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Vacinas pneumocócicas independentes de sorotipo: papel das proteínas PspA e Pneumolisina

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Vacinas pneumocócicas independentes de sorotipo: papel das proteínas PspA e Pneumolisina

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"A vacina não pode ser propriedade do laboratório que a encontrou ou de um grupo de países aliados só por isso. A vacina é um patrimônio da humanidade, de toda a humanidade, é universal porque a saúde é um bem comum. É um patrimônio comum, pertence ao bem comum e esse deveria ser o critério"

(Papa Francisco,2021)

RESUMO

Streptococcus pneumoniae é um importante patógeno responsável por elevadas taxas de morbidade e mortalidade em todo o mundo. As vacinas atualmente disponíveis, baseadas em polissacarídeos capsulares, oferecem proteção limitada a determinados sorotipos e apresentam alto custo de produção, o que dificulta sua implementação em regiões menos desenvolvidas. Apesar da introdução de vacinas conjugadas em vários países, a bactéria ainda causa cerca de 9 milhões de infecções e mais de 1,1 milhão de mortes anualmente, mantendo-se como um problema de saúde pública global. Nesse contexto, proteínas pneumocócicas têm sido investigadas como alternativas promissoras para o desenvolvimento de vacinas mais amplas e acessíveis. Entre as candidatas mais estudadas estão a PspA, altamente imunogênica, porém estruturalmente variável, e a pneumolisina (Ply), que é conservada entre diferentes cepas, mas apresenta toxicidade para o hospedeiro. Considerando essas limitações, este estudo propôs o desenvolvimento de uma vacina quimérica, combinando o fragmento N-terminal de PspA com um derivado não tóxico da pneumolisina (PlD1). A imunização de camundongos com a formulação PspA_PlD1 conferiu proteção contra cepas pneumocócicas que expressam variantes heterólogas de PspA, correlacionando-se com o aumento da deposição de C3 do sistema complemento na superfície de diferentes cepas, mediada por anticorpos. Experimentos com cepas mutantes para PspA ou pneumolisina confirmaram que ambas as proteínas contribuem para essa ativação do complemento. Os resultados demonstram que a proteína quimérica PspA_PlD1 amplia a resposta imunológica protetora, promovendo a deposição de complemento em múltiplas cepas de *S. pneumoniae*, o que sugere seu potencial como uma candidata a vacina de amplo espectro, capaz de induzir proteção cruzada eficaz.

Palavras chaves: *Streptococcus pneumoniae.*Vacinas. PspA. Pneumolisina.

ABSTRACT

Streptococcus pneumoniae is a major pathogen responsible for high morbidity and mortality rates worldwide. Currently available vaccines, based on capsular polysaccharides, offer limited protection against specific serotypes and are costly to produce, which hampers their use in less developed regions. Despite the introduction of conjugate vaccines in several countries, the bacterium still causes approximately 9 million infections and over 1.1 million deaths annually, remaining a global public health concern. In this context, pneumococcal proteins have been investigated as promising alternatives for the development of broader and more accessible vaccines. Among the most studied candidates are PspA, which is highly immunogenic but structurally variable, and pneumolysin (Ply), which is conserved among different strains but toxic to the host. Considering these limitations, this study proposed the development of a chimeric vaccine combining the N-terminal fragment of PspA with a non-toxic derivative of pneumolysin (PlD1). Immunization of mice with the PspA_PlD1 formulation conferred protection against pneumococcal strains expressing heterologous PspA variants, correlating with increased C3 complement deposition on the surface of different strains, mediated by antibodies. Experiments using PspA- or pneumolysin-deficient strains confirmed that both proteins contribute to antibody-mediated complement activation. The results demonstrate that the chimeric PspA_PlD1 protein enhances the protective immune response by promoting complement deposition across multiple *S. pneumoniae* strains, indicating its potential as a broad-spectrum vaccine candidate capable of inducing effective cross-protection.

Key words: *Streptococcus pneumoniae.* Vaccines. PspA. Pneumolysin.

LISTA DE ABREVIAÇÕES

$Al(OH)_3$- Hidróxido de alumínio

C3- Componente 3 do sistema complemento

CFU/mL- *Colony forming units per milliliter*

CEMIB- Centro Multidisciplinar para Investigação Biológica

IAL- Instituto Adolfo Lutz, São Paulo, Brasil

mg/mL- Miligrama por mililitro

mL- Mililitro

nm- Nanômetro

O.D.600nm- *Optical density at 600nm*

OMS- Organização Mundial da Saúde

PCR- *Polymerase chain reaction*

PdT- Toxoide derivado de pneumolisina, com três mutações

PlD1- Toxoide derivado de pneumolisina

Ply- Pneumolisina

PspA- Proteína de superfície A de pneumococo

PspC- Proteína de superfície C de pneumococo

rPspA- Fragmento recombinante N-terminal da PspA

THY- Meio Todd Hewitt com 0,5% de extrato de levedura

UAB- Universidade do Alabama em Birmingham

UFG- Universidade Federal de Goiás, Goiânia, Brasil

WT- *Wild type*

µg- Micrograma

µg/mL- Micrograma por mililitro

µL- Microlitro

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1 INTRODUÇÃO

1.1 *Streptococcus pneumoniae*

Streptococcus pneumoniae é uma bactéria Gram positiva isolada e identificada primeiramente por Louis Pasteur em 1881, apresenta forma esférica (cocos) e se organiza em pares, coloniza primariamente a nasofaringe (Allegrucci e Sauer, 2007; Subramanian *et al.*, 2019). Globalmente, é a principal causa de morbidade e mortalidade entre as infecções do trato respiratório inferior, estudos afirmam que essa bactéria é responsável por aproximadamente 9 milhões de infecções a cada ano, com mais de 1,1 milhão de mortes relacionadas (Wahl *et al.*, 2018; Feldman e Anderson, 2020). Devido à sua elevada taxa de mortalidade global, foi classificado pela Organização Mundial da Saúde (OMS) como patógeno de prioridade média para pesquisa e desenvolvimento de novos antibióticos(Jesudason, 2024).

Em certas condições, como durante a coinfecção com o vírus influenza ou em indivíduos com deficiências nos mecanismos de defesa, pode se disseminar para outros sítios, causando infecções locais e/ou sistêmicas, como pneumonia, bronquite, abscesso cerebral, otite média, septicemia e meningite, isso resulta em reações inflamatórias graves (Beiter *et al.*, 2008; Jindal *et al.*, 2015).

Conforme relatório da Agência Nacional de Vigilância Sanitária (ANVISA) de 2018, as infecções causadas por pneumococo afetam principalmente crianças, indivíduos imunocomprometidos e idosos. Em 2008, um estudo conduzido pela OMS revelou que, de um total de 8,8 milhões de óbitos decorrentes de doenças pneumocócicas, 476.000 ocorreram em crianças menores de cinco anos. A Comissão Nacional de Incorporação de Tecnologias no Sistema Único de Saúde publicou dados que demonstram que no Brasil, estima-se que ocorram, por ano, 57.716 casos de doença pneumocócica e 701 mortes por pneumonia e meningite causadas por pneumococo em todas as faixas de idade (BRASIL, Ministério da Saúde, CONITEC, 2025)

As infecções provocadas pelo pneumococo são graves e frequentemente resultam em óbito e atualmente, além disso, observa-se um aumento da resistência desta bactéria aos antibióticos como penicilinas e eritromicinas, que são os mais comuns no tratamento dessas infecções, além de outros menos comuns, como sulfametoxazol com trimetroprima e tetraciclinas. Atualmente, aproximadamente 30% dos casos graves de infecção causada por essa bactéria demonstram resistência a um ou mais antibióticos clinicamente relevantes (Ventola, 2015).

A transmissão de *S. pneumoniae* ocorre predominantemente por meio de aerossóis, pois a bactéria reside na nasofaringe (Pereira *et al.*, 2022) . A versatilidade do penumococo em causar infecção em diferentes locais ocorre devido a variedade de interações com o hospedeiro, que variam conforme a região da infecção e a gravidade da doença. Para colonizar a nasofaringe, apresenta a capacidade de formar biofilmes na mucosa do trato respiratório superior, causando inflamação que facilita a eliminação da bactéria pelas secreções, promovendo sua transmissão (Zafar *et al.*, 2017; Subramanian *et al.*, 2019; Pereira *et al.*, 2022).

Em relação a sua estrutura *S. pneumoniae* apresenta sua superfície composta por três principais estruturas: a membrana plasmática, que é conservada e contém moléculas de ácido lipoteicóico; a parede celular, composta por polissacarídeos e ácido teicóico, onde diversas proteínas de superfície estão localizadas; e a cápsula polissacarídica, que tem uma espessura de 200-400 nm e uma estrutura química variada (Kadioglu *et al.*, 2008)

A cápsula é vital para a sobrevivência dos pneumococos, pois protege a bactéria da ação do sistema complemento e da destruição por fagócitos, sabe-se que essa bactéria apresenta mais de 100 tipos de polissacarídeos capsulares, que são classificados com base nos componentes e ligações glicanos específicos de cada sorotipo. Desses, 23 sorotipos são responsáveis por 80–90% das infecções pneumocócicas invasivas em nível global (Ganaie *et al.*, 2020; Masomian *et al.*, 2020; Micoli *et al.*, 2023). A cápsula polissacarídica é um dos principais determinantes da virulência pneumocócica, pois estabelece os diferentes sorotipos da bactéria, estimula uma resposta imune protetora específica para cada sorotipo e constitui o principal antígeno utilizado nas vacinas pneumocócicas. Os polissacarídeos capsulares são altamente imunogênicos e formam a base das vacinas pneumocócicas atualmente em uso que incluem múltiplos polissacarídeos capsulares, contemplando formulações que abrangem 10, 13, 15, 20 ou 23 sorotipos (Prymula e Schuerman, 2009; Geno *et al.*, 2015; Zhang *et al.*, 2019; Almeida *et al.*, 2024). No Brasil, a vacina pneumocócica conjugada 10-valente foi incorporada ao Programa Nacional de Imunizações em 2010 e permanece como parte do esquema de vacinação infantil de rotina(Almeida *et al.*, 2024) .

Um estudo distribuição de sorotipos de *S. pneumoniae* no Brasil antes (2008–2009) e após (2012–2013) a introdução da vacina pneumocócica conjugada 10-valente (PCV10). No período pré-vacinal, os sorotipos mais frequentes eram majoritariamente aqueles incluídos na PCV10, destacando-se os sorotipos 14, 6B, 19F e 9V, que representavam a maior parte dos isolados de doença pneumocócica invasiva, especialmente em crianças menores de cinco anos. Após a

implementação da PCV10, observou-se redução expressiva desses sorotipos vacinais e, paralelamente, um aumento de sorotipos não contemplados pela vacina. No período pós-vacina, os sorotipos 3 e 19A emergiram como os mais prevalentes entre crianças menores de cinco anos, sendo o 19A particularmente associado à resistência antimicrobiana. Em indivíduos com cinco anos ou mais, houve predomínio dos sorotipos 12F, 8 e 9N. Esses dados evidenciam um padrão claro de substituição sorotípica após a vacinação, com diminuição dos sorotipos-alvo e expansão de sorotipos não vacinais no país(Almeida *et al.*, 2021).

Tem sido amplamente discutido que, embora as vacinas conjugadas contra o pneumococo tenham reduzido significativamente a carga dessa doença, a substituição de sorotipos exige constantemente a adição de novos sorotipos às formulações, além disso, combinada com os desafios técnicos na produção de vacinas, limita a eficácia da cobertura vacinal (Converso *et al.*, 2020).

Estudos realizados destacam-se entre os cinco sorotipos emergentes, apenas dois foram incorporados na nova vacina PCV15, o que reforça a necessidade de uma vacina 18-valente; vacinas 20-valentes já estão sendo desenvolvidas (Moore *et al.*, 2016; Pichichero, 2017; Converso *et al.*, 2020) . Além disso, há um aumento na incidência de *S. pneumoniae* não encapsulado, responsável tanto por doenças invasivas quanto por casos de otite média (Okade *et al.*, 2014; Jochems *et al.*, 2017; Converso *et al.*, 2020).

Além da cápsula, diversas proteínas de superfície do pneumococo também são fatores de virulência, que ajudam na proteção da bactéria contra a defesa do hospedeiro, como exemplo, a proteína de superfície A (PspA) que esta presente em todos os isolados de *S. pneumoniae* e a pneumolisina, uma proteína essencial para a patogênese do pneumococo (Periselneris *et al.*, 2022).

1.2 A proteína A de superfície pneumocócica (PspA)

A proteína de superfície pneumocócica A (PspA) é um importante fator de virulência em *S. pneumoniae*. É o membro mais prevalente da família de proteínas de ligação à colina, que inclui moléculas de superfície ligadas às moléculas de fosforilcolina dos ácidos teicóicos na parede celular (Crain *et al.*, 1990; Lane *et al.*, 2022).

A PspA é encontrada em todas as cepas pneumocócicas, localiza-se na superfície celular do pneumococo e estudos de transcriptoma indicam que sua expressão ocorre em todos os locais e órgãos anatômicos do hospedeiro durante a infecção (D'mello *et al.*, 2020; Lane *et al.*, 2022).

Apresenta o peso molecular variando de 65 a 99 kDa, sendo um fator de virulência exposto, com sorologia variável que protege as bactérias da ativação do(Darrieux *et al.*, 2007; Hiller *et al.*, 2007; Goulart *et al.*, 2011; Gisch *et al.*, 2013; Goulart *et al.*, 2013; Cornick *et al.*, 2017; Lane *et al.*, 2022).

Estruturalmente, a PspA contém três regiões distintas: um domínio alfa-helicoidal N-terminal, um domínio rico em prolina e um domínio de ligação à colina no C-terminal. O primeiro domínio é a parte exposta da molécula e a porção mais variável da molécula, inclui uma região definidora de clado usada para classificar a PspA em 3 famílias e 6 clados. A família 1 inclui os clados 1 e 2; a família 2 inclui os clados 3, 4 e 5; e a família 3 inclui o clado 6 (Hollingshead *et al.*, 2000). As sequências primárias de aminoácidos dentro do mesmo clado apresentam identidade ≥90% na CDR, enquanto entre famílias diferentes, essa identidade é ≤55% (Hollingshead *et al.*, 2000; Roche *et al.*, 2003). A maioria das cepas pneumocócicas expressa PspA das famílias 1 ou 2. O nível de reatividade cruzada varia entre diferentes PspAs, com maior reatividade dentro de proteínas da mesma família. A região rica em prolina está localizada entre o segmento helicoidal N-terminal e o domínio C-terminal e é caracterizada por repetições curtas, nas quais resíduos de prolina ocorrem a cada três ou quatro aminoácidos. Em aproximadamente metade das PspAs descritas, essas repetições são interrompidas por uma sequência altamente conservada de aminoácidos sem prolina, conhecida como bloco não prolina, que é considerado um importante fragmento antigênico. A região C-terminal da PspA é responsável pela interação da proteína com resíduos de colina na membrana bacteriana, formando um domínio estrutural conservado presente em todas as variantes dessa proteína, seguido por uma cauda hidrofóbica curta (Hollingshead *et al.*, 2000; Roche *et al.*, 2003; Daniels *et al.*, 2010; Goulart *et al.*, 2011; Converso *et al.*, 2017).

A figura 1 apresenta a representação esquemática da estrutura da PspA.

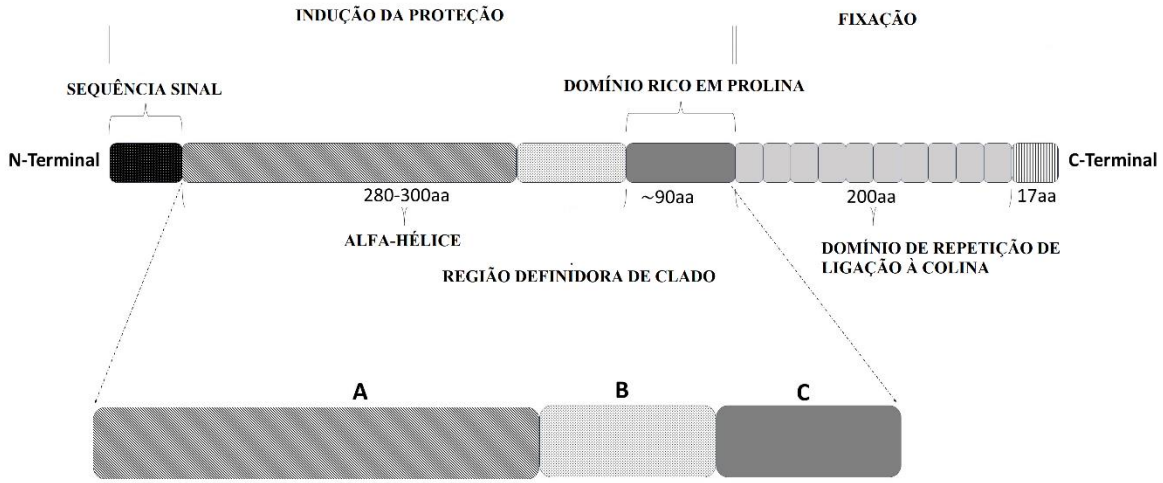

FIGURA 1- Representação esquemática da estrutura da PspA. São apresentados a sequência sinal N-terminal, a região α-helicoidal com 280–300 aa (A) incluindo a região definidora de clado (B), o domínio rico em prolina com aproximadamente 90 aminoácidos (C), o domínio de repetição de ligação à colina com cerca de 200 aa e uma curta cauda C-terminal de 17 aa. Fonte: Próprio autor

O potencial imunogênico da PspA foi identificado há mais de três décadas, com a demonstração de que dois anticorpos monoclonais contra a PspA protegeram camundongos da infecção fatal com três diferentes cepas pneumocócicas que expressam dois sorotipos capsulares (Crain et al., 1990; Mcdaniel et al., 1991).

A presença de PspA reduz a deposição de proteínas do Sistema Complemento na superfície bacteriana, um mecanismo importante pelo qual o hospedeiro elimina a bactéria; tal processo limita o reconhecimento e a eliminação por fagócitos (Ren *et al.*, 2004; Darrieux *et al.*, 2007; Darrieux *et al.*, 2008), além disso atua também como uma adesina, ligando-se a proteínas do hospedeiro, como a gliceraldeído-3-fosfato desidrogenase nas células pulmonares em processo de morte e a lactato desidrogenase (LDH), que auxilia o crescimento bacteriano ao aumentar a produção de energia durante a infecção (Hammerschmidt *et al.*, 1999).

Estudos utilizando um mutante de *S. pneumoniae* para o gene pspA mostraram que essa proteína é essencial para a virulência bacteriana, em que o mutante apresentou uma maior ativação do sistema complemento no soro de camundongos em comparação com a resposta observada na bactéria selvagem (positiva para PspA) e foi eliminado da circulação de forma mais rápida (Ren *et al.*, 2004).

Em humanos, observou-se que a infecção por pneumococo induz a produção de anticorpos anti-PspA, sugerindo que essa proteína é expressa durante o curso da infecção. Da mesma forma,

PspA nativa e fragmentos recombinantes que incluem a região aminoterminal são altamente imunogênicos (Kong *et al.*, 2013; Schachern *et al.*, 2014) e como candidato vacinal foi observada a produção de citocinas como IL-1 e IL-6 no baço e pulmão de animais vacinados (Dos Santos *et al.*, 2022).

Devido ao seu papel relevante na virulência pneumocócica, observa-se que a PspA tem sido investigada em inúmeros estudos como a principal candidata em vacinas independentes de sorotipo, particularmente utilizando sua região N-terminal, que contém a maioria dos epítopos protetores (Daniels *et al.*, 2010). A imunização de camundongos com PspA proporciona proteção contra sepse, pneumonia e, quando administrada com adjuvantes de mucosa, contra colonização. Em chinchilas, a PspA também demonstrou proteção contra otite média (Schachern *et al.*, 2014). Recentemente, o potencial vacinal da PspA foi avaliado em um modelo de estudo de vacina nasal utilizando primatas não humanos, com resultados encorajadores (Nakahashi-Ouchida *et al.*, 2021). Além de sua eficácia protetora bem documentada em modelos animais, a PspA avançou para ensaios clínicos de fase I (Hollingshead *et al.*, 2000; Ren *et al.*, 2004; Briles *et al.*, 2019), reforçando sua promessa como candidata a vacina contra *S. pneumoniae*.

A partir dos resultados de pequisas que avaliavam a imunização com PspA, observou-se que ocorreu a indução da produção elevada de anticorpos IgG, que ativam a via clássica do sistema complemento, resultando em aumento da deposição de C3b e promovendo a opsonofagocitose da bactéria. Esse efeito é amplificado pela ativação da via alternativa do sistema complemento, já que os anticorpos anti-PspA bloqueiam os efeitos inibitórios dessa proteína sobre essa via (Ren *et al.*, 2004; Darrieux *et al.*, 2008).

Outra característica importante da PspA reside em sua capacidade de interagir com a lactoferrina humana, um componente do sistema inato de defesa da mucosa. Essa interação reduz a eficácia bactericida da apo-lactoferrina (Apo-hLf) – a forma livre de ferro da molécula, promovendo a sobrevivência do pneumococo (Hammerschmidt *et al.*, 1999; Shaper *et al.*, 2004). Foi demonstrado que a presença de PspA na superfície bacteriana inibe a atividade lítica da lactoferrina, protegendo o patógeno, enquanto anticorpos anti-PspA potencializam o efeito bactericida da lactoferrina contra *S. pneumoniae* (Mirza et al., 2011; Andre et al., 2015; Nakahashi-Ouchida et al., 2021). Estudos recentes também demonstraram que a PspA pode proteger *S. pneumoniae* contra os efeitos microbicidas da indolicidina, um peptídeo antimicrobiano catiônico pertencente à família das catelicidinas. Análises de espectrometria de massa demonstraram uma

interação direta entre PspA e indolicidina, apoiando a hipótese de que PspA funciona como uma rede molecular. Ao sequestrar peptídeos catiônicos, impede efetivamente seu acesso à membrana bacteriana, mitigando assim a ruptura da membrana e preservando a integridade celular (Waz *et al.*, 2024).

Diversas formulações vacinais baseadas em PspA têm sido investigadas como estratégias para prevenir infecções sistêmicas causadas por *S. pneumoniae*. Essas formulações incluem fragmentos isolados de proteínas, combinações ou fusões com outros antígenos, bem como o uso de vetores e adjuvantes vacinais distintos (Zhang *et al.*, 2019; Girgis *et al.*, 2020; Tada *et al.*, 2021; Afshari *et al.*, 2023; Kono *et al.*, 2023; Milani *et al.*, 2023; Trentini *et al.*, 2024). As investigações iniciais da PspA como candidata a vacina contra infecção sistêmica empregaram a proteína de comprimento total isolada de cepas não encapsuladas de *S. pneumoniae*. Essa abordagem se mostrou eficaz em modelos murinos de sepse, conferindo proteção mesmo contra altas doses de desafio (Talkington *et al.*, 1992; Briles *et al.*, 1996). No entanto, limitações técnicas associadas à purificação de proteínas nativas — como sua propensão à agregação — impulsionaram a exploração de novas formulações vacinais(Talkington *et al.*, 1991; Briles *et al.*, 1998).

Estudos subsequentes demonstraram que fragmentos derivados da região N-terminal da PspA, particularmente aqueles que compreendem os primeiros 245 aminoácidos, mantiveram sua imunogenicidade protetora (Yother e Briles, 1992; Yother *et al.*, 1992; Briles *et al.*, 1998). Essas descobertas impulsionaram o desenvolvimento de vacinas recombinantes baseadas em porções específicas da PspA, visando principalmente seus domínios mais imunogênicos (Talkington *et al.*, 1992; Mcdaniel *et al.*, 1994; Briles *et al.*, 1998; Roche *et al.*, 2003). Com o tempo, o uso de PspA nativo de comprimento total foi substituído por formulações que incorporavam regiões distintas da molécula (Briles *et al.*, 1998). A combinação de regiões conservadas da PspA surgiu como uma estratégia promissora para ampliar a cobertura de vacinas que têm como alvo essa proteína (Darrieux *et al.*, 2008; Kristian *et al.*, 2016; Shafaghi *et al.*, 2023). Técnicas de triagem de peptídeos sobrepostos confirmaram que fragmentos de 100 aminoácidos na região N-terminal podem induzir anticorpos funcionais contra epítopos lineares e conformacionais, tornando-os alvos preferenciais para formulações com múltiplos epítopos (Lima-Junior *et al.*, 2011; Vadesilho *et al.*, 2012; Vadesilho *et al.*, 2014).

1.3 Pneumolisina

A pneumolisina (Ply) é uma citolisina dependente de colesterol, assim como a PspA também é um fator de virulência do *S. pneumoniae*. Está presente em praticamente todos os isolados pneumocócicos, tendo um papel crucial na colonização, invasão e inflamação bacteriana. Sua característica central é a capacidade de formar poros transmembrana em membranas que contêm colesterol, resultando na lise ou disfunção celular, dependendo da concentração da toxina (Kadioglu *et al.*, 2008; Marshall *et al.*, 2015).

A interação patógeno-hospedeiro mediada pela Ply ocorre em todos os estágios da patogênese do *S. pneumoniae*. É considerada uma toxina capaz de formar poros, assim promovendo o rompimento da membrana plasmática da célula hospedeira, promovendo um fluxo descontrolado de íons, pequenas moléculas e proteínas; além disso, a formação de poros interfere em várias vias de transdução de sinal celular. O dano na membrana plasmática depende da concentração da Ply e a sobrecarga intracelular de Ca2+, as células hospedeiras podem ativar vias de morte celular ou de sobrevivência e reparo de danos. Em níveis líticos, os poros induzidos podem sobrecarregar os mecanismos homeostáticos celulares, desencadeando tanto vias de sinalização pró-inflamatórias quanto lesões celulares irreversíveis que resultam na liberação de moléculas que aumentam a inflamação (Van Pee *et al.*, 2016; Pereira *et al.*, 2022).

A Ply em *S. pneumoniae* está localizada principalmente no citoplasma, mas também pode ser exportada para a parede celular de forma independente da lise celular. Sabe-se que essa citolisina dependente de colesterol encontra-se no citosol bacteriano, ligada não covalentemente à parede celular. Devido à ausência de um peptídeo sinal N-terminal que facilite a secreção de proteínas, acreditava-se que Ply seja liberada apenas por autólise, no entanto, a autólise pode não ser o único mecanismo de secreção, portanto, é provável que a secreção de seja regulada por vários mecanismos. Pesquisas recentes mostram que ela pode ser liberada no meio extracelular sem que ocorra autólise, o que contradiz o modelo anterior que associava sua liberação à destruição celular. Além disso, a Ply presente na parede celular mantém sua atividade, sendo acessível a proteases e removível com detergentes (Berry *et al.*, 1989; Balachandran *et al.*, 2001; Spreer *et al.*, 2003; Price *et al.*, 2012; Greene *et al.*, 2015; Pereira *et al.*, 2022).

A Ply é uma toxina proteica de 53 kDa, estruturalmente, possui quatro domínios funcionais. Os domínios 1 e 3 são ligados via domínio 2 ao domínio C-terminal de detecção de membrana 4. Assim como outras citolisinas dependente de colesterol, a Ply contém uma sequência de

aminoácidos altamente conservada, conhecida como alça rica em triptofano, e um par de aminoácidos treonina-leucina, que são essenciais para o reconhecimento e ligação ao colesterol da membrana(Kelly e Jedrzejas, 2000; Farrand *et al.*, 2010; Marshall *et al.*, 2015; Nishimoto *et al.*, 2020; Periselneris *et al.*, 2022). A figura 2 apresenta a estrutura da Ply.

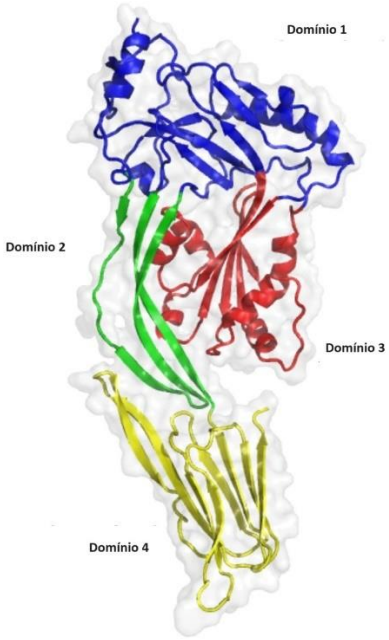

FIGURA 2- Estrutura da pneumolisina. A pneumolisina, é uma toxina proteica de 53 kDa, estruturalmente, possui quatro domínios funcionais. Os domínios 1 e 3 são ligados via domínio 2 ao domínio C-terminal de detecção de membrana 4. Fonte: Imagem adaptada de (Marshall *et al.*, 2015).

Em concentrações elevadas, aa Ply promove a formação de oligômeros nas membranas celulares, causando lesões que permitem à bactéria invadir e estabelecer uma infecção. Em concentrações mais baixas, a Ply é capaz de induzir a produção de citocinas inflamatórias como TNFα e IL-1β em monócitos humanos. Estudos em animais indicaram que a imunização com Ply pode atrasar ou evitar a morte de camundongos expostos a pneumococos virulentos. Essa proteína desempenha um papel crucial na sepse nas primeiras horas após a infecção, permitindo que os pneumococos provoquem sepse aguda em vez de uma bacteremia crônica (HUO et al., 2004). Estudos indicam que anticorpos contra a Ply foram identificados tanto no soro quanto nas secreções mucosas de humanos, desenvolvendo-se com a idade e conforme ocorre a exposição aos pneumococos (Rapola *et al.*, 2000; Simell *et al.*, 2001; Zhang *et al.*, 2006).

A Ply, para perder sua toxicidade em sua forma original, pode ser submetida a processos de detoxificação, seja por mutagênese dirigida ou por desintoxicação química, resultando em formas modificadas conhecidas como pneumolisoides. Versões detoxificadas da pneumolisina foram amplamente estudadas em animais quanto aos efeitos protetores como agente imunogênico(Paton e Ferrante, 1983; Alexander *et al.*, 1994; Musher *et al.*, 2001; Ogunniyi *et al.*, 2001; Garcia-Suarez Mdel *et al.*, 2004; Denoel *et al.*, 2011; Lu *et al.*, 2014; Hermand *et al.*, 2017; Nishimoto *et al.*, 2020).

Esses pneumolisoides foram testados quanto à sua imunogenicidade e capacidade de proteção em modelos animais(Goulart *et al.*, 2013). Entre os mais estudados estão o PdB, que apresenta uma substituição do aminoácido triptofano (Trp) por fenilalanina (Phe) na posição 433 (Paton *et al.*, 1991) e o PdT, um mutante triplo com três substituições: aspartato (Asp) na posição 385 por asparagina (Asn), cisteína (Cys) na posição 428 por glicina (Gly), e triptofano (Trp) na posição 433 por fenilalanina (Phe). O PdT apresentou resultados limitados contra desafios letais intraperitoneais e o PdB isolado e coadministrado com ou proteína pneumocócica se mostrou eficaz em proteger contra desafios nasais com algumas cepas de pneumococos (Berry *et al.*, 1995). A Ply detoxificada (PlD1) nesse estudo foi obtida por mutagênese dirigida ao sítio do gene *ply* da cepa D39 de *S pneumoniae* através da técnica de PCR, conforme os métodos descritos por Nelson e Long (1989) e Ho et al (1989). A mutação introduzida resultou em uma substituição do aminoácido histidina (His) por arginina (Arg) na posição 367 conforme descrito por (Goulart *et al.*, 2013; Milani *et al.*, 2023).

A Ply exibe pouca variação entre diferentes cepas e sorotipos e os efeitos citotóxicos da Ply podem ser reduzidos com substituições simples de aminoácidos. Sabe-se que a substituição do aminoácido na posição 460 foi alterado de leucina para aspartato para eliminar a toxicidade. (L460D) interrompe o reconhecimento do colesterol, eliminando a citotoxicidade da Ply, agora denominada com um toxoide (Boulnois *et al.*, 1991; Farrand *et al.*, 2010; Chen *et al.*, 2015; Han e Zhang, 2019; Nishimoto *et al.*, 2020). Devido à importância da Ply no reconhecimento imunológico, o toxoide de Ply vem sendo estudado com um elemento-chave em muitas formulações de vacinas baseadas em proteínas (Malley *et al.*, 2003; Witzenrath *et al.*, 2011; Nishimoto *et al.*, 2020).

Estudos clínicos com vacinas baseadas em Ply em humanos mostraram sucesso na demonstração de proteção e imunogenicidade (Bologa *et al.*, 2012; Seiberling *et al.*, 2012;

Berglund *et al.*, 2014; Nishimoto *et al.*, 2020). Um derivado desintoxicado de Ply administrado a adultos saudáveis apresentou aumento nos títulos de IgG e na atividade de anticorpos neutralizantes (Kamtchoua *et al.*, 2013). Diferentes estudos afirmam que a eficácia e imunogenicidade de vacinas que utilizam o toxoide pneumolisoide ainda precisam ser mais avaliadas (Nishimoto *et al.*, 2020). Estudos pré-clínicos exploraram vacinas que combinam o toxoide Ply com outros antígenos de superfície, por exemplo, uma vacina que une o toxoide Ply à proteína A de ligação à colina (CbpA ou PspC, sendo PspC a nomenclatura atual) demonstrou proteção ampla contra infecções pneumocócicas em modelos animais de sepse, meningite, otite média e pneumonia (Mann *et al.*, 2014; Nishimoto *et al.*, 2020).

1.4 Pneumolisina e a proteína A de superfície de pneumococo como candidatos vacinais

Tanto a PspA nativa quanto fragmentos recombinantes contendo a região aminoterminal são altamente imunogênicos, provocando uma alta produção de anticorpos específicos em camundongos, coelhos e chinchilas (Kong *et al.*, 2013; Schachern *et al.*, 2014). Além disso, há aumento na produção de citocinas como IL-1 e IL-6 no baço e pulmão de animais vacinados com PspA . A imunização de camundongos com PspA proporciona proteção contra sepse, pneumonia e, quando administrada com adjuvantes de mucosa, contra a colonização. PspA também demonstrou eficácia protetora contra otite média em chinchilas (Schachern *et al.*, 2014). Além de seu potencial protetor em modelos animais, PspA já foi testada em um ensaio clínico de fase I (Briles, Hollingshead, King*, et al.*, 2000).

A variabilidade estrutural da PspA pode ser um fator limitante, para superar esse desafio estratégias eficazes estão sendo investigadas, na qual incluir mais de uma molécula garante a cobertura vacinal abrangente contra o pneumococo. Estudos sobre a reatividade cruzada entre diferentes PspAs indicam que pelo menos uma molécula da família 1 e uma da família 2 devem ser incluídas na vacina para assegurar proteção contra uma variedade maior de isolados de pneumococo, além disso, associar a PspA com outras proteínas de superfíccie pneumocócicas, como PhtD, CbpA ou a pneumolisina (Goulart *et al.*, 2011; Converso *et al.*, 2020; Milani *et al.*, 2023).

A Ply é uma outra proteína pneumocócica que vem sendo investigada como candidata portencial de vacina (Rapola *et al.*, 2000; Holmlund *et al.*, 2006; Francis *et al.*, 2009). Essa proteína exibe pouca variação entre diferentes cepas e sorotipos (Han e Zhang, 2019; Nishimoto *et al.*, 2020), além disso, os seus efeitos citotóxicos podem ser facilmente reduzidos por meio de substituições simples de aminoácidos (Boulnois *et al.*, 1991; Nishimoto *et al.*, 2020). Sabendo que essa proteína é uma citolisina dependente de colesterol, para impedir seu efeito tóxico é realizada uma substituição do aminoácido L460D, que altera o reconhecimento de colesterol, e com isso elimina os efeitos citotóxicos (Farrand *et al.*, 2010; Chen *et al.*, 2015; Nishimoto *et al.*, 2020). Ao produzir esse toxoide e sabendo do papel da Ply no reconhecimento imunológico, essa proteína passou a ser investigada em diversos estudos de vacinas(Malley *et al.*, 2003; Witzenrath *et al.*, 2011; Nishimoto *et al.*, 2020). Desde então, tanto a Ply quanto o toxoide PLY modificado têm sido amplamente investigados em modelos animais, demonstrando efeitos protetores como agentes imunogênicos (Paton *et al.*, 1984; Alexander *et al.*, 1994; Musher *et al.*, 2001; Ogunniyi *et al.*,

2001; Garcia-Suarez Mdel *et al.*, 2004; Sanders *et al.*, 2010; Denoel *et al.*, 2011; Lu *et al.*, 2014; Hermand *et al.*, 2017; Nishimoto *et al.*, 2020).

Estudos clínicos em humanos utilizando vacinas baseadas em Ply também demonstraram sucesso em termos de proteção e imunogenicidade (Bologa *et al.*, 2012; Seiberling *et al.*, 2012; Berglund *et al.*, 2014; Nishimoto *et al.*, 2020) . Um derivado desintoxicado de Ply administrado a adultos saudáveis mostrou aumento nos títulos de IgG contra o toxoide Ply e maior atividade de anticorpos neutralizantes (Kamtchoua *et al.*, 2013; Nishimoto *et al.*, 2020). No entanto, até o momento, uma vacina pneumocócica contendo toxoide Ply não demonstrou proteção em humanos. Ensaios de fase II não mostraram proteção adicional contra transporte nasofaríngeo em crianças, otite média aguda ou pneumonia quando o toxoide Ply foi adicionado em combinação com outros antígenos (Odutola *et al.*, 2017; Hammitt *et al.*, 2019; Nishimoto *et al.*, 2020). Estes resultados sugerem que a toxicidade de PLY pode não ser um fator central na patogênese de doenças da mucosa, embora o debate permaneça em aberto, uma vez que é geralmente aceito como um componente importante em futuras vacinas de proteína. À medida que o desenvolvimento da vacina avança, a eficácia, segurança e imunogenicidade de vacinas baseadas em Ply devem ser reavaliadas não apenas para sua eficácia na prevenção de doenças e transporte, mas também para mudanças na resposta imune mediada por Ply (Nishimoto *et al.*, 2020).

A imunização com fusões de PLY-peptídeo ampliou o reconhecimento dos epítopos da PLY, sugerindo que essas fusões podem melhorar a proteção ao facilitar a resposta imune a epítopos que normalmente não provocam uma resposta robusta de anticorpos (Mann *et al.*, 2014). Embora os anticorpos contra Ply sejam importantes na proteção contra *S. pneumoniae*, é necessário que as vacinas desencadeiem respostas contra vários antígenos proteicos, destacando a importância de vacinas multicomponentes (Wilson et al., 2017).

PspA e Ply são consideradas como principais candidatas para inclusão em vacinas proteicas contra *S. pneumoniae* (Darrieux et al., 2015). Especificamente, a combinação dessas proteínas demonstrou proteger contra a infecção por diferentes cepas de pneumococo (Briles, Hollingshead, Nabors*, et al.*, 2000; Chen *et al.*, 2015).

A figura 3 apresenta a representação esquemática da organização celular de *S. pneumoniae*, destacando a presença da pneumolisina e associada ao espaço periplasmático e parede celular e a PspA está localizada na superfície bacteriana,.

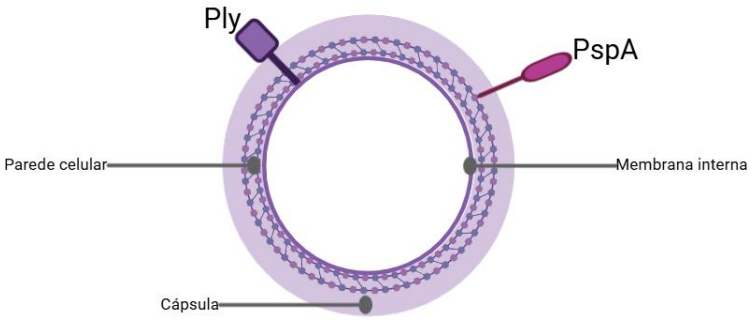

FIGURA 3- Representação esquemática da organização celular de *S. pneumoniae*, destacando dois importantes fatores de virulência. A Pneumolisina (Ply) é mostrada associada ao espaço periplasmático e parede celular; A PspA está localizada na superfície bacteriana, externamente à parede celular e revestida pela cápsula polissacarídica. Estão indicadas também estruturas: membrana interna, parede celular e cápsula. Fonte: Próprio autor

Estudos pré-clínicos também avaliaram vacinas baseadas em proteínas que combinam o toxoide Ply com outros antígenos de superfície. Um exemplo é uma vacina que utiliza uma fusão entre o toxoide Ply e a proteína PspC, um importante fator de virulência pneumocócica, que mostrou proteção significativa contra infecções pneumocócicas em modelos de transporte e infecção nasofaríngea de camundongos, além de sepse, meningite, otite média e pneumonia (Mann *et al.*, 2014; Nishimoto *et al.*, 2020).

As vacinas pneumocócicas que utilizam proteínas são particularmente promissoras porque os antígenos não dependem do sorotipo capsular, podendo assim oferecer uma proteção mais abrangente em comparação com as vacinas pneumocócicas atuais, que são baseadas em sorotipos, ou aumentar a eficácia quando conjugadas ao polissacarídeo capsular (Nishimoto *et al.*, 2020).

Pesquisas anteriores realizadas por nosso grupo investigaram a imunogenicidade e a eficácia protetora de vacinas híbridas contendo a região N-terminal da PspA fusionada com mutantes desintoxicados da pneumolisina (PlD) (Goulart *et al.*, 2013). Estas formulações demonstraram a indução de anticorpos específicos contra ambas proteínas, e proteção contra pneumonia em camundongos (Dos Santos *et al.*, 2022).

O presente estudo buscou aprofundar a avaliação da fusão PspA_PlD1, determinando a capacidade da proteína quimérica de conferir proteção a camundongos contra um desafio letal (sepse) com duas cepas de *S. pneumoniae* de sorotipos diferentes que expressavam PspAs da família 1 e 2. A proteção observada foi associada à deposição de C3 mediada por anticorpos na superfície bacteriana e casou o aumento da opsonofagocitose de pneumococos revestidos por

anticorpos pelas células peritoneais dos camundongos, e indicam que a híbrida é capaz de ampliar a cobertura vacinal das proteínas individuais.

2. OBJETIVO

2.1 Objetivo Geral

O objetivo desse estudo foi avaliar a o potencial imunogênico e protetor da proteína quimérica rPspA1_PlD1 contra um desafio letal com uma cepa heteróloga de *S. pneumoniae* em modelo murino.

2.2 Objetos Específicos

1. Determinar os efeitos da vacinação com as proteínas recombinantes na produção de anticorpos específicos.
2. Avaliar a ligação dos anticorpos produzidos às proteínas nativas expressas na superfície de *S. pneumoniae*
3. Investigar a capacidade dos anticorpos vacinais de promover a deposição de proteínas do sistema complemento na superfície bacteriana.
4. Avaliar a proteção conferida pela vacinação contra desafio letal com *S. pneumoniae*.
5. Avaliar a contribuição de cada proteínas para a proteção observada, comparando-se mutantes que não expressam PspA ou PLY.

3. CAPÍTULO 1: Artigo publicado: Fusion of PspA to detoxified pneumolysin enhances pneumococcal vaccine coverage

Este capítulo descreve os mecanismos responsáveis pela maior cobertura vacinal da quimérica PspA-PlD1 em comparação às proteínas isoladas, em modelo murino. Camundongos imunizados com a proteína quimérica apresentaram elevada produção de anticorpos específicos contra as duas proteínas incluídas na formulação. Os anticorpos foram capazes de aumentar a deposição de proteínas do Sistema Complemento na superfície de pneumococos de diferentes sorotipos e expressando moléculas de PspA de famílias distintas. A redução na deposição de complemento mediada pelos anticorpos vacinais em bactérias mutantes que não expressam PspA ou Ply indica que as duas proteínas contribuem para o efeito observado. Por fim, observou-se que a vacinação com a proteína quimérica conferiu maior proteção contra desafio letal com pneumococo virulento, quando comparada às proteínas isoladas. Em conjunto, os dados sugerem que a vacina quimérica PspA_PlD1 é uma estratégia promissora, induzindo a produção de anticorpos que atuam favorecendo a deposição de proteínas do sistema complemento na superfície de bactéria, favorecendo sua eliminação por fagocitose. Também se demonstrou que as duas proteínas, PspA e pneumolisina, contribuem para a proteção conferida pela vacina.

O estudo foi publicado na revista PLOS One; ISSN: 1932- Fator de impacto (*JCR ou Scopus Citescore*): 5,4; *Highest percentile* (JCR ou Scopus): 86% Q1 *Scopus*; Qualis da revista de acordo com o percentil: conceito A1 na Medicina 1- Anexo I. Published: December 14, 2023 https://doi.org/10.1371/journal.pone.0291203.

PLOS ONE

RESEARCH ARTICLE

Fusion of PspA to detoxified pneumolysin enhances pneumococcal vaccine coverage

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Abstract

Despite the implementation of conjugate vaccines in several countries, *S. pneumoniae* continues to pose a great burden worldwide, causing around 1 million annual deaths. Pneumococcal proteins have long been investigated as serotype-independent vaccines against this pathogen, with promising results. However, it is a consensus that one antigen alone will not be sufficient to provide long-term protection with wide coverage. Amongst the most well studied pneumococcal proteins are PspA and pneumolysin (Ply), two major virulence factors required by the bacterium for successful invasion of host tissues. PspA is highly immunogenic and protective, but it is structurally variable; pneumolysin is conserved among different pneumococci, but it is toxic to the host. To overcome these limitations, N-terminal PspA fragments have been genetically fused to non-toxic pneumolysin derivatives (PID) to create PspA_PID chimeras. Mouse immunization with these fusions confers protection against pneumococcal strains expressing heterologous PspAs, which correlates with antibody-induced complement C3 deposition on the surface of multiple pneumococcal strains. Analysis of mutant strains lacking PspA or Pneumolysin shows that both proteins contribute to the antibody-mediated enhancement in complement deposition induced by the fusion. These results expand previous data evaluating PspA_PID and demonstrate that the fusion combines the protective traits of both proteins, inducing antibodies that efficiently promote complement deposition on multiple strains and cross-protection.

Introduction

Streptococcus pneumoniae is an opportunistic pathogen that colonizes the nasopharynx and oropharynx of healthy individuals. Although colonization is commonly asymptomatic, under certain conditions it may progress to local or systemic diseases; which classifies this microbe as the second most common cause of bacterial mortality, responsible for one of the greatest problems of public health worldwide [1, 2].

The current vaccines used in prophylaxis against pneumococcal diseases are based on capsular polysaccharides conjugated with carrier proteins which, although effective against

invasive infections, tend to lose efficacy overtime due to serotype replacement [3, 4]. The conjugate vaccines have high production costs, which further limit their implementation in developing countries, where the disease burden is highest [3]. Thus, protein-based, serotype independent vaccines emerge as a promising alternative to provide greater coverage at reduced costs [5].

Pneumococcal surface protein A (PspA) and Pneumolysin are among the top candidates to be included in protein vaccines against *S. pneumoniae* (revised in [6]). In particular, the combination of these proteins is protective against infection with different pneumococcal isolates [7–11]. Previous work from our group evaluated the immunogenicity and protective efficacy of hybrid vaccines containing the N-terminal region of PspA fused to detoxified pneumolysin (PlD) mutants [12]. In that study, the chimeric protein rPspA1_PlD1 was able to protect mice against lethal challenge with two pneumococci of different serotypes expressing PspAs of family 1. Protection was associated with antibody-mediated C3 deposition on the bacterial surface, and increased opsonophagocytosis of antibody-coated pneumococci by mouse peritoneal cells.

Despite its high immunogenicity and prevalence among clinical isolates of pneumococci, PspA exhibits structural and serological variability, especially in the N-terminal, exposed half of the protein [13], which could limit the efficacy of PspA-based vaccines. Analysis of the sequence variations in PspA identified a domain including 100 aminoacids within the N-terminal half of the molecule, named clade-defining region, which was used to classify PspAs in three families and 6 clades. Families 1 and 2 (clades 1 to 5) are present in most clinical isolates [13, 14]. Different PspAs exhibit variable degrees of cross-reactivity, which roughly follow the levels of similarity among the aminoacid sequences; however, studies investigating the cross reactivity of different molecules within each major PspA family found great variations, with a few sequences being more cross-reactive than others [15, 16]. Based on those studies, we have selected a clade 1 PspA that induced the production of antibodies with the greatest cross-reaction among heterologous molecules, for inclusion in the chimeric protein formulation. To test the level of cross-reactivity and cross-protection induced by rPspA1_PlD1, we evaluated the protective efficacy of the vaccine against infection with pneumococcal strains bearing heterologous PspAs; the mechanisms underlying cross-protection were determined, as well as the contribution of each individual protein to the protection conferred by the chimera.

Materials and methods

Bacterial strains and growth conditions

The pneumococcal strains used in this work are shown in Table 1. The bacteria were kept as frozen stocks (-80 ºC) in Todd-hewitt medium supplemented with 0,5% yeast extract (THY) and 15% glycerol; when necessary, the bacteria were thawed, plated on blood agar and incubated at 37 ºC overnight in microaerophilic conditions. On the next day, the colonies were transferred to liquid THY medium and cultured until they reached an optical density at 600 nm between 0.4–0.5, corresponding to approximately 10^6 CFU/ml.

Recombinant proteins

A gene fragment encoding the N-terminal region of *pspA* clade 1 and the proline-rich domain, was amplified from the genomic DNA of strain St 245/00, cloned into pAE-6xHis vector and expressed in *E. coli* as described previously [16]. PlD1 was obtained through site directed mutagenesis of the *ply* gene from D39 strain by PCR–according to the method proposed by Nelson and Long (1989) [21] and Ho *et al* (1989) [22] and contains a His-Arg substitution at position 367, as described in [12]. PspA1-PlD1 hybrid was produced by genetic fusion of

Table 1. Pneumococcal strains used in this study.

Strain	Serotype	PspA Clade	Source	Reference
245/00	14	1	IAL	[16]
St P1153	9V	3	UFG	[14]
3JYP2670	3	4	UAB	[17]
St P490	14	4	UFG	[14]
St 255/00	6A	5	UFG	[14]
St P865	23F	5	UFG	[14]
D39[#]	2	2	UAB	[18]
ΔPspA [*#]	2	-	UAB	[19]
D39_ΔPly[#]	2	2	UAB	[20]

IAL: Instituto Adolfo Lutz, São Paulo, Brazil

UAB: University of Alabama at Birmingham

UFG: Universidade Federal de Goiás, Goiânia, Brazil

[*]ΔPspA is a mutant PspA negative strain derived from D39.

[#] D39, ΔPspA and D39_ ΔPly were kindly provided by Dr Anders Hakansson from Lund University in Malmö, Sweden.

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pspA1 and *plD1* through their cohesive ends. The *plD*1 gene fragment amplified by PCR was first ligated into pGEMT-easy vector (Promega), generating pGEMT-easy_plD1. Next, *plD1* was cut out of the vector by endonuclease digestion (using the restrictions enzymes *Xho*I and *Eco*RI–Thermo Fisher, whose target DNA sequences had been inserted in the primers for *plD*1 amplification) and ligated into pAE-*pspA* which had been previously digested with the same endonucleases. The final product was pAE_*pspA-plD1*. A schematic representation of the final construct can be found in S1 Fig. The recombinant DNA was expressed in competent *E. coli BL21DE3* cells and purified through Nickel affinity chromatography.

Animals and immunization

All experiments employing mice have been approved by the ethics committee at Universidade São Francisco, Bragança Paulista–SP (CEUAUSF, permit n 001.08.12). Female BALB/c mice from CEMIB (Campinas, Brazil) were immunized subcutaneously with 3 doses of 8 μg of rPspA1, 11.2 μg of rPlD1, 20 μg of co-administered proteins or 20 μg of the hybrid PspA1-PlD1 at 10-day intervals, diluted in 0.9% saline solution with 50 μg of Al(OH)3 as adjuvant. The adjuvant alone was used as a control. Two weeks after the last immunization, blood was collected from the retro-orbital plexus, centrifuged at 500 x g for 10 minutes, and serum was stored at -20ºC.

Antibody binding and antibody-mediated complement deposition

Antibody binding and complement deposition were performed according to the protocols described by Goulart et al (2013) [12] and Converso et al 2017 [23]. Briefly, pneumococcal strains expressing family 2 PspAs (Table 1) were grown in THY up to the mid-log phase, washed with PBS, and incubated in the presence of heat-inactivated pooled sera from mice immunized with the recombinant proteins at a final concentration of 5%. Following another wash, the samples were incubated with 100 μL of PBS containing FITC-conjugated anti-mouse IgG (MP Biomedicals) at 1:1000 dilution, washed two more times with PBS, resuspended in 1% formaldehyde and analyzed by flow cytometry, using FACS Canto II (BD Biosciences).

For the complement deposition assay, after incubation with antisera, the samples received 10% of BALB/c NMS (normal mouse serum) as a complement source and were incubated at 37 ºC for another 30 min. The samples were washed two times with PBS and incubated with FITC-conjugated anti-C3 (MP Biomedicals) at a 1:500 dilution in 100 μL of PBS on ice. Complement deposition was also evaluated in presence of anti-PspA1_PlD1 antibodies in *S. pneumoniae* D39 and its isogenic mutants lacking PspA (ΔPspA) or Ply (D39_ΔPly), using the same protocol.

Challenge

Two weeks after the last immunization, the animals were challenged intravenously with 10^6 CFUs of *S. pneumoniae* strain 3JYP2670 diluted in PBS to a final volume of 50 μl per mouse. The animals were monitored for 12 days, and moribund mice were euthanized by anesthesia using 300 mg/kg of ketamine and 30 mg/kg of xylazine (ten times the dose necessary to anesthetize the mice). At the endpoint, all surviving animals were euthanized.

Statistical analysis

For the antibody binding experiments, statistical analysis was performed using one way ANOVA with Dunnet's posttest for comparison of multiple groups against the control, or Student t test for comparison between two groups, when indicated. For the complement deposition assays, statistical analysis was performed using ANOVA with Tuckey's posttest for comparison of multiple groups, or Student t test for comparison between two groups, when indicated. In both cases, values of $p \leq 0,05$ were considered statistically significant.

For the challenge experiments, the survival times in immunized and control groups were compared using log rank test. p values $\leq 0,05$ were considered statistically significant.

Results

Antibody binding onto pneumococcal surface

The ability of antibodies against the recombinant proteins to recognize and bind to the native proteins on the bacterial surface was analyzed by flow cytometry using pneumococcal strains 3JYP2670 and St 255/00, which express PspAs from family 2, clades 4 and 5, respectively. Antisera induced by immunization with the hybrid, PspA_-PlD1, exhibited strong binding to both pneumococcal strains, with a percentage of FITC positive bacteria significantly superior to the control (Fig 1). The group injected with the co-administered proteins also presented an increased binding to the pneumococci, but the percentage of positive cells was lower than those incubated with antibodies against the hybrid. In the case of St 255/00, the proportion of positive bacteria after incubation with anti-PspA1_PlD1 was twice as high as that of the co-administered proteins. Sera from mice injected with the isolated proteins, on the other hand, did not bind efficiently to the pneumococci.

Antibody mediated complement deposition

Antibodies induced by immunization with the recombinant proteins were also evaluated for their capacity to promote complement C3 deposition onto the surface of pneumococci expressing heterologous PspA molecules. Five strains were used in the assay: one strain expressing a PspA of clade 3 (St P1153), two of clade 4 (3JYP2670 and P490) and two of clade 5 (St P865 and St 255/00). In accordance with the antibody binding results, sera from mice injected with PspA1_PlD1 induced an increase in the amount of C3 deposited on the surface of four out of five pneumococci tested, in comparison with the control (Fig 2). Furthermore,

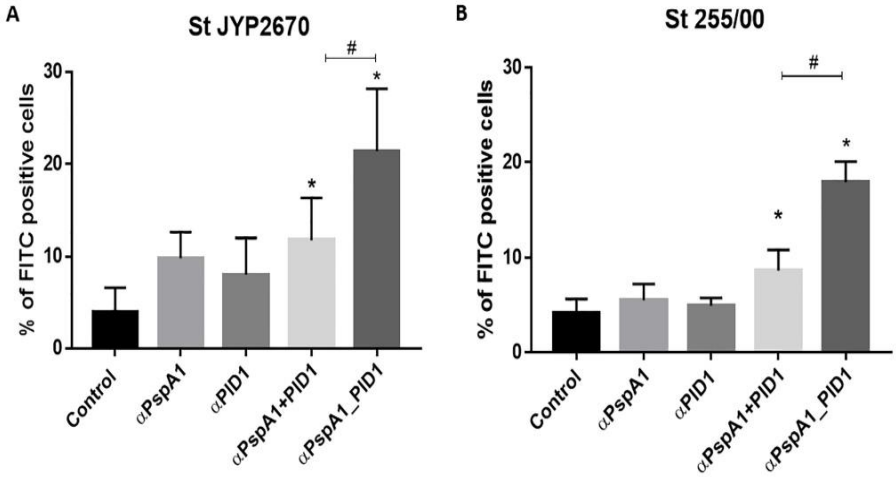

Fig 1. Antibody binding on the surface of *Streptococcus pneumoniae*. Pneumococcal strains 3JYP2670 (A) and St 255/00 (B) were treated with serum from mice immunized with PspA1, PlD1, the proteins mixed, the fusion PspA1_PlD1 or adjuvant alone (control) and FITC conjugated anti-mouse IgG. The percentage of FITC positive bacteria (representing antibody binding) is shown for each group. The error bars represent the standard deviation of the replicates from the mean percentage of FITC positive cells. Statistical analysis was performed using ANOVA with Dunnet's posttest. *$p \leq 0,05$ in comparison with the control; #$p \leq 0,05$ when comparing the proteins mixed and fused.

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incubation with antibodies against the hybrid resulted in close to 100% positive cells in all but one pneumococcal strain. The only exception was the clade 3 strain P1153, which showed around 75% bacteria positive for C3 deposition against 50% in the control group and did not reach statistical significance. Meanwhile, similarly to the antibody binding results, antibodies against the isolated proteins did not promote complement activation and deposition on any of the strains tested.

To examine the contributions of each individual protein to complement deposition on pneumococci, the strain D39 and its isogenic mutants that do not express PspA or Ply were incubated with serum from mice which had been immunized with PspA1_PlD1. As shown in Fig 3, antibodies against the hybrid promoted higher levels of C3 deposition on the wild-type strain in comparison with the $PspA^-$ and Ply^- mutants ($p<0,0001$). The histograms showing the effect of antibodies in complement deposition are included in S2 Fig.

Vaccine-induced protection against pneumococcal infection

For evaluation of cross-protection induced by the recombinant proteins, mice immunized with rPspA1, rPlD1, rPspA1_PlD1 fusion or the co-administered proteins were challenged intravenously with 10^6 CFUs of the pneumococcal strain 3JYP2670, expressing a clade 4 PspA (Table 1). Mice injected with PBS and $Al(OH)_3$ were used as a control. After three days of infection, all control mice had died (Fig 4). In contrast, the group immunized with PspA1_PlD1 fusion showed 90% survival after 12 days. PspA1 alone and the PspA1 + PlD1 mixture were also protective against infection; however, only 50% of the mice survived the challenge in each of these groups. Furthermore, when comparing the survival curves of the immunized mice, those immunized with the hybrid had longer survival times, with all mice alive until day 10. PspA and the protein mixture had similar survival curves, with 5 mice dead

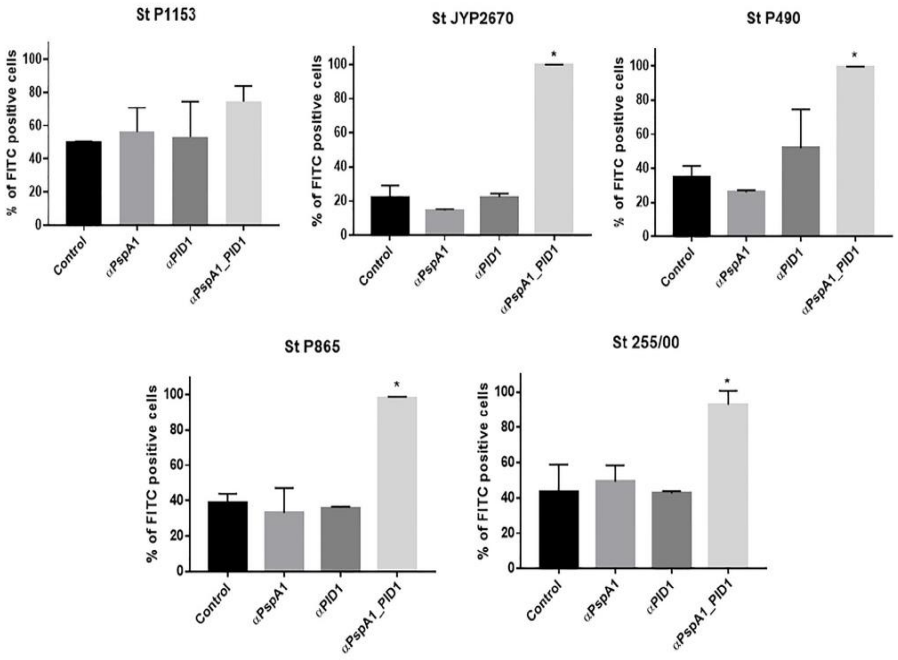

Fig 2. Complement deposition on the surface of *Streptococcus pneumoniae* in presence of specific antibodies. Pneumococcal strains St 255/00, 3JYP2670, P490, P865 and P1153 were treated with serum from mice immunized with PspA1, PlD1, the fusion PspA1_PlD1 or adjuvant alone (control), NMS as a complement source and FITC conjugated anti-mouse C3. The percentage of FITC positive bacteria (representing C3 binding) is shown for each group. The error bars represent the standard deviation of the replicates from the mean percentage of FITC positive cells. Statistical analysis was performed using ANOVA with Dunnet's posttest. *p≤0,05 in comparison with the control.

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after four days of challenge. PlD1 alone did not confer protection against sepsis; all mice died within four days of infection.

Discussion

PspA and Ply are two widely studied pneumococcal proteins displaying key roles in pneumococcal disease progression. Both proteins contribute to immune evasion and invasion of host tissues; Ply promotes cell damage and inflammation, while PspA limits opsonization and killing by complement molecules and antimicrobial peptides [5]. It can also bind to GAPDH on dying lung cells, facilitating bacterial dissemination during pneumonia [24].

For their important contributions to pneumococcal infections, PspA and Ply have been investigated as potential candidates in serotype-independent vaccines in several disease models [3, 25] and clinical trials [26]. The combination of PspA and pneumolysin derivatives has been particularly effective against severe outcomes like pneumonia [27] and bacteremia [8, 12, 28].

Previous work has shown that immunization with a PspA1-PlD1 chimera protects mice against systemic infection with virulent pneumococci bearing PspAs from family one [12]. However, PspA shows some level of variability, especially on the N-terminal, surface exposed region of the molecule (also known as the alfa-helical portion of the molecule), resulting in a mosaic-like sequence pattern. Since these variations impact the coverage of vaccines including

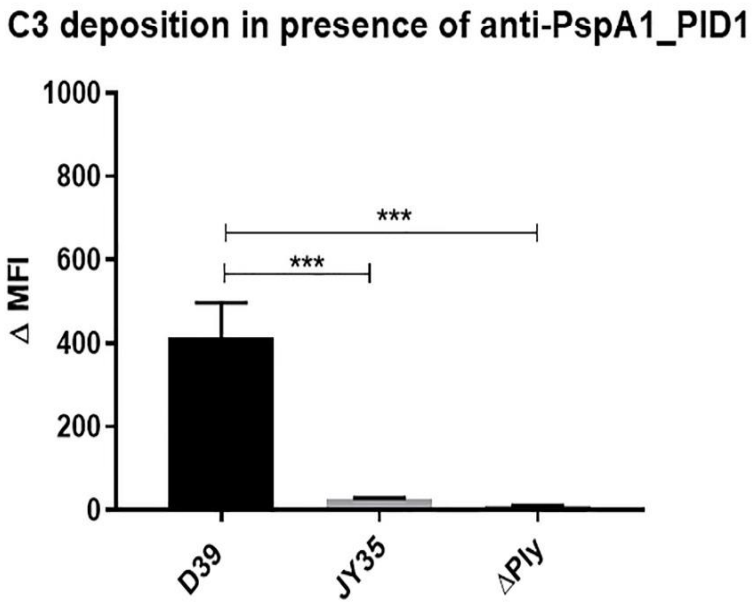

Fig 3. Comparison of antibody-induced complement deposition on wild-type and mutant *S. pneumoniae*. Pneumococcal strains D39 (wt), ΔPspA (PspA negative) and D39_ΔPly were incubated with anti-PspA1_PlD1 antisera or control sera (from sham immunized mice), NMS and FITC conjugated anti-mouse C3. The graph shows the ΔMFI (median of fluorescence intensity) of each sample subtracting the MFI of the control. The error bars represent the standard deviation of the replicates from the mean value. Statistical analysis was performed using ANOVA with Tuckey's posttest. *p≤0,05 comparing wt and mutant bacteria.

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PspA as an immunogen, we sought to evaluate the cross-protection induced by the rPspA1_PlD1 chimera against challenge with a pneumococcal strain bearing a heterologous PspA belonging to family 2. The construct included the N-terminal region plus the proline-rich domain of a clade 1 PspA molecule. Despite inducing similar IgG levels as each individual protein [12], the fusion protein was highly protective against this strain, with 90% of the mice surviving challenge. Interestingly, the co-administered proteins induced the same protection as PspA alone, indicating the fusion of PspA and Ply is important to increase the protective efficacy of this vaccine formulation. This could be due to modifications in the structure of the molecule resulting from the fusion, leading to exposure of protective epitopes. Although this hypothesis needs further investigation to be confirmed, a similar effect has been described for PspA in fusion with capsular polysaccharide 23F [29].

Next, we evaluated the mechanism underlying the increased protection observed with the rPspA-PlD1 chimera. Production of opsonic antibodies has long been identified as an important process for pneumococcal clearance in the host and a correlate of protection in current vaccines [30, 31]. Therefore, we tested if antibodies produced in response to immunization could bind to and increase complement deposition on bacteria bearing diverse PspAs. In accordance with the protection data, sera from mice injected with the rPspA-PlD1 hybrid showed increased binding to PspA family 2 strains, including the challenge isolate, 3JYP2670. Similarly, this antiserum led to higher levels of C3 deposition on the surface of different family two expressing pneumococci, in comparison with the individual proteins. In four out of five

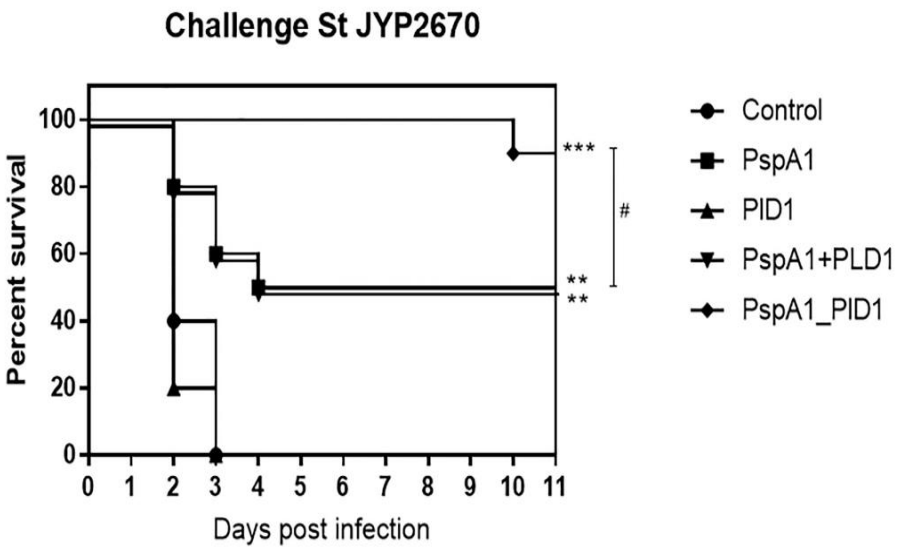

Fig 4. Survival of vaccinated mice after i.v. challenge with *S. pneumoniae*. Mice were immunized s.c. with three doses of PspA1, PlD1, the co-administered proteins or the PspA1-PlD1 hybrid, challenged i.v. with 10^6 CFUs of *S. pneumoniae* strain 3JYP2670 and monitored for 12 days. The control group received adjuvant diluted in saline solution. Survival times are shown individually in the different immunization groups. Statistical analysis was performed using log rank analysis. *p≤0,05 in comparison with the control; # p≤0,05 when comparing different immunization groups.

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strains tested, the percentage of positive bacteria was close to 100%, attesting the ability of such antibodies to activate complement deposition and opsonization. Moreover, similar results were observed in bacteria producing different capsules, suggesting that the formulation is effective against multiple pneumococcal serotypes.

Finally, analysis of C3 deposition on mutant pneumococci lacking PspA or Pneumolysin showed a marked reduction when compared with the wild-type strain, indicating that both proteins contribute to the induction of opsonic and complement activating antibodies.

Anti-PspA antibodies have been shown to contribute to pneumococcal clearance through different mechanisms (reviewed in [32]), including increased complement deposition and opsonophagocytic killing by neutrophils [33] enhancing the bactericidal action of lactoferrin [34–36] and facilitating pneumococcal killing by neutrophil extracellular traps [37]. Although the present study only investigated complement activation by these antibodies, all these mechanisms could aid in preventing pneumococcal disease in vaccinated mice. Pneumolysin neutralization by antibodies prevent binding to cholesterol as well as its subsequent cytotoxic and platelet disrupting effects [38, 39]. Anti-ply antibodies also play a role in limiting biofilm formation and colonization by pneumococci [40–42]. Although immunization with PlD1 alone was not protective in the present study, the adjuvant capacity of pneumolysin derivatives could also contribute to the induction of amplified immune responses with higher cross-reactivity.

Conclusion

The present study confirms the potential of rPspA1-PlD1 as a serotype independent pneumococcal vaccine and expands the previous results, demonstrating that the PspA-PlD1 fusion

combines the protective traits of both proteins (i.e., the high immunogenicity of PspA and the conservation of Pneumolysin among pneumococci) inducing antibodies that efficiently promote complement deposition on multiple strains and cross-protection. The present data contributes to the development of serotype-independent, protein-based pneumococcal vaccines with high coverage, without the limitations of the current polysaccharide-based vaccines.

Supporting information

S1 Fig. Schematic representation of rPspA-PlD1. PspA1 N- and C-terminal domains are shown. The arrow in PlD1 marks the His_{367}-Arg aminoacid replacement in the final protein. The chimeric protein includes the N-terminal domain of PspA1 fused to the complete PlD1 sequence.
(TIF)

S2 Fig. Complement C3 deposition on wild-type and mutant pneumococci. D39 and its isogenic $PspA^-$ (JY53) and Ply^- (D39_ΔPlt) mutant strains were incubated with sera from mice vaccinated with the hybrid protein, rPspA-PlD1, NMS and FITC-conjugated anti-mouse C3. The median fluorescence intensity (MFI) is shown for each bacterium.
(TIF)

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Project administration: Luciana C. C. Leite, Michelle Darrieux.

Supervision: Michelle Darrieux.

Writing – original draft: Barbara Milani, Thiago R Converso, Michelle Darrieux.

Writing – review & editing: Sheila Oliveira, Greiciely O. André, Luciana C. C. Leite, Thiago R Converso, Michelle Darrieux.

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4. CAPÍTULO 2: Artigo Submetido: The Case for PspA: A Comprehensive Review of a Leading Candidate in Pneumococcal Vaccine Research

Este capítulo buscou avaliar o potencial vacinal da proteína PspA por meio de uma revisão de literatura acerca das diferentes estratégias empregando esta proteína em modelos animais de sepse, pneumonia, otite média e colonização. Os resultados foram compilados em um artigo de revisão, em análise na revista ACS Infectious Diseases.

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The Case for PspA: A Comprehensive Review of a Leading Candidate in Pneumococcal Vaccine Research

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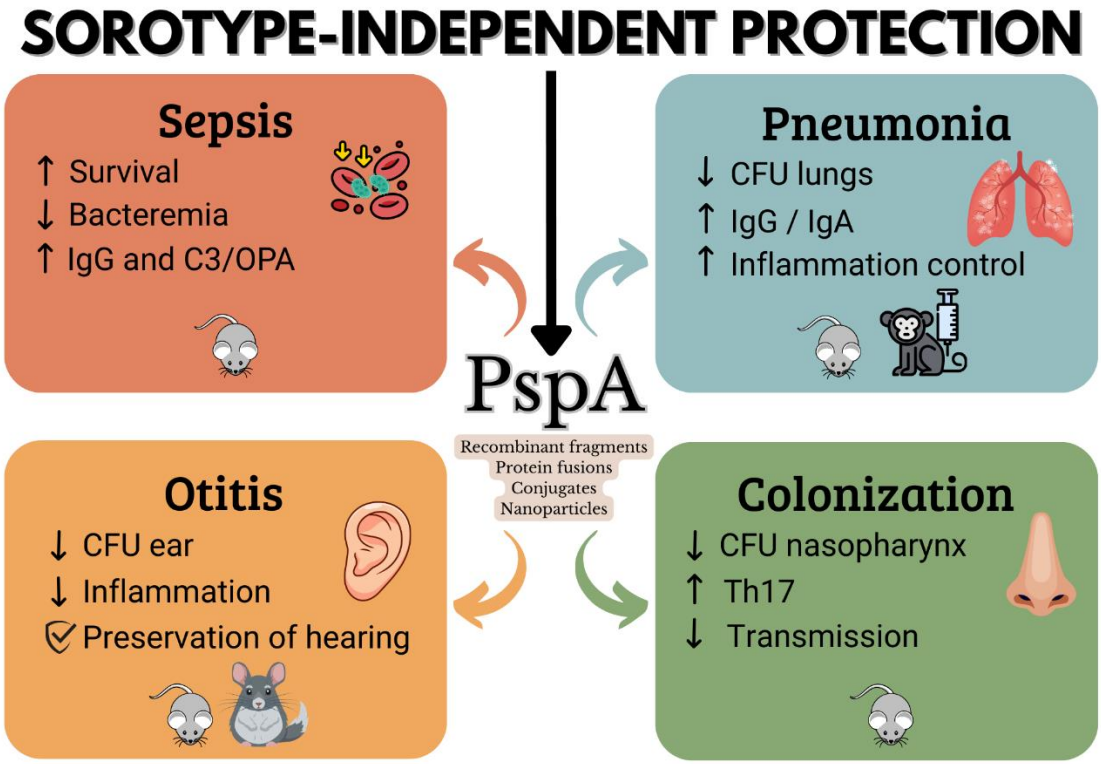
Graphical Abstract



Abstract

Streptococcus pneumoniae remains a leading cause of morbidity and mortality worldwide, with current polysaccharide-based vaccines offering limited serotype coverage, high production costs, and reduced efficacy in vulnerable populations. These limitations have prompted the search for conserved pneumococcal proteins as universal vaccine candidates. Among them, Pneumococcal Surface Protein A (PspA) stands out as a major virulence factor, present in virtually all clinically relevant strains, and capable of interfering with complement activation, opsonophagocytosis, and host defense mechanisms. Over three decades of research have demonstrated PspA's strong immunogenicity, protective efficacy in multiple animal models, and safety in early-phase clinical trials. Here, we critically review advances in PspA-based vaccine development, including recombinant protein fragments, fusion constructs, nanoparticle formulations, and live-vector platforms. We highlight the structural and immunological determinants underlying its protective potential, while discussing major challenges such as

antigenic variability and cross-reactivity. By integrating recent experimental and translational findings, this review outlines the opportunities and obstacles for the implementation of serotype independent PspA-based vaccines

Keywords: *Streptococcus pneumoniae*; pneumococcal vaccines; Pneumococcal Surface Protein A (PspA); animal model.

Introduction

Streptococcus pneumoniae (pneumococcus), is an extracellular pathogen that primarily colonizes nasopharynx, from where it can spread to other sites, causing local and/or systemic infections such as pneumonia, bronchitis, brain abscess, otitis media, septicemia, and meningitis (1, 2) . Globally, *S. pneumoniae* is the leading cause of morbidity and mortality among lower respiratory tract infections (LRTIs); it is responsible for approximately 9 million infections each year, with over 1.1 million related deaths (3-5), primarily affecting children, immunocompromised individuals, and the elderly (6, 7). Due to its high global mortality rate, it has been classified as a medium-priority pathogen by the World Health Organization (WHO) for research and development of new antibiotics (8). The increasing prevalence of antibiotic-resistant strains exacerbates the situation, limiting therapeutic options. Currently, approximately 30% of severe infections caused by pneumococci show resistance to one or more antibiotics (9, 10) . Prevention of pneumococcal infections is currently achieved through immunization using capsular polysaccharides from *S. pneumoniae,* alone or conjugated with carrier proteins (7, 11); however, the high variability of the capsule, combined with technical challenges in vaccine production, such as high costs and difficulties in implementation in developing countries, limits vaccine effectiveness and coverage (7, 12-14). Therefore, serotype-independent, protein-based formulations have been investigated against *S. pneumoniae* (3, 7, 11, 15).

Pneumococcal surface Protein A (PspA) is an important virulence factor in *S. pneumoniae.* It is the most prevalent member of the choline-binding protein family (CBPs), which includes surface molecules attached to the phosphorylcholine moieties of teichoic acids in the cell wall (11, 16). The immunogenic potential of PspA was identified over three decades ago, with the demonstration that two monoclonal antibodies against PspA protected mice from fatal infection with three different pneumococcal strains expressing two capsular serotypes (16, 17).

Structurally, PspA contains three distinct regions (Figure 1): an N-terminal alpha-helical domain, a proline-rich domain, and a choline-binding domain at the C-terminus. The first domain

is the exposed part of the molecule, and the most variable portion of the molecule. It includes a clade-defining region (CDR) used to classify PspA into 3 families and 6 clades. Family 1 includes clades 1 and 2; family 2 includes clades 3, 4, and 5; and family 3 includes clade 6 (18). Primary amino acid sequences within the same clade show ≥90% identity in the CDR, while between different families, this identity is ≤55% (18-21). Most pneumococcal strains express PspA from families 1 or 2. The level of cross-reactivity varies among different PspAs, with higher reactivity within proteins of the same family. The proline-rich region (PRR, or region C) is located between the N-terminal helical segment and the C-terminal domain, and is characterized by short repeats, in which proline residues occur every three or four amino acids. In approximately half of the described PspAs, these repeats are interrupted by a highly conserved sequence of amino acids lacking proline, known as the non-proline block (NPB), which is considered an important antigenic fragment (19, 22, 23). The C-terminal region of PspA is responsible for the protein interaction with choline residues in the bacterial membrane, forming a conserved structural domain present in all variants of this protein, followed by a short hydrophobic tail.

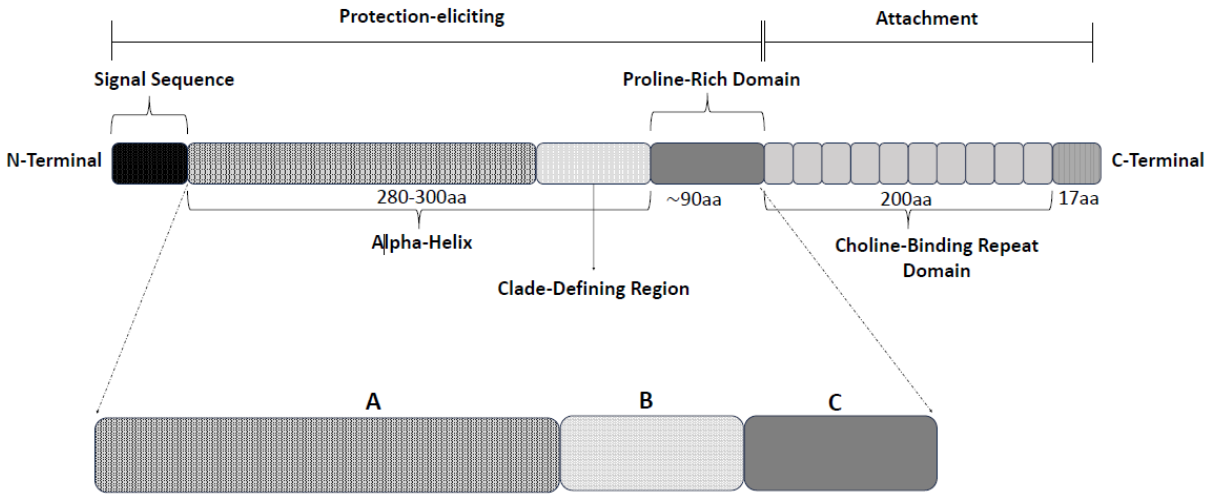


Figure 1 – Schematic representation of the PspA structure. The N-terminal signal sequence, the α-helical region with 280–300 aa (A) including a clade-defining region (B), the proline-rich domain of approximately 90 amino acids (C), the choline-binding repeat domain with about 200 aa, and a short C-terminal tail of 17 aa are shown.

PspA contributes to pneumococcal virulence through multiple mechanisms. It protects the bacterium from phagocytosis and interferes with complement system activation, reducing opsonophagocytosis (24, 25). PspA also acts as an adhesin by binding to host proteins such as

glyceraldehyde-3-phosphate dehydrogenase on dying lung cells and lactate dehydrogenase (LDH), which supports bacterial growth by enhancing energy production during infection (26). Another important trait in PspA relies on its ability to interact with human lactoferrin, a component of the innate mucosal defense system. This interaction reduces the bactericidal efficacy of apo-lactoferrin (Apo-hLf) - the iron-free form of the molecule, promoting pneumococcal survival (26, 27). It has been shown that the presence of PspA on the bacterial surface inhibits the lytic activity of lactoferrin, protecting the pathogen, whereas anti-PspA antibodies enhance the bactericidal effect of lactoferrin against *S. pneumoniae* (20, 28, 29). Recent studies have also demonstrated that PspA can protect *S. pneumoniae* against the microbicidal effects of indolicidin, a cationic antimicrobial peptide belonging to the cathelicidin family. Mass spectrometry analyses have demonstrated a direct interaction between PspA and indolicidin, supporting the hypothesis that PspA functions as a molecular net. By sequestering cationic peptides, it effectively prevents their access to the bacterial membrane, thereby mitigating membrane disruption and preserving cellular integrity (30).

Due to its relevant role in pneumococcal virulence, PspA has been investigated in numerous studies as the leading candidate in serotype-independent vaccines, particularly using its N-terminal region, which contains most of the protective epitopes (31). Immunization of mice with PspA provides protection against sepsis, pneumonia, and, when administered with mucosal adjuvants, against colonization. In chinchillas, PspA has also demonstrated protection against otitis media (32). Recently, the vaccine potential of PspA was evaluated in a nasal vaccine study model using non-human primates, with encouraging results (20). Beyond its well-documented protective efficacy in animal models, PspA has advanced to phase I clinical trials (18, 33, 34), underscoring its promise as a vaccine candidate against *S. pneumoniae.*

Given the considerable vaccine potential demonstrated by PspA, this review aims to critically examine the immune responses elicited by this surface protein in various formats—including as a standalone antigen, in fusion constructs, or in combination with other pneumococcal components. Furthermore, the review seeks to analyze the quality and breadth of the immune responses generated, while also addressing the current limitations and challenges associated with the use of PspA as a vaccine candidate. The following topics describe different infection models using PspA as a vaccine candidate, which represent the main diseases caused by this pathogen.

Sepsis

Various vaccine formulations based on PspA have been investigated as strategies to prevent systemic infections caused by *S. pneumoniae*, particularly in murine models of sepsis. These formulations include isolated protein fragments, combinations or fusions with other antigens, as well as the use of distinct vaccine vectors and adjuvants (35-41). Table 1 summarizes the studies evaluating the protective role of PspA against sepsis.

Initial investigations of PspA as a vaccine candidate against systemic infection employed the full-length protein isolated from non-encapsulated *S. pneumoniae* strains. This approach proved effective in murine sepsis models, conferring protection even against high challenge doses (42, 43). However, technical limitations associated with native protein purification—such as its aggregation propensity—prompted the exploration of novel vaccine formulations (43, 44). Subsequent studies demonstrated that fragments derived from the N-terminal region of PspA, particularly those comprising the first 245 amino acids, retained their protective immunogenicity (43, 45, 46). These findings drove the development of recombinant vaccines based on specific PspA portions, primarily targeting its most immunogenic domains (21, 43, 44, 47). Over time, the use of native full-length PspA was replaced by formulations incorporating distinct regions of the molecule (43). The combination of conserved PspA regions has emerged as a promising strategy to broaden the coverage of vaccines targeting this protein (7, 48, 49). Overlapping peptide screening techniques confirmed that fragments of 100 amino acids within the N-terminal region can induce functional antibodies against both linear and conformational epitopes, making them preferred targets for multi-epitope formulations (50-52).

A few studies have explored the proline-rich region of PspA as a vaccine candidate against systemic *S. pneumoniae* infections, given its conserved and immunogenic epitopes (31, 36, 53). This approach was first evaluated by Girgis et al. as an isolated antigen, with BALB/c mice receiving three subcutaneous doses of recombinant rPR protein adsorbed into aluminum hydroxide ($Al(OH)_3$) followed by intraperitoneal challenge with a clinical serotype 19F strain. Passive immunization with anti-rPR antibodies - despite generating high antibody titers – failed to provide significant protection (0% survival in all vaccinated groups). This outcome was attributed to the strain's thick polysaccharide capsule potentially masking PspA epitopes and impairing humoral response efficacy (36). Building on these findings, Tamborrini et al. investigated synthetic virus-like particles (SVLPs) containing proline-rich region epitopes in a murine systemic sepsis model using *S. pneumoniae* serotype 1 (strain 1577). NMRI mice were immunized with SVLPs containing

either: (i) CCL-PR1 (N-terminal proline-rich region epitopes), CCL-NPB (conserved non-proline block), or their combination; or (ii) heterologous epitopes CCL-PR2/N (N-terminal region variant) or CCL-PR2/C (C-terminal variant), where CCL refers to the Coiled-Coil-Like nanoparticle platform. Following two immunizations, intravenous challenge with 10^4 CFU of strain 1577 revealed significant morbidity delay in CCL-PR1, CCL-NPB, and CCL-PR1+CCL-NPB, but not in recombinant rPspA controls. Among heterologous epitopes, only CCL-PR2/C significantly improved survival, suggesting protective potential for specific fragments like NPB and C-terminal variants (53).

The investigation by Daniels et al. examined the immunogenicity and protective efficacy of distinct fragments from the proline-rich region in CBA/N mice using a systemic pneumococcal infection model. Three recombinant fragments were evaluated: PR+NPB (containing both the proline-rich region and the Non-Proline Block subregion), PR-NPB (comprising only the proline-rich region without NPB), and NPB alone (the isolated Non-Proline Block). Mice received three intraperitoneal doses of 10 µg protein adsorbed to $Al(OH)_3$, followed by lethal challenge with heterologous encapsulated strains of serotype 3 (WU2 and 3JYP2670) and serotype 6A (BG12730), which express different PspA variants. The PR+NPB fragment induced significant protection (70-80% survival across all three strains), whereas PR-NPB and NPB alone failed to confer effective protection. These results establish that the NPB subregion, when maintained in its native context within the proline-rich region, is essential for eliciting functional protective immunity, identifying it as a conserved epitope of interest for C-terminal-based PspA vaccine formulations (31).

The efficacy of multivalent formulations combining PspA fragments with CbpA protein and a mutated pneumolysin toxin (L460D) was evaluated in murine sepsis models. Among the PspA fragments tested, two were particularly noteworthy: H70 (containing the SM1 peptide and proline-rich region) and CD2 (comprising only the proline-rich region). These fragments were fused with either the YLN fusion protein (L460D linked to two CbpA domains, YPT and NEEK) or L460D alone. CD1 mice immunized intraperitoneally with these formulations were challenged with strains D39 (serotype 2), P9 (serotype 6A), or 1861 (serotype 1). The trivalent H70+YLN formulation provided significant protection against all tested strains, particularly against P9 - reducing meningitis incidence from 50% in control mice to 20% in immunized animals. In intravenous sepsis models using strains DBL6A (serotype 6B) and A66.1 (serotype 3), H70+YLN

again demonstrated superior protection compared to individual antigen components(54). Collectively, these studies highlight the proline-rich region's potential, particularly through conserved NPB fragments and multivalent formulations, for inducing protective immunity in pneumococcal sepsis models. The strategic combination of this region with other immunogenic proteins and adjuvants appears crucial for achieving broad efficacy against diverse *S. pneumoniae* serotypes (53).

Antigen combination strategies have similarly demonstrated efficacy. In their study, Afshari et al. employed a bivalent vaccine formulation containing the chimeric protein PspA1-5c+p - which incorporates epitopes from regions B and C of the five major PspA clades - combined with the highly conserved PhtD protein. BALB/c mice immunized intraperitoneally with this formulation exhibited 100% survival following intraperitoneal challenge with strain ATCC 49619 (serotype 19F), along with complete bacterial clearance in the spleen and significant reduction in blood bacterial load. When compared to formulations containing either antigen alone, the PspA+PhtD combination showed superior efficacy, clearly demonstrating the advantage of this multi-epitope approach (55).

The potential of passive immunization through maternal transfer was investigated using a model where BALB/cByJ female mice were intranasally immunized with rPspA (PspA2/TIGR4) combined with cholera toxin subunit B (CTB). Following intraperitoneal challenge with strain TIGR4, offspring born to and nursed by immunized mothers demonstrated approximately 70% survival and complete absence of bacteremia. The functional immune response involved production of IgG, IFN-γ, and IL-17A, indicating the induction of protective immunological memory (37).

In addition to passive immunization strategies, various active approaches have been employed to enhance antigenic coverage. Afshari et al. evaluated the chimeric protein PspA1-5c+p administered in a three-dose intraperitoneal regimen. BALB/c mice challenged with three distinct *S. pneumoniae* strains exhibited high cross-reactivity and significant functional activity in both Serum Bactericidal Assay (SBA) and Opsonophagocytic Assay (OPA). Although survival rates were not detailed, the data suggest robust protection, with over 50% bactericidal and opsonic activity against the tested variants (35).

Studies have analyzed a chimeric formulation combining the N-terminal region of PspA with detoxified pneumolysin, demonstrating high efficacy of this vaccine candidate, since

detoxification eliminated the cytolytic activity of the toxin while preserving its immunogenicity (38, 56). In the study by Goulart et al. (2013), PspA was fused to PdT, a detoxified version of pneumolysin obtained by site-directed mutagenesis of the ply gene from strain D39, in which the substitution of leucine (Leu) at position 460 by aspartic acid (Asp) (L460D) abolished cytolytic activity. In contrast, in the study by Milani et al. (2023), the genetic fusion involved the N-terminal region of PspA1 and the detoxified pneumolysin PlD1, generated by site-directed mutagenesis that replaced histidine (His) at position 367 with arginine (Arg) (H367R). In the study of Goulart et al. (2013), the evaluation of the PdT derivative focused on a lethal sepsis model in BALB/c mice, immunized subcutaneously with three doses at 14-day intervals and subsequently challenged intravenously with virulent strains of S. pneumoniae 491/00 (clade 1 of PspA) and A66.1 (clade 2 of PspA). The results showed that immunization with rPdT alone did not confer protection against the lethal challenge, indicating that although immunogenic, this antigen by itself was not sufficient to induce systemic protective responses. However, when fused to the N-terminal region of PspA2 (rPspA2-PdT), the detoxified mutant contributed to increased survival rates in mice, surpassing both the control group and PdT administered alone. In the publication of Milani et al. 2023 BALB/c mice immunized subcutaneously with three doses of the rPspA1-PlD1 fusion protein achieved 90% survival following intravenous challenge with strain 3JYP2670 (serotype 3, clade 4). This survival rate surpassed that observed with PspA1 alone or co-administered with PlD1 (both yielding 50% protection), underscoring the synergistic effect between these antigens. The formulation further induced efficient C3 deposition and generated cross-reactive (38). These findings indicate that detoxified pneumolysin plays a relevant role when genetically combined with PspA, enhancing the efficacy of the immune response in sepsis models (38, 56).

In the same vein, Zane et al. evaluated the impact of including either flexible or rigid linkers between the PspA and PdT proteins. The authors investigated how inserting peptide linkers—a rigid one (RL) or a flexible one (FL)—between two fused pneumococcal proteins (PspA and PdT), using the N-terminal region of PspA, could influence the structural stability and production of the candidate vaccine. Using both *in silico* and experimental approaches, the researchers compared three versions of the protein: without a linker, with a rigid linker, and with a flexible linker, and assessed their performance in production models and protective efficacy in specific pathogen-free (SPF) female BALB/c mice. The animals were immunized subcutaneously in a three-dose regimen, spaced 15 days apart, with either rPspA-FL-PdT or rPspA-RL-PdT, using sterile 0.9% saline and

$Al(OH)_3$ as an adjuvant. The adjuvant alone in saline served as a negative control. Twenty-one days after the third dose, the animals were challenged intranasally with *S. pneumoniae* strain A66.1. Specific IgG antibody levels were measured 15 days after each immunization. The results showed that, for anti-PspA IgG, antibody levels against all fusion proteins were similar, with more noticeable differences between the first and second doses, reaching a plateau after the second dose. The third dose increased anti-PspA antibody levels only in the groups immunized with rPspA-FL-PdT. For anti-PdT IgG, marked differences were observed, with higher levels after the third dose. Immunization with rPspA-PdT without a linker showed stability issues that compromise large-scale production, making the inclusion of linkers a promising strategy to overcome this limitation. Both versions provided 100% protection against a lethal intranasal challenge with *S. pneumoniae*, showing that linker inclusion—especially the flexible type—enhances pharmacological traceability without impairing immunological efficacy, representing a step forward in producing safe and stable fusion vaccines(57). Thus, the production of rPspA-FL-PdT enables the delivery of two antigens in a single process, which is advantageous from both economic and bioprocessing standpoints, while also yielding a more stable molecule compared to the linker-free version. Further studies are needed to refine this vaccine approach (38, 57).

Moving on to mucosal formulations, an intranasal vaccine containing recombinant PspA (clade 2) combined with the pCA adjuvant was tested in a lethal pneumonia/sepsis model using the Xen10 strain (serotype 3). Immunization of BALB/cCrSlc mice with three weekly doses resulted in over 70% survival, with robust systemic and mucosal immune responses, marked by the presence of IgG and IgA, in addition to a predominant Th2 profile. The combined formulation significantly outperformed each component administered individually, reinforcing the potential of the nasal vaccine(39).

Of particular interest, the most distinct approach among the models analyzed involved a combination of systemic and mucosal immunization based on PspA variants. BALB/c mice subjected to a prime/boost regimen with subcutaneous application followed by an intranasal booster achieved 100% survival against intranasal challenge with two highly lethal strains—ATCC 10813 (clade 2) and BAA-334 (clade 3). In contrast, exclusively systemic vaccination conferred only 30% protection against the clade 2 strain, while the isolated mucosal route resulted in 80% survival. The combined formulation induced high levels of IgG, IgA, and IL-17A, highlighting the importance of mucosal immunity in protecting against lethal pneumococcal infection (41).

Converso et al. developed a recombinant vaccine based on a chimeric protein containing resulting from the genetic fusion of the N-terminal region plus the proline-rich region of PspA (St P490 strain, family 2) with the PotD protein (St 540/99 strain), named rPspA-PotD. Immunization was performed in BALB/c mice subcutaneously, with three doses at 14-day intervals, where the immunizing groups were the chimeric protein rPspA-PotD, rPspA, rPotD, and the control group, all adsorbed into $Al(OH)_3$. Two weeks after the last dose, the animals were challenged intranasally with two virulent *S. pneumoniae* strains, ATCC6303 (serotype 3, PspA clade 5) and A66.1 (serotype 3, PspA clade 2). The rPspA-PotD vaccine conferred significant protection, with a survival rate of 90% against ATCC6303 and 90% against A66.1, demonstrating similar efficacy to isolated PspA protein, and superior to isolated PotD, which conferred no protection. Furthermore, the hybrid formulation induced high levels of specific IgG antibodies, induced antibodies that recognized different PspA variants across various pneumococcal strains and promoted increased in vitro phagocytosis. A reduction in nasopharyngeal colonization after challenge was also observed. These data confirm the ability of the rPspA-PotD chimeric protein to induce a comprehensive protective immune response, both against lethal systemic infection and mucosal colonization (58).

Seeking to further broaden coverage, Yu et al. tested a bivalent formulation composed of the PsaA-PspA23 fusion protein (with epitopes from clades 2 and 3) co-administered with the PspA4 protein (clade 4), both adsorbed into $Al(OH)_3$. Female BALB/c mice were immunized subcutaneously with three doses of the vaccine formulation at two-week intervals. Two weeks after the last immunization, the animals were challenged intranasally with virulent *S. pneumoniae* strains representing PspA clades 1 to 5, including strains ATCC 6312 (clade 1), ATCC 6304 (clade 1), ATCC 10813 (clade 2), ATCC BAA-334 (clade 3), and ATCC 6303 (clade 5). The group vaccinated with the PsaA-PspA23 + PspA4 formulation showed over 50% survival in all challenges, with significantly greater protection compared to control groups (PBS and PPV23), regardless of the challenging strain's clade of origin. These results demonstrate that co-administration of PspA antigens from different families, along with PsaA, can provide robust cross-protection against lethal systemic pneumococcal infection (59).

One of the main limitations of PspA as a vaccine candidate is its high structural variability, which can compromise the breadth of protection when only a single fragment or variant of the protein is used. As a strategy to overcome this barrier, different groups have investigated the

development of PspA fusion proteins, in which immunogenic fragments are combined to broaden cross-reactivity. In the study by Darrieux et al. formulations containing the N-terminal region of PspA from different clades were constructed, resulting in the antigens PspA1ABC (clade 1), PspA3ABC (clade 3), as well as the hybrids PspA1ABC-4B (clade 1 fused to fragment B of clade 4) and PspA1ABC-3AB (clade 1 fused to fragments A and B of clade 3). BALB/c mice immunized subcutaneously with these formulations and challenged with representative strains of clades 1, 2, 3, and 4 showed protection directly related to the similarity between the vaccine fragment and the clade expressed by the bacterium, with particular emphasis on the hybrids, which conferred broader protection and correlated with increased C3 deposition on the bacterial surface (60). Similarly, Piao et al. (2014) evaluated PspA fusion proteins constructed from fragments of the N-terminal region (α-helical domain and proline-rich region) of different clades from families 1 and 2. The experimental design was comparable, differing only in the vaccination scheme (the combined use of CpG ODN and $Al(OH)_3$ as dual adjuvants in C57BL/6J mice) and in the intranasal challenge with strains expressing PspA from clades 1 to 5. Results showed that, as observed by Darrieux et al., protection depended on the choice of fragments included in the chimeric protein: the PspA3+2 formulation conferred protection against all five clades tested, whereas PspA2+4 and PspA2+5 failed against strains from clades 1 and 3.These findings indicate that the inclusion of fragments from clades 3 and 2 in the PspA3+2 construct resulted in a broader range of cross-reactivity and protection in the murine pneumococcal sepsis model (61).

To overcome this limitation, an innovative strategy utilized a recombinant *Mycobacterium bovis* BCG strain, genetically modified to express a fusion protein between PspA (clade 2) and detoxified pneumolysin (PdT). Neonatal mice received a single intraperitoneal dose of rBCG-PspA-PdT on the fifth day of life, followed by a booster with the aluminum-adjuvanted recombinant protein on the 12th day. Intranasal challenge with the WU2 strain (serotype 3; clade 2) at 21 days resulted in 100% survival, demonstrating the formulation's ability to induce functional protective immunity at a critical phase of immune development (40).

Finally, Goulart et al. investigated the use of BCG as a live vaccine vector expressing pneumococcal proteins for protection against lethal *S. pneumoniae* infection. C57BL/6 mice were immunized with recombinant BCG (rBCG) strains individually expressing SP0148, SP2108, or the PspA-PdT fusion protein, or with a mixture of these strains (rBCG Mix), followed by a booster dose containing the respective recombinant proteins (rMix). The SP0148 and SP2108 proteins,

individually expressed by recombinant *M. bovis* BCG strains, are not variants of PspA but rather distinct pneumococcal surface proteins associated with transport and adhesion mechanisms, respectively. Only the rBCG PspA-PdT construct expresses pneumococcal surface protein A (PspA) fused to detoxified pneumolysin (PdT). The sepsis model was induced by pulmonary aspiration of the virulent WU2 strain (serotype 3), and animals were monitored for 15 days to assess survival rate. Results showed that only co-administration of multiple pneumococcal antigens expressed in a live vector followed by a booster with recombinant proteins (rBCG Mix/rMix), was able to significantly protect mice against lethal challenge, with 90% survival, compared to total mortality observed in the control group (62). These findings underscore the potential of the multivalent approach based on recombinant proteins delivered via BCG vector as a promising serotype-independent vaccine candidate for the prevention of pneumococcal sepsis (62, 63).

Figueiredo et al. evaluated the efficacy of chitosan-incorporated nanoparticles (NPs) as adjuvants in different PspA-based formulations: (1) PGA-co-PDL/HCl-CS polymeric NPs with surface-adsorbed PspA, and (2) PLGA/HCl-CS NPs encapsulating PspA. Prior to vaccination, mice were anesthetized intraperitoneally with a xylazine/ketamine solution (20 mg/kg xylazine and 100 mg/kg ketamine). Female BALB/c mice received pulmonary mucosal vaccination with either empty NPs or NPs containing 2 μg or 6 μg PspA4Pro. Control groups included subcutaneous (sc) injection of saline or purified PspA4Pro (2 μg or 6 μg in 100 μL), plus pulmonary instillation of purified PspA4Pro (2 μg or 6 μg in 50 μL, administered to one nostril under anesthesia). Mice were immunized twice at 14-day intervals and challenged 21 days post-final immunization. Results demonstrated that both formulations induced PspA-specific serum IgG production but with distinct functional profiles. Mice immunized with encapsulated PspA (PLGA/HCl-CS) showed 100% survival after lethal *S. pneumoniae* challenge, versus 83% survival with adsorbed PspA (PGA-co-PDL/HCl-CS). The encapsulated formulation more efficiently activated dendritic cells, with elevated expression of activation markers (CD40 and MHC class II), suggesting enhanced antigen presentation. Structural analysis revealed that encapsulated PspA maintained structural integrity post-release, while surface adsorption impaired functional properties like lactoferrin-binding capacity. These findings demonstrate that pulmonary immunization with PspA-containing nanoparticles - particularly encapsulated formulations - provides robust and durable protection against lethal pneumococcal infection (64).

Other studies have investigated the use of genetically inactivated bacteria as vaccine delivery systems. Castro et al. explored the potential of recombinant *Bordetella pertussis* expressing PspA4Pro as a delivery system/adjuvant for immunization against *S. pneumoniae*. For this purpose, *B. pertussis* strains producing PspA from clade 4 (PspA4Pro), fused to the N-terminal region of filamentous hemagglutinin (Fha44), were used to generate the wP^PspA4Pro formulation. Female BALB/c SPF mice were immunized subcutaneously under different regimens: wP^PspA4Pro alone; purified PspA4Pro protein; or the fusion protein Fha44:PspA4Pro. In the prime–boost strategy, animals received the recombinant wP^PspA4Pro followed by booster doses with purified PspA4Pro or saline. The Fha44:PspA4Pro group received two subcutaneous doses of the fusion protein, 15 days apart. Isolated PspA4Pro and Fha44 were used as controls. Twenty-one days after the final immunization, mice were challenged intranasally with *S. pneumoniae* ATCC6303 (serotype 3). The results showed that immunization with wP^PspA4Pro induced low anti-PspA4 IgG levels and did not protect against the lethal pneumococcal challenge. Purified PspA4Pro induced higher antibody levels and greater protection against pneumococcal infection than the prime–boost strategies, with sera from PspA4Pro-immunized mice being the only ones to promote significant C3 complement deposition on the pneumococcal surface. Finally, purified Fha44:PspA4Pro induced high anti-PspA4Pro IgG titers but failed to confer protection, suggesting that antibodies elicited by the fusion protein were not directed against protective epitopes (65).

To evaluate the protection conferred by a combination of pneumococcal Choline-Binding Proteins - CBPs, including PspA, PspC, LytA, B and C, as well as other less prevalent proteins), the study by Dion & Ashurst investigated the immunization of BALB/c mice with native proteins extracted from different *S. pneumoniae* strains, including non-encapsulated mutant strains that expressed different PspAs as well as PspA-negative strains. The animals were immunized subcutaneously, without the use of adjuvants, with three doses containing 1 µg of CBPs eluted with 2% choline chloride from strains cultured in THY medium. Three weeks after the last immunization, mice were challenged by intranasal aspiration with strains A66.1 (serotype 3, PspA clade 2) or ATCC 6303 (serotype 3, PspA clade 5). Against the A66.1 challenge, survival rates were 100% for Rx1 CBPs, 92% for Rx1pspA4, 83% for Rx1ΔpspA, 75% for Rx1pspA1, and 42% for rPspA4, while the control group (saline) showed only 8% survival. In the ATCC 6303 challenge, the highest survival rates were observed in groups immunized with Rx1pspA4 (58%) and Rx1ΔpspA (42%) CBPs, followed by Rx1pspA1 (33%), rPspA4 (17%), Rx1 (8%), and saline

(0%). These data indicate that immunization with native CBPs, even in the absence of adjuvant, can confer significant protection against pneumococcal sepsis, with variable efficacy depending on the antigenic composition of proteins extracted from each strain (66). Furthermore, the higher survival rates found in groups immunized with CBPs including PspA reinforces its contribution to protective immune responses.

In an innovative approach, Suzuki et al. developed a fusion vaccine combining PspA with the receptor-binding domain (RBD) of parvovirus B19. The RBD corresponds to the VP1 unique region (VP1u) of the viral capsid, which contains the main neutralizing epitopes and is essential for viral attachment and entry into host cells, making it a strategic target for vaccine development. The parvovirus B19 is a single-stranded DNA human pathogen that can cause different clinical manifestations, ranging from infectious erythema in children to severe complications. Considering that both B19 and *S. pneumoniae* are relevant respiratory mucosal pathogens capable of causing serious diseases, the fusion of the B19 RBD with pneumococcal PspA was proposed as an innovative approach to induce protective immunity against two distinct agents in a single formulation. The chimeric protein RBD-PspA was expressed in *E. coli*, purified, and used for intramuscular immunization of BALB/c mice in three doses administered at two-week intervals. The aim was to evaluate whether the formulation could induce protective immunity against two distinct pathogens. To assess protection against pneumococcal pneumonia, two weeks after the final dose animals were inoculated intranasally with *S. pneumoniae* serotype 3 (strain ATCC6303) and monitored for 14 days. Mice immunized with RBD-PspA showed 100% survival, similar to the group immunized with PspA alone, whereas all unvaccinated control animals succumbed to infection. Moreover, the RBD-PspA formulation induced high serum IgG levels against both RBD and PspA, accompanied by class switching from IgM to IgG, an effect not observed with RBD alone. *In vitro* assays using splenocytes indicated that immunization with the chimeric protein activated PspA-specific helper T cells, which secreted IL-5 and facilitated class switching also against RBD, thereby conferring dual immunity to both antigens (67).

In conclusion, multiple studies support PspA as a versatile serotype-independent vaccine candidate against invasive pneumococcal disease. Its protective efficacy has been consistently demonstrated across multiple formulations, offering broad protection against multiple bacterial strains. Strategies that combine conserved PspA fragments, particularly from the N-terminal and proline-rich regions, with other pneumococcal antigens or adjuvants, have significantly enhanced

immune responses and survival rates in murine sepsis models. Collectively, these findings reinforce the critical role of PspA-based vaccines as a promising path toward broad and effective protection against systemic *S. pneumoniae* infections.

Table 1. Studies evaluating PspA as a vaccine candidate against pneumococcal sepsis

Reference	Model	Formulation	Results
Trentini et al., 2024(40)	Neonatal C57BL/6 mice	rBCG PspA-PdT (prime) followed by rPspA-PdT + $Al(OH)_3$ (boost)	100% survival; IgG1→IgG2c switching; ↑ memory B/T cells; ↑ pro-inflammatory cytokines (IL-6, IL-17, TNF-α, IFN-γ).
		rBCG PspA-PdT (prime only)	Partial protection; high-affinity antibodies; ↑ memory B/T cells; ↓ total serum Ig.
		WT-BCG (prime) followed by rPspA-PdT + $Al(OH)_3$ (boost)	Partial protection via BCG-trained immunity; ↓ memory B cells vs. rBCG PspA-PdT.
		rPspA-PdT + $Al(OH)_3$ (two doses)	Low/non-significant protection; high IgG1 but poor bacterial binding.
Girgis et al., 2020(36)	BALB/c mice	Recombinant proline-rich region (rPR) of PspA + Alum adjuvant (active immunization) or anti-rPR polyclonal antibodies (passive immunization)	No survival benefit after lethal challenge (S. pneumoniae 19F, 1×10^7 CFU); all animals succumbed; vaccination induced anti-rPr IgG titers.
Zhang et al., 2019(41)	BALB/c mice	Recombinant PspA fused to transferrin-binding protein B (PspA-TbpB) + aluminum adjuvant; two intramuscular	Significant protection against lethal challenge (S. pneumoniae ATCC 6303); 100% survival; ↓ bacteremia; ↑ specific IgG.

Afshari et al., 2023(35)	BALB/c mice	Chimeric protein PspA1-5c+p	High cross-reactivity covering different PspA variants. ↑ Serum Bactericidal and opsonophagocytic activities >50% bactericidal and opsonic activity against the tested strains.
Kono et al., 2023(37)	BALB/cByJ mice	Recombinant PspA (PspA2/TIGR4) intranasally administered to adult females before mating, with cholera toxin subunit B adjuvant (first 2 weeks)	Offspring of immunized mothers: ↑ anti-PspA IgG-producing splenocytes; ↑ IgM, IgG1, IgG2a, IgG2b titers; ↑ IFN-γ, IL-17A; prolonged survival after lethal challenge.
		Recombinant PspA (PspA2/TIGR4) intranasally administered to adult females before mating, with cholera toxin subunit B adjuvant (first 2 weeks)	Offspring without maternal immunization or only breastfeeding: no ↑ anti-PspA IgG/cytokines; no improved survival.
Zane et al., 2023(57)	C57BL/6 mice	Recombinant PspA (clade 2) fused to flagellin (FliC) as intrinsic adjuvant, intranasally administered	↑ Serum and mucosal anti-PspA IgG/IgA; ↑ IL-17A; 100% survival after lethal S. pneumoniae (clade 2) challenge; partial cross-protection against clade 3.
Castro et al., 2020(65)	Female SPF BALB/c mice	wPPspA4Pro (inactivated B. pertussis expressing Fha44:PspA4Pro)	Low anti-PspA4Pro IgG; no binding to native PspA; no complement deposition; no protection against lethal challenge.

		Purified PspA4Pro	High anti-PspA4Pro IgG; binding to native PspA; complement deposition; 60% survival after lethal challenge.
		Prime-boost protocol with wPPspA4Pro followed by PspA4Pro	Antibody levels similar to wPPspA4Pro; no improved binding or complement deposition; no protection.
		Purified Fha44:PspA4Pro	Similar IgG levels as PspA4Pro; weaker binding to native PspA; low complement deposition; no protection.
Figueiredo et al., 2022(64)	BALB/c mice	PspA encapsulated or adsorbed in hybrid nanoparticles of PLGA/HCl-CS or PGA-co-PDL/HCl-CS	PLGA/HCl-CS/encapsulated PspA: 100% protection against lethal challenge; PGA-co-PDL/HCl-CS/adsorbed PspA: 83% protection; both formulations activated dendritic cells and preserved PspA integrity.
Chen et al., 2015(54)	CD1 mice	PspA fragments: H70 (SM1 peptide + proline-rich region); CD2 (proline-rich region only). Tested formulations: 1- PspA fragments fused to the chimeric YLN protein (L460D + two CbpA domains: YPT and NEEK). 2- PspA	Protection against lethal challenge. Reduction in meningitis incidence ↑ protection with H70+YLN formulation.

		fragments fused to L460D alone.	
Afshari et al., 2023(55)	BALB/c mice	Chimeric protein PspA1-5c+p fused with PhtD	100% survival, increased protection when compared to individual antigens ↑ bacterial clearance in the spleen. Reduction in bacterial load in blood.
Milani et al., 2023(38)	Female BALB/c mice	Chimeric vaccine: N-terminal PspA fragments fused to non-toxic pneumolysin derivative (PlD)	↑ Cross-protection against strains with heterologous PspAs; ↑ complement C3 deposition on multiple strains; antibodies mediated complement activation; both PspA and Ply antigens contributed to protective effect.
Daniels et al., 2010(31)	CBA/N mice	Recombinant proline-rich (PR) regions of PspA and PspC; passive immunization with anti-PR or anti-NPB monoclonal antibodies	Protection against lethal infection; PR epitopes antibody-accessible on pneumococci; survivors had ↑ anti-PR antibodies; PR/NPB regions conserved and cross-reactive → potential vaccine targets.

Tamborrini et al., 2015(53)	BALB/c mice	PspA(295–307)–CRM197 + alum	Complete protection against lethal challenge with S. pneumoniae; peptide alone did not protect.
Tada et al., 2021(39)	BALB/cCrSlc mice	Intranasal vaccine rPspA + pCA adjuvant	↑ survival in BALB/cCrSlc mice; Robust systemic and mucosal immune responses (IgG and IgA), predominant Th2 profile; combined formulation significantly superior to individual components.
Converso et al., 2017(58)	BALB/c mice	Chimeric protein rPspA-PotD + alum (s.c.)	Protection against lethal challenge with two pneumococcal strains (St A66.1 and ATCC6303); survival comparable to rPspA alone; rPotD alone not protective.
Yu et al., 2018(59)	BALB/c mice	Bivalent vaccine: fusion protein PsaA-PspA23 (clades 2 & 3, family 1 & 2) + recombinant PspA4 (clade 4, family 2) + alum (s.c.)	Protection against lethal challenge with PspA clades 1–5; survival >50% for all strains, up to 100% for some; significantly higher than PPV23 and PBS controls.
		Same bivalent vaccine (PsaA-PspA23 + PspA4 + alum)	Bacterial loads in blood reduced to <10 CFU/ml; near complete clearance for multiple clades; superior to PPV23.

Darrieux et al., 2007 (60)	BALB/c mice	PspA fragments; PspA hybrids PspA1ABC-4B and PspA1ABC-3AB + alum (i.p.)	Cross-protection; ↑ C3 deposition on the bacterial surface.
Piao et al., 2014(61)	C57BL/6J mice	Fusion proteins PspA2+4, PspA2+5, or PspA3+2 + CpG ODN + alum (s.c.)	PspA3+2: significant protection against strains from clades 1–5; sera showed >60% IgG binding to all five clades. PspA2+4 and PspA2+5: protection against clades 2, 4, 5, but failed against clades 1 and 3.
	C57BL/6J mice (sera tested)	Same fusion proteins (PspA2+4, PspA2+5, PspA3+2)	Antiserum from PspA3+2 vaccination bound strongly to isolates of clades 1–4, but weakly to clade 5; PspA2+4 and PspA2+5 sera had stronger binding to clade 5 isolates.
Majunder et al., 2024(63)	Swiss Webster and C57BL/6 mice	Outer membrane vesicles (OMVs) from engineered *Y. pseudotuberculosis* expressing PspA (OMV-PspA, i.m.)	100% survival against secondary Spn D39 challenge (short-term); 80% long-term protection at day 205; sera transfer conferred 80% survival, confirming antibody-mediated protection.
			90% survival against secondary Spn A66.1 (serotype 3) challenge; cross-protection confirmed against multiple clinical isolates (high OPK activity).

Goulart et al., 2017(62)	C57BL/6 mice	rBCG strains expressing PspA-PdT, SP0148, SP2108 (prime) + recombinant proteins (boost)	90% survival after lethal challenge (vs 100% mortality controls).
Goulart et al., 2013(56)	BALB/c mice	Subcutaneous recombinant fusion proteins: PspA1-PlD1, PspA1-PlD2, PspA2-PdT (fusions of PspA clades 1 or 2 with detoxified pneumolysin mutants), with alum	↑ anti-PspA & anti-Ply antibodies; ↑ complement deposition; ↑ opsonophagocytosis (heterologous PspA strains); sera neutralized Ply hemolysis; ↑ mouse survival; PspA1-PlD1 & PspA1-PlD2 → 100% protection
Dion e Ashurst, 2025(66)	BALB/c mice	s.c. Choline-binding proteins (CBPs) extracted from S. pneumoniae including or excluding PspA, and	100% survival with CBPs including PspA. Efficacy varies according to the antigenic composition of the proteins extracted from each strain.
Suzuki et al., 2021(67)	BALB/c mice	Chimeric RBD-PspA protein (i.m.)	100% survival; high serum IgG levels against RBD and PspA. Class switching from IgM to IgG; Activation of PspA-specific helper T cells

Pneumonia

Community-acquired pneumonia remains a significant public health concern, with estimated hospital costs reaching up to 9 billion dollars annually. The 30-day hospital mortality rate can reach 22%, making it the leading cause of death among infectious diseases. Before the advent of antibiotics, this bacterium was estimated to account for about 95% of cases. Currently, although this proportion has decreased, pneumococcus is still identified in up to 15% of cases in the United States and approximately 27% globally (66). Vaccines utilizing PspA have emerged as promising alternatives to conjugate vaccines due to their ability to induce cross-protection against different serotypes (68). Given that *S. pneumoniae* has historically been the bacterial pathogen most frequently associated with pneumonia worldwide, understanding *S. pneumoniae* pulmonary infection models and experimentally evaluating the efficacy of PspA-containing vaccine formulations is crucial for developing more comprehensive immunological strategies. These strategies should provide effective protection against diverse and potentially invasive strains (66). Table 2 includes a summary of PspA-based vaccines in pneumonia.

The study by Rodrigues et al. (2024) developed an experimental pneumococcal pneumonia vaccine based on nanocomposite microparticles (NCMPs) carrying liposomes (LPs) containing two PspA variants: clade 1 (PspA1) and clade 4 (PspA4Pro). Notably, the researchers used recombinant protein fragments corresponding to the mature N-terminal region through the proline-rich domain, encompassing PspA's major immunogenic epitopes. The liposomes consist of lipid bilayer vesicles that encapsulate antigenic proteins, protecting them from degradation while enabling controlled release. The nanocomposite microparticles represent solid structures formed through liposome drying, creating a stable powder ideal for mucosal delivery. This approach aimed to create a serotype-independent vaccine capable of protecting against diverse *S. pneumoniae* strains by stimulating pulmonary immunity through intranasal administration. Formulations were produced via microfluidics and converted into NCMPs, which demonstrated excellent stability and protein activity preservation. Female BALB/c mice received intranasal immunization with LP/NCMP suspensions (6 μg total protein). Control groups included saline solution, empty LP/NCMPs (antigen-free), and subcutaneous immunization with purified PspA1, PspA4Pro, or their mixture without nanoparticles. Animals received two doses at 15-day intervals, followed by intranasal challenge 21 days post-boost with *S. pneumoniae* strains ATCC 6303 (ST3, PspA5) or A66.1 (ST3, PspA2). Subcutaneous immunization failed to induce mucosal antibodies, whereas

LP/NCMPs generated low but detectable respiratory and vaginal mucosal IgA/IgG. Both individual and combined LP/NCMP formulations demonstrated cross-protection and binding to pneumococcal strains expressing different PspA clades. A balanced IgG1/IgG2a response was observed. Lung-targeted α-GalCer-containing LP/NCMPs induced pulmonary resident memory T cell ($CD4^+$ TRM) formation (69). These results suggest that mucosal immunization with microparticles harboring PspA is a promising approach to reduce pneumococcal pneumonia via induction of protective antibodies.

Roberts et al. progressed to coinfection models simulating more complex clinical scenarios, evaluating the protective potential of PspA as a vaccine antigen in a murine model of secondary *S. pneumoniae* infection following influenza A infection. This strategy was chosen considering that *S. pneumoniae* is one of the main agents of post-influenza complications, with synergy between the two pathogens. The study employed a respiratory coinfection model where BALB/c mice were initially infected with influenza A virus and then challenged intranasally with *S. pneumoniae* during the recovery phase, mimicking the increased susceptibility to bacterial infection observed in humans after influenza. The primary objective was to compare the coinfection effect in mice previously immunized with Prevnar (a vaccine containing capsular polysaccharides from 7 or 13 *S. pneumoniae* serotypes) or with PspA (family 1, clade 2), using the complete recombinant PspA protein. The mice were vaccinated intramuscularly, received a booster 3 weeks after administration, and antibodies were extracted at week 4. Two weeks post-vaccination, the animals were intranasally infected with 10-15 PFU of H1N1 strain A/Puerto Rico/8/1934 (PR8). Body weight was monitored daily, and between days 8-10 post-viral infection - when mice began regaining weight - they were challenged with 1.5×10^4 CFU of *S. pneumoniae* type 2 (strain D39) or with 5×10^2, 5×10^3, or 5×10^4 CFU of *S. pneumoniae* type 3 (strain A66.1), also via intranasal administration. The results indicate that PspA immunization induced higher IgG levels compared to mice vaccinated with Prevnar or unvaccinated controls. It was also observed that protection conferred by both vaccines depended on the bacterial dose used for challenge. At a concentration of $20 \times LD_{50}$ of strain A66.1, all vaccinated mice were protected. PspA immunization also resulted in significant reduction of bacterial load in bronchoalveolar lavage and lung tissue, along with complete bacterial clearance in blood. Finally, unlike Prevnar, PspA conferred protection against *S. pneumoniae* D39, a serotype not included in the conjugate vaccine formulation at the time of the study (70).

Similarly, Majumder et al. tested a vaccine based on outer membrane vesicles expressing PspA (OMV-PspA), also aimed at preventing pneumococcal infection following influenza. *Yersinia pseudotuberculosis* (YptbS46) containing the Asd pSMV92 plasmid can synthesize the α-helical region of PspA, corresponding to amino acid residues 3 to 285, along with monophosphoryl lipid A as an adjuvant, leading to production of outer membrane vesicles (OMVs) containing high amounts of recombinant protein, designated OMV-PspA. Six-week-old male and female Swiss Webster mice were immunized intramuscularly with OMVs in PBS, with a booster dose after 21 days. As controls, they used rPspA adsorbed to Alhydrogel or PBS alone. Intranasal challenge occurred two times. The first challenge was performed on day 36 after the primary dose, with intranasal infection by H1N1 A/California/04/2009 (CA04); nine days later (day 45), the animals were challenged with *S. pneumoniae* (strain D39). A second, later challenge protocol involved influenza infection on day 196 and *S. pneumoniae* challenge on day 205, using the same strains. The OMV-PspA immunization provided 80% protection against secondary *Spn* challenge, while rPspA immunization provided only 20% protection, and the OMV-NA and PBS groups showed no protection. Monitoring revealed that in the OMV-PspA immunized group, IL-6 and IL-1β levels at 1 DPV (days post-vaccination) were highest, decreasing from 2 DPV onward, with higher serum CRP levels compared to other groups at 1 DPV. The OMV-PspA vaccine induced high titers of anti-PspA IgG, mainly in mucosa, with no detectable IgA titers. There was also a significant increase in lung PspA-specific $CD4^+$ and $CD8^+$ T cell populations compared to other groups. Thus, OMV vaccination may provide non-specific protection against influenza viral infection while inducing less lung damage. However, this combination has safety limitations. OMV-PspA caused substantial weight loss and prolonged elevation of alveolar macrophages in mice, requiring further investigation to identify the specific OMV components responsible for this reactogenicity (63).

Complementing the analysis of mucosal vaccine strategies, Ortiz Moyano et al. investigated the use of bacterium-like particles (BLPs) derived from *Corynebacterium pseudodiphtheriticum* strain 090104 (Cp 090104) as adjuvants for pneumococcal vaccines containing either PspA protein or the commercial polysaccharide vaccine Pneumovax® 23 (PPV). The aim was to enhance both mucosal and systemic immune responses. For this purpose, the authors developed two distinct experimental models using three-week-old female Swiss albino mice, a strain selected for its susceptibility to pneumococcal infection. To test primary and secondary infection, different approaches were employed. In the first model, animals received either live Cp 090104 or BLPs

intranasally for five consecutive days. On day six, they were nasally challenged with *S. pneumoniae* serotype 6B or 19F to evaluate whether Cp or BLPs alone, without prior immunization, could induce protection against primary infection. In the second model, mice were immunized in a prime-boost regimen with three intranasal doses on days 0, 14, and 28, using either the commercial Pneumovax® 23 vaccine or the recombinant chimeric protein vaccine PSPF (PsaA-Spr1875-PspA-FliC), combined with either Cp 090104 or BLPs. On day 30, Poly(I:C), a TLR3 agonist, was administered to induce viral-like pulmonary inflammation. On day 33, animals were intranasally challenged with *S. pneumoniae* serotype 19F to simulate secondary pneumococcal infection. Key findings showed that both Cp 090104 and derived BLPs stimulated airway innate immunity and served as effective adjuvants for both vaccines. The PSPF formulation, containing fragments of PsaA, Spr1875 and PspA proteins fused to flagellin FliC (as an immunomodulatory adjuvant), induced strong immune responses. The study documented increased pneumococcal-specific IgA and IgG production in bronchoalveolar lavage (BAL) and serum; elevated IFN-γ and IL-4 levels (indicating Th1 and Th2 cell activation); and enhanced antibody deposition in respiratory mucosa, particularly with Cp 090104. Vaccination reduced bacterial lung load in both primary and secondary infections. Thus, mucosal adjuvants with either PPV or PSPF containing PspA proved effective, with the advantage that PspA promoted broader immune responses against more conserved proteins and provided higher serotype coverage (71).

Still focusing on complex respiratory infections, Kramskaya et al. proposed a combined vaccination strategy involving influenza and a chimeric protein containing pneumococcal antigens. The authors studied a combined immunization with live attenuated influenza vaccine (LAIV) and a recombinant pneumococcal chimeric protein (PSPF), composed of PsaA, PspA and Shr1875 - three *S. pneumoniae* surface proteins - associated with flagellin, a potent activator of innate immune response. Eight to ten-week-old female mice were immunized in different ways. One group received intranasal inoculation with live influenza vaccine PBS (LAIV), PSPF vaccine containing the recombinant PSPF polypeptide diluted in PBS, mixed LAIV + PSPF vaccine or PBS; the control group received single viral and bacterial infection. The vaccine was repeated after 21 days. On days 53 and 54 of the experiment, animals were challenged with different protocols: one group was initially infected with *S. pneumoniae* (strain 73 of serotype 3) and, 24 hours later, with influenza virus A/South Africa/3626/13 (H1N1), configuring secondary influenza infection. Another group received viral infection first, followed by *S. pneumoniae*, representing secondary

pneumococcal infection. Both infections were performed intranasally with sublethal pathogen doses. In the pneumonia model, viral and bacterial loads in lungs were analyzed 48 hours after influenza infection (i.e., 24 hours after bacterial superinfection). Results demonstrated that mice vaccinated with the LAIV + chimeric protein combination showed significant reduction in pulmonary viral load, with virus isolation only at minimal titers. In contrast, groups receiving only chimeric protein or PBS showed high viral load. Similarly, the lowest bacterial lung load was observed in the virus-bacteria vaccinated group, while other groups showed high levels of *S. pneumoniae* in lungs. Furthermore, vaccination with the chimeric protein, alone or combined with LAIV, induced high levels of IgG specific against bacterial components, with the most robust humoral response in the combined formulation group, suggesting a synergistic effect. The LAIV-only vaccinated group showed no significant IgG response against bacterial antigens. Thus, combined immunization prevented severe respiratory infections caused by viral-bacterial coinfection, reducing pulmonary pathogenic load and progression risk (72). These findings support the potential of a combined vaccination strategy employing LAIV and a chimeric pneumococcal protein to prevent secondary bacterial and viral respiratory infections (ou to prevent severe outcomes associated with influenza–*S. pneumoniae* coinfections).

Wiedinger et al. investigated molecular strategies to target PspA to antigen-presenting cells by developing a vaccine that fused the N-terminal region of PspA (family 1, clade 2, comprising 303 amino acids) to the Fc portion of murine IgG2a (IgG2a Fc-PspA). The Fc region represents the constant domain of immunoglobulins responsible for interactions with immune receptors (FcγR). Specifically, the IgG2a isotype exhibits high affinity to Fcγ receptors (FcγRI and FcγRIII), promoting enhanced uptake by antigen-presenting cells (APCs) such as macrophages and dendritic cells. This approach aimed at boosting immune responses by facilitating antigen internalization and presentation. Fcγ receptors (FcγR) serve as primary regulators of IgG effector functions *in vivo*, controlling critical processes including antigen presentation, antibody-dependent cellular cytotoxicity (ADCC), phagocytosis, and activation/proliferation of myeloid cells. The strategy focused on delivering PspA to APCs via FcγR, leveraging the distinct immunomodulatory properties of different IgG subtypes. For immunization, mice were divided into groups of five and administered either 20 µl of PBS, 10 µg of IgG1 Fc or IgG2a Fc fusion proteins, or 5 µg of PspA alone. All groups received booster immunizations on days 14 and 28. Two weeks after the final dose, animals were challenged with 8×10^6 colony-forming units (CFU) of *S. pneumoniae* strain

A66.1 and monitored for 21 days. Results demonstrated that immunization with IgG2a Fc-PspA induced significantly higher levels of PspA-specific antibodies across all evaluated isotypes, including IgG and IgA. In contrast, fusion with IgG1 Fc failed to enhance PspA immunogenicity compared to the protein alone. The IgG2a Fc-PspA-induced immune response showed a predominant Th1 profile, with elevated concentrations of IL-2, IFN-γ, and TNF-α in mouse lungs. Additionally, researchers observed increased numbers and frequency of AM1 alveolar macrophages, characterized by a proinflammatory phenotype and production of IL-12, IL-23, and nitric oxide. Functionally, bacterial load in lungs was significantly lower in the IgG2a Fc-PspA group at 24 hours post-challenge, while groups immunized with PspA alone or IgG1 Fc-PspA showed high pulmonary bacterial loads comparable to controls. These findings reinforce PspA's central role as a vaccine antigen and demonstrate how antigen presentation format and the specific IgG isotype used for fusion directly influence the magnitude and quality of protective immune responses. Thus, the Fcγ receptor-targeting strategy - particularly through IgG2a - proved especially promising to promote protective Th1-based immune responses (73).

In addition to mucosal strategies, several subcutaneously administered vaccine formulations have been proposed, particularly those based on fusion proteins combining PspA with other pneumococcal antigens (57, 65, 74, 75). Santos et al. evaluated the immune response and protection induced by a vaccine composed of a fusion protein between PspA (pneumococcal surface protein A) and a detoxified derivative of pneumolysin (PlD1) in a murine model of lobar pneumococcal pneumonia. The chimeric construct used the N-terminal region of PspA from a serotype 14 strain (St 245/00), classified as PspA1 (family 1), containing predominantly the α-helical domain (αHD), which harbors the main immunogenic epitopes of the protein. Female BALB/c mice were immunized subcutaneously with three doses of the chimeric protein rPspA1-PlD1, administered at 14-day intervals, using $Al(OH)_3$ as an adjuvant. The control group received only the adjuvant. Two weeks after the last immunization, the animals were challenged intranasally with *S. pneumoniae* serotypes 14 (St 245/00) and 19F (P854), both considered to have low invasiveness, while colonizing the lungs and replicating the hallmarks of lobar pneumonia. Seven days after challenge, pulmonary bacterial loads, cytokine levels in the bronchoalveolar lavage fluid (BALF), and airway inflammation were assessed. The results showed that the rPspA1-PlD1 vaccine conferred significant protection against pneumococcal pneumonia, associated with an early and controlled local inflammatory response. There was a significant increase in TNF-α and IL-6

levels in BALF, with rapid mobilization of immune cells within 6 hours of infection, peaking at 12 hours, and followed by a marked reduction at 24 hours. Lung integrity was preserved in immunized mice, which showed only mild leukocytic infiltration, whereas control animals presented moderate inflammation, congestion, and alveolar hyperplasia. Pulmonary bacterial loads in vaccinated animals remained very low throughout the experiment. Furthermore, antibodies generated by the vaccine recognized heterologous *S. pneumoniae* strains, suggesting potential for cross-protection. The study concluded that the rPspA1-PlD1 formulation was capable of inducing opsonophagocytic antibodies, promoting a balanced local inflammatory response, and providing protection against both pulmonary and systemic infection, with higher survival rates in immunized mice compared to the control group (74).

Yokota et al. adopted a distinct strategy, applying a prime–boost approach with different immunization routes using fragments from the N-terminal region of PspA The formulation employed an intramuscular priming followed by a mucosal booster, aiming to simultaneously stimulate systemic and mucosal, immune responses. In mice, the booster was administered intranasally, whereas in monkeys the booster was delivered intratracheally. aiming to simultaneously stimulate systemic and mucosal immune responses. In BALB/c mice, the PspA3 + 2 protein was emulsified in a water-in-oil-in-water (WOW) system and adjuvanted with curdlan (a β-glucan derived from bacteria) and CpG-ODN. Immunization induced the production of serum IgG and secretory IgA (SIgA) in bronchoalveolar lavage fluid (BALF) and conferred significant protection against respiratory infection caused by *S. pneumoniae* serotype 6A (family 1, clade 2) compared with control groups. The preclinical model also included *Macaca fascicularis* monkeys, which, after the prime–boost regimen with PspA3 + 2/WOW adjuvanted with curdlan alone or in combination with CpG-ODN, exhibited detectable levels of lung-specific SIgA, indicating the potential of this formulation to elicit effective immune responses in nonhuman primates (76).

Also combining different forms of antigen presentation, Goulart et al. employed a recombinant BCG strain expressing PspA–PdT, administered via *Mycobacterium bovis* BCG (rBCG PspA–PdT). The vaccine construct contained the N-terminal region of PspA, which comprises the immunogenic α-helical domains. Female C57BL/6 mice were immunized subcutaneously with a primary dose of the recombinant BCG expressing the chimeric protein, followed 30 days later by a booster dose of recombinant PspA–PdT (rPspA–PdT) adsorbed into $Al(OH)_3$. Control groups received wild-type BCG (WT-BCG), rPspA–PdT alone, or saline. To

assess the immune response, animals underwent intranasal instillation with *S. pneumoniae* serotype 3 (strain WU2, PspA^{+}) in a murine aspiration pneumonia model. The heterologous immunization regimen (rBCG PspA–PdT / rPspA–PdT) promoted early bacterial clearance from the lungs, with reduced bacterial loads in bronchoalveolar lavage fluid (BALF) as early as 12 hours post-challenge, and no detectable bacteria after 48 hours. The vaccination scheme induced both humoral and cellular immune responses, including increased production of anti-PspA IgG, greater C3 deposition on the bacterial surface, reduced neutrophil influx, and lower inflammatory cytokine levels, while preventing tissue damage and improving animal survival (77). These findings reinforce the efficacy of BCG as an effective antigen delivery platform for vaccines targeting respiratory pathogens, with induction of robust antibody and cellular responses.

Reflecting its strong protective efficacy against systemic infection, PspA-based formulations consistently conferred significant protection against pneumococcal pneumonia, even in complex coinfection settings with influenza. These findings support the use of PspA as a strong candidate to enhance protection against community-acquired pneumonia and reduce disease burden, which may be superior to the current serotype-specific conjugate vaccines.

Table 2. Studies evaluating the protective efficacy of Pspa against pneumococcal pneumonia

Reference	Model	Formulation	Results
Goulart et al., 2020(77)	C57BL/6 mice	rBCG expressing PspA-PdT (prime dose) followed by rPspA-PdT + $Al(OH)_3$ (boost dose)	↓ Bacterial load in BALF (~10^2 CFU at 12h; clearance at 48h); no blood dissemination; ↑ neutrophil influx; ↑ $CD4^+$ lymphocytes; ↑ anti-PspA IgG1/IgG2c; higher complement deposition.
Ortiz Moyano al., 2024(71)	C57BL/6 mice	Recombinant $PspA_4Pro$ (2 µg) + curdlan adjuvant; two intranasal doses 14 days apart	↓ Lung bacterial load (~2 log10 CFU at 48h); ↑ anti-PspA IgG/IgA in serum and respiratory tract; ↑ $CD4^+/CD8^+$ lung recruitment.
Dos Santos et al., 2022(74)	BALB/c mice	Fusion protein rPspA-PlD1 (N-terminal PspA + pneumolysin derivative PlD1) with $Al(OH)_3$ adjuvant	↓ Lung bacterial load (day 7, 1/7 animals bacteria-free); ↑ IL-6, TNF-α; ↓ leukocyte infiltration vs. control.
		PspA alone with $Al(OH)_3$ adjuvant	Trend toward ↓ bacterial load (not significant); no significant change in BALF cellular infiltrate.
		PlD1 alone with $Al(OH)_3$ adjuvant	Similar lung bacterial loads vs. control; ↑ BALF leukocytes (6–24h); no significant protection.
Rodrigues et al., 2024(69)	BALB/c mice	Recombinant PspA (clade 1) with aluminum adjuvant, intramuscular administration	↑ Anti-PspA IgG (mainly IgG1/IgG2a); ↓ lung bacterial load and tissue inflammation after intrapulmonary clade 1 challenge; ↓

			nasopharyngeal load after homologous (clade 1) challenge; partial protection against heterologous (clade 2).
Roberts et al., 2019(70)	BALB/c mice	rPspA or Prevnar (PCV7/PCV13), (i.m.)	↑ IgG levels in PspA 100% protection against A66.1 PspA significantly reduced bacterial load in BAL and lungs, with complete clearance in blood; ↑ protection against D39 with PspA
Kramskaya et al., 2019(72)	Female BALB/c mice	Live attenuated influenza vaccine (LAIV), recombinant chimeric PSPF protein (PsaA, PspA, and Shr1875 associated with flagellin), i.n.	The combined LAIV + PSPF formulation significantly reduced pulmonary viral and bacterial loads; ↑ IgG levels.
Wiedinger et al., 2020(73)	C57BL/6 mice	PspA-IgG2a-Fc (adjuvant-free)	Effective protection; ↑ AM1; ↑ lung DC subsets; ↑ Th1 cytokines; ↑ PspA-specific IgG/IgA.
		PspA-IgG1-Fc (adjuvant-free)	Minimal benefit vs. PspA alone; inhibitory FcγRIIB interaction.
	C57BL/6 mice (animal deficient for FcγRIIB)	PspA-IgG1-Fc in FcγRIIB KO	Enhanced protection; ↑ B cell maturation and proliferation.
Yokota et al., 2023(76)	Mouse C57BL/6	Fusion PspA + CpG and/or curdlan (prime i.m. + boost i.n.)	↑ Serum IgG; ↑ Bronchoalveolar IgA; effective prevention of pneumococcal infection.

	Cynomolgus macaque	Fusion PspA + CpG and/or curdlan (prime i.m. + boost intratracheal)	↑ Serum IgG; ↑ Bronchoalveolar IgA; effective prevention of pneumococcal pneumonia
Majunder et al., 2024(63)	Swiss Webster and C57BL/6 mice	Outer membrane vesicles (OMVs) from engineered *Y. pseudotuberculosis* expressing PspA (OMV-PspA, i.m.)	100% protection (influenza–S. pneumoniae coinfection); ↓ lung damage; ↓ bacterial burden; balanced Th1/Th2; strong lung CD4+/CD8+ T-cell responses; ↑ opsonophagocytic activity

Otitis

Otitis media (OM) is an inflammation of the middle ear, most common in children, associated with infectious, allergic, anatomical, genetic, and environmental factors. *S. pneumoniae* represents the primary bacterial pathogen involved in this disease The acute form of otitis (AOM) typically occurs following upper respiratory tract infections (URTIs) and causes fever, otorrhea, irritability, and in severe cases, hearing loss. Otitis media with effusion (OME) is characterized by non-purulent fluid behind the tympanic membrane without pain (78, 79) , while the chronic form (COM) involves pain, persistent discharge, and hearing impairment (79). In the U.S., annual OM-related costs exceed $5 billion (79, 80) , and globally, the disease accounts for approximately 28,000 deaths and nearly half of permanent hearing loss cases (78, 81). Multiple studies have investigated PspA's role in OM pathogenesis and its potential as a vaccine target, employing distinct experimental models including chinchillas, rats, and mice, along with *in vitro* approaches (78, 82-86), as summarized in table 3.

The study published by Schachern et al. employed an experimental model involving bilateral otitis media induction in adult chinchillas, using *S. pneumoniae* strain D39 (serotype 2, NCTC 7466) to evaluate the role of surface proteins PspA and PspC in virulence. Four experimental groups were bilaterally inoculated in the middle ear with: (1) Wild-type D39 (with

intact *pspA* and *pspC* genes); (2) PspA⁻ mutant (lacking *pspA* gene); (3) PspC⁻ mutant (lacking *pspC* gene); and (4) PspA⁻/PspC⁻ double mutant (deficient in both proteins). Inoculation was performed via intrabullar injection (directly into the tympanic bulla) with 0.5 ml bacterial suspension per middle ear under ketamine hydrochloride/acepromazine maleate anesthesia. Forty-eight hours after inoculation, animals were euthanized for sample collection: middle ear effusions for bacterial colony-forming unit (CFU) counts; cochleae and round window membranes (RWM) for histological analysis including quantification of inflammatory infiltrate (polymorphonuclear and mononuclear cells) in toluidine blue-stained sections examined by light microscopy. Effusions showed high bacterial loads in wild-type and PspC⁻ groups, while PspA⁻ and double mutant groups had undetectable bacteria. Histologically, wild-type and PspC⁻ groups exhibited intense inflammatory infiltration, whereas PspA-deficient groups showed reduced inflammatory responses. These findings demonstrate PspA's critical role in pneumococcal otitis media (84).

Complementing those results, a study comparing wild-type strains with isogenic *pspA*⁻ or *ply*⁻ mutants in a chinchilla otitis media model showed that loss of PspA led to rapid clearance of *S. pneumoniae*, with no viable bacteria detected in middle ear samples and substantially reduced inflammatory infiltrate. Meanwhile, both wild-type and double mutant *Ply*⁻/*PspA*⁻ strains exhibited marked bacterial growth with significantly higher counts compared to the initial inoculum. Furthermore, animals infected with the PspA-negative strain showed preserved middle and inner ear histology without round window membrane thickening or bacterial presence in the scala tympani. These results confirm PspA as a critical virulence factor in pneumococcal otitis media and underscore its potential as a vaccine target for preventing middle ear infection and inflammation (85).

Schachern et al. also investigated how the absence of PspA, PsaA, and pneumolysin affects the structure and permeability of the round window membrane (RWM) - the primary interface between middle and inner ear. PspA deletion resulted in markedly reduced virulence: only one animal died, and surviving subjects showed no bacteria in either RWM or scala tympani. Inflammation was limited to the RWM epithelium in just two animals. These findings demonstrate PspA's direct contribution to pneumococcal penetration into cochlear structures, underscoring its role in OM progression to deeper infections (32).

A study by Tsuprun et al. investigated the role of PspA protein in inducing sensorineural hearing loss associated with the disease. The research was based on the observation that, although

OM is generally linked to conductive hearing loss, there is growing evidence that bacterial products and inflammatory mediators can cross the round window membrane, reach the inner ear, and cause cochlear damage. To explore this hypothesis, healthy chinchillas were inoculated in the tympanic bulla with either *S. pneumoniae* serotype 2 (wild-type D39 strain) or isogenic strains deficient in pneumolysin or PspA, as well as a control group that received only phosphate-buffered saline. Each animal received 0.5 mL of the bacterial suspension, and auditory function was assessed before and 28 days after infection using auditory brainstem responses (ABRs) at frequencies from 1 to 32 kHz, followed by histopathological analysis of the cochlea. The results showed that infection with the PspA^{-} strain completely prevented sensorineural hearing loss and caused no cochlear damage, even when administered at a higher dose than the wild-type strain. In contrast, the D39 strain caused a significant elevation in hearing thresholds and structural alterations in the cochlea. These findings highlight the contribution of PspA to OM pathogenesis and its relevance as a promising immunogen for vaccine development aimed at preventing hearing-related complications (86).

The protective efficacy of PspA immunization against pneumococcal AOM was evaluated by Habets et al. in a mouse model of coinfection with influenza A (H3N2 strain) followed by *S. pneumoniae* (serotype 19F) infection—a clinically relevant condition often associated with the progression of pneumococcal colonization to middle ear infection. Female mice aged 6 to 8 weeks from BALB/c, C57BL/6 (B6), B6.μMT$^{-/-}$ (antibody-deficient), and B6.IL17RA$^{-/-}$ (lacking the IL-17 receptor) strains were used. Animals were immunized intranasally with three doses of purified rPspA (5 μg per dose), obtained from the *S. pneumoniae* TIGR4 strain. The formulation was administered with or without the adjuvant cholera toxin subunit B (CTB). Three weeks after the final immunization, mice were challenged intranasally with influenza A virus (strain A/Udorn/307/72 – H3N2). Three days after viral infection—allowing sufficient time to simulate virus-induced respiratory mucosal dysfunction—animals were infected intranasally with 10^4 colony-forming units (CFU) of *S. pneumoniae* BHN100 strain (serotype 19F). Three days later, mice were euthanized for quantification of bacterial load in nasal washes and middle ear homogenates. Intranasal vaccination with recombinant PspA co-administered with the CTB adjuvant conferred significant protection against pneumococcal OM, reducing bacterial loads in the middle ear. Protection was associated with the induction of local IgA and IgG antibodies and dependence on IL-17–mediated signaling. In contrast, administration of PspA alone did not confer protection, highlighting the importance of immune co-stimulation for vaccine efficacy. These

results reinforce the potential of PspA as a vaccine antigen against otitis media, particularly when combined with adjuvants that stimulate mucosal antibody and cellular immune responses (82).

Similarly, a study aimed to investigate the efficacy of active immunization with PspA in preventing AOM, using an experimental model in adult Sprague-Dawley rats immunized subcutaneously with three doses of PspA formulated in Freund's adjuvant. Infection was induced by direct inoculation of 5×10^6 CFU of a serotype 6A strain into the middle ear cavity with a bacterial suspension, following surgical exposure of the tympanic bulla. Infection was monitored by otomicroscopy, with OM defined as the presence of purulent exudate in at least one evaluation. Active immunization with PspA conferred protection against purulent otitis media in rats challenged with *S. pneumoniae*. None of the immunized animals developed infection, in contrast to the control groups, which showed a high incidence of otitis. Protection was associated with the induction of high levels of specific IgG, indicating that the humoral response generated by PspA was sufficient to prevent middle ear infection. These findings further highlight the efficacy of PspA as a vaccine candidate against pneumococcal otitis media (78).

Finally, Li-Korotky *et al*. employed an *in vitro* model using human middle ear epithelial cells, to evaluate the differential expression of PspA in opaque (O) and transparent (T) variants of *S. pneumoniae*, focusing on their adaptation and virulence during middle ear infections. These variants represent morphologically distinct bacterial forms: opaque variants exhibit higher expression of polysaccharide capsule, conferring greater resistance to opsonophagocytosis and being commonly associated with invasive infections such as sepsis. In contrast, transparent variants express less capsule but higher amounts of surface proteins, such as PspA; they display greater adhesion to epithelial cells and predominate in mucosal colonization. The *S. pneumoniae* strains used were derived from human clinical isolates, which present distinct gene expression patterns and colony morphologies. To explore the behavior of these variants under conditions that mimic the inflammatory environment of otitis media, human middle ear epithelial cells were exposed to pneumococci under conditions simulating physiological dysfunctions of the middle ear, such as Eustachian tube obstruction (ETO) and the presence of tympanostomy tubes (TT). This model allowed the assessment of differential expression levels of virulence genes in response to the pathological microenvironment. PspA was analyzed exclusively as an endogenous virulence factor, with emphasis on its differential expression among phenotypic variants. The study demonstrated that PspA expression was significantly higher in the transparent (T) variants of *S. pneumoniae*,

both under basal conditions and after epithelial adhesion. In the *in vitro* human middle ear epithelial cell model, *pspA* gene expression was significantly upregulated in T variants adhered to the epithelium under simulated tympanostomy tube (TT) conditions, compared to opaque (O) variants. These findings indicate that PspA is strongly associated with the phenotype most adapted to colonization and persistence in the middle ear mucosa—key traits for the development of otitis media. The differential expression profile of PspA, combined with its well-known role in immune evasion, further supports its relevance as a promising vaccine candidate, particularly for the prevention of non-invasive pneumococcal infections such as otitis media, where adhesion and interaction with the epithelium are critical events in pathogenesis (83).

In summary, these studies demonstrate that PspA plays a critical role in the pathogenesis of otitis media by promoting bacterial persistence, inflammation, and inner ear damage. Moreover, vaccination with PspA induces protective local and systemic immune responses—especially when combined with appropriate adjuvants— reinforcing its potential as a vaccine candidate for preventing pneumococcal otitis media and its complications.

Table 3. Studies investigating PspA as a vaccine against otitis media

Reference	Model	Formulation	Results
Schachern et al., 2014(32)	Chinchilla	Infection with wild-type S. pneumoniae D39 and isogenic mutants (ΔpspA, ΔpspC, ΔpspA/ΔpspC)	ΔpspA and ΔpspA/ΔpspC: no bacterial growth in middle ear effusion, strongly attenuated infection. ΔpspC: reduced CFUs vs WT but still viable. Wild-type caused highest inflammation.
White et al., 1999 (78)	Sprague-Dawley rats	Infection with S. pneumoniae serotype 6A into the middle ear cavity after surgical exposure of the tympanic bulla	None of the immunized animals developed otitis, whereas control groups showed a high incidence; protection was associated with high levels of specific IgG, demonstrating that the humoral response induced by PspA was

		Immunization with rPspA in Freund's adjuvant, (s.c.)	sufficient to prevent middle ear infection.
Li-Korotky et. al., 2010(83)	Human middle ear epithelial cell line (HMEEC)	Expression analysis of pneumococcal virulence genes (NanA, HylA, PspA, CbpA) under simulated middle ear conditions: normal, Eustachian tube obstruction (ETO), and tympanostomy tube (TT) placement	Transparent (T) phenotype: higher PspA expression; opaque (O) phenotype: higher CbpA. Pathological conditions (ETO, TT) enhanced NanA, HylA, and PspA expression. Suggests PspA is a signature virulence factor of T variants during OM pathogenesis.
Habets et al., 2016(82)	BALB/c and C57BL/6 mice (WT, IL-17RA–/–, and antibody-deficient μMT	Intranasal recombinant PspA (from TIGR4) + cholera toxin subunit B (CTB)	Significant reduction of pneumococcal load in middle ear and nasal cavity (>1 log). Protection required IL-17 signaling but not antibodies; PspA alone induced antibodies but no protection.
Schachern et al., 2008(84)	Chinchillas	Intrabullar inoculation with *S. pneumoniae* D39 Wild-type S. pneumoniae D39 (serotype 2) and isogenic mutants (ΔpspA, ΔpspC, ΔpspA/ ΔpspC)	↑ bacterial loads and inflammation in wild-type and $PspC^{-}$ groups; undetectable bacteria and reduced inflammatory responses in PspA-deficient groups. Findings demonstrate the critical role of PspA in pneumococcal otitis media.

Schachern et al., 2013(85)	Chinchilla	Wild-type S. pneumoniae D39 (serotype 2) and isogenic mutants (ΔpspA, Δply, ΔpspA/Δply)	ΔpspA: no viable bacteria detected in middle ear at 48 h, rapid clearance, least virulent. Δply: CFUs remained near inoculum level, attenuated pathology. ΔpspA/Δply: similar virulence to WT, no attenuation. WT: highest CFU and inflammation.
Tsuprun et al., 2008(86)	Chinchilla	Inoculation of middle ear with WT S. pneumoniae D39 or isogenic mutants (ΔpspA, Δply)	WT caused permanent hearing loss (10–15 dB at 4–32 kHz, >20 dB at 1–2 kHz) and inner ear pathology. ΔpspA or Δply mutants caused no significant hearing loss.

Colonization

Pneumococcal colonization of the nasopharynx represents an initial and crucial step in invasive pneumococcal disease pathogenesis, influenced by carriage dynamics and competition between different serotypes (87). *S. pneumoniae* frequently colonizes the human nasopharynx asymptomatically but can also cause diseases including sinusitis, otitis media, pneumonia, sepsis, and meningitis. High carriage rates have been reported among children, particularly in low-income countries, where colonization in children under five years ranges from 20% to 93.4% (88). Furthermore, concurrent carriage of two or more pneumococcal serotypes is common, reaching up to 50% of carriers (87-89). Therefore, understanding interactions between different serotypes and PspA-induced immune responses is essential for developing effective vaccine strategies against colonization. Studies investigating the effect of PspA-based vaccines on pneumococcal colonization are listed in table 4.

Kuipers K et al. (2017) evaluated the impact of genetic background across different mouse strains on nasal colonization by *S. pneumoniae* and the efficacy of a PspA-based vaccine in this process. Seven-week-old female C57BL/6J, BALB/c, and CB6F1 (BALB/c × C57BL/6J hybrids) mice were administered intranasally with 10 μg of recombinant PspA from TIGR4 using 4 μg of cholera toxin B subunit (CTB) as adjuvant.. The scheme included three vaccine doses at two weeks

intervals between each immunization. Three weeks post-final immunization, mice were intranasally challenged with 10^6 CFU of strain TIGR4, and colonization was assessed five days later by counting CFU recovered from nasal washes. Among controls (CTB alone), BALB/c mice showed higher colonization density than C57BL/6 and CB6F1. PspA vaccination significantly reduced colonization across all strains, with the greatest reduction in CB6F1 hybrids where nearly all vaccinated animals showed undetectable colonization. Intra-group variation was lowest in BALB/c, while CB6F1 and C57BL/6 exhibited wider bacterial count dispersion. These findings indicate that while intranasal CTB-adjuvanted PspA provides protection across genetic backgrounds, the magnitude of colonization protection varies by host strain. The authors suggest CB6F1's enhanced resistance may stem from combined Th1 (C57BL/6-dominant) and Th2 (BALB/c-dominant) immune responses, yielding a more balanced cellular/humoral immunity that more effectively reduces pneumococcal load (90). This result demonstrates PspA's ability to prevent pneumococcal colonization in mice, while highlighting the importance of a balanced Th1/Th2 response to protection.

Conjugate vaccines including PspA fused to polysaccharides have also been tested in murine models of colonization. The work from Kaplonek et al. evaluated the protective efficacy of PspA4Pro conjugated to capsular polysaccharide serotype 14 (forming PS14-mPspA4Pro conjugate) against nasal colonization by *S. pneumoniae*. Female BALB/c mice were immunized subcutaneously with three doses (days 0, 14, 28) of PS14-mPspA4Pro conjugate or the co-administered unconjugated antigens. Control mice received Alum diluted in PBS. On day 42, blood was collected for serological analysis, followed by intranasal challenge with 1×10^7 CFU of *S. pneumoniae* strain 0603 (serotype 6B, clade 1). Nasal washes were collected seven days post-challenge for bacterial counting. Both conjugate-vaccinated and co-administered antigen groups showed significant reduction in nasal colonization when compared to controls. The protective efficacy was similar between vaccinated groups, demonstrating that PspA4Pro conferred cross-clade protection against colonization with a heterologous strain. These findings support PspA's potential as a vaccine antigen capable of cross-clade protection, whether free or conjugated to polysaccharide (91).

Carneiro et al. investigated the protective efficacy of *S. pneumoniae* extracellular vesicles (pEVs) against nasal colonization and invasive infection. pEVs are membrane-derived structures secreted into extracellular space, carrying selective cargo including key pneumococcal virulence

antigens such as pneumolysin (Ply), maltose/maltodextrin ABC transporter (MalX), chaperone PrsA, pneumococcal surface protein C (PspC), and pneumococcal surface protein A (PspA), capable of inducing immune responses when administered to hosts. Specific pathogen-free (SPF) female BALB/c and C57BL/6 mice were subcutaneously immunized with 1 μg, 2.5 μg, or 5 μg of pEVs derived from strain R6 (WT pEVs) or R6 ΔpspA (ΔpspA pEVs - PspA-deficient), diluted in 100 μL saline. Control groups received saline alone. Three doses were administered at 15-day intervals. For intranasal immunization, mice received three doses of 750 ng WT or ΔpspA pEVs in 20 μL saline following the same schedule. Challenges were performed 21 days post-final immunization for both pneumonia and colonization models. For nasal colonization challenges, C57BL/6 mice were inoculated with 10 μL containing 1×10^6 CFU of strain 0603 (ST6B, PspA1). pEVs demonstrated significant protection against nasal colonization, even across different capsular types and heterologous PspA variants. While PspA contributed to protection against invasive infections, it was non-essential for preventing nasal colonization. Post-colonization assays revealed that intranasal immunization with R6 pEVs protected mice against pneumococcal nasal colonization despite low circulating antibody titers against PspA, PrsA, and MalX, indicating that high antibody levels are not required for this protection (92).

The importance of vaccine strategies in preventing co-colonization has also been addressed in studies combining PspA with other components. Colichio et al. established a murine model of nasopharyngeal co-colonization using combinations of vaccine-type (VT) and non-vaccine-type (NVT) *S. pneumoniae* strains, genetically marked with erythromycin (erm) or spectinomycin (spec) resistance through *iga* insertion without affecting virulence in mice. The vaccine formulation consisted of recombinant PspA proteins from clades 1 and 4 (PspA1 + PspA4) combined with whole-cell pertussis vaccine (wP) as adjuvant, administered intranasally in three 10 μg doses at two-week intervals. PCV13 and PCV13 combined with PspA1+PspA4+wP were also evaluated. Five days after intranasal challenge with 5×10^5 CFU of each strain, colonization was quantified by selective plating of nasal washes. In the VT4+NVT33 model, PCV13 significantly reduced VT4 but showed a trend toward increased NVT33 colonization, whereas PspA1+PspA4+wP reduced both strains, achieving significant reduction of NVT33 compared to wP alone. The combination PspA1+PspA4+wP+PCV13 reduced both strains compared to PCV13 alone. In the VT23F+NVT15B/C model, PCV13 did not reduce VT23F (PspA2), consistent with lack of serological recognition, while PspA1+PspA4+wP - either alone or combined with PCV13 -

significantly reduced both strains. The wP adjuvant alone also showed protective effects, though with varying efficacy between strains. These results indicate that immunization with PspA1+PspA4+wP, either alone or combined with PCV13, provides broader protection against co-colonization by both VT and NVT strains compared to PCV13 alone, without promoting serotype replacement (93). This result suggests a protective role for PspA in controlling concomitant infections with different pneumococcal strains - a common scenario especially in children.

In another study, Tostes *et al.* compared different combinations of PspA from clades 1, 2, 3, and 4 associated with the whole-cell pertussis vaccine (wP) in protection against pneumococcal co-colonization. Four *S. pneumoniae* strains were used: St491/00 (serotype 6B, PspA1) and St472/96 (serotype 6B, PspA4, trimethoprim-resistant), as well as SPEC 6B (serotype 6B, PspA3, spectinomycin-resistant) and EMC 23F (serotype 23F, PspA2). The vaccination strategy consisted of intranasal immunization of five- to seven-week-old female specific-pathogen-free C57BL/6 mice with 5 μg of recombinant PspA protein combined with 1/8 of the wP dose, administered in two doses 14 days apart; in groups receiving a mixture of two proteins, each was given at 2.5 μg. Mice were anesthetized intraperitoneally with 200 μl of a solution containing 0.2% xylazine and 0.5% ketamine before immunization. Three weeks after the final dose, the animals were anesthetized again with the same solution and challenged intranasally with a mixture of two pneumococcal strains (5×10^5 CFU of each strain), inoculated into both nostrils. Five days post-challenge, the mice were euthanized intraperitoneally with a lethal dose of xylazine/ketamine, and nasal colonization was assessed by plating nasal washes on blood agar with or without selective antibiotics, enabling differential quantification of each strain in the co-colonization model. The results showed that the formulations rPspA1 + wP, rPspA3 + wP, and rPspA4 + wP significantly reduced colonization by both serotype 6B strains compared to the saline group, with rPspA1 + wP and rPspA4 + wP showing additional reductions against their homologous strains compared to the wP group. Combined strategies (rPspA1 + rPspA4 + wP or a heterologous regimen of rPspA1/wP followed by rPspA4/wP) promoted greater reductions in colonization by both strains than immunization with a single antigen. In the challenge with 23F OPKA (PspA2) and 6B OPKA (PspA3), rPspA1 + wP reduced colonization by 23F OPKA, whereas rPspA4 + wP reduced colonization by both strains compared to saline (94).

Following the hybrid protein approach, Converso et al. (2017) analyzed a chimeric protein combining PspA fused to PotD and its potential for protection against invasive pneumococcal infection and nasopharyngeal colonization in mice. PotD, like PspA, is a membrane protein of *S. pneumoniae* capable of binding polyamines and transporting them from the extracellular medium into the cytoplasm. For immunization, a hybrid protein was generated through the genetic fusion of the gene fragment encoding the N-terminal region plus the proline-rich region of PspA from strain St P490 with the *potD* gene from strain St 540/99, followed by PCR amplification. Animals were immunized subcutaneously with three doses of 10 µg rPspA, 12 µg rPotD, or 22 µg rPspA-PotD at 14-day intervals, using 0.9% sterile saline with 100 µg $Al(OH)_3$ as an adjuvant. The adjuvant alone diluted in saline was used as a control. Fourteen days after the last immunization, two challenges were performed. In the colonization challenge, animals were inoculated into one nostril with 1×10^7 CFU of the noninvasive pneumococcal strain St 0603 diluted in 10 µL of sterile PBS. Results demonstrated that the inclusion of PotD reduced nasopharyngeal colonization, an effect not previously observed with subcutaneous immunization containing PspA alone. Immunization with the chimeric protein led to production of antibodies with increased binding capacity to pneumococcal strains of various serotypes and genetic backgrounds, enhanced opsonophagocytosis, and IL-17 secretion by splenocytes. The rPspA-PotD hybrid was highly immunogenic and induced stronger IgG responses than the individual proteins, reducing nasopharyngeal colonization, which decreases transmission and prevents disease, thus demonstrating the potential and efficacy of the PspA-PotD fusion in hampering disease progression (58)**.**

Goulart et al. (2017) used recombinant *M. bovis* BCG strains expressing pneumococcal antigens, including PspA-PdT, in a prime-boost regimen against nasal colonization by *S. pneumoniae*. C57BL/6 mice were immunized in a prime-boost scheme, in which the first dose consisted of recombinant BCG expressing SP 0148, SP 2108, or the fusion protein PspA-PdT (PspA with broad cross-reactivity + detoxified pneumolysin), followed by a boost with the corresponding purified recombinant protein; the combination of the three rBCG strains (rBCG Mix) followed by the three proteins (rMix) was also tested. The challenge strain used was *S. pneumoniae* St 603 (serotype 6B), administered intranasally three weeks after the last vaccine dose. Seven days post-challenge, colonization was assessed by retrograde nasal wash, with CFU counting

on blood agar. The rBCG 0148/rSP 0148 and rBCG 2108/rSP 2108 regimens induced high levels of IL-17A and IFN-γ after in vitro stimulation and significantly reduced nasal colonization compared to the saline and WT-BCG/rMix groups. The rBCG PspA-PdT/rPspA-PdT formulation showed no significant effect on colonization, whereas the rBCG Mix/rMix combination maintained the protection observed for SP 0148 and SP 2108 individually. These results indicate that SP 0148 and SP 2108, when delivered by rBCG, elicit robust Th17 responses and confer protection against pneumococcal colonization by serotype 6B, whereas the PspA-PdT fusion protein, under the tested conditions, did not demonstrate the same protective effect (62).

Finally, Kuipers et al. (2017) used laboratory strains and human clinical isolates of *S. pneumoniae*, including the TIGR4 strain and strains from the Pneumococcal Bacteraemia Collection Nijmegen (PBCN), to evaluate a murine model of nasal colonization. The vaccine formulation was based on outer membrane vesicles (OMVs) from attenuated *Salmonella Typhimurium* (SL3261ΔtolRAΔmsbB) displaying on their surface the α1α2 fragment of PspA derived from TIGR4. This region, located between the signal sequence and the proline-rich region (PRR), has been identified as responsible for inducing Th17-mediated protection. Mice were intranasally immunized with OMVs containing PspA α1α2 or with control OMVs (lacking antigen) and subsequently challenged intranasally with 10^6 CFU of pneumococcus in 10 µL of PBS, using either the homologous TIGR4 strain or heterologous strains selected based on their *in vitro* IL-17A induction profile (“high IL-17A” or “low IL-17A”) determined in an ex vivo assay. Colonization challenge was assessed by counting CFUs recovered from nasal washes. Results showed that vaccination with PspA α1α2-OMVs conferred significant protection against colonization by the homologous strain and by heterologous strains from the “high IL-17A” group, but not against those from the “low IL-17A” group. This protection correlated with elevated IL-17A levels in the nasal mucosa, demonstrating that the Th17 response induced by the α1α2 fragment is critical for protection, and that the ex vivo IL-17A assay may predict vaccine efficacy in reducing pneumococcal colonization (95).

Collectively, these studies highlight the central role of PspA in preventing *S. pneumoniae* colonization in different experimental models, through induction of protective immune responses—particularly those involving balanced Th1/Th2 or Th17 pathways. Importantly, PspA-based immunization offers cross-clade protection and reduces co-colonization by vaccine-type and non-vaccine-type strains, offering a safe and effective vaccine platform for decreasing

pneumococcal transmission and thereby extending indirect protection to unvaccinated individuals through herd immunity.

Table 4. PspA as a vaccine candidate against pneumococcal colonization

Reference	Model	Formulation	Results
Converso et al., 2017(58)	BALB/c mice	Chimeric protein rPspA-PotD + alum (s.c.)	Significant reduction of nasopharyngeal colonization (~1 log); effect not seen with rPspA alone; rPotD also reduced colonization.
Goulart et al., 2017(62)	C57BL/6 mice	Same rBCG mix + protein boost	Significant reduction of colonization load vs controls.
Kaplonek et al., 2022(91)	Female BALB/c mice	PS14-mPspA4Pro conjugate (PspA4Pro + capsular polysaccharide serotype 14) or co-administered unconjugated antigens; (s.c.)	Reduction in nasal colonization; Cross-protection with PspA4Pro against colonization by a heterologous strain.
Carneiro et al., 2025(92)	BALB/c mice	pEVs (WT or ΔpspA) (i.n.)	Both WT and ΔpspA pEVs reduced nasal colonization (~1 log) vs controls; PspA not essential for colonization protection.
Tostes et al., 2017(94)	C57BL/6 mice	Intranasal rPspA1, rPspA2, rPspA3, rPspA4 (isolated or combined) + whole-cell pertussis vaccine (wP)	Significant reduction in colonization; best results with rPspA1 or rPspA4 + wP; mixture rPspA1+rPspA4+wP showed stronger reduction than single antigens; no strain replacement observed.

Kuipers et al. 2017(90)	C57BL/6 mouse (WT and IL-17R–/–)	Intranasal OMVs from Salmonella Typhimurium (LPS-detoxified, ΔmsbB) displaying PspA α1–α2 fragment (strain TIGR4)	Significant reduction of nasal colonization in WT mice; no protection in IL-17R–/– mice, showing IL-17A dependence; cross-reactive Th17 response against selected heterologous strains; estimated 19.1% coverage among 1,352 carriage isolates.
Kuipers et al., 2017(95)	C57BL/6, BALB/c, and CB6F1 mice	Intranasal recombinant PspA (strain TIGR4) + cholera toxin subunit B (CTB)	Significant reduction of nasal colonization in all strains; strongest effect in CB6F1, moderate in BALB/c, lowest in C57BL/6; protection correlated with nasal IgG and IL-17A responses.
Colichio et al., 2020(93)	C57BL/6 mice	Intranasal rPspA1 + rPspA4 + whole-cell pertussis vaccine (wP); compared with PCV13 (s.c.) and combination (PspA1+4+wP + PCV13)	PCV13: reduced VT strain, but gave competitive advantage to NVT. PspA1+4+wP: reduced both VT and NVT strains. Combination (PspA1+4+wP + PCV13): reduced both strains, overcoming serotype replacement.

Final remarks and perspectives

PspA-based vaccine formulations, whether used in isolation, as fusion proteins, in combination with other antigens, delivered via live or particulate vectors, or associated with mucosal adjuvants, have demonstrated reduced bacterial burden, increased survival, and induction of functionally relevant humoral and cellular responses in different contexts, including sepsis,

pneumonia, nasopharyngeal colonization, and otitis media, and across various species, Evidence from phase I clinical trials further supports its initial immunogenicity and safety. A limitation that must be highlighted is the antigenic variability among PspA families and clades, reinforcing the need for multivalent formulations. Several studies have included components from families 1 and 2 to enhance cross-reactivity; moreover, fragment selection should prioritize epitopes with proven protective efficacy, such as the initial ~100 amino acids of the N-terminal region and elements of the PRR in their native context, such as the NPB, as well as chimeric constructs capable of enhancing complement activation and opsonophagocytic activity. Mucosal strategies, such as systemic+mucosal prime–boost regimens, liposomes/nanoparticles, and the use of specific adjuvants, have shown promise in reducing colonization and transmission, although they require standardization and long-term safety assessment, particularly in the case of OMVs and recombinant BCG. In summary, PspA represents a relevant vaccine candidate, especially as a component of multivalent protein-based vaccines and/or in combination with polysaccharides, with the potential to broaden serotype-independent protection. Validation in phase II/III clinical trials will be pivotal to confirm its impact on public health.

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5. CONCLUSÃO

Os dados do presente estudo permitem concluir que:

- Vacinas quiméricas baseadas na fusão entre PspA e PlD1 foram imunogênicas, induzindo a produção de anticorpos capazes de se ligar à superfície de diferentes isolados de *S. pneumoniae;*
- Os anticorpos vacinais foram capazes de promover a deposição do componente C3 do Sistema Complemento, contribuindo com a opsonização do patógeno;
- A vacinação com a proteína quimérica conferiu proteção, nos camundongos, contra desafio letal com *S. pneumoniae*;
- A proteção induzida pela proteína quimérica foi superior às proteínas isoladas, indicando uma ampliação do efeito protetor pela fusão;
- A análise de cepas mutantes revelou que ambas as proteínas contribuem com os efeitos protetores observados.
- Vacinas baseadas na proteína PspA representam estratégias vacinais promissoras contra infecção pelo pneumococo, ativando respostas protetoras com ampla cobertura.

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