Universidade Estadual de Campinas
Faculdade de Odontologia de Piracicaba

HASSAN NAVEED

ROLE OF *CPPA* IN COMPLEMENT MEDIATED IMMUNE EVASION OF *STREPTOCOCCUS SANGUINIS*

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Thesis presented to the Piracicaba Dental School of the University of Campinas in partial fulfillment of the requirements for the degree of Doctor in Oral and Dental Biology, in Microbiology and Immunology area.

Tese apresentada a Faculdade de Odontologia de Piracicaba da Universidade Estadual de Campinas como parte dos requisitos exigidos para a obtenção do título de Doutor em Biologia Buco-Dental, Area de Microbiologia e Imunologia.

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ESTE EXEMPLAR CORRESPONDE À VERSÃO FINAL DA TESE DEFENDIDA PELO ALUNO HASSAN NAVEED E ORIENTADA PELA PROFA. DRA. RENATA DE OLIVEIRA MATTOS GRANER

Piracicaba
2023

Ficha catalográfica
Universidade Estadual de Campinas
Biblioteca da Faculdade de Odontologia de Piracicaba
Marilene Girello - CRB 8/6159

N229r

Naveed, Hassan, 1992-
Role of *cpp*A in complement mediated immune evasion of *Streptococcus sanguinis* / Hassan Naveed. – Piracicaba, SP : [s.n.], 2023.

Orientador: Renata de Oliveira Mattos Graner.
Coorientador: Lívia Araújo Alves.
Tese (doutorado) – Universidade Estadual de Campinas, Faculdade de Odontologia de Piracicaba.

1. *Streptococcus sanguinis*. 2. Peptídeos hidrolases. 3. Fatores de virulência. 4. Fator H do complemento. I. Mattos-Graner, Renata de Oliveira, 1971-. II. Alves, Lívia Araújo, 1988-. III. Universidade Estadual de Campinas. Faculdade de Odontologia de Piracicaba. IV. Título.

Informações Complementares

Título em outro idioma: Papel do *cpp*A na evasão imune mediada pelo complemento de *Streptococcus sanguinis*
Palavras-chave em inglês:
Streptococcus sanguinis
Peptide hydrolases
Virulence factors
Complement factor H
Área de concentração: Microbiologia e Imunologia
Titulação: Doutor em Biologia Buco-Dental
Banca examinadora:
Renata de Oliveira Mattos Graner [Orientador]
Thaís Larissa Araujo de Oliveira Silva
Eliane Namie Miyaji
Ana Paula de Souza
Marlise Inêz Klein Furlan
Data de defesa: 03-03-2023
Programa de Pós-Graduação: Biologia Buco-Dental

Identificação e informações acadêmicas do(a) aluno(a)
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- Currículo Lattes do autor: http://lattes.cnpq.br/3765257183456730

UNIVERSIDADE ESTADUAL DE CAMPINAS
Faculdade de Odontologia de Piracicaba

A Comissão Julgadora dos trabalhos de Defesa de Tese de Doutorado, em sessão pública realizada em 03 de março de 2023, considerou o candidato HASSAN NAVEED aprovado.

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A Ata da defesa, assinada pelos membros da Comissão Examinadora, consta no SIGA/Sistemade Fluxo de Dissertação/Tese e na Secretaria do Programa da Unidade.

“As pessoas que vencem neste mundo são as que procuram as circunstâncias de que precisam e, quando não as encontram, as criam”.

George Bernard Shaw

DEDICATÓRIA

Dedico esta tese a meus pais, a minha mãe **Riaz Bibi** (*in memoriam*) e ao meu pai **Ghafoor Ur Rehman**. Que com todo seu amor e zelo, sempre me deram esperanças para alcançar o que desejei. Agradeço todo incentivo e ajuda em absolutamente tudo o que eu precisei. Agradeço por sempre estarem comigo, me acompanhando em todas as jornadas. E por serem o meu maior exemplo e com certeza a minha fonte de inspiração e amor.

Ao meu pai Ghafoor Ur Rehman**,** especialmente, que sempre me ensinou a nunca desistir e sempre estudar, conquistando tudo aquilo que sonhei. A ele que sempre foi e será meu exemplo de vida e persistência e pelo amor que nunca me deixou faltar.

Eu gostaria de agradecer especialmente a Profª Drª Renata de Oliveira Mattos-Graner e a Drª. Livia Araújo Alves, por todo auxílio que elas me deram durante todo período, pois com a ajuda delas hoje estou apto a completar meu doutorado.

A familiares e amigos, e a todos que sempre torceram pelo meu crescimento pessoal e profissional. Com muito amor lhes agradeço e dedico essa conquista a vocês.

AGRADECIMENTOS

À Deus pela minha vida, por sua presença a todo momento nos meus caminhos me ajudando de a melhor forma trilhá-los e por todos os desafios até hoje experenciados.

À Universidade Estadual de Campinas, através do Reitor Prof. Dr. Antônio José de Almeida Meirelles, e à Faculdade de Odontologia de Piracicaba, através do diretor Prof. Dr. Flávio Henrique Baggio Aguiar, pela oportunidade de crescimento profissional e pessoal nesta conceituada Universidade.

A Coordenadoria da Pós-Graduação, especialmente aos coordenadores do Programa de Biologia Buco-Dental. À Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES), por proporcionar a minha formação profissional com seu apoio e garantir o benefício da bolsa de estudos de doutorado outorgada.

A Profª Drª Renata de Oliveira Mattos-Graner por aceitar me orientar no doutorado, bem como pela confiança, por sua dedicação e orientação em meu desenvolvimento profissional.

Ao Drª. Livia Araújo Alves pelas experiências vivenciadas, pelos conselhos e orientações e todo apoio durante minha estadia no Programa.

Ao Prof Dr. José Francisco Höfling, Prof. Dr. Marcelo Fabiano Gomes Boriollo e Profª Drª. Marilse Inêz Klein Furlan pela cooperação e auxílio. Agradeço por todo desenvolvimento na área acadêmica e por me proporcionarem ótimas experiências.

Gostaria de agradecer ao Dr. Edgard Graner e à Dra. Débora Campanella Bastos, do Departamento de Patologia, que com muita paciência me ajudaram muito no manuseio e registro das análises de citometria de fluxo.

Agradeço ao Dr. Tsute Chen, Diretor do Centro de Bioinformática do Forsyth Institute, Cambridge, E.U.A., pela pronta cooperação com este estudo, através das análises genômicas das cepas bacterianas estudadas.

Aos técnicos de laboratório da Faculdade: Valéria Alessandra Defávari Franco, Laboratório de Microbiologia e Imunologia Oral, que sempre me ajudou e incentivou muito em tudo o que precisei dentro e fora do laboratório.

Aos examinadores titulares que compuseram a banca de defesa da tese e auxiliaram em como aprimorar nossos estudos: Profª Drª Renata de Oliveira Mattos-Graner, Profª Drª Marlise Inêz Klein Furlan, Prof. Dra. Juliana Campos Junqueira e Prof. Dra. Thais Larissa Araujo de Oliveira Silva e aos membros suplentes: Profª Drª Ruchele Dias Nogueira e a Prof Dr. Marcelo Rocha Marques.

Aos companheiros do Laboratório de Microbiologia e Imunologia pelo convívio de sempre e por todos os momentos agradáveis e memoráveis. Agradeço imensamente a todos que colaboraram com o desenvolvimento desse trabalho direta ou indiretamente.

Este estudo foi subvencionado pela FAPESP (proc. nº. 2018/02054-4; proc. 2021/13074-9) e pela Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) – Código financeiro 001 e bolsa de doutorado.

ABSTRACT

Streptococcus sanguinis is an abundant commensal species of the oral microbiome, which commonly causes opportunistic cardiovascular infections, including infective endocarditis. Although *S. sanguinis* survival in the bloodstream and in extra-oral tissues are required for its systemic virulence, the mechanisms involved in evasion of this species to complement-mediated immunity are unknown. *In silico* analysis of *S. sanguinis* genomes revealed the gene *cppA*, encoding for a protein (CppA) annotated as a degrading protease of C3, a central effector component of the complement. The aim of this study was to investigate the role of CppA in *S.sanguinis* evasion to complement-mediated immunity. To this end, *cppA* expression was initially assessed by RT-qPCR in nine previously characterized *S. sanguinis* strains, including SK36 and clinical strains isolated from the oral cavity or from the bloodstream. Then, a nonpolar isogenic mutant of *cppA* (called SKcppA) was constructed by homologous recombination in strain SK36. SKcppA was also transformed with a shuttle plasmid harboring a full copy of *cppA* to yield the complemented strain SKcppA+, used as control. Flow cytometry assays were then applied to compare SKcppA, SK36 and SKcppA+ regarding their binding to C3b, to the complement activating proteins C1q and SAP, and to complement down-regulators (fator H and C4BP), after treatment with human serum (20%) or with PBS or heat-inactivated serum (negative controls). The frequencies of bacterial phagocytosis by neutrophils (PMN) isolated from human peripheral blood were assessed in the same strains previously labeled with FITC fluorophore and exposed to PMN in medium supplemented or not with 20% human serum. Persistence of strains in human blood were also assessed in *ex vivo* assays. Additionally, the frequencies of natural transformation with a shuttle-plasmid were determined for strain comparisons. Significant increase in C3b deposition was observed in SKcppA as compared to SK36 and SKcppA+ (Kruskal-Wallis, $p<0,05$). SKcppA did also show increased binding to C1q and SAP, whereas significant reductions in binding to C4BP e FH. Consistently, SKcppA showed significant increases in opsonophagocytosis by PMN ($p <0,05$) and significant reductions in *ex vivo* survival in blood after 4 to 42 h of incubation, when compared to SK36 and SKcppA+ (Kruskal-Wallis, $p<0,05$). Slight increase in transformation efficiency (20% in relation to SK36) was observed in SKcppA ($p <0,05$). These findings show that CppA functions in evasion to C3b deposition and opsonophagocytosis by PMN, affecting bacterial binding to C1q and SAP, and recruiting complement down-regulator proteins. CppA is also required for *S. sanguinis* persistence in human blood and shows slight influence on *S. sanguinis* competence phenotype. The important role of CppA in virulence-associated functions in *S. sanguinis* here reported support the application of this protease as a therapeutic target for controlling systemic infections by oral streptococci.

Keywords: *Streptococcus sanguinis*, protease, virulence factor, complement system, Factor H (FH), SAP, C1q, C4BP, opsonophagocytosis, blood viability, natural transformation.

RESUMO

Streptococcus sanguinis é uma espécie comensal abundante do microbioma bucal, a qual comumente causa infecções oportunistas cardiovasculares, incluíndo-se a endocardite infecciosa. Embora a sobrevivência de *S. sanguinis* na corrente sanguínea e nos tecidos extra-bucais seja necessária para que esta espécie cause estas infecções sistêmicas, os mecanismos de escape desta espécie à imunidade mediada pelo sistema complemento não são conhecidos. Análises *in silico* dos genomas de *S. sanguinis* revelaram a presença do gene *cppA*, o qual codifica uma proteína (CppA) anotada como protease degradadora de C3, componente efetor central do complemento. O objetivo deste estudo foi investigar a participação de CppA no escape de *S. sanguinis* à imunidade mediada pelo sistema complemento. Para isto, inicialmente a expressão de *cppA* foi investigada através de ensaios de RT-qPCR em nove cepas previamente caracterizadas de *S. sanguinis*, incluíndo-se SK36 e cepas clínicas isoladas da cavidade bucal e da corrente sanguínea. A seguir, foi construída por recombinação homóloga uma cepa mutante isogênica *cppA* a partir da cepa SK36 (nomeada SKcppA). SKcppA foi ainda transformada com um plasmídeo auto-replicativo contendo uma cópia de *cppA*, para gerar a cepa complementada SKcppA+, utilizada como controle. Ensaios de citometria de fluxo foram então realizados para comparar SK36, SKcppA, e SKcppA+ quanto à ligação a C3b, a proteínas ativadoras do complemento (C1q e SAP) e a proteínas inibidoras do complemento (Fator H e C4BP), após tratamento com soro humano (20%) ou com PBS e soro inativado (controles negativos). Comparações na frequência de fagocitose por neutrófilos (PMN) isolados de sangue periférico humano foram realizadas com as mesmas cepas previamente marcadas com fluoróforo (FITC) e expostas aos PMN em meio acrescido ou não de 20% de soro, sendo as proporções de PMN com bactérias intracelulares determinadas por citometria de fluxo. A sobrevivência das cepas em sangue humano foi também avaliada em ensaios *ex vivo*. As frequências de transformação natural com plasmídeo auto-replicativo também foi comparada. Aumento significativo na deposição de C3b foi observado em SKcppA em comparação com SK36 e SKcppA+ (Kruskal-Wallis, $p<0,05$). SKcppA também mostrou maior ligação a C1q e SAP, além de redução significativa na ligação a C4BP e FH. Consistentemente, SKcppA apresentou aumento significativo na opsonofagocitose por PMN ($p <0,05$) e redução significativa (da sobrevivência) *ex vivo* após 4 a 42 h de incubação em sangue humano, em comparação à SK36 e à SKcppA+ (Kruskal-Wallis, $p<0,05$). Moderado aumento na eficiência de transformação (20% relativo à SK36) foi observado em SKcppA ($p <0,05$). Os resultados deste estudo demonstram que CppA é uma proteína de escape à deposição de C3b e opsonofagocitose por PMN, interferindo na ligação bacteriana a C1q e SAP e recrutando proteínas inibidoras do complemento. CppA é ainda requerida para a sobrevivência de *S. sanguinis* em sangue humano, apresentando pequena influência no fenótipo de competência desta espécie. O papel importante de CppA nos fenótipos de *S. sanguinis* associadas à virulência reportados neste estudo suportam a utilização desta protease como alvo terapêutico para controle de infecções sistêmicas oportunistas por estreptococos.

Palavras-chave: *Streptococcus sanguinis*, protease, fator de virulência, sistema complemento, Fator H (FH), SAP, C1q, C4BP, opsonofagocitose, sobrevivência em sangue, transformação natural.

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LIST OF ABBREVIATIONS

LP: Lectin pathway

CP: Classical pathway

AP: Alternative pathway

PMN: Polymorphonuclear neutrophils

PRP: Pattern recognition proteins

CppA: C3-complement degrading protease

IE: Infective endocarditis

AEP: Acquired enamel pellicle

NETs: Neutrophils extracellular traps

Swan: Streptococcal cell-wall anchored nuclease

SrpA: Serine-rich protein A

PAAP: Platelet-aggregation-associated protein

MAC: Membrane attack complex

°C: Celsius grade.

µg: microgramа.

10% CO_2: 10% carbondioxide

16S rRNA: 16S ribosomal ribonucleic acid

A550nm: Absorbance at 550 nanometers wavelength

BHI: Brain Heart Infusion (Agar or Broth)

BLAST: Basic Local Alignment Search Tool. (BLASTP for protein)

cDNA: DNA obtained after reverse transcription of RNA

DNA: Deoxyribonucleic Acid

dNTP: Deoxynucleotide triphosphates

erm^R: Erythromycin resistance

mid log phase: Mid logarithmic phase of bacterial growth

g: Grams

gDNA: genomic DNA

h: Hours

LB: Luria-Bertani Medium

min: minute

ml: Millilitre

mM: milliMolar

ORF: Open Reading Frame

bp: Basepairs

PCR: Polymerase Chain Reaction

pDNA: Plasmid DNA

pH: potential of hydrogen

qPCR: quantitative polymerase chain reaction

qRT-PCR: Real-Time Quantitative Reverse Transcription PCR

RNA: Ribonucleic acid

PMN: polymorphonuclear leukocytes

SUMMARY

1 INTRODUCTION

Streptococcus sanguinis is a primary colonizer of tooth surface and an abundant member of human oral microbiome (Huttenhower *et al*., 2012; Zhu *et al*., 2018), known for being an opportunistic pathogen of cardiovascular infections, such as atherosclerosis and infective endocarditis (IE), in susceptible hosts (von Reyn *et al*., 1981; Douglas *et al*., 1993; di Filippo *et al*., 2006; Nakano *et al*., 2006). The annual incidence of infective endocarditis (IE) is 3–10 per 100,000 people (Cahill TJ *et al*., 2016), with a mortality frequency of approximately 30% (Rajni R *et al*., 2020). The individuals receiving health-care assistance are more susceptible to IE, accounting for 25 to 34% of total cases (Rezar R *et al*., 2021). The epidemiology of IE is however changing, apparently due to increases in the number of individuals receiving implanted cardiovascular devices and increasing life expectancy (Rajni R et al., 2020). The susceptibility of hosts having chronic kidney disease, cancer, and human immunodeficiency virus (HIV) further contribute to increases in the incidence of IE. About 80–90% of the cases of IE involves infection by *Staphylococcus aureus* (about 26.5%), viridans group *streptococci* (approximately 19%), other *streptococci* (17.5%) and *enterococci* (10.5%) (Rajni R *et al*., 2020).

Molecular mechanisms of virulence of *S. sanguinis* leading to cardiovascular infections remains to be elucidated. According to genetic screening in rabbit models of infective endocarditis, genes involved in promotion of cardiac vegetations were identified and included genes involved in cell wall biogenesis (*SSA_099*, *murB, bacA*,), metabolic function (*purB* and *thrB*, respectively for purines and threonine synthesis), and adaptation to anaerobic conditions (*nrdD*) (Paik *et al*., 2005; Martini *et al*., 2020). Screenings of genes involved in *S. sanguinis* fitness in heat-inactivated serum also revealed genes involved in metal transport and purine metabolism (Zhu *et al*., 2022). However, functions of *S. sanguinis* involved in persistence in human blood are required and not addressed in animal models of infection or in studies with heat-inactivated serum, given the crucial role of the complement system in host immune blood clearance.

The complement system can be activated by three different pathways, the lectin (LP), classical (CP) and the alternative pathway (AP), each of them produce effector proteins responsible for blood clearance mechanisms such as opsonization, chemotaxis and activation of phagocytes, as was as immune adherence by erythrocytes. Spontaneous C3 hydrolysis

activates AP, whereas Pattern recognition proteins are required for recognition and further activation of LP and CP, whereas both pathways can be amplified that spontaneous hydrolysis of C3 in the AP. Sortase, NADH-oxidases and cell-wall associated nucleases expression may play role in complement/PMN immunity evasion by mechanisms not entirely understood (Yamaguchi *et al*., 2006; Morita *et al*., 2014). Complement system evasion mechanisms of *S. sanguinis* are largely unknown, although strains of this species show higher resistance to deposition of major complement effector component (C3b) and downstream functions, when compared to other commensal streptococcal species of the oral cavity (Alves *et al*., 2019). On the other hand, *S. sanguinis* strains show high diversity in C3b deposition and binding to pattern recognition proteins (PRP) and to complement host down-regulators (Alves *et al*., 2022). Moreover, invasion of cardiovascular endothelial cells by *S. sanguinis* appears to be mediated by complement system (Alves *et al*., 2022). Thus, molecular mechanisms of complement evasion of *S. sanguinis* are crucial to be elucidated in the particular capacity of *S. sanguinis* to promote cardiovascular infections.

Human C3 is a 186 kDa protein, comprised of a 111 kDa α chain and a 75 kDa β chain linked by disulfide bonds. Pathogenic species of streptococci express proteases associated to the cell wall capable of degrading C3 (Lambris *et al*., 2008; Marquart, 2021). *S. pneumoniae* expresses at least two proteases potentially involved in C3 degradation, CppA and PhtA, although proteolytic activity of CppA on C3 has not been published , but reported in a patent (Hostetter *et al*., 2004; reviewed in Marquart, 2021). CppA is conserved among streptococcal species of the nasopharynx and oral cavity, including *S. sanguinis* (Lynskey *et al.*, 2017; Agarwal *et al*., 2021; Alves *et al*., 2022; Mattos-Graner *et al*., 2023). However, the proteolytic activity of CppA proteins could not be experimentally demonstrated so far. In *S. pyogenes*, although total protein bacterial extracts could degrades C3, recombinant CppA protein did not show proteolytic activity on C3 (Lynskey *et al.*, 2017). In *S. sanguinis*, we found that *cppA* might be regulated by the two-component system SptRS, shown to be required for persistence in whole saliva and complement evasion (Carmargo *et al*., 2018). CppA was also found to be a key protein involved in *Streptococcus pneumoniae* virulence and in the strain capacity to be transmitted from mothers to pups in mice and ferret models of infection (Rowe *et al*., 2019).

2 REVIEW OF LITERATURE

2.1 ROLE OF *STREPTOCOCCUS SANGUINIS* IN SYSTEMIC DISEASES

Streptococcus sanguinis is an abundant bacterial specie of the oral cavity. It is a primary colonizer of tooth surface (Carlsson *et al*., 1970; Kreth *et al*., 2016), and influences microbial succession of dental biofilms. This species is present at higher proportion in health-associated dental biofilms (Ge *et al*., 2008; Giacaman *et al*., 2015). Production of H_2O_2 by *S. sanguinis* enables it to inhibit colonization by *Porphyromonas ginigivalis* and *Streptococcus mutans* (pathogens of periodontal disease and dental caries, respectively) in dental biofilms. It enlightens the importance of better understanding of molecular mechanisms of *S. sanguinis* in modulating oral colonization, being a commensal specie of oral cavity (Kreth *et al*., 2005; Kreth *et al*., 2009). On the other hand, *S. sanguinis* is known for its common involvement in infective endocarditis (IE) (Di *et al*., 2006; Douglas *et al*., 1993; von Reyn *et al*., 1981). Abundance of these microorganisms in dental biofilms might be a reflection of involvement of *S. sanguinis* in systemic infections. However, in comparison to species of other oropharyngeal streptococci, *S. sanguinis* (SK36) genome analysis unfolds remarkably high number of surfaces adhesins and cell wall proteins (Xu *et al*., 2007). Expression of diverse adhesins on surface of *S. sanguinis* might enhance its competitive capability to bind to tooth surface for colonization (Nobbs *et al*., 2009), but molecular mechanisms leading to this competitiveness are yet to be unfolded. A film composed of host and microbial salivary glycoproteins, serum glycoproteins bound to tooth surface termed acquired enamel pellicle (AEP) is the initial point of attachment,high-affinitygh affinity binding of *S. sanguinis* to the components of AEP is required (Lee *et al*., 2013). H_2O_2 production is induced following the pioneer adhesion of *S. sanguinis* resulting in inhibition of competing species and inducing release of genomic DNA, a key component of biofilms extracellular matrix (Kreth *et al*., 2009; Moraes *et al*., 2014).

In dental biofilms, sucrose is utilized to produce extracellular polysaccharides (EPS) of insoluble nature by the caries pathogen *Streptococcus mutans* for its establishment. Differently, the majority of *S. sanguinis* strains does not rely on production of insoluble glucans (Hamada *et al*., 1980). Similarly, to the way capsules are anti-opsonizing, cell surface bound EPS of *S. mutans* inhibits the deposition of opsonin's of the complement system (Alves *et al*., 2016). *S. sanguinis* does not produce similar insoluble EPS, but most strains of this species show reduced susceptibility complement mediated opsonization when compared to other oral

streptococci (Alves *et al*., 2019). However, complement evasion factors of *S. sanguinis* are still unknown.

2.2 *STREPTOCOCCUS SANGUINIS* SYSTEMIC VIRULENCE FACTORS

S. sanguinis strains are known to be involved in infective endocarditis (IE) with high frequency (Douglas *et al*., 1993; von Reyn *et al*., 1981) and also in atheromatosis (Nakano *et al*., 2006), but the mechanism of systemic virulence is not well understood yet. Currently few known virulence factors are limited to only some strains (Kreth *et al*., 2016). After bloodstream invasion from the oral cavity, bacteria may avoid immune clearance by host defenses and eventually, cause extra-oral host tissue infections. C-reactive protein and fibrinogen are observed to be in elevated levels upon bacteremia's caused by oral bacteria (Wu *et al*., 2000) and are risk factors of cardiovascular diseases (Corrado *et al*., 2006). Injured endothelium results in formation of platelets and fibrin networks which are infected by bacteria upon entering the bloodstream, resulting in vegetation formation in cases of infective endocarditis (IE). The high repertoire of cell wall and surface lipoprotein proteins found in the *S. sanguinis* genome might link this species to IE pathogenesis thought binding to host tissues for infection and/or evasion to host immune responses (Xu *et al*., 2007). In mice models, other oral streptococci such as *S. mutans*, *Streptococcus anginosus* and *Streptococcus sobrinus* promote higher activation of of CD4+ and CD8+ T lymphocytes, when compared to *S. sanguinis* (Kreth *et al*., 2016). *Streptococci* are divided into four major groups namely Mutans, Anginosus, Salivaris, and Mitis (*S. sanguinis* is part of Mitis group). Consistently, *S. sanguinis* strains are less susceptible to deposition of complement system comparing to other species of mitis group (Alves *et al*., 2019). Better understanding of mechanisms *S. sanguinis* evasion to complement mediated immunity needs to be unfolded for developing strategies to control streptococcal bacteremia and to understand how *S. sanguinis* dominates in state of health in oral cavity. Saliva and gingival crevicular fluid contain complement proteins as defense factors (Andoh *et al*., 1997; Huynh *et al*., 2015).

Streptococcus sanguinis appears to evade intracellular and extracellular microbicidal factors of neutrophils (PMN) through the expression of systemic virulence genes. These include *swan* (streptococcal cell-wall anchored nuclease) (Morita *et al*., 2014) affects neutrophils extracellular traps (NETs), which is majorly built of DNA. *Streptococcus sanguinis*

further express oxidative stress resistance gene termed *nox*, likely involved in resistance to oxygen-dependent microbicidal functions of PMN (Ge *et al*., 2016). Thus, *S. sanguinis* systemic virulence seems to be dependent on escape of PMN. Resistance to phagocytosis and killing by human blood PMN is reported, as reduced by inactivation of *srtA* in SK36, which codes enzyme SrtA (cell wall anchoring protein) (Yamaguchi *et al*., 2006). These findings indicating that opsonophagocytosis susceptibility depends on cell surface proteins of *S. sanguinis*. SrtA of S. *sanguinis* SK36, is involved in anchoring total of 33 proteins to cell wall, but in IE rabbit models none of them were found to be involved in virulence (Turner *et al*., 2009). Systemic virulence factors are tracked back using extensive screening studies using competitive animal models of IE (Turner *et al*., 2009; Ge *et al*., 2008; Callahan *et al*., 2011; Ge *et al*., 2016). One potential limitation of these models is that, as mutant and parent strains are simultaneously inoculated in the same niche, thus mutant strains may be favored by wild type secreted factors (Ge *et al*., 2008).

Along with blood evasion factors of PMN, *S. sanguinis* strains associated with human IE cases also show increased ability to promote platelet aggregation (Herzberg *et al*., 1990). Platelet aggregation is known to be induced by several oral streptococcal species (Chia *et al*., 2004). The ability of *S. sanguinis* strains to induce platelet aggregation was observed to be approximately 60% (Herzberg, 1996). In heart valves, vegetation development can be highly promoted by platelet aggregation and aids in evasion to host immune factors (Fitzgerald *et al*., 2006). For this process in *S. sanguinis* species, SrpA (serine-rich protein A) and PAAP (Platelet-aggregation-associated protein) are known surface glycoproteins. Studies on an *S. sanguinis* homologue of *S. gordonii* SrpA, GspB, indicate that SrpA/GspB glycoproteins are responsible for binding to salivary mucin (Plummer *et al*., 2005). SrpA/GspB glycoprotein is reported to be involved in binding to platelet receptors, which is an initial step of platelet aggregation (Fitzgerald *et al*., 2006). PAAP glycoprotein consists of collagen-like domain and is rhamnose rich and reported to be involved in initial interactions with platelet receptors and induction of aggregation (Gong *et al*., 1995; Herzberg *et al*., 2005). Studies using strain NCTC 7863 from IE patient indicate that complement activation is needed for *S. sanguinis* capacity to promote platelet aggregation (Ford *et al*., 1996). Among different donors, platelet aggregation does not depend on polymorphisms of platelet receptor but varies based on serum components (Fitzgerald *et al*., 2006; Ford *et al*., 1996). Hence, role of complement system is evident in PMN functions induction in blood and seems to modulate platelet aggregation by *S. sanguinis*,

strengthening the need of molecular studies to identify the mechanisms through which *S. sanguinis* modulate complement functions.

2.3 BACTERIAL CONTROL BY THE COMPLEMENT SYSTEM

In innate immunity, microorganisms of blood and tissues are battled by one of its key components termed the complement system. This system further aids in adaptive immune regulation and amplification of functions of B and T lymphocytes and further plays roles in maintaining homeostasis in tissues (Walport, 2001; Zipfel and Skerka, 2009). The complement system includes approximately 30 soluble proteins, as well as receptors on several cells (red blood cells, platelets, phagocytes, and others) and membrane regulatory proteins (Zipfel and Skerka, 2009). Opsonizing factors of the complement (C3b/iC3b, C4b) are produced upon cascade of proteolytic nature. These cascades further generate membrane attack complexes (C5 to C9), and inflammatory and chemotactic factors (the C3a, C4a, C5a anaphylatoxins) (Walport, 2001). Complement receptors (CR) on red blood cells, phagocytes and numerous nucleated cells include receptors for C3b and complement anaphylatoxins. Once bound to the microbial surface, C3b works as an opsonin for phagocytosis, as well as for binding to red blood cells expressing C3b/iC3b receptors (CR1, CR3, CR4, CRIg), which aids on microbial removal by red blood cells to the phagocytic system of the liver and spleen, a process termed Immune Adherence (Walport, 2001; Dunkelberger and Song, 2010). On the other hand, regulatory complement proteins (including cell-membrane and fluid phase proteins) protect host cells from destructive complement functions. Different complement pathways are down-regulated by different sets of these regulatory proteins such as the fluid-phase glycoproteins, factor H, factor HL1 (factor H-like 1 protein), C4b-binding protein (C4BP), C1 inhibitory protein C1-INH (C1-esterase inhibitor) (Zipfel and Skerka, 2009; Merle *et al*., 2015) (Figure 1).

There are three pathways of complement cascade activation, the classical, the lectin and the alternative pathways (Figure 1). Different proteins activate each of these pathways. The activation of the classical pathway is initiated through binding to the C1 complex (consisting of C1q, C1r and C1s) to IgM or IgG antibodies (on antigen-antibody complexes), pentraxins (SAP, CRP) of acute phase on the microbial surfaces or directly to particular microbial components. Binding of C1q results in activation of the C1r/s serine proteases, which in turn cleaves C2 and C4 complement proteins, generating the C4b and C2b fragments which are

assembled into the C3 convertase C4b2b. The lectin pathway is initiated by the mannose-binding lectin (MBL) protein which binds to mannose-containing surface components of microorganisms, resulting in activation of MASP1/2 proteases that cleave C2 and C4 and resulting in formation of the C3 convertase C4b2b (Zipfel and Skerka, 2009; Matsushita, 2010). Differently, the alternative pathway is initiated with spontaneous hydrolysis of C3, generating C3b-like reactive compounds, C3(H_2O) which binds to fluid-phase Factor B and cleaved by Factor D at the Factor B part, forming the C3 convertase C3bBb; C3bBb convertases may stabilized and/or recruited to the foreign surfaces by properdin (Zipfel and Skerka, 2009). Once C3 convertases are generated they further cleaves additional C3 complement proteins which combined to C3 convertases form the C5 convertases either of the classical/lectin pathways (C4b2b3b) or of the alternative pathways (C3bBbC3b). The C5 convertases of all pathways cleave C5, of which C5b combines with C6, C7, C8, and C9 resulting in formation of membrane attack complex (MAC) (C5b-C9). Complement effector molecules generated during complement proteolytic cascades play multiple functions such as opsonization, chemotaxis, anaphylatoxin, cytolysis and enhanced antibody production. Opsonization is the process in which phagocytosis of microbes is enhanced in the presence of opsonin (C3b) because phagocytes possess C3b receptors on their surface. The fragments C3a, C4a and C5a act as chemoattractant, by attracting phagocytes and induce degranulation of mast cells (C3a, C4a and C5a), which results in release of histamine aiding in enhanced vascular permeability and contraction of smooth muscles. Cytolysis is caused by the MAC insertion into membrane of different cells (including bacteria and tumor cells). C3b is further degraded into C3d, which is bound through C3d receptors by dendritic cells and B cells, further stimulating antibody responses.

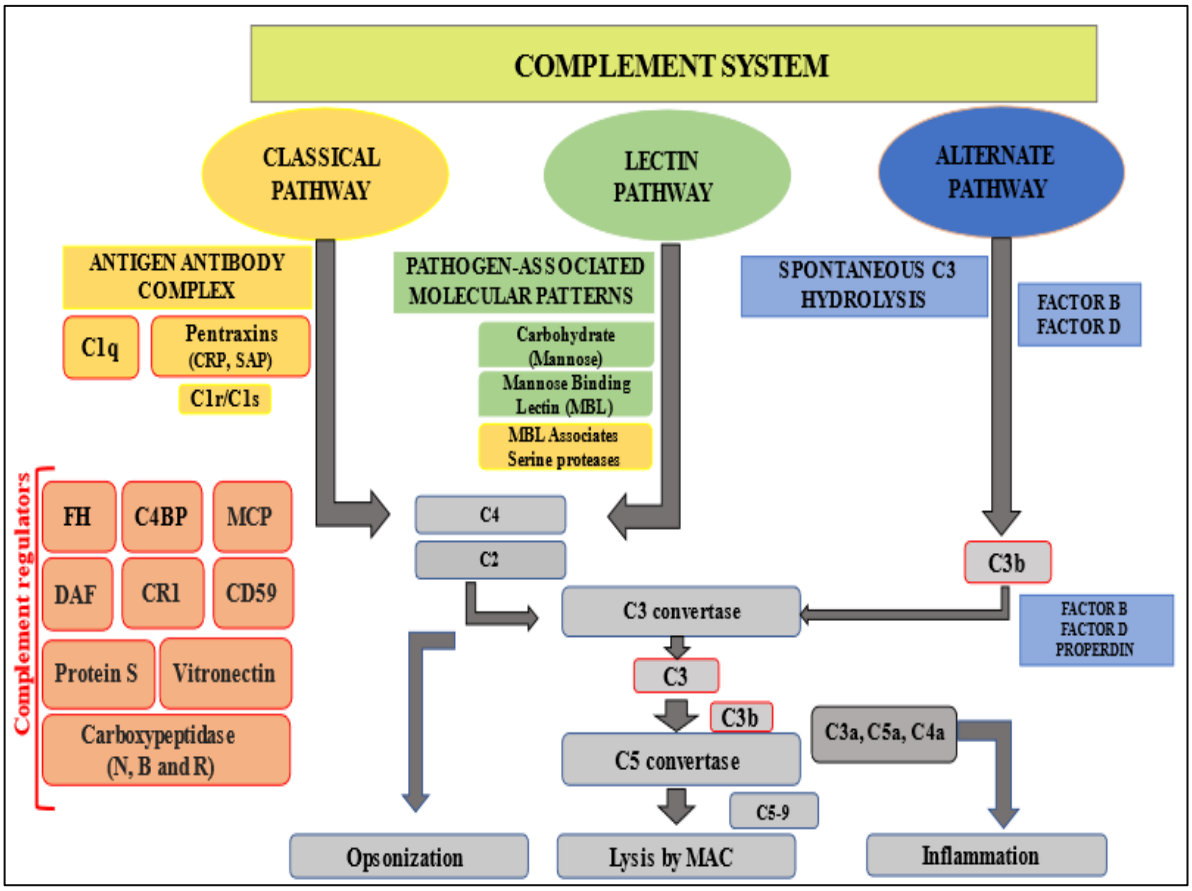

Figure 1 - Activation and regulation of three pathways of the complement system. The complement system can be activated by three pathways named Classical, Lectin, and Alternate pathways. The classical pathway (CP) and Lectin pathway (LP) are activated once the target structure has interacted with pattern recognition molecules C1q, which recognizes surface-bound IgG/IgM antibodies, pentraxins (CRP, SAP) or directly bind to the target surface. The LP is triggered by mannose-containing carbohydrates recognized by collectins, e.g, Mannose Binding Lectins (MBL), and ficolins. Once pattern recognition occurs, serine proteases associated with C1q (C1r/C1s) or MBL (MASP1/2) are activated and cleave C4 and C2, generating fragments that are combined into a C3 convertase (C4b2a). The AP is spontaneously activated upon slow hydrolysis of the thioester bond of C3, generating a C3b-like form, C3(H_2O), which can bind to factor B (FB). C3(H_2O)FB is then cleaved by a serine protease (Factor D), forming C3 convertase [C3(H_2O)Bb or C3bBb]. C3-convertases cleave C3, releasing C3b and C3a effector fragments involved in downstream defense functions. Host surfaces are protected by complement regulators. These include the serine protease factor I, complement receptor 1 (CR1), membrane cofactor protein (MCP; CD46), C4-binding protein (C4BP), and fluid phase Factor H, decay accelerating factor (DAF), vitronectin, and membrane-bound CD59. Among those, C4BP present in fluid-phase or associated to the surface, binds to multiple C4b fragments dissociating the C3-convertases of the CP/LP and favoring downstream C4b cleavage. On the other hand, soluble FH binds to C3b, working as a co-factor for C3b degradation by FI and avoiding FB binding. FH also promotes dissociation of C3bBb convertase of the AP.

The efficiency of activation of each pathway of the complement on bacterial species varies among species and/or strains, in part because not all bacterial species are recognized by the same recognition proteins (Yuste *et al*., 2006; Yuste *et al*., 2008; Mattos-Graner *et al*., 2023). For example, teichoic acid of *S. pneumoniae* have phosphorylcholine to which CRP binds and activates the classical pathway (Hakenbeck *et al*., 2009), whereas phosphorylcholine is absent in *S. gordonii* and *S. sanguinis* surfaces**.** Consistently, CRP does not bind to *S. sanguinis* surfaces (Alves *et al*., 2019). There are significant differences in binding of the short pentraxin SAP (serum amyloid protein) amongst *S. sanguinis* strains (Alves *et al*., 2022). One of the major effector functions of the complement for elimination of Gram-positive bacteria relies on deposition of C3b on the cell walls. Phagocytes recognize the C3b (iC3b) opsonin's on surface of bacteria by CR1 and other C3b receptors. Moreover, C3a and C4a fragments are chemotactic in nature which attracts phagocytes once released at site of infection (Lambris *et al*., 2008; Walport, 2001). Although MAC has a known effect on Gram-negative bacterial because of targeting the external membrane, it has been reported that MAC could also act on cell division septa of Gram-positive bacteria as well (Berends *et al*., 2013). Complement system plays significant role in oral cavity by controlling microorganisms (Mattos-Graner *et al*., 2023). Complement proteins are present in the serum-containing gingival crevicular fluid (GCF) released at the gingival sulcus. Increased volumes of GCF are promoted by gingival inflammation promoted by microbial dental biofilms (Huynh *et al*., 2015). Protection by the complement system to saliva exposed supragingival surfaces has however received limited consideration. Although at lower concentration compared to serum and GCF, there is evidence that whole saliva of healthy individuals has complement active proteins (Andoh *et al*., 1997). Thus, complement proteins present in GCF and in saliva seems to contribute to the homeostasis of the oral microbiota (Mattos-Graner *et al*., 2023). It is thus possible that the apparent resistance of *S. sanguinis* to complement activation underlies that capacity of this species to pioneering colonize dental surfaces and other oral niches. This ability might contribute to the capacity of *S. sanguinis* to persist in the bloodstream and to cause extra-oral infections.

3 GENERAL OBJECTIVE

The major aim of this study was to investigate the roles of CppA in the *S. sanguinis* capacity to evade complement-mediated immunity.

3.1 SPECIFIC OBJECTIVES

a) To investigate the expression of *cppA* in *S. sanguinis* strains isolated from the oral cavity or from the blood stream using qRT-PCR.

b) To construct a deletion mutant of *cppA* in the *S. sanguinis* strains SK36 (SKcppA) and a complemented mutant with an episomal copy of cppA (SKcppA+) to be used as control of polar effects due to chromosomal deletion of *cppA*.

c) To compare C3b deposition between the parental strain SK36, SKcppA and SKcppA+.

d) To compare bacterial binding to complement activator proteins (SAP and C1q) and to complement down-regulators (Factor H and C4BP) between parent strain SK36, SKcppA, and SKcppA+.

e) To compare the susceptibilities of SK36, SKcppA, and SKcppA+ to phagocytosis by PMN isolated from peripheral human blood in the presence and absence of serum.

f) To compare the *ex vivo* survival in human blood between parent strain SK36, SKcppA, and SKcppA+.

g) To compare transformation efficiency of *S. sanguinis* strains and SKcppA to reference strain SK36.

4 MATERIAL AND METHODS

4.1 *S. SANGUINIS* STRAINS USED IN THIS STUDY

The *S. sanguinis* strains used in this study are shown in Table 1. The strain SK36 was used for constructing the deletion isogenic mutant of *cppA* (SKcppA). This mutant was then complemented with an episomal copy of *cppA*, to obtain the complemented strain SKcppA+.

Table 1 - *S. sanguinis* strains used in current study with their isolation site, genome accession number and references.

Strains	Isolation site	Genome accession number	Source / Reference
		Streptococcus sanguinis	
SK36	Dental biofilm	GCA_000960035.1	ATCC 10556; Xu *et al*., 2007
SK49	Dental biofilm	GCA_000212815.1	Mogens Kilian; Kilian *et al*., 1989
SK72	Dental biofilm	GCA_000192185.1	Mogens Kilian; Kilian *et al*., 1989
SK115	Dental biofilm	GCA_000192205.1	Mogens Kilian; Kilian *et al*., 1989
SK160	Dental biofilm	GCA_000192245.1	Mogens Kilian; Kilian *et al*., 1989
SK330	Oral cavity	GCA_000195025.1	Mogens Kilian; Kilian *et al*., 1989
SK353	Oral cavity	GCA_000191085.1	Mogens Kilian; Kilian *et al*., 1989
SK678	Blood	GCA_000212835.1	Mogens Kilian; Kilian *et al*., 1989
SK1056	Blood	GCA_000191125.1	Mogens Kilian; Kilian *et al*., 1989
		Mutant constructed using SK36	
SKcppA	-	-	Current study
		Complement constructed using SKcppA	
SKcppA+	-	-	Current study

4.2 CONSTRUCTION OF ISOGENIC MUTANT (SKcppA) AND COMPLEMENT STRAIN (SKcppA+)

The isogenic mutant (SKcppA) was constructed in *S. sanguinis* strain SK36 by using PCR ligation methodology (Figure 2 and Table 2), as described previously (Camargo *et al*., 2018). Plasmid pVA838 (Macrina *et al*., 1982) was used to obtain an erythromycin resistance cassette (Erm^r; 979bp), which was used to replace internal sequence of *cppA* (544 bp) with recombinant allele constructed using PCR ligation strategy. The recombinant allele was transformed into SK36, as described previously (Moraes *et al*., 2013), and transformants recovered in agar BHI (BHI with erythromycin) and then verified by PCR and DNA sequencing.

Table 2 - Oligonucleotides (constructed using Primer 3; (https://primer3.ut.ee/)) used for construction of mutant and complement strains (underlined sequences are restriction sites in sequences of primers).

Mutant Construction SKcppA		
Primer	**Sequence**	**Size (bp)/Tm**
P1	TAAGGAGAAAGAGGCGGTTG	20bp / 57ºC
P2 Asc 1	TTGGCGCGCCTTCATGCCCAGCTGATTTAC	30bp / 56ºC
P3 Xho 1	TTCTCGAGCAAAGCTCAGGTCAATCGTT	28bp / 57ºC
P4	TGAAAATAAGCCAGCACTACC	21bp / 57ºC
Erythromycin Cassette		
E1 Asc 1	TTGGCGCGCCTGGCGGAAACGTAAAAGAAG	30bp / 58°C
E2 Xho 1	TTCTCGAGGGCTCCTTGGAAGCTGTCAGT	29bp / 58°C
Complement construction		
C1 SacI	TTGAGCTCTTCGATTATTGGGCAGGCTG	28bp / 62ºC
C2 BamHI	TTGGATCCCCTTTCCTTCTTGCGGCTCA	28bp / 62ºC

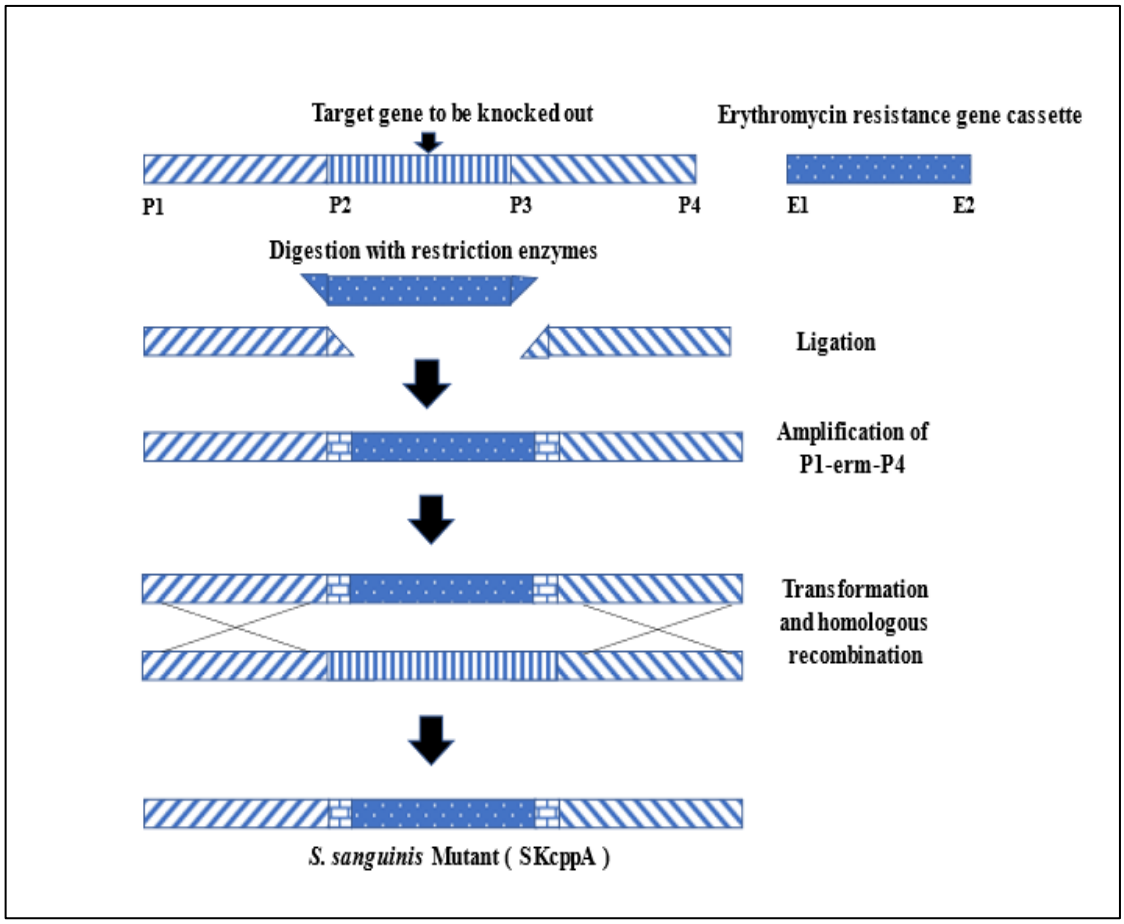

Figure 2 - Strategy for knocking out *cppA* using PCR based mutagenesis. Primers were used for PCR amplification of the SK36 chromosome segments (P1, P2, P3 and P4) and the erythromycin cassette from plasmid pVA838 (E1 and E2). PCR amplicons were then ligated and thus used as template in PCR reactions with used P1-P4 to yield the recombinant allele used for homologous recombination.

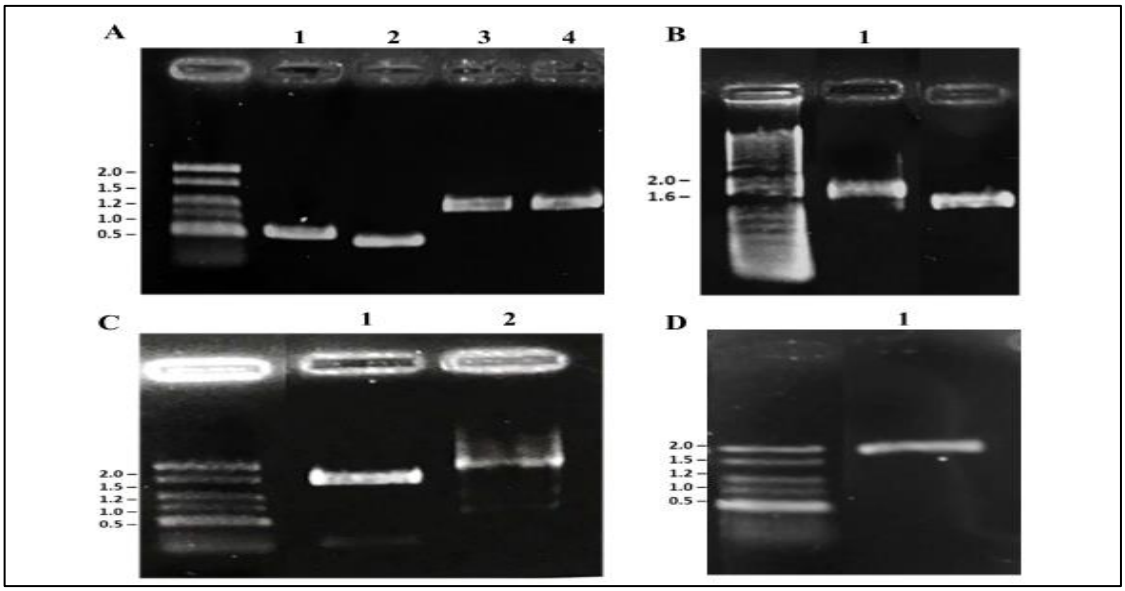

Figure 3 - Agarose gel (1%) containing PCR products for mutant construction SKcppA. (A) Lane 1 shows digested P1/P2 product (530 pb) by AscI, lane 2 shows digested P3/P4 product (400 pb) by XhoI and lanes 3 and 4 show digested products of E1/E2 with AscI and XhoI, respectively. B) Lane 1 shows ligated P1/E2 product (1509 pb) and lane 2, the ligated E1/P4 product (1379 pb). C) Lane 1 shows *cppA* P1/P4 fragment (1.433 pb) without Erm^r, amplified from SK36; lane 2 shows recombinant *cppA* P1/P4 (1.909 pb) with Erm^r. D) Gel purified recombinant *cppA* P1/P4 product (1.909 pb) with Erm^r used for transformation of SK36 to obtain the mutant SKcppA.

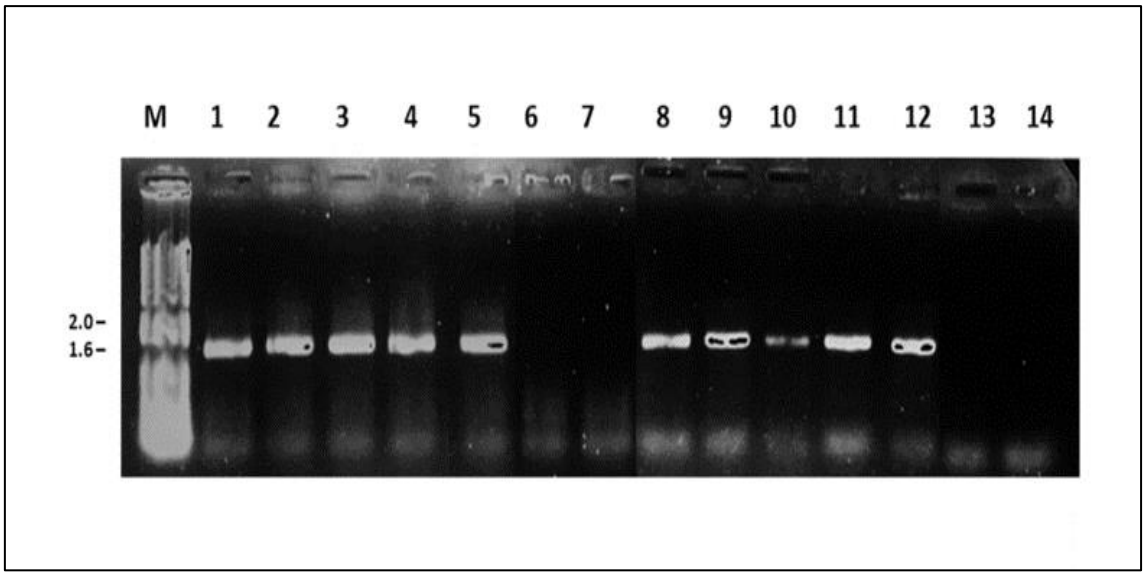

Figure 4 - Transformants obtained for SKcppA selection were tested by performing confirmatory PCR to yield P1/E2 and E1/P4 products. Lane 1-4 shows bands for P1/E2 product PCR amplified from transformants DNA, lane 5 is positive control ligation product (P1/E2-*cppA*), lane 6 is negative control of P1/E2 using SK36 DNA and lane 7 is negative control of P1/E2 without DNA. Lane 8-11 shows bands for E1/P4 product PCR amplified from transformants DNA, lane 12 is positive control ligation product (E1/P4-*cppA*), lane 13 is negative control E1/P4 using SK36 DNA and lane 14 is negative control of E1/P4 without DNA.

For complement (SKcppA+) strain construction, SKcppA was transformed shuttle plasmid pDL278 (LeBlanc *et al*., 1992) harboring full-length *cppA* including its promoter region. The respective complemented mutant (SKcppA+) was grown in BHI agar supplemented with spectinomycin (200 µg/ml), and expression of *cppA* confirmed by RT-qPCR analysis.

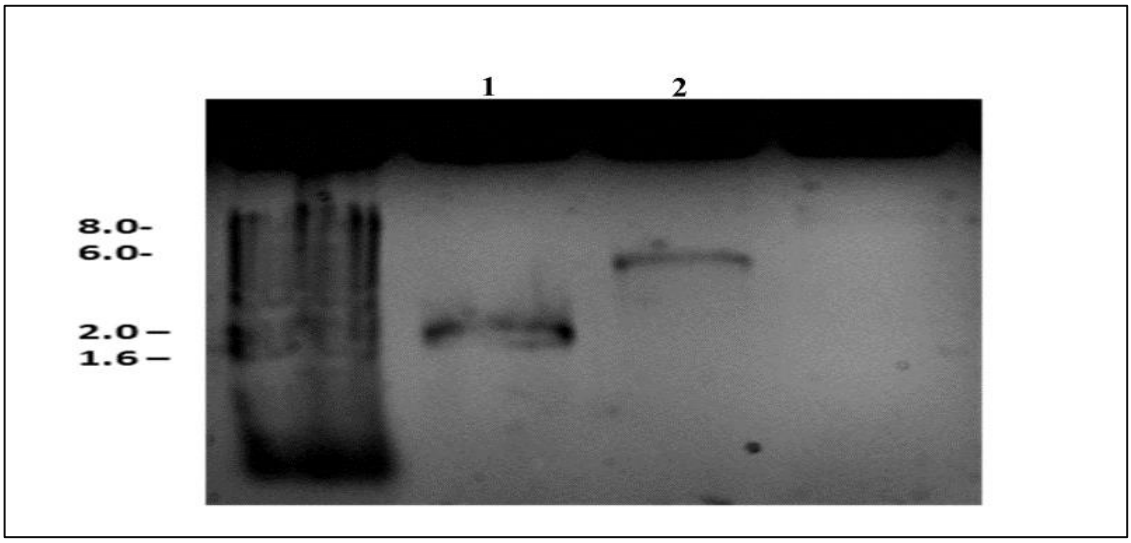

Figure 5 - Complement (SKcppA+) construction using pDL278. Lane 1 show band for digested (C1-C2) product, lane 2 show band for digested plasmid pDL278 and lane 3 is negative control.

4.3 GROWTH CURVES FOR MUTANT (SKcppA) AND COMPLEMENT STRAINS (SKcppA+)

Frozen stocks of strains were used to grow on brain heart infusion (BHI) agar (BD Difco, USA) (37°C; 10% CO_2 atmosphere). In case of mutant (SKcppA) and complement strains (SKcppA+), BHI medium was supplemented with erythromycin (10 µg/ml) or spectinomycin (200 µg/ml) respectively. Fresh BHI media was inoculated from overnight (18h) BHI cultures adjusted based on absorbance (A_{550nm} 0.03) and incubated (37°C; 10% CO_2 atmosphere). Hourly intervals (1h to 8h) were set for determining A_{550nm} of cultures. Three independent experiments were performed to plot their data. Morphological analysis of strains was also assessed in cells collected from BHI cultures at $A_{550\ nm}$ of 0.3 and 0.7, which were Gram-stained and analyzed using a light microscope (Nova Optical Systems – NOVA 800-T).

4.4 RNA ISOLATION OF *S. SANGUINIS* STRAINS AND TRANSCRIPTIONAL ANALYSIS OF *cppA* AMONG ALL *S. SANGUINIS* STRAINS

Strains grown in BHI (37ºC, 10% CO_2 in air) until the A_{550nm} 0.3, and the cells harvested by centrifugation (Centrifuge Eppendorf® 5810R; 5000rcf, 5 min, 4ºC) for RNA purification using RNeasy kit (Qiagen, USA) as previously described (Camargo *et al*., 2018). Briefly, for total RNA extraction, cells were lysed using 0.16g of zirconia beads (0.1mm diameter (Biospec, EUA)), 200ul of TE (Tris-HCL 10mM pH 8,0; EDTA 1mM pH 8,0) in Mini-bead beater (Biospec, EUA) with maximum force (2 cycles of 45 sec with 45 sec interval on ice). RNA samples were treated with turbo DNase (Ambion, USA) (Camargo *et al*., 2018), concentration and purity of RNA samples was determined using NanoDrop® (Thermo Scientific, EUA), and samples were considered pure having value A260nm/A280nm of 1.8 or higher. RNA integrity was analyzed by gel electrophoresis, 5ul of samples were run on 1.2% of agarose gel (containing 1,8% de formaldehyde and 0,15µl/ml of ethidium bromide) in running buffer (20mM MOPS, 5mM sodium acetate and 1 mM EDTA). Gels visualized under UV containing 2 bends of ribosomal RNAs (23S e 16S) were referred to integrity of RNA samples. Samples of cDNA were obtained from samples of 1µg of total RNA, using the SuperScript III system (Life Technologies, USA) and random primers (Stipp *et al*., 2008), according to manufacturer instructions. Levels of transcripts were then accessed by qPCR using primers (Table 3), constructed using region of *cppA* conserved among all the strains tested in StepOne™ Real-Time PCR System (Life Technologies, USA). Reaction mixtures for qPCR (10 µl of total volume) included 10 µM of each primer, cDNA (10 ng), and 1× Power SYBR® Green PCR Master Mix (Lifetech, USA). For absolute quantification of gene transcripts, a standard curve of genomic DNA (ten-fold serially dilutes 0.003 to 300 ng) were used. 16S rRNA gene was used for normalization of transcript levels of *cppA* gene (Camargo *et al*., 2018).

Table 3 - Oligonucleotides used for qPCR for transcriptional analysis of *cppA* in *S. sanguinis* strains.

CppA primers for qPCR		
Primer	Sequence	Size (bp) / Tm
qPCR CppA forward	AAATTAGCCAGAATCG	16 / 44
qPCR CppA reverse	AAGTCCTCTTGCTTTTC	17 / 48
16S primers for *S. sanguinis* strains		
16S forward primers	GGAAACTGTTTAACTTGAGTGCA	23 / 57.76
16S reverse primers	GGCCTAACACCTAGCACTCA	20 / 59.10

4.5 SAMPLES OF HUMAN BLOOD AND SERUM

Serum and blood samples were collected from one volunteer in good general health as described previously (Alves *et al*., 2016), according to Ethics Committee approved protocols of Piracicaba Dental School, University of Campinas (CEP/FOP-UNICAMP/ CAAE 83140418.0.0000.5418).

4.6 C3B DEPOSITION AND BINDING TO C4B-BINDING PROTEIN (C4BP), C1Q, FATOR H (FH) AND SERUM AMYLOID PROTEIN (SAP)

Complement proteins binding to bacterial surface was determined as described previously (Alves *et al*., 2022). Bacteria were collected in mid log phase of growth (A_{550}nm 0.3; 1x 10^7 CFU/ml) and washed twice with PBS. Then, bacterial cells were treated with 20% human serum (in PBS) followed by incubation for 30 min at 37ºC. Cells were then washed twice with PBS-Tween 0.05% (PBST) and incubated with specific antibodies diluted in PBST. These include anti-human C3 IgG (Polyclonal goat) conjugated to FITC (fluorescein isothiocyanate) (ICN, USA) (1:300; 40 min. on ice), anti-human C4BP FITC conjugated (1:225; 60 min at 25°C) (LSBio, USA), or anti-human C1q FITC conjugated (1:300; 60 min. at 37°C) (LSBio, USA) (Domenech *et al*., 2013). For detection of surface-bound FH, bacterial cells were initially incubated with primary anti-human FH IgG antibody (1:100, 30 min. at 37°C) (Calbiochem) followed by incubation with secondary FITC-conjugated anti-goat IgG (1:1.000; 4°C, for 40 min). For detection of SAP binding, cells were incubated with FITC-

conjugated polyclonal anti-human SAP (1:200; 37°C for 60 min) (LSBio, EUA) (Yuste *et al*., 2007). After antibody probing, cells were washed with PBST and then treated with 3% paraformaldehyde in PBS. FACS Calibur flow cytometer (BD Biosciences) was used for the analysis of samples. Forward and side scatters were set for 25,000 bacterial cells to be gated. Geometric means fluorescence intensity (MFI) was determined, and fluorescence index of fluorescence (FI) were calculated by multiplying the MFI values by the percentage of positive cells (Alves *et al*., 2022). Cells treated with PBS instead of serum were used as reference control. Bacterial cells treated with heat inactivated serum (56°C; 20 min.) were also used as controls in preliminary experiments.

4.7 ISOLATION OF PMN FROM HUMAN PERIPHERAL BLOOD AND PHAGOCYTOSIS ASSAYS

PMN were isolated from fresh human blood collected in heparin tubes. PMN were isolated using 1119 and 1083 density Histopaque (Sigma-Aldrich) to form double gradient by centrifugation, as described previously (Alves *et al*., 2022). PMN were suspended in RPMI 1640 (GIBCO, Life Technologies, NY, USA) medium supplemented with inactivated 10% fetal bovine serum. May-Grunwald Giemsa and trypan blue exclusion staining were used to determine purity (>95%) and cell viability (>98%). Bacteria used in phagocytosis assays were previously labeled with FITC as previously described (Alves *et al*., 2022). Phagocytosis assays were performed in 96-well plates with 50 µl of RPMI medium (supplemented or not with 20% human serum) with 2 x 10^5 PMNs per well and 10^7 CFU/ml FITC-labeled bacteria per well (MOI =1:200), incubated at 37ºC for 5 min (gentle shaking, 10% CO_2). Afterwards, 3% paraformaldehyde was used for fixation of PMN, and samples analyzed by flow cytometry using a FACS Calibur (BD Biosciences) flow cytometer. A total of 10,000 PMN were analyzed, for determining the number of PMN with intracellular bacteria (Alves *et al*., 2022).

4.8 *EX VIVO* PERSISTENCE IN HUMAN BLOOD

Ex vivo persistence of *S. sanguinis* in human blood was analyzed as previously described (Alves *et al*., 2022). *S. sanguinis* strains were grown in BHI until mid-log phase (A_{550nm} 0.3). Bacterial cells from 1 ml of the cultures were then harvested (11000rpm, 4ºC, 2 min), washed twice with PBS and resuspended in 1ml of fresh peripheral human blood. Initial blood counts (CFU/ml at time 0) are obtained upon collection immediately after initial mixture to access blood-mediated aggregation, while remaining sample was incubated at 37°C (in aerobic orbital shaker) during 42h. Aliquots of these suspensions were then collected for bacterial counts on BHI agar at selected intervals.

4.9 TRANSFORMATION EFFICIENCY

S. sanguinis strains and SKcppA strains were grown in THB media until reached an A_{660}nm of 0.7 – 0.8. Then, volumes of 300ul of the cultures were then collected, added of 1µg of shuttle plasmid PDL 278, and incubated for 90 min. at 37°C, under shaking (160 rpm). After incubation, samples were plated on BHI and BHS (supplemented with spectinomycin 200µg/ml) agar and incubated (37°C; 10% CO_2 atmosphere) for 48h. Colonies obtained were counted and percent transformants were counted.

4.10 ANALYSIS OF *CPPA* POLYMORPHISMS IN *S. SANGUINIS* GENOMES

To identify *cppA* polymorphism among *S. sanguinis* strains used in our study, genome sequences were retrieved from GenBank and annotated using PROKKA in cooperation with Dr. Tsute Chen (The Forsyth Institute, U.S.A.). Multiple sequence alignments were performed for investigation of DNA and protein polymorphisms of *cppA* among *S. sanguinis* strains using Clustal W (ClustalW; http://www. ebi. ac. uk/ Tools/msa/ clustalw2/) (Madeira *et al*., 2019). Phylogenetic comparisons of CppA of *S. sanguinis* strains and representative homologues of additional streptococcal species were performed using NGPhylogeny (https://ngphylogeny. fr/) (Lemoine *et al.*, 2019).

4.11 DATA ANALYSIS

Strain comparisons with the reference strain SK36 were performed using nonparametric Mann-Whitney U-test. Flow cytometry data (MFI, FI and frequencies of phagocytosis by PMN) were compared using Kruskal-Wallis with *post hoc* Dunn's test. Three independent experiments were performed for all assays except the *ex vivo* blood survival assay for which four independent experiments were performed. Pearson correlation was used to identify associations between binding to complement proteins. Differences were considered significant when a P value of <0.05 was obtained.

5 RESULTS

5.1 PROTEIN SIMILARITIES, GENOMIC CONTEXT AND DOMAIN ARCHITECTURE OF *cppA* in *S. SANGUINIS*

Figure 6 shows BLAST analysis of SKcppA locus structure. Demonstrating genes upstream *SSA_0330* (hypothetical protein) and downstream *SSA_0330* (serine hydrolase) to *cppA* in *S. sanguinis*, genes upstream *pepX* (Xaa-Pro dipeptidyl-peptidase) and downstream *SMU_399* (peptidase) in *S. mutans* and genes upstream *SGO_RS01165* (membrane protein) and downstream *SGO_RS01175* (serine hydrolase) in *S. gordoni*. Shows percent identity and similarity of *cppA* in *S. sanguinis* (100/100%), *S. mutans* (50/70.59%) and *S. gordoni* (67.09/75.2%) along with their protein's length in amino acids 244, 248 and 244 respectively. Analysis of domain architecture suggested two domains, VOC, and terminal CppA-C. VOC (vicinal oxygen chelate) is located at positions 9 to 130 while CppA-C is located at positions 151-242. VOC is found in a variety of structurally related metalloproteins, including the type I *extradiol dioxygenases, glyoxalase* I and a group of antibiotic resistance proteins. This is the C-terminal domain of the CppA protein found in species of *Streptococcus*. CppA is a putative C3-glycoprotein degrading proteinase, involved in pathogenicity.

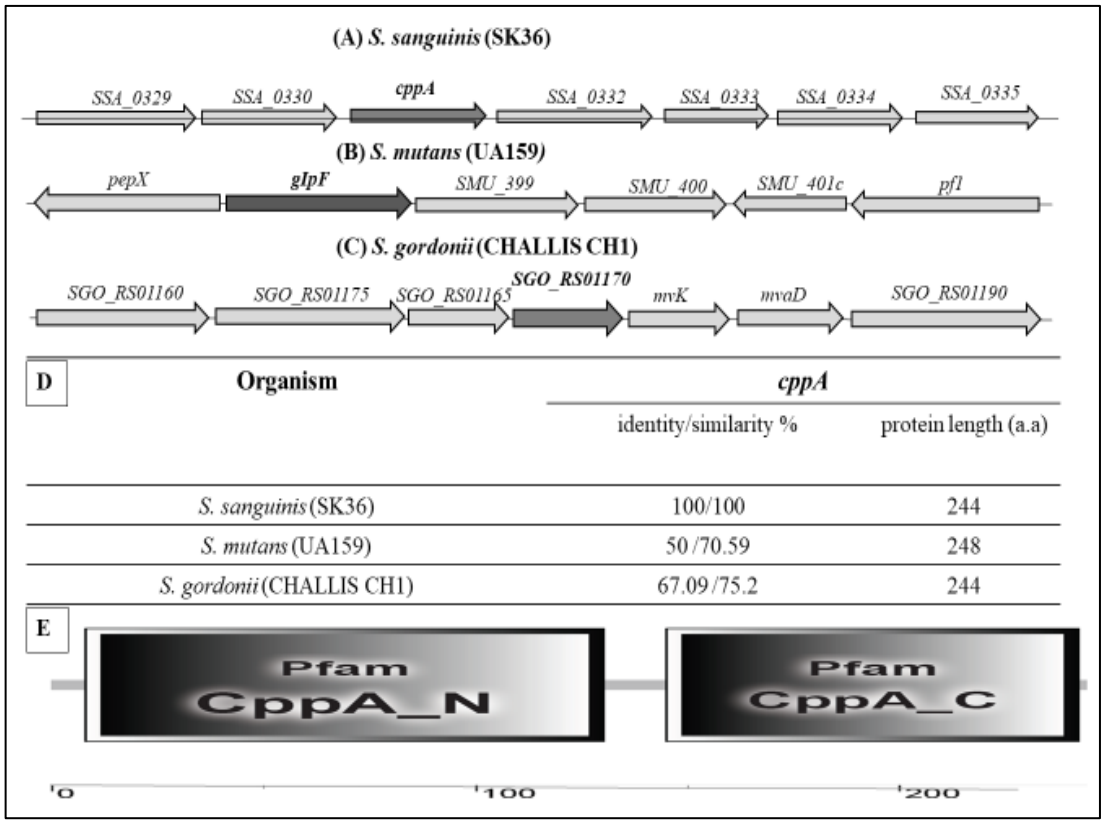

Figure 6 - Genomic context, protein similarities, and domain architecture of *cppAss* of *S. sanguinis*. Schematic representation of the *cppAss* chromosomal loci in S. sanguinis, *S. mutans UA159 and S. gordonii Challis CH1* (A, B and C respectively). Open reading frames (ORFs) are represented by arrows to indicate the direction of transcription; GenBank accession numbers are indicated above each respective arrow. Dark gray arrows represent *cppAss*. Light gray arrows represent genes upstream and downstream to *cppA*. (D) Results of BLASTP analyses of *cppA*. Percent amino acid (aa) identities and similarities were determined by using SK36 sequences as a reference. (E) Schematic representations of domain architectures of *cppA* obtained using SMART research tool (http://smart.embl-heidelberg.de/).

5.2 ANALYSIS OF THE EFFECT OF CPPA DELETION IN S. SANGUINIS PLANKTONIC GROWTH

We performed comparisons of SKcppA mutant with parent SK36 and SKcppA+ to check whether inactivation of *cppA* had some effect on growth of SK36. Because *S. sanguinis* need to cope with higher oxygen tension during initial steps of tooth colonization, growth curves were assessed in BHI (37ºC) under different conditions of O_2 exposure including static incubation in air with 10% CO_2 and incubation under medium aeration (shaking at 160 rpm) using a shaker incubator (NT-712, Nova Technica, Brazil). As shown in Figure 7, no significant

differences were observed in growth curves between SK36 and SKcppA at both atmospheric conditions. Moderate reductions in growth of SKcppA+ were however observed, likely due to spectinomycin supplementation of BHI.

To further analyze differences in strain morphology and/or chain formation, planktonic cells collected at exponential (A_{550nm} 0.3) and stationary (A_{550nm} 0.7) growth phases were Gram stained and analyzed under light microscopy (Figure 8). Varied length of chains of *S. sanguinis* connected with one another were observed in aerobic and microaerophilic conditions at exponential (A_{550nm} 0.3) and stationary (A_{550nm} 0.7) growth phases.

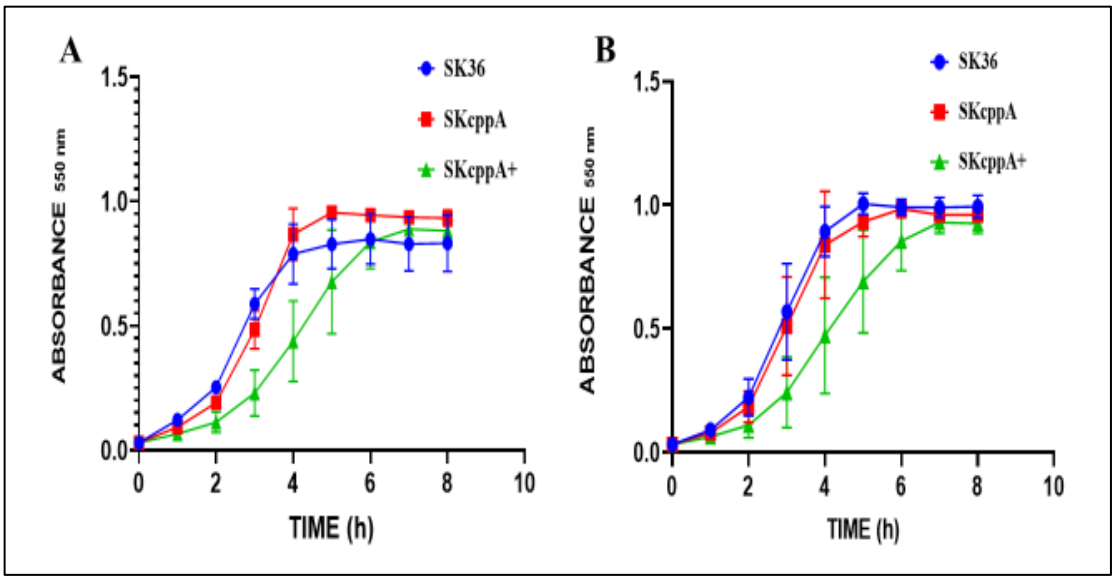

Figure 7 - Growth curves of wild type SK36, mutant SKcppA and complement SKcppA+ strains in aerobic and microaerophilic conditions. (A) Graph showing growth curves of SK36, SKcppA and SKcppA+ in aerobic conditions (37ºC, shaker). Log phase can be observed from 2-5h followed by stationary phase. (B) Graph showing growth curves of SK36, SKcppA and SKcppA+ in microaerophilic conditions (37ºC, 10% CO_2). Log phase can be observed from 2-5h followed by stationary phase for SK36 and SKcppA, while for SKcppA+ after 6h.

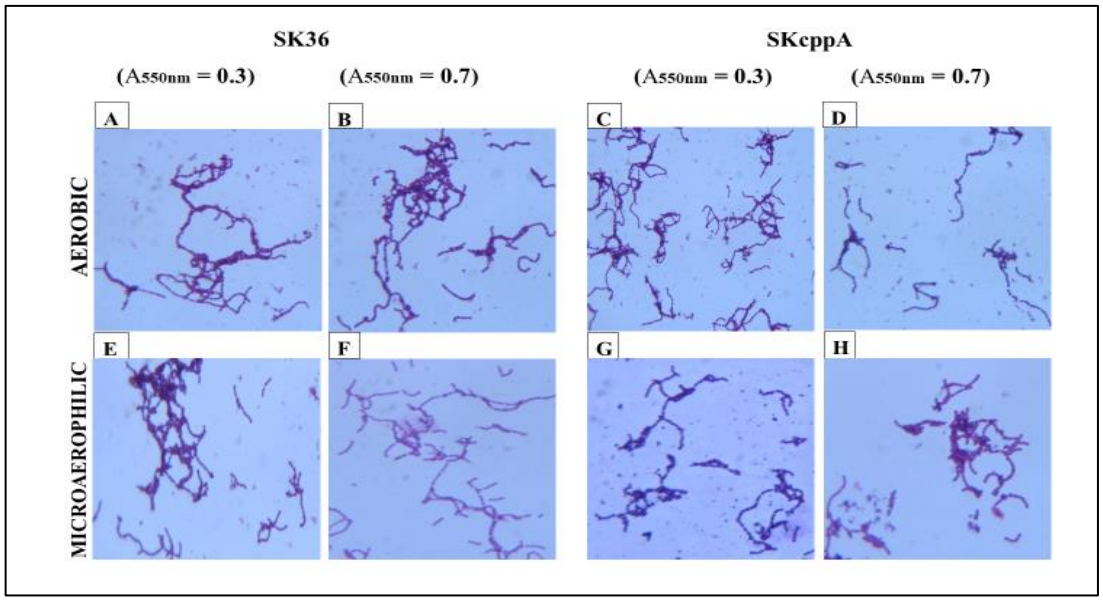

Figure 8 - Gram staining results for wild type (SK36) and mutant (SKcppA) in microaerophilic and aerobic conditions at A_{550nm} 0.3 and 0.7. (A and B) represent gram staining results for SK 36 at A_{550nm} 0.3 and 0.7 in Aerobic conditions, while (E and F) represent gram staining for SK36 grown in Microaerophilic conditions. (C and D) represents Gram staining results for SKcppA mutant in Aerobic conditions at A_{550nm} 0.3 and 0.7 respectively, while (G and H) represent gram staining results for SKcppA grown in Microaerophilic conditions at A_{550nm} 0.3 and 0.7.

5.3 TRANSCRIPTION ANALYSIS OF *cppA* IN *S. SANGUINIS* STRAINS

We used nine *S. sanguinis* strains (SK36, SK49, SK72, SK115, SK330, SK353, SK678 and SK1056) to analyze potential diversity in transcript levels of *cppA*. This gene was expressed by all these strains, although there was a diversity in *cppA* gene transcript levels. As observed in Figure 9, the highest *cppA* transcript levels were observed for SK330, SK36 and SK160, whereas strains SK49, SK72 and SK1056 were amongst the isolates that demonstrated the lowest transcript levels.

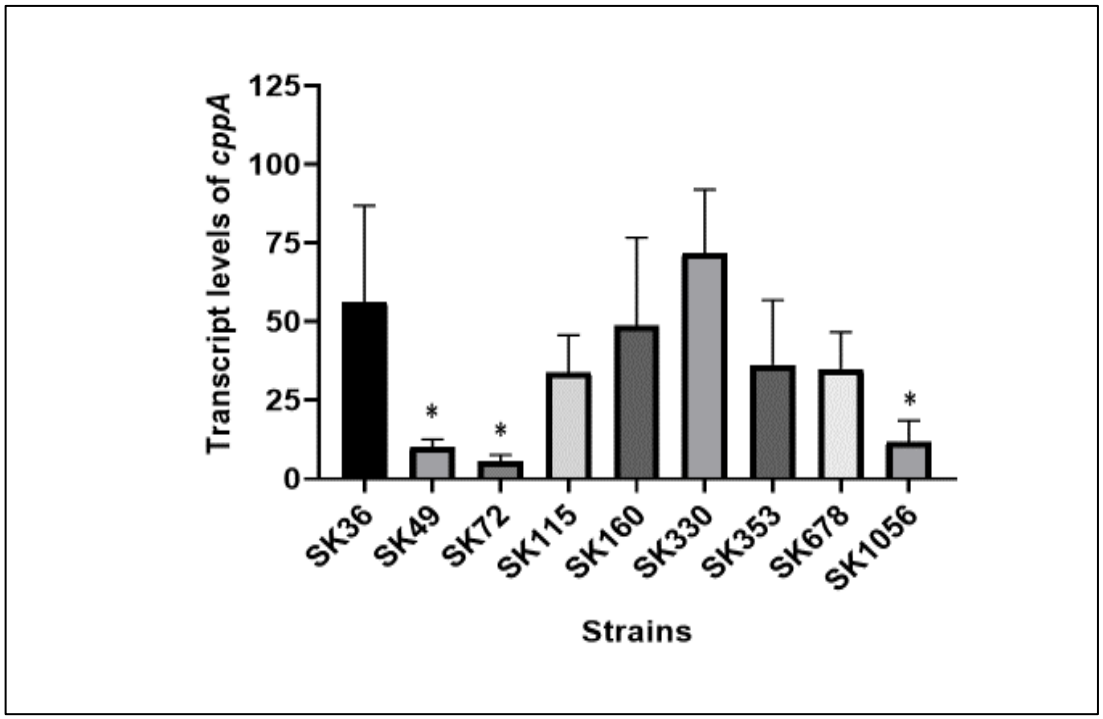

Figure 9 - Transcript levels of *cppA* in *S. sanguinis* strains. Strains were grown in BHI until the mid-log growth phase (A_{550nm} 0.3) (37ºC, 10% CO_2) for RNA purification applied in RT-qPCR analysis. Relative transcript levels of *cppA* were normalized by transcript levels of 16S rRNA which was invariable between strains. Columns represent means of three independent experiments; bars indicate standard deviations. Asterisks indicate significant differences in relation to SK36 reference strain (Kruskal-Wallis with *pos hoc* Dunn's test; $p < 0.05$).

5.4 CppA IS REQUIRED FOR S. SANGUINIS EVASION TO C3B DEPOSITION AND INFLUENCES ON BACTERIAL INTERACTIONS WITH PATTERN RECOGNITION PROTEINS AND HOST COMPLEMENT REGULATORS

The functions of CppA protease in complement evasion of streptococci is not known yet (Lynskey *et al*., 2013; Lynskey *et al*., 2017; Marquart, 2021). To investigate the role of this protease in *S. sanguinis* resistance to complement immunity, we assessed the impact of *cppA* deletion on C3b deposition on SK36. Figure 10A shows, that the isogenic *cppA* mutant SKcppA treated with human serum had clear increases in susceptibility to C3b deposition on its surface as compared to SK36 and the complemented mutant. To investigate whether *cppA* affected *S. sanguinis* binding to proteins activating or inhibiting complement activation, we compared bacterial binding to C4BP, FH, C1q and SAP. SKcppA mutant demonstrated slight but not significant reduction of C4BP binding (Figure. 10B), and a significant reduction in binding to FH (a down-regulator of the alternative pathway), when compared to SK36 and the

complement strain SKcppA+. The SKcppA also showed increased binding to C1q, and SAP as compared to parent and complemented strains. These results indicate the importance of CppA in the evasion of the alternative and classical complement pathways of activation.

Data of binding of wild type clinical strains to the same complement proteins have been published previously (Alves *et al*., 2022). Here we compared transcript levels of *cppA* determined in these strains (Figure 11) with binding intensities to the complement proteins using correlation analysis. Positive correlation was detected between FH binding and transcript levels of *cppA,* compatible with data obtained with SK36 deletion and complemented mutant (Figure 11E). A negative correlation as observed between C4BP binding and *cppA* transcript levels, but without statistical significance (Figure 11D). Hence transcriptional activities of *cppA* of seems to modulate strain-specific interactions with complement proteins.

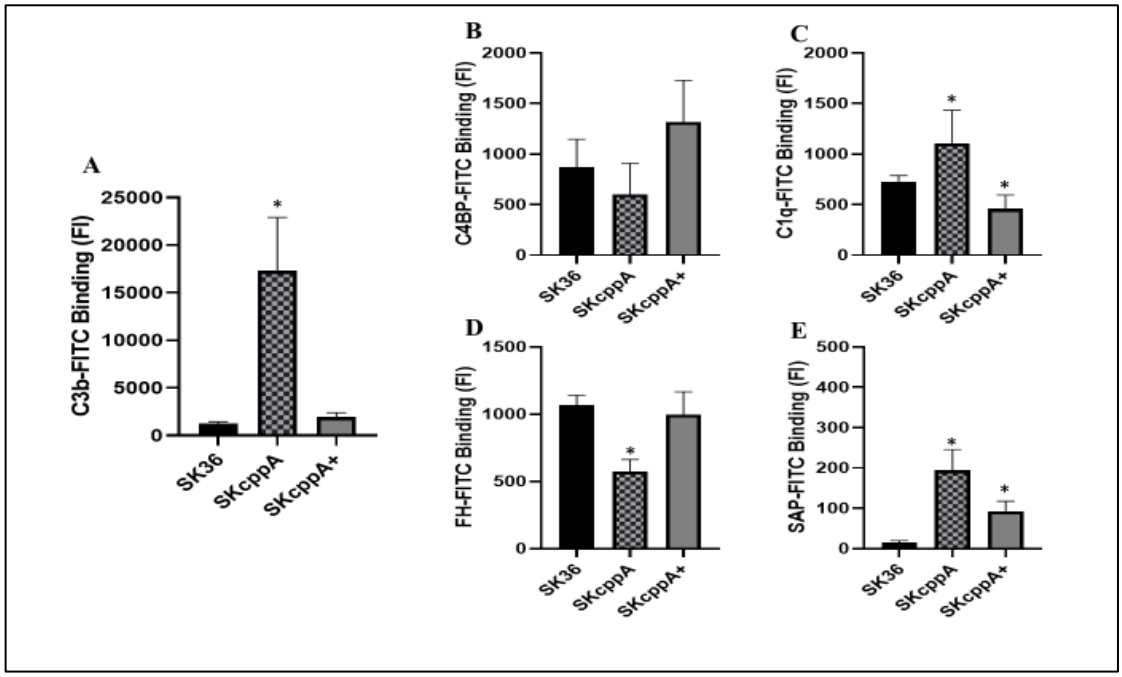

Figure 10 - Binding of *S. sanguinis* strains to C3b and to fluid-phase host proteins involved in complement activation or downregulation. Bacterial binding to C3b, C4BP, FH, C1q and SAP were determined by flow cytometry and expressed as fluorescence index (FI). (A) C3b binding o bacterial surfaces. (B) C4BP binding bacterial surface. (C) C1q binding. (D) Factor H (FH) binding. (E) SAP binding. Columns represent means of three independent experiments; bars indicate standard deviations. Asterisks indicate significant differences in relation to SK36 for SKcppA and SKcppA+ (Kruskal-Wallis with pos hoc Dunn's test; $p < 0.05$).

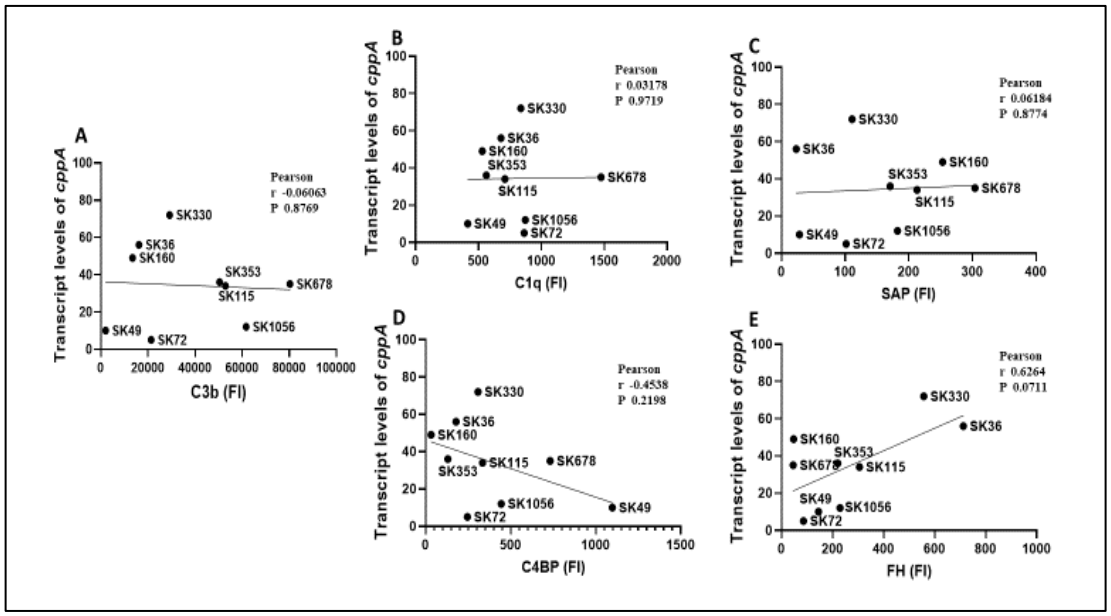

Figure 11 - Correlation of transcript levels of *cppA* in *S. sanguinis* isolates used *versus* fluorescence intensities (FI) of binding to complement proteins. (a) Represents correlation between C3b (FI) vs *cppA* transcript levels, (b) C1q binding (FI) vs *cppA* transcript levels, (c) representing SAP binding (FI) vs *cppA* transcript levels. (d) Shows C4BP binding bacterial surface (FI) vs *cppA* transcript levels and (e) Factor H (FH) binding (FI) vs *cppA* transcript levels.

5.5 DELETION OF CPPA INCREASES *S. SANGUINIS* SUSCEPTIBILITY TO OPSONOPHAGOCYTOSIS BY HUMAN PMN

Because C3b is a major opsonin used by PMN in the bloodstream, we conducted experiments to evaluate effects of *cppA* in *S. sanguinis* SK36 susceptibility to phagocytosis by human PMN isolated from human peripheral blood. We observed that serum mediated opsonophagocytosis was significantly increased in SKcppA as compared to SK36, while fully restoration of phenotype was observed for complemented strain SKcppA+ (Figure 12).

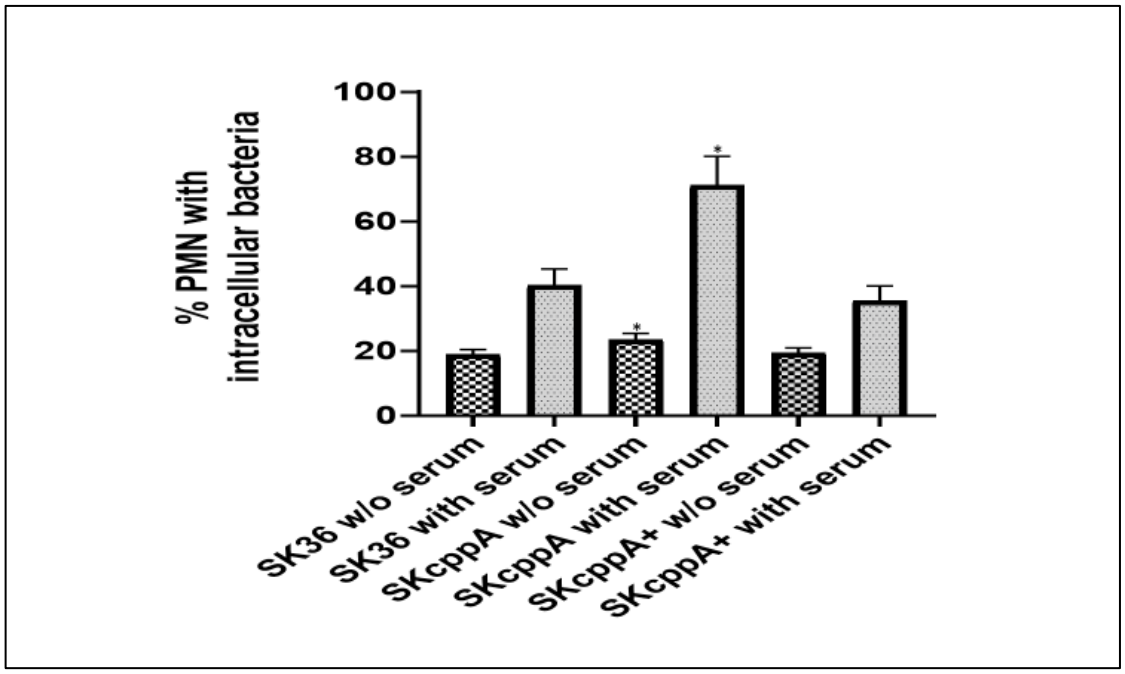

Figure 12 - Phagocytosis of SK36, SKcppA and SKcppA+ by human PMN. Strains previously probed with FITC were incubated with PMN in medium supplemented or not with 20% human serum and PMN were analyzed by flow cytometry for determining the percentages of PMN having intracellular bacteria. Columns represent means of three independent experiments; bars indicate standard deviations. Asterisk indicates significant difference in relation to SK36 for SKcppA and/or SKcppA+ (Kruskal-Wallis with *pos hoc* Dunn's test; $p < 0.05$).

5.6 DELETION OF *cppA* REDUCES *S. SANGUINIS* PERSISTENCE IN HUMAN BLOOD

Because the findings showing increased susceptibility of SKcppA mutant to complement-mediated immunity, we next evaluated the role of *cppA* in persistence of *S. sanguinis* in human blood. As shown in Figure 13, the SKcppA mutant showed reduced capacity to persist in human blood from 4 to 42 h of *ex vivo* incubation as compared to SK36. Importantly, the complemented mutant showed complete recover of the capacity to persist in blood.

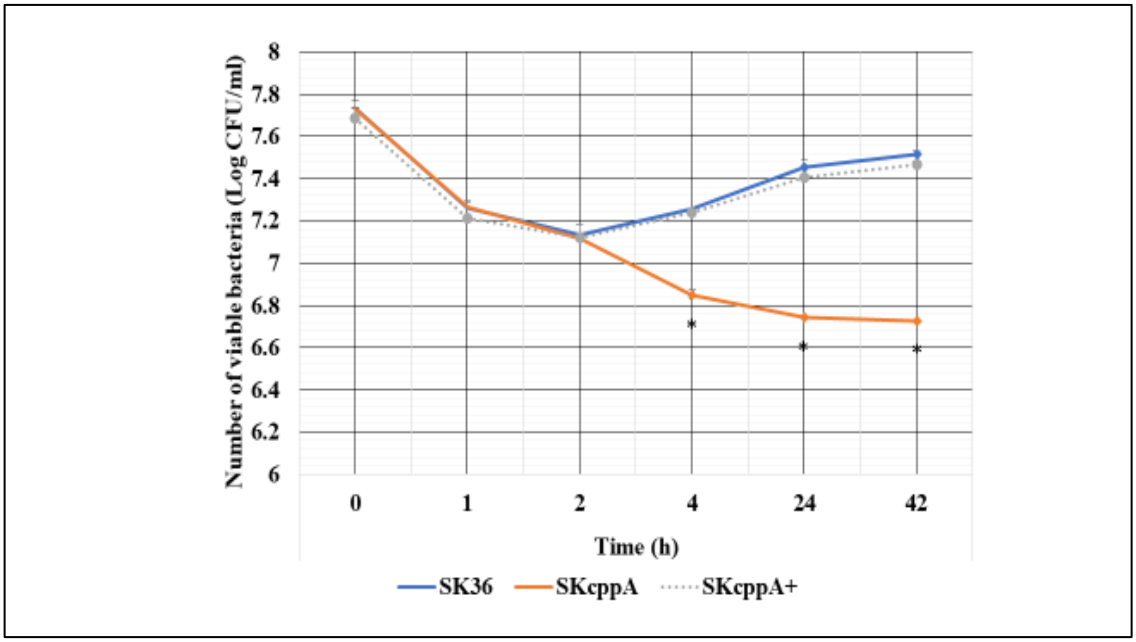

Figure 13 - *Ex vivo* persistence of *S. sanguinis* strains in human blood. Equal numbers of bacterial cells of SK36, SKcppA and SKcppA+ were suspended into fresh human blood, and bacterial counts (log CFU/ml) determined at each time point of incubation (37°C, orbital shaker). Data was obtained in one representative experiment of four independent experiments performed with different blood samples. Asterisks indicate significant differences in relation to parent strain at each time point (Kruskal-Wallis with *post hoc* Dunn's test using correction for repeated measures; $p < 0.05$).

5.7 DELETION OF *cppA* INCREASES TRANSFORMATION EFFICIENCY OF *S. SANGUINIS*

In *S. pyogenes*, a protease of the complement proteins, PepO, was shown to impair competence by degrading short-hydrophobic peptides inducers of competence (Wilkening *et al.*, 2016). We thus assessed the effect of *cppA* deletion in transformation efficiency. Moderate increase in transformation efficiency of SKcppA was observed, when compared to SK36 (Fig. 14A). We further assessed transformation efficiency of wild type clinical strains and observed that all these strains showed reduced transformation efficiency, when compared to SK36, which precluded further analysis between *cppA* transcription and transformation efficiency (Figure 14B). Therefore, we could not detect obvious influence of CppA expression in competence phenotypes of *S. sanguinis*.

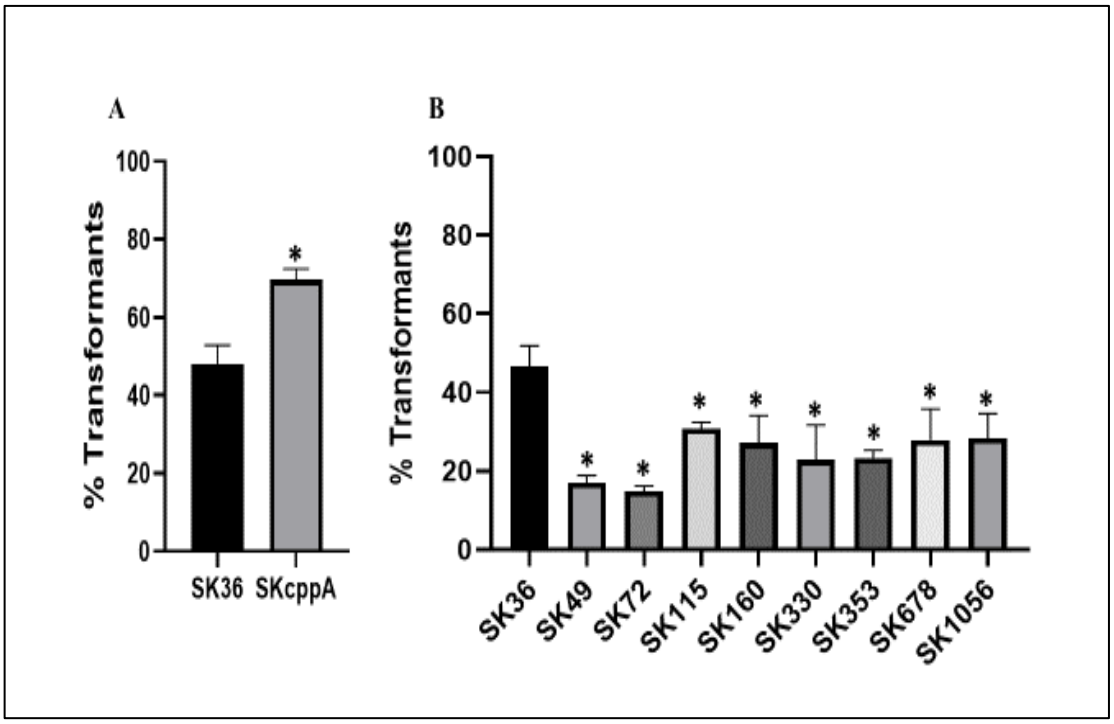

Figure 14 - Transformation efficiency of *S. sanguinis* strains. (A) Comparison of transformation efficiency between SKcppA (transformed with shuttle plasmid pDL27) and SK36. (B) Transformation efficiencies among different wild type strains of *S. sanguinis*.

5.8 POLYMORPHISMS OF *cppA* IN SK36 AND OTHER *S. SANGUINIS* ISOLATES AND SIMILARITIES WITH CPPA STREPTOCOCCAL HOMOLOGUES

Mutation in genes of complement evasion were reported to be involved in evolution of host-pathogen interactions (Cagliani *et al*., 2018; Aleru *et al*., 2020). Hence, we used nine strains to assess genetic polymorphisms of *cppA*, using SK36 as a reference sequence. Ninety-six single nucleotide polymorphisms (SNPs) were found for *cppA* among the nine strains analyzed, which included 71 missense mutations (Figure 15). These missense mutations affect properties such as hydropathy, volume, physicochemical, charge and polarity. These mutations were mostly found near to the C-terminal part of CppA (244 amino acids in length). Thus, although CppA is conserved amongst *S. sanguinis* strains, natural *cppA* mutations promoting polymorphisms at specific regions of CppA are relatively common. Phylogenetic comparisons of *S. sanguinis* CppA with homologues of other streptococcal species of the oral cavity and nasopharynx, indicates closer phylogenetic similarity of *S. sanguinis* CppA with homologues of streptococci of the Mitis group (*S. mitis* and *S. pneumoniae*) than with *S. gordonii* species of the *Sanguinis* group (Figure 16). Differently, *S. mutans* CppA is more closely related CppA

homologues of *S. salivarius* and *S. pyogenes*, whereas more distantly related to *S. sanguinis* CppA.

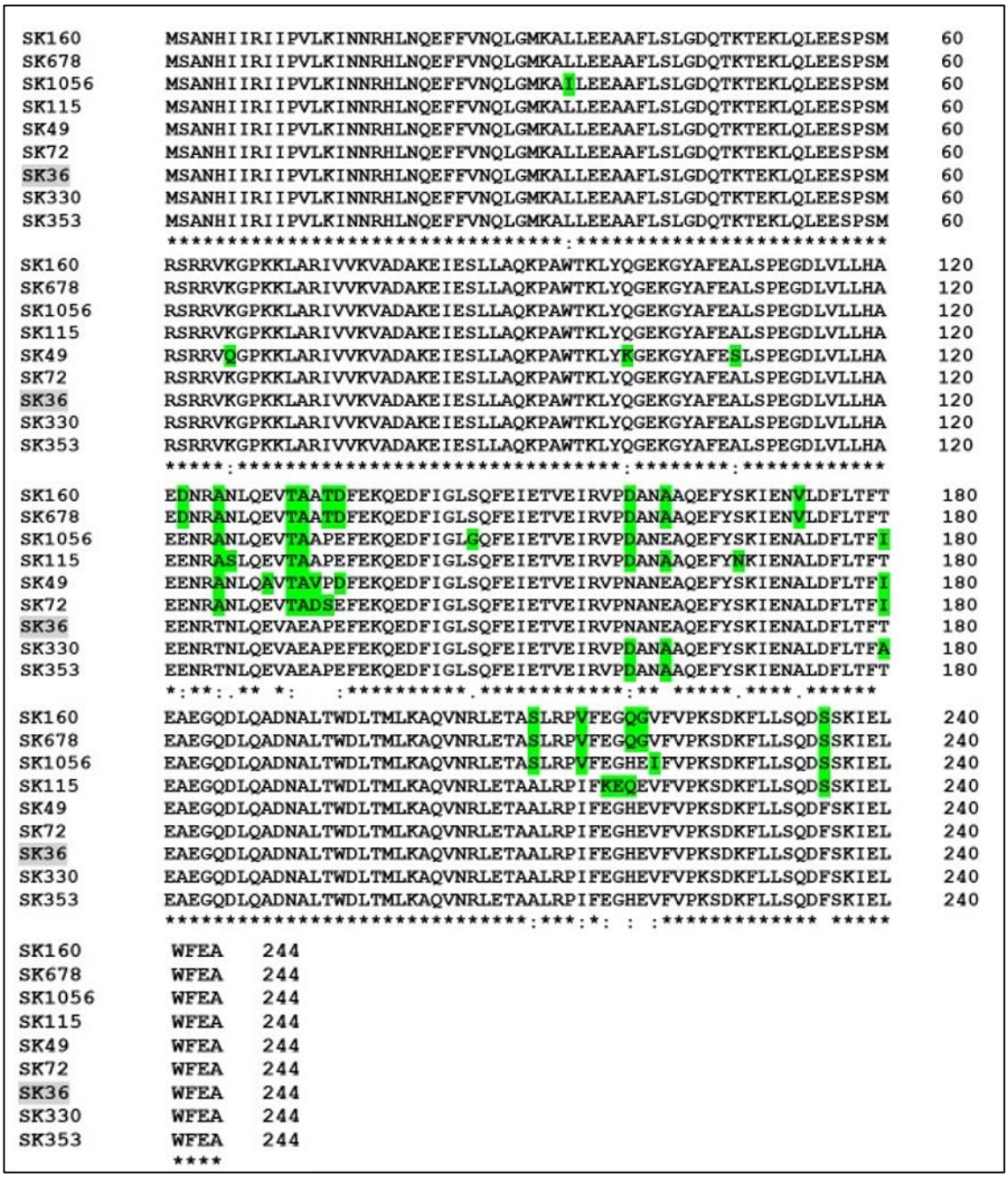

Figure 15 - Comparison of CppA polymorphism in *S. sanguinis* strain. Protein multiple sequence alignment showing amino acid polymorphisms amongst *S. sanguinis* wild type clinical strains. Amino acid changes are marked with red color (Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/))

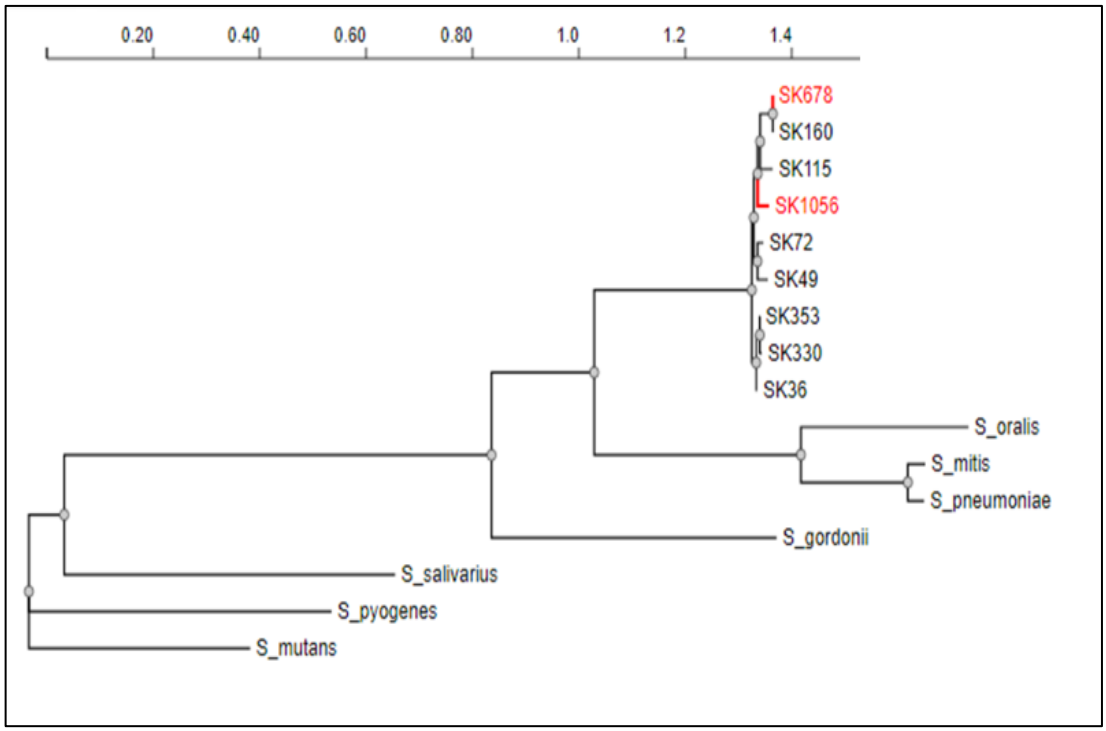

Figure 16 - Phylogenetic comparisons of CppA of *S. sanguinis* strains and representative homologues of additional streptococcal species (NGPhylogeny; (https://ngphylogeny. fr/)).

6 DISCUSSION

As a pioneer colonizer of tooth surfaces, and abundant commensal member of dental biofilms, *S. sanguinis*, might be capable of surviving gingival crevicular fluid and saliva derived immune stresses by expressing immune evasion functions. This ability of *S. sanguinis* might not only help in its survival in the oral cavity against immune components such as complement system, but also in the cardiovascular system, once *S. sanguinis* reaches the bloodstream from oral sites, which might explain in part its ability to cause opportunistic cardiovascular infections. This study shows that *S. sanguinis* expressed the protease CppA and that contributed to immune evasion by diminishing C3b deposition and opsonin-mediated phagocytosis by human blood PMNs. Our data shows that CppA impairs C3b deposition by reducing recognition of major activator proteins of the classical pathway (SAP and C1q) and by recruiting FH down-regulator of the alternative pathway (FH). Our results further indicate that *cppA* is expressed by most *S. sanguinis* isolates, although diversity in *cppA* activities exists. These data enlighten its use as target for therapies for *S. sanguinis* that could be effective control of *S. sanguinis* infections and furthermore of other oral streptococci expressing CppA homologues (Alves *et al*, 2020). There was no significant correlation between C3b binding and *cppA* transcription might be due to conditions applied to analyze *cppA* transcription (mid-log phase on BHI medium). Moreover, bacterial pathogens with increased capacity to evade the complement system typically express multiple complement evasion proteins as compared to less pathogenic strains (Mattos-Graner *et al*., 2023). Thus, correlation analysis of *cppA* expression individually might not reflect the entire repertoire of complement evasion functions expressed by the nine strains analyzed. In addition, it would be important to investigate mechanisms regulating *cppA* expression in conditions simulating blood/serum conditions, which could also influence patterns of *cppA* expression. This is important, since C3b deposition measured in our previous studies of the nine strains were performed with strains treated with human serum (Alves *et al*., 2022). In *S. mutans*, we have found that bacterial exposure to human serum promotes up-regulation of genes for complement evasion in a strain-specific fashion (Alves *et al*., 2019). Despite the lack of significant correlation between *cppA* expression by the nine strains and levels of C3b deposition, we have detected that *cppA* expression levels of strains tended to positively correlate (Pearson correlation; r= 0.63; p = 0.071) with strain binding to factor H, an important down-regulator of the alternative pathway of complement activation, which is crucial for amplification of all the complement activation cascades (Mattos-

Graner *et al*., 2023). This finding is further consistent with the impaired binding to factor H observed in the SKcppA. Thus, one important function of CppA in complement evasion seems to recruit factor H to the *S. sanguinis* surface to avoid the activation of the alternative pathway.

In *S. pyogenes*, CppA, proteolytic ability was not detected though its annotation as C3-degrading protease (Lynskey *et al.*, 2017) or streptococci studied in another study (Marquart, 2020). In *S. pneumoniae* infection, *cppA* has been identified for requirement in transmission (mothers to offspring's), host adaptability and virulence, using ferret and/or mice models for genetic screenings (Carter *et al*., 2014; Rowe *et al*., 2019), which supports that *S. sanguinis* needs CppA gene to persist in blood in our *ex vivo* experiments. Fitness of *S. sanguinis* in human blood might rely on CppA role partially on its complement evasion ability as shown in our study that increase in C3b deposition and opsonophagocytic susceptibility was observed for our isogenic mutant SK*cppA*. That could be one of the crucial factors in evading immune stresses. Screening of *S. sanguinis* genes involved in persistence in heat inactivated human blood did not indicate *S. sanguinis* need of *cppA* (Zhu *et al*., 2020), implying complement-mediated immune evasion function of CppA of *S. sanguinis*. Detail explanation of molecular mechanisms for CppA involvement in providing *S. sanguinis* defense from C3b deposition are thus important. Based on our results, FH complement down-regulators seems to be recruited by *S. sanguinis* by CppA. In addition, CppA seems to interfere in C1q and SAP binding to the *S. sanguinis* surface, resulting in activation of classical pathway.

In this current study, transcription analysis of *cppA* was done for all the studied strains of *S. sanguinis*, constructing pair of primers using *cppA* sequences conserved among all the strains. We found that SK36 was one of the strains with highest levels of *cppA* transcripts. Thus, this strain background was suitable for investigating CppA functions using *cppA* gene inactivation and molecular analyses. Differences in transcript levels of *cppA* observed among strains could be due to different activities of transcription regulators of *cppA*, as observed for other complement evasion genes in *S. mutans* (Alves *et al*., 2016; Alves *et al*., 2019; Alves *et al*., 2020). Differences in *cppA* activities detected among strains highlight the need for addressing *cppA* regulation. In M18 *S. pyogenes* virulent strain which lacks RocA (a Regulator of CovR), *cppA* is up-regulated (Lynskey *et al*., 2017). However, SK36 does not possess RocA and we could not find *rocA* gene in the partial genomes of other studied strains. In *S. sanguinis* CovR roles is unrevealed yet. We have found in the *cppA* promoter regions, binding sites for CovR and VicR (data not shown), but regulation of *cppA* by CovR and VicRK regulators was not supported by *cppA* transcription in comparisons to parent strain SK36 and *covR and vicK*

isogenic mutants we have constructed (data not shown). There is also evidence of species-specificities of CovR regulatory functions. For example, the CovR of *S. pyogenes* and *S. mutans*, have certain amino acid residues required for CovR phosphorylation (threonine T-65 and aspartate D-35) which are lacking in *S. sanguinis* CovR (Mattos-Graner & Duncan, 2017). On the other hand, in *S. sanguinis* SK36, *cppA* is a potential target of the TCS SptRS, which is required for immune evasion, competence, cell wall homeostasis and *ex vivo* survival in saliva (Camargo *et al*., 2018). This emphasizes that in pathogenic and commensal states, proteases seem to have played significant role in *S. sanguinis*-host interactions, modulating *S. sanguinis* capacity to cope with host immune defenses.

Susceptibility of SKcppA mutant to complement mediated immunity was further confirmed by its *ex vivo* persistence in human blood. Our data shows that SKcppA mutant was unable to persist after 4 hours and its counts remained reduced as compared to SK36 and SKcppA+, that were able to recover upon initial decline. This finding indicates the importance of *cppA* for *S. sanguinis* persistence in blood. We further evaluated whether *cppA* has any role in *S. sanguinis* competence, because the protease *pepO* of *S. pyogenes* is reported to be involved in degrading short-hydrophobic peptides resulting in impairing competence, thus disrupting community behavior (Wilkening *et al*., 2016). Moderate increase in transformation efficiency was observed for SKcppA as compared to SK36, thus the role of *cppA* in competence inhibition is uncertain. In addition, all the other wild type of clinical strains demonstrated reduced transformation efficiency in comparison to SK36, which could not be correlated with transcriptional activities of *cppA*. The activities of CppA genetic polymorphism of nine strain were assessed using SK36 as reference strain, revealing ninety-six single nucleotide polymorphisms (SNPs) were found for *cppA* among the nine strains analyzed, which included 29 missense mutations. Mutation in genes of complement evasion were reported to be involved in evolution of host-pathogen interactions (Cagliani *et al*., 2018; Aleru *et al*., 2020). Though CppA is conserved among *S. sanguinis* strains but naturally *cppA* mutations are relatively common. Phylogentic analysis of *S. sanguinis cppA* reveals its closer to Mitis group (*S. mitis* and *S. pneumoniae*).

In summary, we identified that *S. sanguinis* strains express protease termed as CppA, which have significant role in complement-mediated immune evasion and persistence in human blood. *S. sanguinis* expression of CppA modulates binding to host proteins required for complement regulation (factor H, C4BP) and activation (SAP, C1q) for evasion to C3b deposition and opsonophagocytosis by PMN. Tranformation efficiency of SKcppA is

moderately enhanced while lower in other clinical stains than SK36. Mutations observed in *cppA* of *S. sanguinis* might play role in slight difference in functions of different strains.

7 CONCLUSION

The findings reported in this thesis indicate that CppA is a protein applied by *S. sanguinis* to evade the complement system. Based on the presented findings, it was possible to conclude that:

- The gene *cppA* is expressed by all strain of *S. sanguinis* included in this study, although *cppA* activities are strain specific.
- Deletion of *cppA* in SK36 increases its susceptibility to C3b deposition, and to serum-mediated phagocytosis by PMN from human peripheral blood.
- CppA avoids SK36 binding to major host proteins activating the classical pathway of the complement (C1q and SAP), whereas it promotes bacterial binding to the complement down-regulator FH.
- CppA is needed for *S. sanguinis* persistence in human blood.

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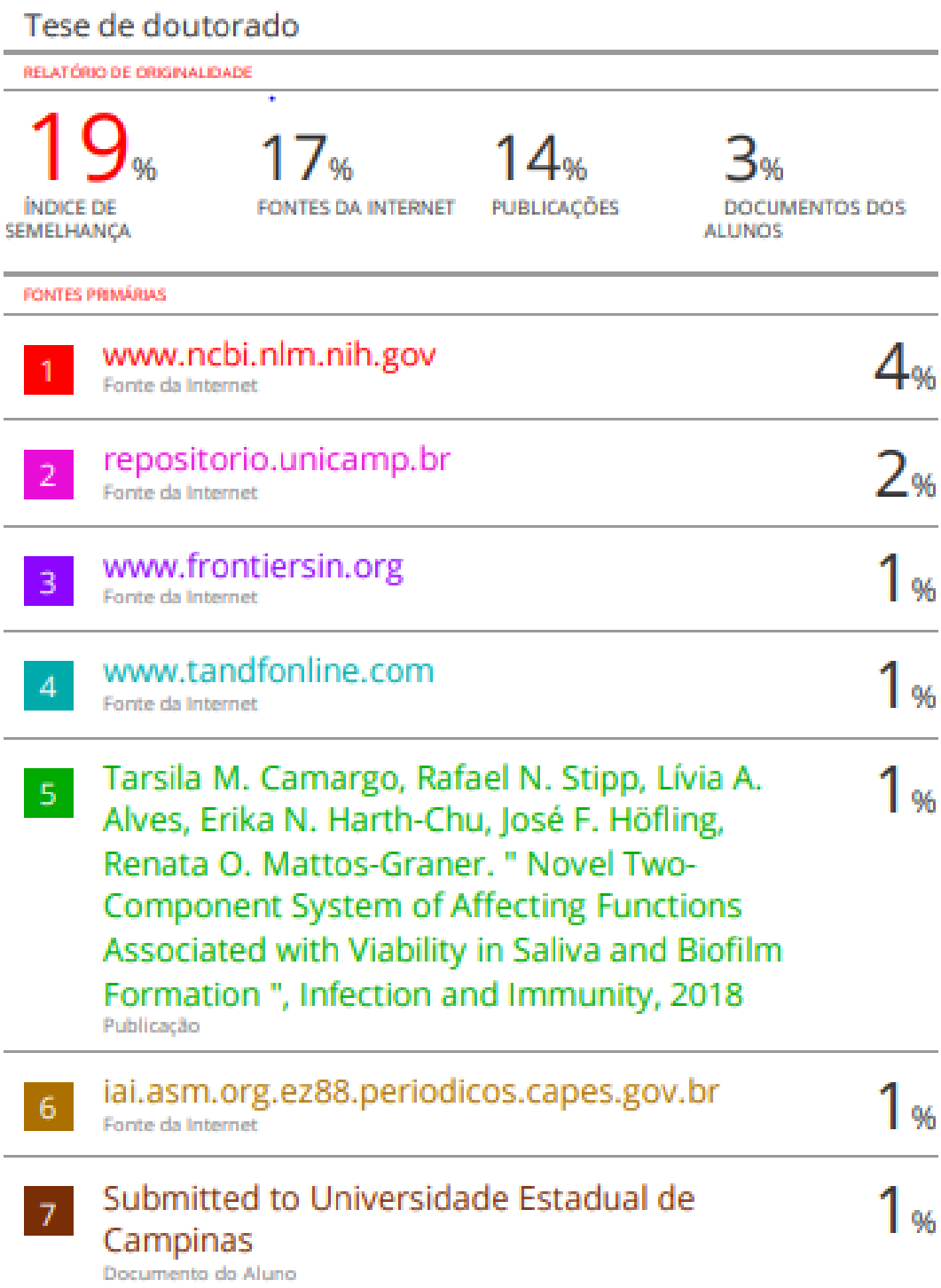
ATTACHMENTS

ATTACHMENT 1 – PLAGIARISM CERTIFICATE



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ATTACHMENT 2 – CIBio CERTIFICATE

CIBioFOP

UNIVERSIDADE ESTADUAL DE CAMPINAS
FACULDADE DE ODONTOLOGIA DE PIRACICABA
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OF-CIBio-007/2019

Piracicaba, 21 de outubro de 2019.

Pesquisador responsável: **Renata de Oliveira Mattos Graner**

Prezado Professor,

Informamos que seu Requerimento de Autorização nº **003/2019**, para Atividades em Contenção com OGM e seus Derivados, para realização do projeto intitulado "Análises da participação dos genes envolvidos na produção de DNA extracelular e exopolissacarídeos na formação de biofilmes por *Streptococcus sanguinis* ", foi analisado e aprovado pela CIBio – FOP/Unicamp. Este projeto deverá ser executado nos laboratórios credenciados para trabalho com OGM e derivados e durante o período de vigência estabelecido, conforme o requerimento aprovado.

Informamos que como responsável por este projeto, estará sob sua responsabilidade:

1. Assegurar a plena capacitação da equipe de trabalho e o cumprimento das resoluções normativas da CTNBio.
2. Manter toda a documentação sobre o projeto e sobre a capacitação da equipe de trabalho em arquivo de pronto acesso, para visitas e inspeções pela CIBio – FOP/Unicamp e/ou órgãos competentes.
3. Comunicar à CIBio-FOP/Unicamp através de novo requerimento, eventuais alterações no projeto, na equipe de trabalho ou de seu local de realização.
4. Encaminhar à CIBio-FOP/Unicamp, o relatório das atividades deste projeto em formulário específico no dia 01 de fevereiro de cada ano e/ou quando do encerramento do projeto.

Atenciosamente,

Profa. Renata de Oliveira Mattos Graner
Presidente CIBio – FOP/UNICAMP
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