhã seguinte e duas no almoço. Entretanto, a paciente não compreendeu bem a prescrição e ingeriu toda a dose prescrita para um mês em uma semana. A paciente foi hospitalizada com múltiplas úlceras intra e extra orais, associadas a dor intensa, disfagia, dificuldade de fala entre outros sinais e sintomas, impedindo alimentação e uso de medicação por via oral. Durante a hospitalização, a paciente recebeu tratamento com medicamentos de uso tópico e laserterapia para controle da dor e da inflamação. A paciente teve alta hospitalar após nove dias de internação para continuar seu tratamento ambulatorial. Esse relato de caso mostra a importância de uma boa comunicação entre profissional de saúde e paciente, especialmente no que diz respeito à compreensão da prescrição medicamentosa.

Palavras-chave: efeitos colaterais e reações adversas relacionados a medicamentos; eritema multiforme; doenças da boca; assistência odontológica, lúpus sistêmicos; metotrexato.

Introduction

Methotrexate is a disease-modifying antirheumatic drug (DMARD) used since 1980 as the first line treatment for rheumatoid arthritis (RA).¹ Initially used as an antimetabolite medication in the treatment

of childhood leukemia, methotrexate prevents cell proliferation by inhibiting the dihydrofolate reductase enzyme.² At lower doses, an effect described as "steroid-like" has been shown in the treatment of RA and other rheumatic diseases. The mechanism of action in RA involves adenosine signaling, reduction of the expression of adhesion proteins and pro-inflammatory cytokines.³



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1

Although methotrexate is not the treatment of choice for systemic lupus erythematosus (SLE), its effect in controlling symptoms in patients who do not respond to hydroxychloroquine is well known.³ Adverse effects of methotrexate include ulcerative stomatitis, leukopenia, nausea, malaise, fatigue, fever, chills, dizziness, low resistance to infections and, in high doses, it usually causes erythema multiforme (EM).⁴

EM is an acute mucocutaneous immune reaction that can be triggered by infection, certain medications, or antigenic stimuli.⁵ Lesions of the oral mucosa are manifested as painful erosive blisters where thick hemorrhagic crusts can affect speech, diet and liquid intake.⁶ It can last from 3 to 6 weeks, and requires immediate discontinuation of the medication and treatment according to the severity of the signs and symptoms.⁵

Case report

A 39-year-old female patient with rheumatologic disease under investigation presented multiple ulcerations of the oral mucosa, crusts and fissures on the lips, fever, ulcerations on the hands and feet, gastralgia, dysphagia and diarrhea, making it impossible for her to eat and use oral medications, leading to her hospitalization at the University of Brasília Hospital.

In the past two years, the patient has reported arthralgia, hands, elbows and knees oedema, painful diffuse erythematous plaques, oral and genital ulcerations, alopecia and photosensitivity. She had the following diagnostic hypotheses: systemic lupus erythematosus, systemic vasculitis, and undifferentiated disease of the connective tissue, whose treatment included methotrexate, prednisone and azathioprine.

During an outpatient visit, she was prescribed a weekly oral dose of 25 mg methotrexate, with 2 capsules of 2.5 mg with each meal until 10 capsules were taken, followed by a one-week break and resumption of the regimen. However, the patient took two capsules with each meal, without any interval until completing 40 capsules, a dose that should be taken divided into four weeks. After some days, multiple extraoral and intraoral ulcerative lesions were developed, compatible with the diagnosis of EM.

On the first hospitalization day, extraoral lesions located on the nose and on the lower lip were observed, as well as intraoral ulcers on the border of the tongue and oral mucosa (Figure 1). Due to lesions in the oral cavity associated with dysphagia, diarrhea and epigastric pain, the patient was unable to ingest solid food, liquids and medications, in addition to being unable to perform oral hygiene. Lesions were also observed in the feet and hands (Figure 2).

Venous hydration therapy was initiated, as well as parenteral administration of medications. Metoclopramide 10 mg three times a day, sodium dipyron 1,000 mg every 8 hours and tramadol 100 mg every 8 hours were prescribed, in addition to the following antibiotics: ciprofloxacin 400 mg every 12 hours and clindamycin 600 mg every 6 hours intravenously due to secondary infection. Topical treatment of the oral ulcers was also performed using nystatin, aluminum hydroxide and corticosteroids. A routine of oral cleaning with 0.12% chlorhexidine digluconate solution, topical application of triamcinolone in oral base on mucosal lesions and dexpanthenol on the lips was established. Oral pasty and liquid food was maintained, which the patient ingested with some difficulty, alleviated by the application of topical anesthetic lidocaine gel 20 mg/g on the oral mucosa.

However, given the absence of a significant improvement after 3 days (Figure 3A), laser therapy was initiated (Figure 3B) to assist in the healing process. The laser applications (Therapy XT, DMC, Brazil) were performed once a day, for 5 consecutive days, using visible red light of 680 nm wavelength and infrared laser of 760 nm wavelength, with a dose of 4 J/cm² and irradiation time of 40 seconds.

On the ninth hospitalization day, the patient was discharged with significant improvement in the oral lesions and dysphagia (Figure 4) and was instructed to continue her rheumatologic monitoring.

Figure 1. (A) Ulcerations on the labial mucosa and nose; (B) Appearance of the lips and intraoral mucosa; (C) Ulceration located on the lateral border of the tongue; (D) Ulceration located on the jugal mucosa.



Figure 2. Clinical examination on the first hospitalization day. Aspect compatible with vasculitis, ulcers located in the fingers, in addition to the cyanotic aspect of the fingers



Figure 3. Partial improvement of the aspect of the oral lesions (A, B) and laser therapy (C) on the 3rd hospitalization day.

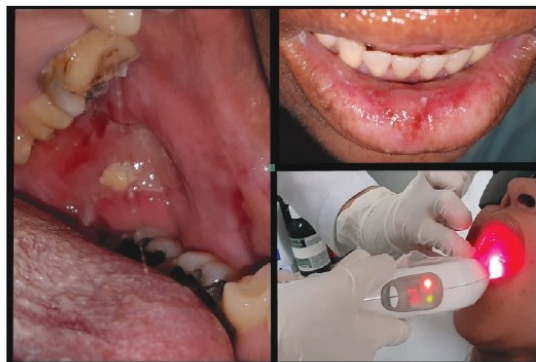


Figure 4. Clinical examination on the 9th hospitalization day, showing a significant resolution of the intraoral and extraoral lesions.



Discussion

Here we present a report of oral manifestations induced by accidental methotrexate overdose. Reports of adverse events with the use of methotrexate are not uncommon, although it is important to discuss the context in which these effects were observed. In therapeutic doses, methotrexate is an effective medication, although toxicity may be a problem for some patients.¹

We observed that, in addition to the correct prescription, it is necessary to ensure that the patient understands how to use the medication. It is important to train and empower patients to manage their medication, mainly in the management of chronic diseases.⁷ Generally, methotrexate is prescribed at a dose of 7.5 – 25 mg once a week.⁸ Unfortunately, our patient misunderstood the prescription, which ended up causing her hospitalization.

Inadequate communication between health professional and patient can be a cause of poor adherence to treatment⁷ or, as in the case presented, it can trigger a severe toxicity reaction. Although it was clear that the prescription was properly done, the patient's understanding regarding the medication dosage was inappropriate.

On the first hospitalization day, a topical use medication was prescribed for the treatment of the intraoral and extraoral lesions. As there was no significant improvement, laser therapy was recommended. A number of studies indicate the effectiveness of laser therapy for the prevention and treatment of oral mucositis that is manifested as a result of some cancer treatments.⁹ Low-intensity lasers increase cell metabolism, stimulating mitochondrial activity, acting as an analgesic and anti-inflammatory agent and repairing lesions in the mucosa.¹⁰ In the present case, the effect of laser therapy on pain relief and inflammation modulation was noticeable.

In addition to that, it is important to emphasize the contribution of the multidisciplinary team for the treatment decisions aiming at a better evolution during hospitalization. In the case in question, the dental surgeon plays a fundamental role, since oral lesions generate a significant limitation in the patient's treatment and quality of life.

Conclusion

Effective communication between health professionals and patients is essential in relation to understanding drug prescriptions for the prevention of errors related to the use of medications. The involvement of the multidisciplinary team in health can reduce risks and improve the quality of the treatment offered to the patients. In summary, the aforementioned case exemplifies the importance of communication between the health professional and the patient, as well as among the health professionals themselves.

Collaborators

ACC: Conception and design of the article, data analysis and interpretation, writing of the article, relevant critical review of the content. YPF: Conception and design of the article, data analysis and interpretation, writing of the article, relevant critical review of the content. LSV: Conception and design of the article, data analysis and interpretation, writing of the article, relevant critical review of the content. PTF: Conception and design of the article, data analysis and interpretation, writing of the article, relevant critical review of the content. DBD: Conception and design of the article, data analysis and interpretation, writing of the article, relevant critical review of the content. ENL: Conception and design of the article, data analysis and interpretation, writing of the article, relevant critical review of the content.

Patient's consent

The patient signed the Free and Informed Consent Form, through which she authorized publishing the images and description of her case.

Ethical approval

The case report herein described was approved by the Committee of Ethics in Research with Human Beings of the Medical School at the University of Brasília, under CAAE protocol 32856720.3.0000.558, and opinion No. 4,112,190, dated June 25th, 2020, observing Resolution 466/2012-CONEP/CNS and CNS Circular letter No. 166/2018.

Funding sources

None.

Authors' statement

The authors declare that the case herein reported did not receive funding for its conduction, and that there are no conflicts of interest with the topic addressed. All the authors approved the final version to be published and accept responsibility for all the information contained in the paper.



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ANEXO A – Parecer do Comitê de Ética em Pesquisa



PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Avaliação da resistência à clorexidina da microbiota bucal de pacientes internados no HUB

Pesquisador: ADRIANA SILVA DA COSTA CRUZ

Área Temática:

Versão: 1

CAAE: 87378818.7.0000.5558

Instituição Proponente: Hospital Universitário de Brasília - HUB

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 2.628.620

Apresentação do Projeto:

O objetivo desse estudo é conhecer o estado de saúde bucal e a microbiota bucal adquirida em um período máximo de 7 dias após admissão hospitalar e determinar sua resistência à clorexidina.

A clorexidina é um antimicrobiano largamente utilizado em ambiente hospitalar como desinfetante e antisséptico de uso tópico. É o antisséptico de escolha para descontaminação bucal de pacientes hospitalizados em UTIs europeias que não têm autonomia para realização de sua própria higiene (Rello et al. 2007).

Estudos demonstram a deterioração da saúde bucal como consequência de hospitalização, com aumento do acúmulo do biofilme dentário e inflamação gengival (Terezakis et al. 2011). As condições de saúde bucal e a microbiota residente e adquirida na área após um período de hospitalização podem influenciar a saúde geral, agravando quadros sistêmicos, e podendo até mesmo ser relacionada à pneumonia nosocomial (Sachdev et al. 2013).

Para o estudo, será realizada coleta com swab na mucosa da cavidade bucal de pacientes internados na Enfermaria de Clínica Médica do HUB. As amostras serão levadas ao Laboratório de Análises Moleculares de Patógenos (LAMP) do Instituto de Biologia da Universidade de Brasília, onde serão analisadas para identificação dos microrganismos e determinação de sua resistência à

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DE BRASÍLIA



Continuação do Parecer: 2.628.620

clorexidina.

É importante conhecer as características da microbiota bucal adquirida no meio hospitalar após um período de internação quanto à sua susceptibilidade em relação à clorexidina, pois há indícios de ligação entre o mecanismo de resistência a esta substância e o desenvolvimento de cepas com múltipla resistência a antibióticos (Ferreira et al. 2014).

Hipótese:

A microbiota bucal adquirida durante internação hospitalar apresenta resistência à clorexidina.

Metodologia Proposta:

Os participantes de pesquisa serão selecionados dentre pacientes internados no Hospital Universitário de Brasília, que deverão aceitar participar da pesquisa e assinar Termo de Consentimento Livre e Esclarecido. Inicialmente, dados pessoais (nome, idade, gênero) e de história clínica (motivo da hospitalização, medicamentos prescritos, comorbidades) serão coletados a partir da entrevista pessoal e do prontuário médico. Será realizado exame físico intrabucal por meio da utilização de espátula de madeira e iluminação artificial (por meio do uso de lanterna), no qual serão examinadas a mucosa bucal, dentes e próteses. Serão coletadas amostras microbiológicas de três regiões da boca. A coleta será realizada por meio de fricção de um swab estéril nos seguintes sítios da cavidade bucal: superfície vestibular dos dentes molares inferiores de um dos lados (quando estiverem presentes na boca), mucosa do fundo de saco vestibular de um dos lados na região de molares inferiores e dorso de língua. As amostras serão coletadas em três momentos: 1ª coleta (T1): até 48 horas após admissão hospitalar; 2ª coleta (T2) 48 horas após a 1ª coleta; 3ª coleta (T3) 7 dias após a admissão hospitalar. As amostras serão transportadas em recipiente refrigerado ao Laboratório de Análises Moleculares de Patógenos (LAMP) do Instituto de Biologia da Universidade de Brasília. As amostras biológicas contidas nos swabs serão homogeneizadas em 1 mL de soro fisiológico e serão centrifugadas a 10.000 xG, por 1 minuto e 30 segundos à temperatura ambiente. O volume de 800 microlitros do sobrenadante será descartado e o precipitado contido no volume restante será novamente homogeneizado por pipetagem para a realização da semeadura por esgotamento em meios de cultura apropriados. As placas serão incubadas a 37°C por 20-24 horas para culturas bacterianas e 48-72 horas para culturas de fungos. As colônias serão identificadas através da utilização de meios de cultura seletivos. A

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Continuação do Parecer: 2.628.620

sensibilidade à clorexidina das espécies de microorganismos encontradas será definida através da concentração inibitória mínima (CIM). O ensaio será realizado em placas de 96 poços não aderentes contendo meio de Mueller-Hinton (MH) para os testes envolvendo bactérias e meio YPD para os testes com fungos. Em cada poço, haverá concentrações conhecidas e diferenciadas de clorexidina. Os microorganismos isolados serão colocados em cultura nestes meios e monitorados por um período mínimo de 6 horas, onde em cada hora serão coletados dados referentes a sua densidade óptica. Dessa forma, os dados serão analisados e, assim, será identificada a concentração mínima inibitória para cada espécie de microorganismo.

Critério de Inclusão:

Os critérios de inclusão são apresentar idade igual ou superior a 18 anos, estar hospitalizado há menos de 48 horas na enfermaria de Clínica Médica do HUB e não apresentar qualquer déficit cognitivo ou dificuldade de compreensão.

Critério de Exclusão:

Os critérios de exclusão são: pacientes em condição de imunossupressão, quimioterapia, transplantados, HIV, que tenham passado por hospitalizações recentes (últimos 30 dias), com comprometimento cognitivo ou dificuldade de compreensão. Além disso, pacientes que não concordarem ou não desejarem participar do estudo.

Metodologia de Análise de Dados:

Para o cálculo amostral, partiu-se de uma taxa inicial de prevalência de resistência à clorexidina de 3%, de acordo com a literatura. Foi utilizada estimativa de comparação entre duas proporções por meio de teste unicaudal (Pocock, 1983). Considerando um poder de 80%, erro alfa de 5% e a estimativa de cepas resistentes à clorexidina de 30%, foi calculado um tamanho amostral de 42 participantes. Considerando uma taxa de perda de 10%, serão recrutados 46 participantes.

Objetivo da Pesquisa:

Objetivo Primário:

Conhecer o estado de saúde bucal e comparar a microbiota bucal de pacientes nas primeiras 48

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Continuação do Parecer: 2.628.620

horas de internação e em um período máximo de 7 dias da hospitalização na Enfermaria do Hospital Universitário de Brasília quanto à existência de resistência fúngica e bacteriana à clorexidina.

Objetivo Secundário:

Identificar as espécies de *Candida sp* e espécies bacterianas encontradas na boca de pacientes hospitalizados, no início e ao final de 7 dias de hospitalização.

Determinar a CIM(concentração inibitória mínima) para clorexidina dos isolados.

Avaliar a presença de lesões de mucosa bucal, número de dentes presentes e próteses dentárias.

Avaliar o índice de placa visível, no início e após 7 dias de hospitalização.

Avaliar o índice de saburra lingual, no início e após 7 dias de hospitalização.

Avaliação dos Riscos e Benefícios:

Riscos:

Há um pequeno risco de reação irritativa nos locais da cavidade bucal onde o swab será friccionado para coleta das amostras. Porém, trata-se de um risco baixíssimo pois a ponta do swab é revestida de algodão e estará estéril no momento da coleta. Caso sejam identificados microrganismos multirresistentes a antibióticos e de alto potencial patogênico, será realizada comunicação à CCIRAS(Comissão de Controle de Infecção Relacionada à Assistência à Saúde), responsável por ações de prevenção de infecções no HUB, para que sejam tomadas as providências cabíveis, como por exemplo a colocação de pacientes em isolamento na enfermaria, a critério.

Benefícios:

Todos os pacientes receberão instrução de higiene bucal e caso sejam encontradas necessidades urgentes e básicas de tratamento odontológico, como extrações dentárias, tratamento periodontal, curetagem e selamento de lesões cáries e tratamento de lesões de mucosa bucal, a pesquisadora ofertará o tratamento odontológico, no momento mais adequado ao participante, após discussão do caso com a equipe médica assistente. Portanto, os benefícios individuais serão

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Continuação do Parecer: 2.628.620

as orientações sobre medidas de higiene bucal, conhecimento do estado de saúde bucal e tratamento em caso de necessidades odontológicas urgentes e básicas. Os resultados da pesquisa poderão trazer benefícios à população do ponto de vista da possível necessidade de revisão do uso de biocidas como a clorexidina em protocolos hospitalares para a prevenção de infecções nosocomiais.

Comentários e Considerações sobre a Pesquisa:

O projeto apresenta justificativa da relevância da pesquisa e metodologia clara.

Considerações sobre os Termos de apresentação obrigatória:

O TCLE apresenta linguagem clara e acessível. O termo cumpre toda as obrigações e de fato esclarece os procedimentos que serão realizados caso a pessoa aceite participar da pesquisa.

Conclusões ou Pendências e Lista de Inadequações:

Recomenda-se a aprovação do projeto.

Considerações Finais a critério do CEP:

Projeto apreciado na 4ª Reunião Ordinária do CEP-FM-UnB-2018. Após apresentação do parecer do (a) Relator (a), aberta a discussão para os membros do Colegiado. O projeto foi Aprovado.

De acordo com a Resolução 466/2012-CONEP/CNS, itens X.1.- 3.b. e XI.-2.d, este Comitê chama a atenção da obrigatoriedade de envio do relatório parcial semestral e final do projeto de pesquisa para o CEP-FM, através de Notificações submetidas pela Plataforma Brasil, contados a partir da data de aprovação do protocolo de pesquisa.

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_1051879.pdf	11/04/2018 11:02:43		Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	TCLE_v1_9_4_18_docx.docx	11/04/2018 10:56:49	ADRIANA SILVA DA COSTA CRUZ	Aceito
Projeto Detalhado / Brochura Investigador	PROJETO_ADRIANA_v1_9_4_18_docx.docx	11/04/2018 10:56:08	ADRIANA SILVA DA COSTA CRUZ	Aceito
Outros	Termo_ciencia_HUB.pdf	10/04/2018	ADRIANA SILVA DA	Aceito

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Outros	Termo_ciencia_HUB.pdf	10:55:05	COSTA CRUZ	Aceito
Outros	Concordancia_Institucional_HUB.pdf	10/04/2018 10:53:04	ADRIANA SILVA DA COSTA CRUZ	Aceito
Outros	Termo_concordancia_IB.pdf	10/04/2018 10:49:41	ADRIANA SILVA DA COSTA CRUZ	Aceito
Outros	RESUMO_docx.docx	10/04/2018 10:47:41	ADRIANA SILVA DA COSTA CRUZ	Aceito
Folha de Rosto	Folha_de_rosto.pdf	10/04/2018 10:39:29	ADRIANA SILVA DA COSTA CRUZ	Aceito
Outros	Equipe_de_pesquisadores_pdf.pdf	28/03/2018 20:43:11	ADRIANA SILVA DA COSTA CRUZ	Aceito
Outros	Curriculo_Yara_Peixoto_Fidelis.pdf	28/03/2018 20:38:03	ADRIANA SILVA DA COSTA CRUZ	Aceito
Outros	Curriculo_Herick_Sampaio_Muller.pdf	28/03/2018 20:37:12	ADRIANA SILVA DA COSTA CRUZ	Aceito
Outros	Curriculo_Vicente_de_Paulo_Martins.pdf	28/03/2018 20:36:03	ADRIANA SILVA DA COSTA CRUZ	Aceito
Outros	Curriculo_Eduardo_Barbosa_Coelho.pdf	28/03/2018 20:35:10	ADRIANA SILVA DA COSTA CRUZ	Aceito
Outros	Curriculo_Erica_Negrini_Lia.pdf	28/03/2018 20:34:15	ADRIANA SILVA DA COSTA CRUZ	Aceito
Outros	Curriculo_Adriana_Silva_da_Costa_Cruz.pdf	28/03/2018 20:33:15	ADRIANA SILVA DA COSTA CRUZ	Aceito
Orçamento	PLANILHA_DE_ORCAMENTO_pdf.pdf	28/03/2018 20:29:45	ADRIANA SILVA DA COSTA CRUZ	Aceito
Declaração de Pesquisadores	Declaracao_responsabilidade_pesquisador.pdf	28/03/2018 20:28:15	ADRIANA SILVA DA COSTA CRUZ	Aceito
Outros	CARTA_DE_ENCAMINHAMENTO.pdf	28/03/2018 14:49:51	ADRIANA SILVA DA COSTA CRUZ	Aceito

Situação do Parecer:

Aprovado

Necessita Apreciação da CONEP:

Não

BRASILIA, 30 de Abril de 2018

Assinado por:
Florêncio Figueiredo Cavalcanti Neto
(Coordenador)

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ANEXO B – E-mail de aceite do artigo “Oral Health and the presence of infectious microorganisms in hospitalized patients: a preliminary observational study”

28/07/2022 09:50

Gmail - Annals of Medicine - Decision on MS SANN-2022-OR-0598.R1



Adriana Cruz <sc.adriana@gmail.com>

Annals of Medicine - Decision on MS SANN-2022-OR-0598.R1

6 mensagens

Annals of Medicine <onbehalf@manuscriptcentral.com>

16 de junho de 2022 05:29

Responder a: IANN-peerreview@journals.tandf.co.uk

Para: sc.adriana@gmail.com

16-Jun-2022

Re: SANN-2022-OR-0598.R1 - "ORAL HEALTH AND THE PRESENCE OF INFECTIOUS MICROORGANISMS IN HOSPITALIZED PATIENTS: A PRELIMINARY OBSERVATIONAL STUDY"

Dear Dr. Adriana Cruz,

I am pleased to announce that your revised manuscript entitled "ORAL HEALTH AND THE PRESENCE OF INFECTIOUS MICROORGANISMS IN HOSPITALIZED PATIENTS: A PRELIMINARY OBSERVATIONAL STUDY" has been accepted for publication in Annals of Medicine in its present form.

You will receive notice of final proofs within approximately 4-5 weeks, and instructions on how to download the pdf version of your article and how to return your final corrections will be included in that e-mail. The publisher requests that proofs are checked and returned within 48 hours of receipt of this notice to not delay the publication date of your article.

Articles are usually published online within approximately 1-2 weeks from receipt of final corrections and returned copyright agreement.

Thank you very much for your article and cooperation. Your contribution to our Journal is greatly appreciated.

Sincerely,
Daniele Roberto Giacobbe, MD, PhD
Section Editor
Annals of Medicine

Reviewer(s)' Comments to Author (if any):

Reviewer: 1

Comments to the Author

The authors have addressed the comments and provided clarifying statements or additions to the manuscript. This research opens a new door in understanding the role of oral health in hospital acquired infection and makes an important contribution to the field.

Thank you for the opportunity to review this manuscript.

Reviewer: 2

Comments to the Author

Authors have satisfactorily addressed all issues that have been raised. I don't have to suggest any further revisions.

Associate Editor's Comments (if any):

Associate Editor: 1

Comments to the Author:

I am pleased to inform you that the above manuscript has been accepted for publication

Adriana Cruz <sc.adriana@gmail.com>

17 de junho de 2022 12:40

Para: Erica Negrini Lia <ericalia70@gmail.com>, Vicente de Paulo Martins <vicente.p.martins@gmail.com>, Danielly Mendonça <daniellymsilva98@gmail.com>, Yara Peixoto <yarapeixotof@gmail.com>, Herick Muller <drhsmuller@gmail.com>

Olá pessoal!

Nosso artigo foi aceito para publicação pela revista "Annals of Medicine", conforme e-mail que encaminho abaixo.

<https://mail.google.com/mail/u/0/?ik=7eb096c48b&view=pt&search=all&permthid=thread-f%3A1735779348950606085&siml=msg-f%3A1735779...> 1/4