UNIVERSIDADE SÃO FRANCISCO
Programa de Pós-Graduação *Stricto Sensu* em Ciências da Saúde

BRENDA VIEIRA FERREIRA

IMPORTÂNCIA DO TRANSPORTADOR DE POLIAMINAS PotABCD E POLIAMINAS NA FORMAÇÃO DE BIOFILME POR *Streptococcus pneumoniae*

Bragança Paulista
2023

BRENDA VIEIRA FERREIRA – R.A.:202220897

IMPORTÂNCIA DO TRANSPORTADOR DE POLIAMINAS PotABCD E POLIAMINAS NA FORMAÇÃO DE BIOFILME POR *Streptococcus pneumoniae*

Dissertação apresentada ao Programa de Pós-Graduação *Stricto Sensu* em Ciências da Saúde da Universidade São Francisco, como requisito parcial para obtenção do Título de Mestre em Ciências da Saúde.

Área de Concentração: Ciências da Saúde

Orientador: Prof. Dr. Thiago Rojas Converso

Bragança Paulista
2023

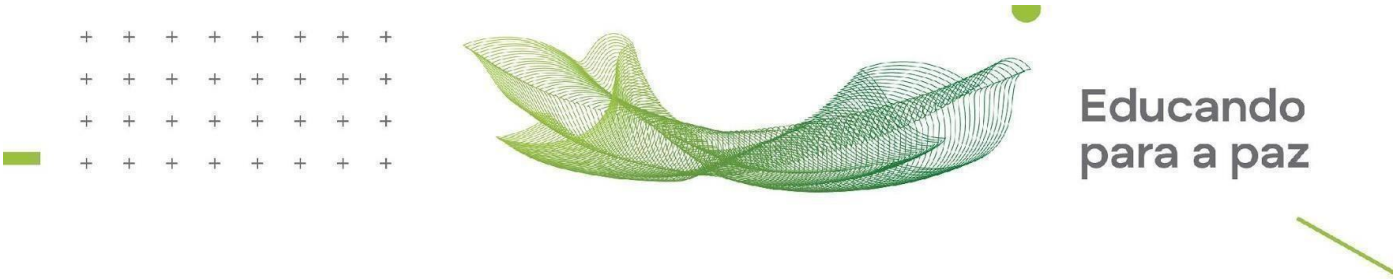
WC 217
F439i

Ferreira, Brenda Vieira.
Importância do transportador de poliaminas potABCD e poliaminas na formação de biofilme por *Streptococcus pneumoniae* / Brenda Vieira Ferreira. – Bragança Paulista, 2023.
39 p.

Dissertação (Mestrado) – Programa de Pós-Graduação *Stricto Sensu* em Ciências da Saúde da Universidade São Francisco.
Orientação de: Thiago Rojas Converso.

1. Streptococcus pneumoniae. 2. Transportador de poliaminas. 3. Biofilm. I. Converso, Thiago Rojas. II. Título.

Sistema de Bibliotecas da Universidade São Francisco – USF
Ficha catalográfica elaborada por: Karen Viana de Oliveira - CRB-8/10956



FERREIRA, Brenda Vieira. "Importância do transportador de poliaminas PotABCD na formação de biofilme por *Streptococcus pneumoniae*". Dissertação defendida e aprovada no programa de Pós-Graduação *Stricto Sensu* em Ciências da Saúde da Universidade São Francisco em 12 de dezembro de 2023 pela Banca examinadora constituída pelos professores:

Prof. Dr. Thiago Rojas Converso - Orientador e Presidente Universidade São Francisco

Profa. Dra. Carina Carvalho dos Santos
Universidade Federal da Bahia

Prof. Dr. Lúcio Fábio Caldas Ferraz Universidade São Francisco

DEDICATÓRIA

Dedico este trabalho a todos os meus ancestrais, familiares, professores, orientadores, colegas de profissão e a todas as pessoas que um dia cruzaram meu caminho para que pudesse ser a pessoa que sou hoje tanto no profissional quanto no pessoal. Dedico também este a minha fé, mas não somente a uma fé de cunho religioso, mas sim aquela fé intrínseca no dia de amanhã, no acreditar que vai dar certo, na fé de não desistir.

AGRADECIMENTOS

O presente trabalho foi realizado com apoio da Coordenação de Aperfeiçoamento de Pessoal deNível Superior - Brasil (CAPES) - Código de Financiamento: 88887.666340/2022-00.

RESUMO

O *Streptococcus pneumoniae* é uma bactéria de significativa relevância global, causador de mais de um milhão de mortes anuais pelo mundo. Geralmente, contraído nos anos iniciais de vida, o pneumococo realiza a colonização de forma assintomática no trato respiratório superior, desenvolvendo biofilmes que mantêm sua presença no local por meses. Porém,em condições que modificam o ambiente bacteriano, como à semelhança de infecções virais, os pneumococos podem romper o biofilme e infiltrar-se em outros tecidos, causando diversas doenças de diferentes gravidades. O transportador de poliaminas a proteína do complexo PotABCD, PotD é um fator crucial de virulênciado pneumococo, sendo que a imunização de murinos com o transportador de poliaminas recombinante reduz eficientemente a colonização bacteriana. Além disso, sugere-se que o PotABCD sejaparticipante crucial no desenvolvimento de biofilmes por pneumococos. Nesse contexto, considerando o papel protetor de PotD na limitação da colonização bacteriana e sua provável contribuição para a formação de biofilme, este estudo visa avaliar o papel de PotABCD, da espermidina e putrescina na produção de biofilme por pneumococo. Na primeira etapa da pesquisa,avaliou-se a formação de biofilmes na presença de poliaminas, produtos captados pelo transportador PotABCD, em comparaçãocom um meio sem adição de poliaminas. Posteriormente, comparou-se a formação de biofilmes em pneumococos selvagens e mutantes que não expressam o transportador PotABCD, utilizando ensaios de formação de biofilme em placas de 24 poços. Dada a relação identificada entre a formação de biofilmes e os pneumococos estar ligada à colonização da nasofaringe, realizou-se um ensaio para avaliar a formação de biofilmes em células. A cepa mutante exibiu uma redução da formação de biofilme *in vitro* e em culturas de célular, em comparação com a estirpe selvagem. No teste de qualidade dos biofilmes, observou-se uma redução de aproximadamente 3-4 logs na viabilidade bacteriana, indicandoa formaçãodebiofilmesestáveis. Além disso, foi observada uma ampliação na formaçãode biofilme pela cepa selvagem após a introdução das poliaminas. Em conjunto,os resultados deste estudo têm o potencial de contribuir para a compreensão dos mecanismos de formação de biofilme em pneumococos e sua potencial associação com o processo de colonização, reforçando a importância de estudos de formação de biofilmes em modelos fisiológicos.

Palavras-chave: *Streptococcus pneumoniae*. transportador de poliaminas. Biofilme.

ABSTRACT

The *Streptococcus pneumoniae* is a bacterium of significant global relevance, causing over a million deaths annually worldwide. Typically contracted in the early years of life, pneumococcus colonizes asymptomatically in the upper respiratory tract, developing biofilms that maintain it s presence at the site for months. However, under conditions that modify the bacterial environment, such as viral infections, pneumococci can disrupt the biofilm and infiltrate other tissues, causing various diseases of different severities. The polyamine transporter protein of the PotABCD complex, PotD, is a crucial virulence factor of pneumococcus, and immunization of mice with the recombinant polyamine transporter efficiently reduces bacterial colonization. Additionally, it is suggested that PotABCD plays a crucial role in the development of biofilms by pneumococci. In this context, considering the protective role of PotD in limiting bacterial colonization and its likely contribution to biofilm formation, this study aims to evaluate the role of PotABCD, spermidine, and putrescine in pneumococcal biofilm production. In the first stage of the research, biofilm formation was evaluated in the presence of polyamines, products captured by the PotABCD transporter, compared to a medium without the addition of polyamines. Subsequently, biofilm formation in wild-type pneumococci and mutants that do not express the PotABCD transporter was compared using biofilm formation assays in 24-well plates. Given the identified relationship between biofilm formation and pneumococcal colonization of the nasopharynx, an assay was conducted to evaluate biofilm formation on cells. The mutant strain exhibited a reduction in in vitro and cell culture biofilm formation compared to the wild- type strain. In the biofilm quality test, a reduction of approximately 3-4 logs in bacterial viability was observed, indicating the formation of stable biofilms. Additionally, an increase in biofilm formation was observed in the wild-type strain after the introduction of polyamines. Overall, the results of this study have the potential to contribute to the understanding of biofilm formation mechanisms in pneumococci and their potential association with the colonization process, reinforcing the importance of biofilm formation studies in physiological models.

Keywords: *Streptococcus pneumoniae. polyamine transporter. Biofilm.*

Lista de Símbolos e Abreviações

Δ*potABCD*	Cepa mutante negativa para os genes *potA, potB, potC* e *potD*
C+Y	Glucose médium
CFU	Unidade formadora de colônia
ml	Mililitro
mM	Milimolar
NESPs	Non-encapsulated pneumococci
NETs	*Neutrophil extracelular traps*
nm	Nanômetro
O.D	Densidade óptica
PBS	Solução salina tamponada
PotABCD	Transportador de poliaminas tipo ABC
rpm	Rotações por minuto
rPotD	Proteína potD recombinante
TSB	Tryptic Soy Broth

Lista de Figuras

SUMÁRIO

INTRODUÇÃO

1.1 *Streptococcus pneumoniae*

O pneumococo, ou *Streptococcus pneumoniae*, é uma bactéria Gram-positiva, podendo apresentar ou não encapsulamento, e é classificada comoum anaeróbio facultativo. Demonstrando a capacidade de colonizar a nasofaringe e a orofaringe de maneira comensal (HENRIQUES-NORMARK; NORMARK, 2010). No entanto, em hospedeiros suscetíveis, essa bactéria pode causar diversas doenças com gravidade variável. Dentre as doenças associadas a ela, destacam-se a pneumonia, a meningite, a otite média e a septicemia (ROSSI et al., 2012; SMITH-VAUGHAN et al., 2006)

A frequência da colonização por pneumococo pode se alterar de acordo com a idade do hospedeiro, em crianças menores de 5 anos o pneumococo é o principal agente causador de pneumonia bacteriana enquanto que em adultos representa de 10% a 30% das pneumonias adquiridas na comunidade (CDC, 2015). Embora a colonização nasofaríngea por pneumococos seja mais comum do que a infecção pneumocócica, é importante destacar que o fator determinante para o pneumococo permanecer restrito à nasofaringe ou causar infecção reside na susceptibilidade do hospedeiro colonizadoe na capacidade patogênica da cepa (KELLER; ROBINSON; MCDANIEL, 2016). O *S. pneumoniae* é responsável pelo falecimento anual superior a um milhão de indivíduos. As taxas de mortalidade estão diretamente ligadas à bacteremia e à meningite, alcançando números mais elevados nos extremos de idade. Entre os idosos, a mortalidade decorrente de bacteremia chega a atingir 60%, enquanto a relacionada à meningite chega a 80% (ORIHUELA; TUOMANEN, 2006).

Na atualidade, há vacinas pneumocócicas disponíveis voltadas para o pneumococo encapsulado, associado a formas mais graves de doenças. Entretanto, os pneumococos não encapsulados, referidos como NESPs (do inglês, *non-encapsulated pneumococci*), ainda apresentam taxas substanciais de infecção, apesar da introdução das vacinas. Isso ocorre porque há vacinas destinadas a esse conjunto de bactérias, que agora apresentam maior incidência em virtude da pressão seletiva exercida sob os *S. pneumoniae* ecapsulados. Adicionalmente, observam-se modificações na

frequência de sorotipos encapsulados; a diminuição na colonização por sorotipos vacinais resulta em um aumento nas infecções provocadas por sorotipos não contemplados pela vacina (HICKSet al., 2007). Globalmente, as informações epidemiológicas destacam a urgência de examinar alternativas em estratégias de vacinaçãocapazes de conferir proteçãocontra uma ampla diversidade de pneumococos, independentementedo sorotipo.

1.2 Função do biofilme nas infecções pneumocócicas

Na cavidade nasal e faringe, o pneumococo tem a capacidade de formar agrupamentos altamente estruturados que exibem uma resistência considerável a agentes antimicrobianos, em comparação com as células em estado planctônico (não estão agrupadas em biofilme). Quando organizado na forma de biofilme, o pneumococo demonstra uma capacidade invasiva reduzida, proporcionando um ambiente propício para a multiplicação bacteriana e uma maior troca de material genético. Além disso, biofilmes também foram identificados em superfícies mucosas durante infecções como sinusite, pneumonia e otite média (CHAO et al., 2014; SHAK; VIDAL; KLUGMAN, 2013). Contudo, mesmo que a forma em biofilme revele uma capacidade invasiva reduzida, estudos evidenciaram que o biofilme de *S. pneumoniae* tem a capacidade de impedir a formação de biofilme de espécies concorrentes, como o *Staphylococcus aureus*, em modelos vivos (REDDINGER et al., 2018).

Os biofilmes em pneumococos funcionam como reservatórios para micro-organismos. Bactérias que adotam a configuração de biofilme, durante a colonização, manifestam menor virulência, mas atuam como fonte de micro-organismos patogênicos que se desprendem do biofilmeem resposta a sinais do ambiente, disseminando-se para diferentes localidades (MARKS; REDDINGER; HAKANSSON, 2012). Tais indicadores abrangem a disponibilidade de nutrientes, flutuações de temperatura e presença de ATP extracelular (CHAO et al.,2014), capazes também de ser desencadeados por uma infecção pelo vírus influenzaem indivíduos previamente colonizados por pneumococo. A interação sinérgica entre infecções virais e a patogênese dos pneumococos foi documentada em diversos achados científicos

(CHERTOW; MEMOLI, 2013; PETTIGREWet al., 2011); nos quais os vírus induzem a exibição a receptores de células do epitélio, como a febre, mudanças na presença de nutrientes (como, glicose), síntese de ATP extracitoplasmático, noradrenalina e liberação de reguladores de respostas inflamatórias. Esses sinais resultam em modificações na dinâmica de síntese do RNA no pneumococo, desencadeando o desbloqueio das cepas patogênicas (CHAO et al2014). Como consequência, observa-se um incremento na proliferação bacteriana e a desintegração do tecido nasofaríngeo (DIAVATOPOULOSet al., 2010; MCCULLERS, 2014). Além disso, desempenha o papel de reservatório para micro-organismos com potencial patogênico, sugere-seque o biofilme *in vivo* funcione como um aparato defesa contra a criação de redes extracelulares de neutrófilos. As NETs têm a capacidade de se ligar e eliminar bactérias Gram-positivas e Gram-negativas, assim como alguns tipos de leveduras patogênicas. Em infecçõespneumocócicas, apesar de se perceber a geraçãode NETs e sua associaçãocom bactérias, sua capacidade bactericida é diminuída (URBAN; LOURIDO; ZYCHLINSKY, 2006).

Além disso, todos os processos de formação de biofilme são regulados por moléculas de sinalização pertencentes ao sistema de detecção de *quorum sensing* (QS). Este sistema é responsável pela comunicação das bactérias que ocorre através da produção, secreção e detecção de pequenas moléculas denominadas AutoIndutores (BALESTRINO et al., 2005) de modo que, uma vez que a quantidade dessas moléculas na matriz extracelular atinge umlimite, os sinais são ativados, alterando o perfil de expressão de determinados genes, modificandoo fenótipo bacteriano, alterando a expressão de fatores de virulência, tolerância ácida e formação de biofilme, outro papel importante do QS está na regulação de biofilmes maduros (ABRAHAM,2016; GUERRA et al., 2022).

1.3 O *operon* de transporte de poliamina (*potABCD*)

A habilidade de colonização do pneumococo é influenciada por diversos fatores bacterianos, sendo o transportador de poliaminas, *potABCD,* um dos principais destaques entre esses fatores (FLEMMINGet al., 2016; PIPKINS;*et al.*, 2017). Compreende-se que as poliaminas desempenham um papel vital no funcionamento das células, sendo moléculas indispensáveis para

O crescimentocelular, a produção de proteínas e moléculas de DNA e RNA (SHAW, SWIATLO, 2006). Dentre as variedades de poliaminas, destacam-se a putrescina, espermidina, espermina e cadaverina. Essas moléculas desempenham funções essenciais em diversos processos fisiológicos, interagindo intensamente com ácidos nucleicos, o que modula as funções de RNA, DNA e trifosfatases nucleotídicas. Além disso, exercem influência na síntese proteica e na produção de substâncias análogas(IGARASHI; KASHIWAGI, 2000). A maioriadas bactérias tem a habilidade não apenas de sintetizar naturalmente, mas principalmente transferir moléculas de poliaminas do meio extracelular para o intracelular, mediante a utilização de um conjunto de proteínas membranares. No contexto do *S. Pneumoniae*, esse transporte de poliaminas é mediado pelo PotABCD, um grupo de proteínas essencialmente expressas em todas as cepas dessa bactéria (IGARASHI; ITO; KASHIWAGI, 2001).

Estudos baseados no alinhamento com proteínas semelhantes de outras bactérias propõem as funções individuais de cada componente do grupo PotABCD (FIGURA 1). A proteína PotA desempenha o papel de prover energia ao conjunto, uma vez que possui um canal de ligação para moléculas de adenosina trifosto. As proteínas PotB e PotC, por sua vez, apresentam características de proteínas transmembrana, conferindo a capacidade de formar canais para o transporte das poliaminas. Por fim, a proteína PotD, especialmente no contexto do *S. pneumoniae*, apresenta um peptídeo sinal distintivo de bactérias Gram-positivas, inidicando uma posicionamento no exterior da membrana. Adicionalmente, o transportador contém sítios de ligação para putrescina e espermidina, indicando que PotD é encarregado de capturar essas poliaminas do meio fora da célula (SHAH et al., 2011).

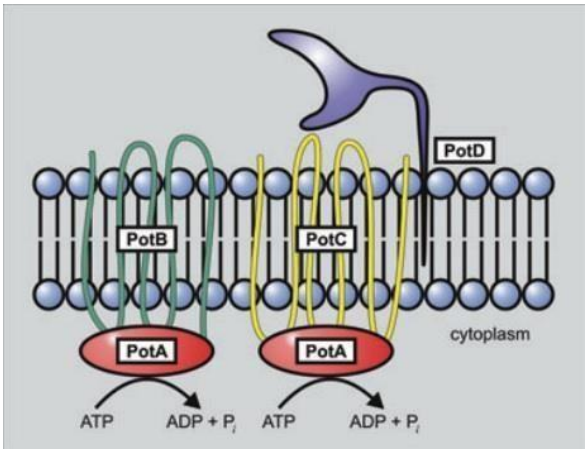


FIGURA 1.**Sugestão de disposição de proteína no complexo PotABCD de *Streptococcus pneumoniae*.** Neste complexo, a proteína PotA tem a função de ATPase, fornecendo energia para o complexo; as proteínas PotB e PotC têm a função de formar um canal no domínio transmembrana para o transporte das poliaminas; a proteína PotD tem a função de se conectar e adquirir as poliaminas da região extracelular, conduzindo-as para a região intracelular por meio do canal formado por PotB e PotC. Fonte: P. Shah and E. Swiatlo, 2008.

Dado o seu posicionamento na membrana, a PotD tem sido alvo de estudos como um potencial candidato vacinal contra o pneumococo em diversos modelos de infecção. Camundongos que foram imunizados com PotD demonstraram proteção efetiva contra a sepse causada por pneumococos virulentos. (SHAH; SWIATLO, 2006). Adicionalmente, evidenciou-se que a vacinação intranasal de murinos com proteína recombinante PotD diminui a colonização nasofaríngea por pneumococos. (MIN et al., 2012; SHAH et al., 2009). Os camundongos vacinados com rPotD demonstraram capacidade de reconhecer o estímulo com a proteína, respondendo com a produção de óxido nítrico (NO) (CONVERSOet al., 2017).

Além da vacinação intranasal, observou-se que a imunização subcutânea de camundongos com PotD apresenta a capacidade de reduzir a colonização por pneumococos. Em síntese, as imunizações utilizando PotD recombinante demonstraram ser eficazes na proteção contra a colonização nasal em camundongos (CONVERSO et al., 2017). Alguns mecanismos de defesa bacteriana, também foram alvos de apuração como: A produção de anticorpos anti-rPotD e a opsonofagocitose mediada por anticorpos específicos; a capacidade dos anticorpos anti-rPotD ligados à superfície de promover a fagocitose e eliminação bacteriana por fagócitos avaliada in vitro; observação de uma diminuição significativa no número de unidade formadora de colônias (UFC) recuperadas quando as bactérias foram incubadas com soro de camundongos imunizados

com rPotD em comparação com o grupo controle (CONVERSO et al., 2017b).

Com a capacidade reduzida da proteína PotD em colonizar o trato respiratório superior como ponto de partida, o propósito deste estudo foi investigar a interação do transportador de poliaminas PotABCD na formação de biofilme pelo S*treptococcus pneumoniae*. Isso foi realizado tanto em pneumococos selvagens quanto em mutantes que não expressam o operon Pot. Além disso, o estudo buscou analisar a formação de biofilme na presença de poliaminas e a colonização intranasal.

2. OBJETIVOS

O objetivo do presente estudo é investigar o papel do transportador de poliaminas PotABCD na formação de biofilmes por *Streptococcus pneumoniae.*

Objetivos específicos:

- Comparar a formação de biofilme, em superfície abiótica e substrato celular, por pneumococos selvagens e mutantes que não expressam o *potABCD.*
- Avaliar a formaçãode biofilme em superfície abiótica por pneumococos selvagens na presença e ausência de poliaminas exógenas.
- Avaliar a formação de biofilme nasofaríngeo *in vivo* por pneumococos selvagem e mutante.

3. CAPÍTULO 1 (Manuscrito submetido a revista – PlosOne)

Role of the polyamine transporter PotABCD during biofilm formation by *Streptococcus pneumoniae*

Neste artigo é descrito os principais aspectos envolvidos na formação de biofilme por *Streptococcus pneumoniae* e sua relação com o transportador de poliaminas PotABCD. Neste trabalho foram utilizadas duas cepas de *Streptococcus pneumoniae* TIGR4, um isolado bacteriano virulento do sorotipo 4, e seu mutante isogênico Δ*potABCD*, no qual os genes *potA*, *potB*, *potC* e *potD* (correspondentes ao operon pot completo) foram removidos. A construção da cepa mutante envolveu a substituição alélica dos genes do operon *potABCD* por um cassete de resistência à trimetoprima, flanqueado pelas regiões antes e depois dooperon. Ensaios de formação de biofilme em superfícies bióticas e abióticas foram realizados afim de comparar as diferenças entre cepas selvagens e mutantes, bem como ensaios deformação com adição de poliaminas exógenas, além de ensaios em substrato celular e modelos murinos de colonização.

Os resultados mostraram que a ausência do *operon* impacta significativamente aprodução de biofilme onde a cepa mutante produz menos biofilme em relação a cepa selvagem. Ea adição de poliaminas exógenas aumentam apenas a formação de biofilme na cepa selvagem, quanto pouco se altera na cepa mutante. Os ensaios de formação de biofilme em substrato celular apresentaram resultadossemelhantes aos ensaios em superfície abiótica. Evento também observadono ensaios *in vivo*, pois os camundongos infectados com a cepa mutante mostraram que a inibiçãodo transporte de poliaminas reduziu a colonização da bactéria no tecido linfóide associado ao nariz dos camundongos. Esses resultados sugerem que a inibição do transporte de poliaminas desempenha um papel importante no processo de formação de biofilme e reforça a imporntância de mais estudos sobre biofilme em modelos fisiológicos.

BRIEF RESEARCH REPORT

Role of the polyamine transporter PotABCD during biofilm formation by Streptococcus pneumoniae

Vieira, B[1], Alcantara, J.B[1], Destro, G [1], Guerra, M.E.S[1], Oliveira, S[1], Lima, C.A[2]; Longato, GB[2], Hakansson, A.P[3], Leite, L.C.C[4], Darrieux, M[1+], Converso, TR[1+]*.

[1] Laboratório de Biologia Molecular de Microrganismos, Universidade São Francisco, Bragança Paulista, Brazil

[2] Laboratório de Farmacologia Molecular e Compostos Bioativos, Universidade São Francisco, Bragança Paulista, Brazil

[3] Division of Experimental Infection Medicine, Department of Translational Medicine, Lund University, Lund, Sweden.

[4] Laboratório de Desenvolvimento de Vacinas, Instituto Butantan, São Paulo, Brazil.

[+] These two authors share senior authorship.

* Corresponding author, mailing address: trconverso@hotmail.com

Keywords: Polyamine transporter**,** Biofilm; *Streptococcus pneumoniae*; pathogenesis

Abstract

Streptococcus pneumoniae is a bacterium of great global importance, responsible for more than one million deaths per year. This bacterium It is typically acquired during the early years of life and colonizes the upper respiratory tract asymptomatically, establishing its presence by forming. However, under conditions that alter the bacterial environment, such as viral infections, pneumococci can escape from the biofilm and infiltrate other environments, resulting in local and systemic disease of varying severity. The polyamine transporter PotABCD is required for optimal survival of the organism in the host. Immunization of mice with recombinant PotD has the potential to decrease subsequent. PotD has also been proposed to play a role in the development of pneumococcal biofilms. Hence, in this study, our objective was to elucidate the role of PotABCD and polyamines in pneumococcal biofilm formation. First, the formation of biofilms was evaluated in the presence of exogenous polyamines – the substrate transported by PotABCD – added to culture medium. Next, a *potABCD*-negative strain was used to determine biofilm formation in different model systems using diverse levels of complexity transitioning from abiotic surfaces to cellular substrates to in vivo animal models and was subsequently compared with its wild-type strain. The results showed that adding more polyamines to the medium stimulated biofilm formation, suggesting a direct correlation between polyamines and biofilm formation. Also, deletion of *potABCD* operon impaired biofilm formation in all models tested. Interestingly, more differences between wild-type and mutant were observed in the more complex the model, which emphasizes the significance of employing more physiological models in studying biofilm formation.

Introduction

Streptococcus pneumoniae, often recognized as pneumococcus, is a Gram-positive, facultative anaerobic bacterium that could be or not encapsulated. It is typically acquired during early childhood and most often colonizes the nasopharynx and oropharynx asymptomatically as biofilms [1, 2]. However, under certain conditions, such as due to viral infections, pneumococci have the ability to break free from the biofilm and infiltrate other niches such as the lungs and internal organs, causing pneumonia, meningitis, and sepsis [3, 4]. Invasive infection arising from bacterial invasion is responsible for more than one million deaths per year [1, 2].

The initial and obligatory events required for colonization of *Streptococcus pneumoniae* occurs in the nasopharynx [5-7]. In this niche, the pneumococci can be found organized as biofilms, highly structured communities that display increased resistance to antimicrobial agents when compared to planktonic cells grown statically in culture medium. Pneumococci grown in biofilms have a lower invasive capacity, and the biofilm environment provides a favorable environment for bacterial multiplication and greater exchange of genetic materials [7]. Bacterial aggregation and biofilms have also been found on mucosal surfaces during middle ear infection, sinusitis, and pneumonia [5, 8].

Many bacterial factors are important for the ability of various organisms to colonize the host. Among these factors, the polyamine transporter, Pot, stands out [9, 10]. It is known that polyamines are molecules critical for the growth and viability of all cells are putrescine, spermidine, spermine, and cadaverine. are examples of the most common polyamines and are linked to several physiological processes, such as interaction with nucleic acids, modulation of the function of RNA, DNA and nucleotide triphosphatases; synthesis of proteins and related substances [11]. Furthermore, a study conducted on an abiotic surface demonstrated that the absence of polyamine transport and the presence of certain proteins negatively influences the biofilm formation by encapsulated pneumococci [10]. Most bacteria can synthesize polyamines, but can also transport them from the extracellular to the intracellular environment using a set of membrane proteins [12].

In *S. pneumoniae*, the transport of polyamines is carried out by the polyamine transporter complex, also known as PotABCD, an ABC transporter expressed in virtually

all pneumococcal strains [13]. Within the complex, the transmembrane protein PotD is responsible for capturing the polyamines from the extracellular environment. Considering its extracellular location, PotD has been explored as a potential vaccine candidate against pneumococcal infection in different models [14-18]. Mice vaccinated with recombinant PotD exhibited reduced nasopharyngeal colonization by the pneumococcus and were protected against invasive challenge [14, 18, 19].

Given the importance of PotD in reducing nasopharyngeal colonization, this work aims to investigate the relationship between the polyamine transporter PotABCD and biofilm formation by *Streptococcus pneumoniae*. Our results support this hypothesis as our *in vitro* and *in vivo* experiments show that addition of extracellular polyamines increased biofilm formation and as the mutant (unable to express PotABCD) produced less biofilm than the wild-type.

Materials and methods

1. Strains and storage conditions

In this study, the *Streptococcus pneumoniae* strains: TIGR4 (a virulent encapsulated bacterial isolate of serotype 4), and its isogenic mutant ΔpotABCD whose genes *potA, potB, potC* and *potD* (corresponding to the entire *pot* operon) were deleted, were used. The mutant strain was constructed by allelic replacement of genes belonging to the *potABCD* operon with a kanamycin resistance cassette, flanked by the upstream and downstream regions of the operon. This strain was produced and provided by Dr. Swiatlo, VA Medical Center, Section for Infectious Disease, Mississippi, USA [10]. The strains were cultivated to an $O.D._{600nm}$ of 0.4 - 0.5 in Trypticase Soy broth (TSB medium). Frozen stocks were produced by centrifuging bacterial culture and resuspending it at 10% of the original volume in TSB with addition of 20% glycerol and stored at -80 °C until use.

2. Comparison of *in vitro* biofilm production by wild-type pneumococci and Pot-negative mutants with and without addition of polyamines

The TIGR4 and ΔpotABCD strains were first cultured to an $O.D._{600nm}$ of 0.3 in TSB medium under anaerobic conditions at 37 °C and the cells were then transferred to 24-well plates. In each well, 10^5 CFU of bacteria were added in 1 ml of TSB medium supplemented with 10% equine serum and incubated for 24 hours at 37 °C.

After 24 hours incubation, the medium was discarded, the wells were washed with PBS once, and dried at room temperature (21°C). Adhered cells were stained with 0.1% crystal violet in distilled water for 30 minutes. Then, the dye was discarded, the cells were washedagain three times with PBS. The crystal violet that adhered to the biofilm was solubilized in 1 ml of 95% ethanol and incubated at room temperature for 15 min at 80 rpm agitation to solubilize the bacterial cells. The absorbance of the samples was measured in a spectrophotometer (Glomax® multi detection system) at 590 nm.

For CFU counting, after 24 hours, the wells were washed with PBS to remove planktonic cells. Fresh PBS (500 µl) was added to each well and the biofilm was scrapedoff using a 20–200 µl pipette tip to release all biofilm cells. The cells were collected diluted, and plated on blood agar plates overnight at 37 ºC in anaerobiotic conditions. The following day, colonies on the plates were counted. To determine biofilm formation in the presence of polyamines, 2 mM, 1 mM or 0.5 mM of either spermidine or putrescine were added to the non-supplemented medium. All other parameters were the same.

3. Biofilm formation on cell substrate

The biofilm evaluation on cell substrate was analyzed as described by Marks et al. [6] with modification. Briefly, human lung epithelial carcinoma cells (A549 cells, ATCC CCL-185), were cultured in DMEM medium with 10% fetal bovine serum at 37 ºC until confluence and fixed with 4% paraformaldehyde in 24-well flat-bottom polypropylene plates. The cells were used as substrate for the bacteria to form biofilm. For that, bacterial stocks were thawed and diluted to a 1:1,000 in TSB, 500 µl of the diluted bacteria were seeded in each well and incubated at the nasopharyngeal temperature of 34 °C in 5% CO_2. For optimal biofilm formation the medium was changed every 12 hours, and the biofilms were left for 60h when the medium was discarded, and the wells washed with PBS to remove planktonic cells. Fresh PBS (500 µl) was added to each well and the biofilm was scraped off using a 20–200 µl pipette tip to release all biofilm cells. The cells were collected, diluted, and plated on blood agar plates overnight at 37 ºC in anaerobiotic conditions. The following day, colonies on the plates were counted.

4. Biofilm formation and colonization *in vivo.*

The experiments described in this work were approved by the ethics committee at São Francisco University, Bragança Paulista, Brazil (CIAEP/CONCEA Nº 01.226.2014, approved in March 2021).

Ten female C57Bl/6 mice (5-7 weeks) were infected as described by Converso *et al.* [20]. Briefly, wild-type and mutant strains were thawed and diluted to a concentration of 1 x 10^9 CFU/ml in sterile PBS and each animal received 10 µL (1 x 10^7 CFUs) in one nostril/nare.

The biofilm/colonization analysis was performed as described by Marks at al. [6]. Nine days post infection, the animals were euthanized, the trachea was exposed and sectioned, and the upper respiratory tract was washed to remove planktonic bacteria. Next, the Nasal Associated Lymphoid Tissue (NALT) was removed and macerated in a homogenizing bag containing 600 µL of PBS. This material was diluted and plated on blood agar plates containing 2.5 µg/mL of gentamicin; the plates were incubated for a

period of 18 h in anaerobic conditions at 37°C for CFU counting.

Results

1. Biofilm formation in abiotic surface

To compare the ability of biofilm formation between TIGR4 wild-type and ΔpotABCD strains, the two strains were added to a polystyrene 24 well plate and the biofilm was allowed to develop for 24 h. Fig 1 A) presents the difference between the strains, showing that the mutant forms almost 40% less biofilm than the wild-type bacterium. On Fig 1 B) the same assay was performed but the biofilm was evaluated by CFU count, confirming the previous result, the mutant produced 3 times less biofilm than the wild-type strain. This result agrees with previous result and confirms our hypothesis.

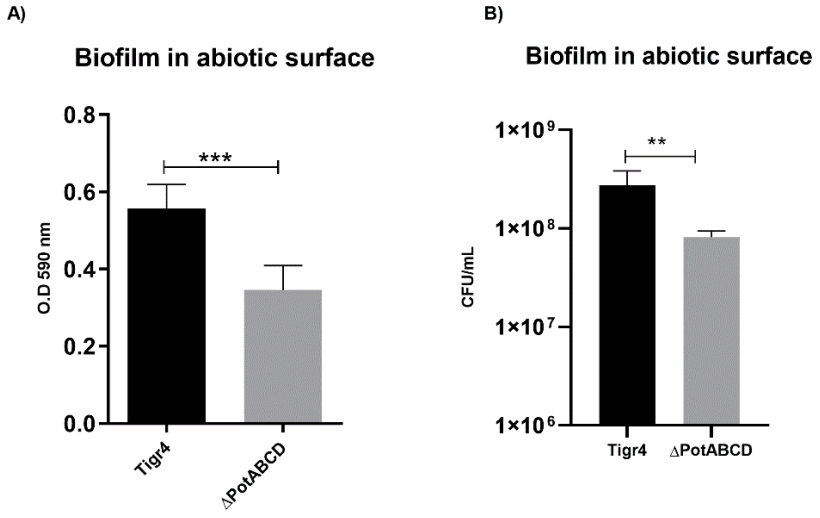


Fig 1. Biofilm formation in abiotic surface.
The biofilm formation was evaluated after 24h of culture in a microplate containing TSB medium supplemented with 10% equine serum. **A)** The biofilm biomass was evaluated by crystal violet staining and plate reader at 590 nm. **B)** The biofilm was evaluated by CFU counting. The results expressed are representative of three independent experiments carried out in decaplicate and the difference was determined by Student's *t*-test where ** =$p<0.01$ and ***=$p<0.001$.

2. Biofilm formation on cell substrate

The A549 cellular substrate was used to more closely mimic the human respiratory environment, the natural pneumococcal colonization niche, a protocol adapted from Chao et al. [21] to compare the biofilm formation between wild-type and *potABCD* negative bacteria (Fig 2). The result is presented as the number of recovered CFU and shows that the wild-type strain produces 10 times more biofilm than the mutant, reinforcing our hypothesis that polyamine transporters are important for biofilm formation even in a more physiological model, simulating the natural niche where the bacteria reside.

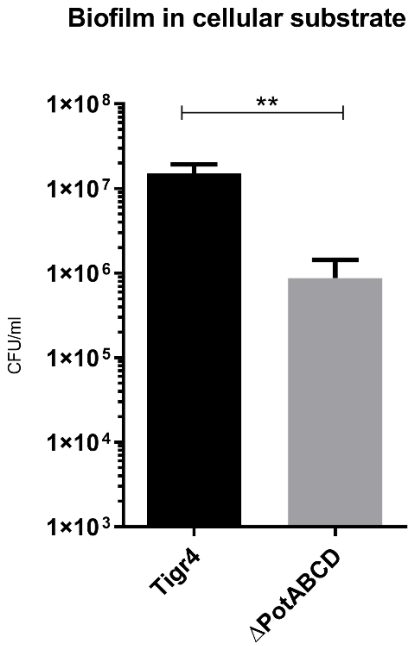


Fig 2. Biofilm formation on cell substrate.
S. pneumoniae wild-type or ΔpotABCD strains were added to a 24 well plates coated with A549 fixed cells, the medium was changed every 12 hours and bacteria were kept for 60 hours for biofilm formation. The biofilm was analyzed by plating serial dilutions on blood agar plates. The results expressed are representative of three experiments carried in octuplicates. The difference was determined by Student's *t*-test where **=$p < 0.01$.

3. Polyamine transporter is important for colonization and biofilm formation *in vivo.*

To determine whether the polyamine transporter is important for biofilm formation *in vivo,* we infected C57Bl/6 animals with the wild-type or the mutant strain and allowed the bacterium to colonize and form biofilm for 9 days. At this time, the Nasal Associated Lymphoid Tissue (NALT) was collected and the recovered CFUs were counted. Similar to that observed on the cell substrate biofilm, the mutant bacterium that was unable to capture polyamines from the environment, and colonized the nasopharynx to a lower degree than PotABCD-positive wild-type bacteria counterpart. The CFUs recovered in mice infected with the wild-type strain showed more than 100 times higher bacterial load in the nasopharynx than the group infected with the mutant strain (39,000 vs 360).

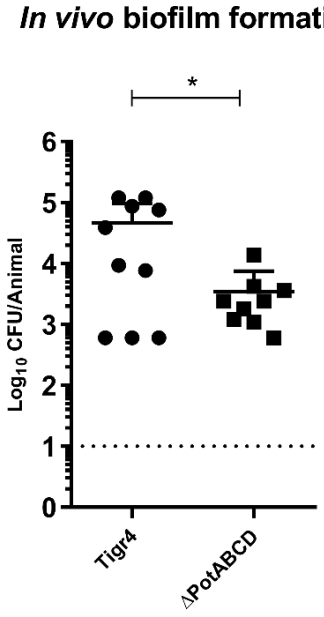


Fig 3. The importance of polyamine transporter on biofilm formation *in vivo*.
C57Bl/6 micewere infectedviaintranasal route with 1.10^7 CFU of TIGR4 or ΔPotABCD strains. On the ninth day post-infection, the Nasal Associated Lymphoid Tissue(NALT) was collected, homogenized, and plated on blood agar plates for CFU counting in each group. Each dot in the graph correspond to the number of bacteriarecoveredin one animal. The dotted line corresponds to the detection limit for this experiment which is 10. This graph is representative of two independent experiments. Difference was analyzed by Student t test where *=p <0.05.

4. Exogenous polyamines improve biofilm formation in *S. pneumoniae.*

To investigate whether the polyamines or their transporters are important for biofilm formation by pneumococci, we added exogenous polyamines to the culture medium and evaluated the biofilm formation (Fig 4). The addition of exogenous putrescine at concentrations of 1 mM and 2 mM led to an increase in biofilm formation in comparison to the untreated control, containing no putrescine (Fig 4A). In this assay, the doses of 0.25 and 0.5 mM did not affect biofilm formation. We observed a similar result with the addition of exogenous spermidine (Fig 4B). There was an increase in biofilm formation after the addition of 0.25, 0.5 and 1 mM of spermidine. Interestingly, the addition of 2 mM spermidine resulted in the same amount of biofilm as observed under untreated control conditions.

This indicates that polyaminesact differently in the formation of biofilms or that the polyamine transport mechanism favors the entry of putrescine in the bacterial cell as compared to spermidine. For the mutant bacterium, the addition of polyamines did not affect biofilm formation, reinforcing the hypothesis that the ability to import polyamines into the cell influences the formation of biofilms by the pneumococcus (Supplementary Fig 4).

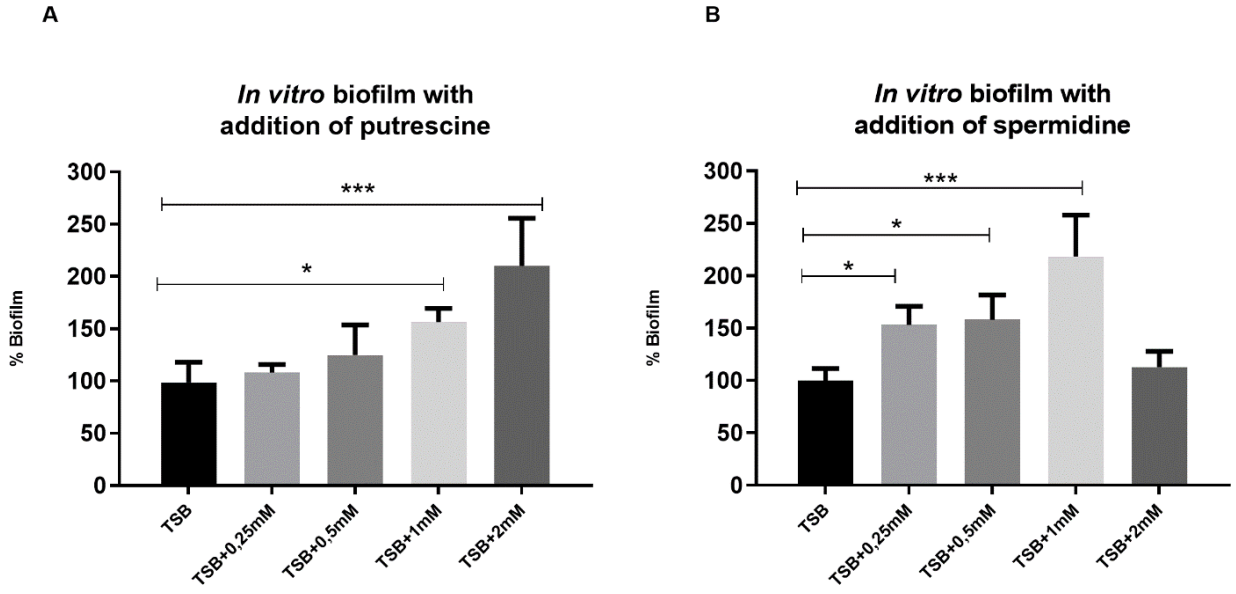


Fig 4. In *vitro* biofilm formation by *S. pneumoniae* with the addition of putrescine and spermidine. The biofilm was evaluated after 24h in microplateassay with addition of 0.25 mM, 0.5 mM, 1 mM and 2 mM of **putrescine (A)** or **spermidine (B).** The biomass was evaluated by crystal violet staining at 590 nm. The absorbance from group TBS was used to calculate the percentages being considered 100%. The results expressedare representative of experiments carried out in quadruplicate. The comparison between groups was performed using the One-way ANOVA followed by Dunnet's test, where ***=p < 0.001, and *=p<0,05

Discussion

Polyamines are essential nutrients required for cells to survive. Putrescine and spermidine are the most common polyamines found in bacterial cells [12]. All cells can synthesize polyamines by metabolic pathways, but they are also capable of transporting them into the cell interior by an ABC transporter called Pot. Several studies have suggested a relationship between polyamines and/or their transporters and biofilm formation in different models [22-25].

A study by Pipkins et al. (2017) demonstrated that the absence of the *potD* operon caused a decrease in biofilm production in an *in vitro* abiotic biofilm assay when compared to the parental wild-type strain [10]. Interestingly, this was only observed in the encapsulated strain, and the opposite effect was observed in the unencapsulated strain [10]. The authors suggested that the presence of the capsule could interfere with

bacterialadhesins, but this was not confirmed in the study. Our work investigated biofilm formationusing a physiological models with epithelial cells as a substrate for the biofilm to develop and examined biofilm formation *in vivo* using a mouse colonization and biofilm model.

While *in vitro* studies from several groups have explored biofilm formation in the nasopharynx by pneumococci [26-28], Marks et al. were the first to demonstrate that the pneumococcus is capable of forming highly structured biofilms during colonization of the upper airways in mice. This study also proposed the use of epithelial cells to mimic the natural environment encountered by pneumococci during *in vivo* colonization [7]. We adapted this model [6], using lung epithelial cells (A549), and our results showed that the mutant strain formed around 10 times less biofilm than the wild-type, a result that agrees with the abiotic observation. This result is interesting because it more closely mimics the natural pneumococcal niche.

Marks et al. (2012) described an *in vivo* mouse model to evaluate biofilm formation by pneumococcus. The group identified that strains with mutations in virulence factors have less potential for biofilm formation on epithelial cells, and this is correlated with their ability to colonize the murine nasopharynx *in vivo* [6]. We used this model to evaluate the impact of *potABCD* deletion on biofilm formation *in vivo*. Our results showed that the mutant strain formed less biofilm than the wild-type *in vivo* and it is interesting to note that the difference between the two groups were even higher using the mouse model than previously observed in the *in vitro* models, reinforcing that more physiological models are closer to the natural environment of colonization/ biofilm development than the simpler *in vitro* models.

Finally, we wanted to investigate if the addition of polyamines to the medium would impact the biofilm formation; we found that supplementing the culture medium with polyamines increased biofilm formation by the TIGR4 strain in a dose-dependent manner. These results support the idea that polyamines are important for biofilm formation. Previous studies have shown that the addition of spermidine and nor-spermidine (a spermidine precursor) can regulate biofilm formation in a Vibrio cholerae model, our results are similar to those observations [23, 29]. Interestingly, Karatan et al. showed that adding spermidine to the medium in concentration higher than 0.5 mM inhibits the biofilm

formation by V. cholerae [23]. In our results this was also observed as the pneumococcus produces less biofilm when the maximum concentration is added (2.0 mM).

Another study investigating the relationship between polyamines and biofilms examined the behavior of a *Yersinia pestis* strain unable to synthesize putrescine. They found that the mutant produced much less biofilm than its wild-type counterpart due to a decreased concentration of intracellular putrescine [24]. Similarly, in *Pseudomonas aeruginosa*, increasing the intracellular putrescine concentration was linked to an increase in biofilm formation [30]. Our study showed that the addition of either putrescine or spermidine impacts biofilm formation *in vitro*, supporting the idea that the absence of the *pot* operon would reduce the amount of polyamines in the intracellular environment and thus impact bacterial ability to form biofilm.

Zhang et al. investigated polyamine transporters and biofilm in *Escherichia coli* and found that a strain modified to overexpress the PotD protein produced much more biofilm than the parental strain [31]. This work supports our results, suggesting that our observation is also true for different organisms.

Our work, however, presents some limitations, with the most notable one being the absence of a complementary strain of the mutant restoring the expressing of the *potABCD* operon. We made numerous attempts to produce this strain, but all were unsuccessful. Nonetheless, this mutant has already been well characterized and used in other publications [10, 15, 32]. Our experiment involving the addition of polyamines demonstrated that the mutant did not respond to the presence of these molecules in the culture medium, maintaining the biofilm at levels identical to the medium without supplementation. This reinforces that the removal of the operon resulted only in the lack of polyamine transport into the cell.

In conclusion, our study highlights the importance of the ABCD polyamine transporter in the biofilm development by *S. pneumoniae*. Additionally, we demonstrate that the addition of exogenous polyamines stimulates the bacterium to produce more biofilm *in vitro*, while the absence of the *pot* operon hinders biofilm formation in different models using different levels of complexity from abiotic surface to *in vivo* animal model. Interestingly, more physiological models such as mice show that the absence of the *pot* operon has a greater impact on biofilm formation. Our findings emphasize the significance of employing more physiological models in studying biofilm formation.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

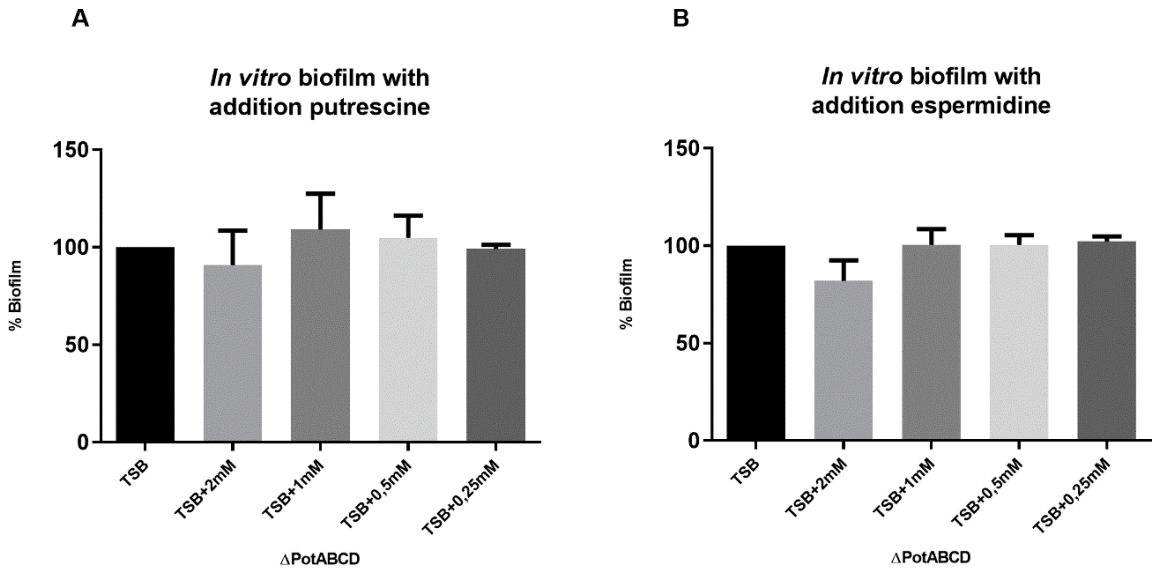
Author Contributions

APH, MD and TRC designed the study. BV, MESG, JB, GD and SO have performed the experiments. GBL and CAL was responsible for maintaining the cell cultures. BV and TRC drafted the manuscript. LCCL, APH and MD have revised the text and figures. All authors read and approved the final manuscript.

Funding

This work was supported by FAPESP grants: 2019/23566-6, 2019/25728-3 and 2017/24832-6. Conselho Nacional de desenvolvimento Científico e Tecnológico (CNPq): 400099/2022-5; Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES): 88887.666340/2022-00; and Swedish Research Council (VR) grant number: 2021-06050.

Suplemmentary figure 4



Supplementary Fig 4. In vitro biofilm formation by *S. pneumoniae* with the addition of putrescine and spermidine.

Thebiofilm was evaluated after 24h in microplateassay with addition of 0.25 mM, 0.5 mM, 1 mM and 2 mM of **putrescine (A)** or **spermidine (B).** The biomass was evaluated by crystal violet staining at 590 nm. The absorbance from group TBS was used to calculate the percentages being considered 100%. The results expressedare representative of experiments carried out in quadruplicate. The comparison between groups was performed using the One-way ANOVA followed by Dunnet's test, where ns=<0,05.

Acknowledgement

We want to acknowledge Dr. Edwin Swiatlo for providing the PotABCD negative strain.

We thank Casa Nossa Senhora da Paz for the financial support with the publication fee.

References

1. Henriques-Normark B, Normark S. Commensal pathogens, with a focus on Streptococcus pneumoniae, and interactions with the human host. Experimental cell research. 2010;316(8):1408-14. Epub 2010/03/17. doi: 10.1016/j.yexcr.2010.03.003. PubMed PMID: 20227406.
2. Converso TR, Assoni L, Andre GO, Darrieux M, Leite LCC. The long search for a serotype independent pneumococcal vaccine. Expert Rev Vaccines. 2020;19(1):57-70. Epub 2020/01/07. doi: 10.1080/14760584.2020.1711055. PubMed PMID: 31903805.
3. Rossi F, Franco MRG, Rodrigues HMdP, Andreazzi D. Streptococcus pneumoniae: sensibilidade a penicilina e moxifloxacina. J bras pneumol. 2012;38(1):66-71. doi: 10.1590/S1806-37132012000100010.

4. Smith-Vaughan H, Byun R, Nadkarni M, Jacques NA, Hunter N, Halpin S, et al. Measuring nasal bacterial load and its association with otitis media. BMC Ear Nose Throat Disord. 2006;6:10. doi: 10.1186/1472-6815-6-10. PubMed PMID: 16686940; PubMed Central PMCID: PMCPMC1479363.

5. Chao Y, Marks LR, Pettigrew MM, Hakansson AP. Streptococcus pneumoniae biofilm formation and dispersion during colonization and disease. Front Cell Infect Microbiol. 2014;4:194. Epub 2015/01/30. doi: 10.3389/fcimb.2014.00194. PubMed PMID: 25629011; PubMed Central PMCID: PMCPMC4292784.

6. Marks LR, Parameswaran GI, Hakansson AP. Pneumococcal interactions with epithelial cells are crucial for optimal biofilm formation and colonization in vitro and in vivo. Infect Immun. 2012;80(8):2744-60. Epub 2012/05/31. doi: 10.1128/IAI.00488-12. PubMed PMID: 22645283; PubMed Central PMCID: PMCPMC3434590.

7. Marks LR, Reddinger RM, Hakansson AP. High levels of genetic recombination during nasopharyngeal carriage and biofilm formation in Streptococcus pneumoniae. mBio. 2012;3(5). doi: 10.1128/mBio.00200-12. PubMed PMID: 23015736; PubMed Central PMCID: PMCPMC3448161.

8. Shak JR, Vidal JE, Klugman KP. Influence of bacterial interactions on pneumococcal colonization of the nasopharynx. Trends Microbiol. 2013;21(3):129-35. doi: 10.1016/j.tim.2012.11.005. PubMed PMID: 23273566; PubMed Central PMCID: PMCPMC3729046.

9. Flemming HC, Wingender J, Szewzyk U, Steinberg P, Rice SA, Kjelleberg S. Biofilms: an emergent form of bacterial life. Nat Rev Microbiol. 2016;14(9):563-75. Epub 2016/08/12. doi: 10.1038/nrmicro.2016.94. PubMed PMID: 27510863.

10. Pipkins HR, Bradshaw JL, Keller LE, Swiatlo E, McDaniel LS. Polyamine transporter potABCD is required for virulence of encapsulated but not nonencapsulated Streptococcus pneumoniae. PloS one. 2017;12(6):e0179159. Epub 2017/06/07. doi: 10.1371/journal.pone.0179159. PubMed PMID: 28586394;
PubMed Central PMCID: PMCPMC5460881.

11. Igarashi K, Kashiwagi K. Polyamines: mysterious modulators of cellular functions. Biochemical and biophysical research communications. 2000;271(3):559-64. Epub 2000/05/18. doi: 10.1006/bbrc.2000.2601. PubMed PMID: 10814501.

12. Shah P, Swiatlo E. A multifaceted role for polyamines in bacterial pathogens. Molecular microbiology. 2008;68(1):4-16. Epub 2008/04/15. doi: 10.1111/j.1365-2958.2008.06126.x. PubMed PMID: 18405343.

13. Igarashi K, Ito K, Kashiwagi K. Polyamine uptake systems in Escherichia coli. Res Microbiol. 2001;152(3-4):271-8. Epub 2001/06/26. doi: 10.1016/s0923-2508(01)01198-6. PubMed PMID:
11421274.

14. Shah P, Swiatlo E. Immunization with polyamine transport protein PotD protects mice against systemic infection with Streptococcus pneumoniae. Infect Immun. 2006;74(10):5888-92. Epub 2006/09/22. doi: 10.1128/IAI.00553-06. PubMed PMID: 16988268; PubMed Central PMCID: PMCPMC1594930.

15. Shah P, Romero DG, Swiatlo E. Role of polyamine transport in Streptococcus pneumoniae response to physiological stress and murine septicemia. Microb Pathog. 2008;45(3):167-72. Epub 2008/06/24. doi: 10.1016/j.micpath.2008.05.001. PubMed PMID: 18572376.

16. Shah P, Briles DE, King J, Hale Y, Swiatlo E. Mucosal immunization with polyamine transport protein D (PotD) protects mice against nasopharyngeal colonization with Streptococcus pneumoniae. Exp Biol Med (Maywood). 2009;234(4):403-9. Epub 2009/01/30. doi: 10.3181/0809-RM-269. PubMed PMID: 19176871.

17. Min X, Zhang X, Wang H, Gong Y, Li M, Xu W, et al. Protection against pneumococcal infection elicited by immunization with glutamyl tRNA synthetase, polyamine transport protein D and sortase A. Vaccine. 2012;30(24):3624-33. Epub 2012/04/03. doi: 10.1016/j.vaccine.2012.03.042. PubMed PMID: 22464966.

18. Converso TR, Goulart C, Rodriguez D, Darrieux M, Leite LC. Systemic immunization with rPotD reduces Streptococcus pneumoniae nasopharyngeal colonization in mice. Vaccine. 2017;35(1):149-55. Epub 2016/11/26. doi: 10.1016/j.vaccine.2016.11.027. PubMed PMID: 27884476.

19. Rojas Converso T, Goulart C, Rodriguez D, Guerra MES, Darrieux M, Leite LCC. Immune response induced in mice by a hybrid rPotD-PdT pneumococcal protein. PloS one. 2022;17(8):e0273017. Epub 2022/08/23. doi: 10.1371/journal.pone.0273017. PubMed PMID: 35994444; PubMed Central PMCID: PMCPMC9394809.

20. Converso TR, Goulart C, Darrieux M, Leite LCC. A protein chimera including PspA in fusion with PotD is protective against invasive pneumococcal infection and reduces nasopharyngeal colonization in mice. Vaccine. 2017;35(38):5140-7. Epub 2017/08/19. doi: 10.1016/j.vaccine.2017.08.010. PubMed PMID: 28818567.

21. Chao Y, Bergenfelz C, Hakansson AP. Growing and Characterizing Biofilms Formed by Streptococcus pneumoniae. Methods Mol Biol. 2019;1968:147-71. Epub 2019/04/01. doi: 10.1007/978-1-4939-9199-0_13. PubMed PMID: 30929213.

22. Goytia M, Dhulipala VL, Shafer WM. Spermine impairs biofilm formation by Neisseria gonorrhoeae. FEMS Microbiol Lett. 2013;343(1):64-9. doi: 10.1111/1574-6968.12130. PubMed PMID: 23506248; PubMed Central PMCID: PMCPMC3651792.

23. McGinnis MW, Parker ZM, Walter NE, Rutkovsky AC, Cartaya-Marin C, Karatan E. Spermidine regulates Vibrio cholerae biofilm formation via transport and signaling pathways. FEMS microbiology letters. 2009;299(2):166-74. Epub 2009/08/22. doi: 10.1111/j.1574-6968.2009.01744.x. PubMed PMID: 19694812.

24. Patel CN, Wortham BW, Lines JL, Fetherston JD, Perry RD, Oliveira MA. Polyamines are essential for the formation of plague biofilm. Journal of bacteriology. 2006;188(7):2355-63. Epub 2006/03/21. doi: 10.1128/JB.188.7.2355-2363.2006. PubMed PMID: 16547021; PubMed Central PMCID: PMCPMC1428407.

25. Ramon-Perez ML, Diaz-Cedillo F, Contreras-Rodriguez A, Betanzos-Cabrera G, Peralta H, Rodriguez-Martinez S, et al. Different sensitivity levels to norspermidine on biofilm formation in clinical and commensal Staphylococcus epidermidis strains. Microb Pathog. 2015;79:8-16. doi: 10.1016/j.micpath.2014.12.004. PubMed PMID: 25549879.

26. Munoz-Elias EJ, Marcano J, Camilli A. Isolation of Streptococcus pneumoniae biofilm mutants and their characterization during nasopharyngeal colonization. Infect Immun. 2008;76(11):5049-61. doi: 10.1128/IAI.00425-08. PubMed PMID: 18794289; PubMed Central PMCID: PMCPMC2573321.

27. Sanchez CJ, Kumar N, Lizcano A, Shivshankar P, Dunning Hotopp JC, Jorgensen JH, et al. Streptococcus pneumoniae in biofilms are unable to cause invasive disease due to altered virulence determinant production. PLoS One. 2011;6(12):e28738. doi: 10.1371/journal.pone.0028738. PubMed PMID: 22174882; PubMed Central PMCID: PMCPMC3234282.

28. Waite RD, Struthers JK, Dowson CG. Spontaneous sequence duplication within an open reading frame of the pneumococcal type 3 capsule locus causes high-frequency phase variation. Mol Microbiol. 2001;42(5):1223-32. doi: 10.1046/j.1365-2958.2001.02674.x. PubMed PMID: 11886554.

29. Lee J, Sperandio V, Frantz DE, Longgood J, Camilli A, Phillips MA, et al. An alternative polyamine biosynthetic pathway is widespread in bacteria and essential for biofilm formation in Vibrio cholerae. The Journal of biological chemistry. 2009;284(15):9899-907. Epub 2009/02/07. doi: 10.1074/jbc.M900110200. PubMed PMID: 19196710; PubMed Central PMCID: PMCPMC2665113.

30. Williams BJ, Du RH, Calcutt MW, Abdolrasulnia R, Christman BW, Blackwell TS. Discovery of an operon that participates in agmatine metabolism and regulates biofilm formation in Pseudomonas aeruginosa. Mol Microbiol. 2010;76(1):104-19. Epub 2010/02/13. doi: 10.1111/j.1365- 2958.2010.07083.x. PubMed PMID: 20149107; PubMed Central PMCID: PMCPMC4227303.

31. Zhang X, Zhang Y, Liu J, Liu H. PotD protein stimulates biofilm formation by Escherichia coli. Biotechnology letters. 2013;35(7):1099-106. Epub 2013/03/30. doi: 10.1007/s10529-013-1184-8. PubMed PMID: 23539287.

32. Shah P, Marquart M, Quin LR, Swiatlo E. Cellular location of polyamine transport protein PotD in Streptococcus pneumoniae. FEMS microbiology letters. 2006;261(2):235-7. Epub 2006/08/16. doi: 10.1111/j.1574-6968.2006.00352.x. PubMed PMID: 16907726.

4. CONCLUSÃO

Em conclusão, este estudo destaca a importância do transportador de poliamina ABC no desenvolvimento de biofilme por *S. pneumoniae*. Além disso, foi demonstrado que a adição de poliaminas exógenas estimula a bactéria a produzir mais biofilme *in vitro*, enquanto a ausência do *operon pot*ABCD dificulta a formaçãode biofilme em diferentes modelos que incluem *invitro* com superfícies abióticas e bióticas. Estas descobertas também enfatizam a importância de empregar mais modelos fisiológicos no estudo da formação de biofilmes.

REFERÊNCIAS

(CDC), C. f. D. C. a. P. Pneumococcal Disease. *In*: **Epidemiology and Prevention of Vaccine-Preventable Diseases, 14th Edition**. Washington, DC: U.S. Department of Health and Human Services, 2015. p. 253-266.

ABRAHAM, W. R. Going beyond the Control of Quorum-Sensing to Combat Biofilm Infections. **Antibiotics (Basel)**, 5, n. 1, Jan 9 2016.

BALESTRINO, D.; HAAGENSEN, J. A.; RICH, C.; FORESTIER, C. Characterization of type 2 quorum sensing in Klebsiella pneumoniae and relationship with biofilm formation. **Journal of Bacteriology**, 187, n. 8, p. 2870-2880, Apr 2005.

CHAO, Y.; MARKS, L. R.; PETTIGREW, M. M.; HAKANSSON, A. P. Streptococcus pneumoniae biofilm formation and dispersion during colonization and disease. **Frontiers in Cellular and Infection Microbiology**, 4, p. 194, 2014.

CHERTOW, D. S.; MEMOLI, M. J. Bacterial coinfection in influenza: a grand rounds review. **JAMA**, 309, n. 3, p. 275-282, Jan 16 2013.

CONVERSO, T. R.; GOULART, C.; RODRIGUEZ, D.; DARRIEUX, M. *et al*. Systemic immunization with rPotD reduces Streptococcus pneumoniae nasopharyngeal colonization in mice. **Vaccine**, 35, n. 1, p. 149-155, 2017/01/03/ 2017.

DIAVATOPOULOS, D. A.; SHORT, K. R.; PRICE, J. T.; WILKSCH, J. J. *et al*. Influenza A virus facilitates Streptococcus pneumoniae transmission and disease. **FASEB Journal**, 24, n. 6, p. 1789-1798, Jun 2010.

FLEMMING, H. C.; WINGENDER, J.; SZEWZYK, U.; STEINBERG, P. *et al*. Biofilms: an emergent form of bacterial life. **Nature Reviews Microbiology**, 14, n. 9, p. 563-575, Aug 11 2016.

GUERRA, M. E. S.; DESTRO, G.; VIEIRA, B.; LIMA, A. S. *et al*. Klebsiella pneumoniae Biofilms and Their Role in Disease Pathogenesis. **Frontiers in Cellular and Infection Microbiology**, 12, p. 877995, 2022.

HENRIQUES-NORMARK, B.; NORMARK, S. Commensal pathogens, with a focus on Streptococcus pneumoniae, and interactions with the human host. **Experimental Cell Research**, 316, n. 8, p. 1408- 1414, May 1 2010.

HICKS, L. A.; HARRISON, L. H.; FLANNERY, B.; HADLER, J. L. *et al*. Incidence of pneumococcal disease due to non-pneumococcal conjugate vaccine (PCV7) serotypes in the United Statesduring the era of widespread PCV7 vaccination, 1998-2004. **Journal of Infection Disease**, 196, n. 9, p. 1346- 1354, Nov 1 2007.

IGARASHI, K.; ITO, K.; KASHIWAGI, K. Polyamine uptake systems in Escherichia coli. **Research Microbiology**, 152, n. 3-4, p. 271-278, Apr-May 2001.

IGARASHI, K.; KASHIWAGI, K. Polyamines: mysterious modulators of cellular functions. **Biochemical and Biophysical Research Communication**, 271, n. 3, p. 559-564, May 19 2000.

KELLER, L. E.; ROBINSON, D. A.; MCDANIEL, L. S. Nonencapsulated Streptococcus pneumoniae: Emergence and Pathogenesis. **MBio**, 7, n. 2, p. e01792, Mar 22 2016.

MARKS, L. R.; REDDINGER, R. M.; HAKANSSON, A. P. High levels of genetic recombination during nasopharyngeal carriage and biofilm formation in Streptococcus pneumoniae. **mBio**, 3, n. 5, 2012.

MCCULLERS, J. A. The co-pathogenesis of influenza viruses with bacteria in the lung. **Nature Review Microbiology**, 12, n. 4, p. 252-262, Apr 2014.

MIN, X.; ZHANG, X.; WANG, H.; GONG, Y. *et al*. Protection against pneumococcal infection elicited by immunization with glutamyl tRNA synthetase, polyamine transport protein D and sortase A. **Vaccine**, 30, n. 24, p. 3624-3633, May 21 2012. Research Support, Non-U.S. Gov't.

ORIHUELA, C. J.; TUOMANEN, E. Streptococcus pneumoniae: invasion and inflammation. *In*: **Gram-Positive Pathogens, Second Edition**: American Society of Microbiology, 2006. p. 253-267.

PETTIGREW, M. M.; GENT, J. F.; PYLES, R. B.; MILLER, A. L. *et al*. Viral-bacterial interactions and risk of acute otitis media complicating upper respiratory tract infection. **Journal of Clinical Microbiology**, 49, n. 11, p. 3750-3755, Nov 2011.

PIPKINS, H. R.; BRADSHAW, J. L.; KELLER, L. E.; SWIATLO, E. *et al*. Polyamine transporter potABCD is required for virulence of encapsulated but not nonencapsulated Streptococcus pneumoniae. **PLoS One**, 12, n. 6, p. e0179159, 2017.

REDDINGER, R. M.; LUKE-MARSHALL, N. R.; SAUBERAN, S. L.; HAKANSSON, A. P. *et al*. Streptococcus pneumoniae Modulates Staphylococcus aureus Biofilm Dispersion and the Transition from Colonization to Invasive Disease. **MBio**, 9, n. 1, Jan 9 2018.

ROSSI, F.; FRANCO, M. R. G.; RODRIGUES, H. M. d. P.; ANDREAZZI, D. Streptococcus pneumoniae: sensibilidade a penicilina e moxifloxacina. **Jornal Brasileiro de Pneumologia**, 38, n. 1, p. 66-71, 2012-02 2012.

SHAH, P.; BRILES, D. E.; KING, J.; HALE, Y. *et al*. Mucosal immunization with polyamine transport protein D (PotD) protects mice against nasopharyngeal colonization with Streptococcus pneumoniae. **Experimental Biology and Medicine (Maywood)**, 234, n. 4, p. 403-409, Apr 2009.

SHAH, P.; NANDURI, B.; SWIATLO, E.; MA, Y. *et al*. Polyamine biosynthesis and transport mechanisms are crucial for fitness and pathogenesis of Streptococcus pneumoniae. **Microbiology**, 157, n. Pt 2, p. 504-515, Feb 2011.

SHAH, P.; SWIATLO, E. Immunization with polyamine transport protein PotD protects mice against systemicinfectionwith Streptococcus pneumoniae. **Infection and Immunity**, 74, n. 10, p. 5888-5892, Oct 2006.

SHAK, J. R.; VIDAL, J. E.; KLUGMAN, K. P. Influence of bacterial interactions on pneumococcal colonizationof the nasopharynx. **Trends Microbiology**, 21, n. 3, p. 129-135, Mar 2013.

SMITH-VAUGHAN, H.; BYUN, R.; NADKARNI, M.; JACQUES, N. A. *et al*. Measuring nasal bacterial load and its association with otitis media. **BMC Ear Nose Throat Disorders**, 6, p. 10, May 10 2006.

URBAN, C. F.; LOURIDO, S.; ZYCHLINSKY, A. How do microbes evade neutrophil killing? **Cellular Microbiology**, 8, n. 11, p. 1687-1696, Nov 2006.