UNIVERSIDADE ESTADUAL PAULISTA – UNESP

CAMPUS DE JABOTICABAL

DESENVOLVIMENTO DE VACINA INJETÁVEL PARA CONTROLE DE *Mycoplasma hyopneumoniae* EM SUÍNOS UTILIZANDO A SÍLICA NANOESTRUTURADA SBA-15 COMO ADJUVANTE

Fernando Antonio Moreira Petri

Médico Veterinário

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UNIVERSIDADE ESTADUAL PAULISTA – UNESP

CÂMPUS DE JABOTICABAL

DESENVOLVIMENTO DE VACINA INJETÁVEL PARA CONTROLE DE *Mycoplasma hyopneumoniae* EM SUÍNOS UTILIZANDO A SÍLICA NANOESTRUTURADA SBA-15 COMO ADJUVANTE

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Tese apresentada à Faculdade de Ciências Agrárias e Veterinárias – Unesp, Câmpus de Jaboticabal, como parte das exigências para a obtenção do título de Doutor em Medicina Veterinária, área: Clínica Médica Veterinária

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REGISTRO DE IMPACTO

A pneumonia enzoótica suína (PES), causada por *Mycoplasma (M.) hyopneumoniae*, permanece como um dos principais desafios sanitários da suinocultura intensiva, devido a sua elevada prevalência, dos prejuízos zootécnicos e associação frequente com o uso de antimicrobianos. Nesse contexto, o estudo contribui para o desenvolvimento de estratégias inovadoras de prevenção ao investigar uma abordagem vacinal baseada em sílica mesoporosa SBA-15, integrando conceitos de nanotecnologia, imunologia de mucosas e microbiologia respiratória.

Do ponto de vista científico e tecnológico, o trabalho amplia o conhecimento sobre a aplicação da SBA-15 como plataforma vacinal, avaliando sua eficácia, imunogenicidade e potencial utilização contra *M. hyopneumoniae*. A utilização de um sistema de liberação baseado em nanomateriais representa um avanço em relação às formulações convencionais, ao possibilitar bom custo-benefício, apresentação antigênica e potencial modulação da resposta imune, fornecendo base para o desenvolvimento de novas estratégias e para aplicações translacionais na área de sanidade animal. No âmbito da saúde animal, há contribuição para o aprimoramento das estratégias de controle da PES, com potencial impacto na redução da colonização e da transmissão de *M. hyopneumoniae* em rebanhos suínos. O fortalecimento da resposta imune por meio de vacinas eficazes pode resultar em menor severidade clínica da doença, promovendo melhorias no bem-estar animal e atendendo às demandas por sistemas produtivos mais eficientes e sustentáveis.

Um aspecto inovador deste estudo está na possível influência da microbiota nasal dos animais sobre a resposta imune. A análise das alterações microbianas associadas à intervenção vacinal amplia a compreensão das interações entre imunidade, patógenos respiratórios e comunidades microbianas, contribuindo para uma visão ampliada de sanidade que considera, além do controle do agente etiológico, a preservação do equilíbrio microbiano. Sob a perspectiva da saúde pública e do conceito de *One Health*, os achados apresentam relevância indireta ao apontar caminhos para a redução do uso de antimicrobianos na suinocultura, além de potenciais impactos econômicos e sociais associados à redução de perdas produtivas, aumento da eficiência dos sistemas de criação, fortalecimento da competitividade da suinocultura brasileira e formação de recursos humanos qualificados.

IMPACT STATEMENT

Porcine enzootic pneumonia (PEP), caused by *Mycoplasma* (*M.*) *hyopneumoniae*, remains one of the main sanitary challenges in intensive swine production due to its high prevalence, negative effects on zootechnical performance, and frequent association with the use of antimicrobials. In this context, the present study contributes to the development of innovative preventive strategies by investigating a vaccine approach based on mesoporous silica SBA-15, integrating concepts of nanotechnology, mucosal immunology, and respiratory microbiology.

From a scientific and technological perspective, this work expands current knowledge on the application of SBA-15 as a vaccine platform by evaluating its efficacy, immunogenicity, and potential use against *M. hyopneumoniae*. The use of a nanomaterial-based delivery system represents an advance over conventional vaccine formulations, as it enables favorable cost-effectiveness, improved antigen presentation, and potential modulation of the immune response. These findings provide a foundation for the development of new vaccination strategies and for translational applications in the field of animal health. In terms of animal health, the study contributes to the improvement of PEP control strategies, with potential impacts on reducing colonization and transmission of *M. hyopneumoniae* in swine herds. Strengthening the immune response through effective vaccination may lead to reduced clinical severity of the disease, promoting improvements in animal welfare and meeting the demands for more efficient and sustainable production systems.

An innovative aspect of this study lies in the possible influence of the nasal microbiota on the immune response. The analysis of microbial changes associated with vaccination broadens the understanding of interactions among host immunity, respiratory pathogens, and microbial communities. This approach contributes to an expanded concept of animal health that considers, in addition to pathogen control, the preservation of microbial balance. From a public health perspective and within the One Health framework, these findings are indirectly relevant by indicating pathways for reducing antimicrobial use in swine production, as well as potential economic and social impacts associated with reduced production losses, increased efficiency of production systems, strengthened competitiveness of the Brazilian swine industry, and the training of qualified human resources.

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CERTIFICADO DE APROVAÇÃO

TÍTULO DA TESE: DESENVOLVIMENTO DE VACINA INJETÁVEL PARA CONTROLE DE Mycoplasma hyopneumoniae EM SUÍNOS UTILIZANDO A SÍLICA NANOESTRUTURADA SBA-15 COMO ADJUVANTE

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Jaboticabal, 02 de dezembro de 2025.

DADOS CURRICULARES DO AUTOR

Fernando Antonio Moreira Petri – nascido em 11 de abril de 1996, na cidade de Sertãozinho, São Paulo – Brasil. Médico Veterinário graduado pela Universidade Estadual Paulista “Júlio de Mesquita Filho” – FCAV/Unesp Jaboticabal em 2021. Durante a graduação foi membro-organizador do Grupo de Estudos em Suinocultura (Suinesp) e integrante da Consultora Agropecuária Júnior (CAP Jr). Além disso, foi bolsista de iniciação científica ICSB PROPe-Unesp (2018-2019) e FAPESP (2019-2020, processo 2019/14122-7), sob orientação do Prof. Dr. Luís Guilherme de Oliveira. Realizou o estágio curricular obrigatório no CEPPA – Centro de Pesquisa e Patologia Animal, em Paulínia – São Paulo e na SF Agropecuária, em Brasilândia – Mato Grosso do Sul, com Trabalho de Conclusão de Curso intitulado “Importância da monitoria patológica em frigorífico no acompanhamento da saúde respiratória dos suínos de granja comercial”. Em seguida, ingressou no programa de Pós-Graduação em Medicina Veterinária da FCAV/Unesp Jaboticabal, como aluno de Doutorado direto, onde inicialmente foi bolsista CAPES e logo bolsista FAPESP (processo 2021/14515-9), sob orientação do Prof. Dr. Luís Guilherme de Oliveira e coorientação do Prof. Dr. Hélio José Montassier. Durante o doutorado, concentrou suas atividades de pesquisa no Laboratório de Medicina de Suínos – Departamento de Clínica e Cirurgia Veterinária da FCAV/Unesp Jaboticabal. Neste período, realizou estágio de pesquisa no exterior por 12 meses (FAPESP, processo 2023/15067-5), no IRTA-CReSA (Catalunha, Espanha) sob supervisão da Dra. Marina Sibila e Dra. Virgínia Aragón. Possui experiência nas áreas de doenças dos suínos, experimentação animal, medicina veterinária preventiva e diagnóstico veterinário.

EPÍGRAFE

Aprendi que a vida não se trata de vencer a tempestade, mas de aprender a dançar sob a chuva.

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Dedico este trabalho aos meus pais, de quem tenho muito orgulho, e cujos valores levarei sempre comigo.

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SUMÁRIO

Página

CAPÍTULO 1 – Considerações gerais 1

1. Introdução 1

2. Revisão de literatura 2

3. Referências bibliográficas 11

[1]CAPÍTULO 2 – Shedding reduction and immunity modulation in piglets with an inactivated *Mycoplasma hyopneumoniae* vaccine encapsulated in nanostructured SBA-15 silica 19

1. Introduction 21

2. Materials and methods 22

2.1 Ethics statement 22

2.2 Mycoplasma hyopneumoniae cultivation and protein extraction 22

2.3 Preparation and characterization of SBA-15-M. hyopneumoniae vaccine 23

2.4 In vitro characteristics of SBA-15-M. hyopneumoniae vaccine 24

2.5 Selection of piglets and experimental design 24

2.6 Sample collection (blood serum, nasal and laryngeal swabs) 25

2.7 Euthanasia of piglets and macroscopic and microscopic lung lesion evaluation 26

2.8 Collection of lung fragments and BALF samples 26

2.9 Quantification of M. hyopneumoniae-specific antibody responses in blood serum 26

2.10 Quantification of M. hyopneumoniae-specific antibody detection in nasal swabs and BALF samples 27

2.11 DNA extraction of lungs and laryngeal swabs 27

2.12 Evaluation of bacterial loads by qPCR assays targeting p102 gene of M. hyopneumoniae 28

2.13 RNA extraction and cDNA synthesis 28

2.14 Evaluation of cytokine gene expression by RT-qPCR 28

2.15 Statistical analysis 29

3. Results 29

3.1. Silica-based M. hyopneumoniae vaccine characterization 29

3.2. Macroscopic and microscopic lung lesion score 30

3.3. Serum IgG and IgA antibody responses against M. hyopneumoniae 34

3.4. Mucosal responses against M. hyopneumoniae 35

3.5. Clinical signs, shedding and colonization of bacteria in lung tissue 38

3.6. Gene expression of cytokines in lung tissue 40

4. Discussion 42

5. References 49

Supplementary material 53

[1]CAPÍTULO 3 – Early-life nasal microbiota composition predicts *Mycoplasma hyopneumoniae* respiratory disease outcomes and is preserved by vaccination in piglets 62

1. Introduction 65

2. Materials and methods 68

2.1 Study design, sampling and clinical evaluation 68

2.2 Quantification of IgG anti-M. hyopneumoniae levels in serum 69

2.3 Clinical parameters and metadata determination of immune responsiveness and disease susceptibility in pigles 70

2.4 DNA extraction and 16S gene amplicon sequencing 70

2.5 Microbiota analysis 71

2.6 Correlation analysis between microbiota diversity and immune outcomes 72

3. Results 73

3.1. Sequencing throughput stats 73

3.2. Alfa and beta diversity description in D0, D21 and D35 by each group 73

3.3. Nasal microbiota at weaning and the longitudinal modulation after vaccination and challenge 74

3.4. Taxonomy identification between timepoints and the core microbiota 75

3.5. Macroscopic lung lesion outcomes and IgG anti-M. hyopneumoniae quantification in serum 84

3.6. Classification of piglets according to IgG anti-M. hyopneumoniae and macroscopic lung lesions 85

3.7. Effect of the weaning-associated microbiota on susceptibility to M. hyopneumoniae-induced lung lesions 86

3.8. Effect of the weaning-associated microbiota on immune responsiveness after challenge 87

3.9. Effect of intramuscular vaccination at D0 and challenge at D21 on the nasal microbiota 89

3.10. Microbiota correlations with vaccination and challenge responses 91

4. Discussion and conclusion 91

5. References 99

Supplementary material 108

CERTIFICADO DA COMISSÃO DE ÉTICA NO USO DE ANIMAIS

UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Câmpus de Jaboticabal

CEUA – COMISSÃO DE ÉTICA NO USO DE ANIMAIS

C E R T I F I C A D O

Certificamos que o projeto de pesquisa intitulado **"Desenvolvimento de vacina injetável para controle de *Mycoplasma hyopneumoniae* em suínos utilizando a sílica nanoestruturada SBA-15 como adjuvante"**, protocolo nº 3897/21, sob a responsabilidade do Prof. Dr. Luís Guilherme de Oliveira, que envolve a produção, manutenção e/ou utilização de animais pertencentes ao Filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica (ou ensino) - encontra-se de acordo com os preceitos da lei nº 11.794, de 08 de outubro de 2008, no decreto 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), e foi aprovado pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA), da FACULDADE DE CIÊNCIAS AGRÁRIAS E VETERINÁRIAS, UNESP - CÂMPUS DE JABOTICABAL-SP, em reunião ordinária de 16 de fevereiro de 2022.

Vigência do Projeto	25/02/2022 a 31/11/2024
Espécie / Linhagem	*Sus scrofa domesticus* / Linhagem comercial (Landrace x Large White)
Nº de animais	50
Peso / Idade	5 - 6kg – 21 dias até 40kg (75 dias)
Sexo	Machos e Fêmeas
Origem	Unidade de criação comercial

Jaboticabal, 20 de fevereiro de 2022.

Fabiana Pilarski

Profª DrªFabiana Pilarski
Coordenadora – CEUA

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DESENVOLVIMENTO DE VACINA INJETÁVEL PARA CONTROLE DE *Mycoplasma hyopneumoniae* EM SUÍNOS UTILIZANDO A SÍLICA NANOESTRUTURADA SBA-15 COMO ADJUVANTE

RESUMO – *Mycoplasma* (*M.*) *hyopneumoniae* é o principal agente etiológico da pneumonia enzootica suína (PES), doença respiratória responsável por grandes perdas econômicas na produção de suínos em todo o mundo. As vacinas comerciais atuais conferem apenas proteção parcial, não prevenindo a transmissão do patógeno nem induzindo imunidade total, o que evidencia a necessidade de estratégias vacinais mais eficazes. Esta tese investigou o uso de nanopartículas mesoporosas de sílica ordenada (SBA-15) como adjuvante em vacinas experimentais contra *M. hyopneumoniae* e avaliou seus efeitos sobre respostas imunes, proteção e microbiota respiratória em leitões. No primeiro estudo, leitões imunizados com a vacina contendo SBA-15 apresentaram respostas imunes humorais e celulares robustas, com produção de IgA e IgG e modulação de citocinas Th17 e Th2, resultando em menor ocorrência de lesões pulmonares comparado com animais não imunizados e redução da eliminação bacteriana em comparação com animais vacinados com a vacina comercial. No segundo estudo, avaliou-se a interação entre vacinação e microbiota nasal. Ambas as vacinas reduziram a gravidade das lesões pulmonares, sendo que a vacina comercial induziu respostas humorais mais intensas. A análise da microbiota mostrou que a vacinação preservou a diversidade microbiana após o desafio, enquanto animais não vacinados apresentaram redução significativa. Notavelmente, maior diversidade ao desmame associou-se a lesões pulmonares menos severas e maior resposta de IgG, e a presença de táxons específicos, como *Alloprevotella* spp., correlacionou-se negativamente com a gravidade da doença. Em conjunto, os resultados demonstram que vacinas baseadas em SBA-15 representam uma estratégia promissora para o controle da PES e reforçam o papel da microbiota respiratória como moduladora da imunidade e potencial biomarcador de eficácia vacinal e resiliência em suínos.

Palavras-chave: adjuvante, doenças infecciosas, ecologia microbiana, imunologia, vacinas bacterianas.

DEVELOPMENT OF AN INJECTABLE VACCINE FOR CONTROL OF *Mycoplasma hyopneumoniae* IN PIGS USING NANOSTRUCTURED SILICA SBA-15 AS AN ADJUVANT

ABSTRACT – *Mycoplasma* (*M.*) *hyopneumoniae* is the primary etiological agent of porcine enzootic pneumonia (PEP), a respiratory disease responsible for major economic losses in pig production worldwide. Current commercial vaccines provide only partial protection, failing to prevent pathogen transmission or induce sterile immunity, highlighting the need for improved immunization strategies. This thesis investigated the use of ordered mesoporous silica nanoparticles (SBA-15) as an adjuvant in experimental vaccines against *M. hyopneumoniae* and evaluated their impact on immune responses, protection, and respiratory microbiota in piglets. In the first study, piglets immunized with SBA-15-loaded vaccines developed robust humoral and cellular immune responses, including IgA and IgG production and modulation of Th17 and Th2 cytokines, leading to reduced lung lesions and bacterial shedding compared to animals immunized with a commercial vaccine. In the second study, we assessed the interplay between vaccination and the nasal microbiota. Both the SBA-15 and commercial vaccines mitigated lung pathology, while the commercial formulation induced stronger systemic antibody responses. Microbiota analysis revealed that vaccination preserved microbial diversity post-challenge, whereas non-vaccinated animals displayed a significant reduction. Importantly, higher diversity at weaning correlated with milder lesions and enhanced IgG responses, and specific taxa, such as *Alloprevotella* spp., were negatively associated with disease severity. Altogether, our findings demonstrate that SBA-15-based vaccines are a promising tool to improve PEP control, while also highlighting the role of the respiratory microbiota as a modulator of immunity and potential biomarker for vaccine efficacy and resilience in pigs.

Keywords: adjuvant, bacterial vaccines, immunology, infectious diseases, microbial ecology.

CAPÍTULO 1 – Considerações gerais

1. Introdução

A produção de carne suína brasileira tem passado por modificações e evolução visando atender às demandas do mercado mundial e aumentar sua competitividade frente aos demais segmentos de produção de proteína animal. Porém, juntamente com a intensificação da produção, surgem cada vez mais novos desafios que interferem no sistema produtivo, como a ocorrência de doenças respiratórias. Além do impacto direto da infecção sobre os animais, também existe o impacto financeiro associado ao controle, prevenção e o impacto ecológico e sanitário devido ao uso de antimicrobianos na tentativa de controle dos patógenos causadores de enfermidades respiratórias. Desta forma, tem-se depositado grande atenção às estratégias de controle destas doenças devido a sua alta ocorrência e aos prejuízos que estas enfermidades são capazes de causar nos sistemas especializados de produção de suínos, como a pneumonia enzootica suína (PES), que tem *Mycoplasma* (*M.*) *hyopneumoniae* como patógeno bacteriano primário. Embora as vacinas comerciais reduzam as lesões pulmonares, elas não previnem totalmente a colonização ou a transmissão do patógeno.

Em convergência com o controle da PES e abordagens inovadoras, a sílica mesoporosa SBA-15 usada como carreador-adjuvante vacinal surgiu como uma estratégia versátil e promissora para aumentar a eficácia das vacinas e as respostas imunes em suínos, oferecendo o uso de novas tecnologias no controle da enfermidade na suinocultura e permitindo abrir novos horizontes para a indústria veterinária. Ainda, a microbiota tem se mostrado um elemento importante na sanidade animal, principalmente na suinocultura, com a ascensão de novos produtos como probióticos e prebióticos, em detrimento da redução do uso indiscriminado de antibióticos.

O principal objetivo deste projeto de pesquisa é avaliar o uso de uma nanotecnologia, a sílica SBA-15, para o controle de *M. hyopneumoniae* em forma de vacinação intramuscular (IM) e investigar as interações entre a vacinação e a microbiota respiratória em suínos. Especificamente, o primeiro estudo desta tese aborda desde a preparação da vacina nanotecnologica utilizando extrato proteico de

M. hyopneumoniae até a eficácia da vacina experimental em leitões desmamados. O segundo estudo apresenta como a vacina experimental intramuscular, composta por sílica SBA-15 carregada com proteínas de *M. hyopneumoniae*, influencia a diversidade microbiana do trato respiratório superior — e como essas comunidades microbianas, por sua vez, podem impactar a resposta imune do animal e a evolução da doença. Ao comparar essa formulação experimental com uma vacina comercial e um grupo controle não vacinado, o estudo busca aprofundar a compreensão da relação recíproca entre a microbiota nasal e a vacinação contra *M. hyopneumoniae*.

2. Revisão de literatura

A carne suína é a segunda proteína mais consumida no mundo, de acordo com os dados mais recentes da Organização das Nações Unidas para a Alimentação e Agricultura (FAO), e sua produção deve crescer mais de 7.000 quilotoneladas entre 2023 e 2032, ultrapassando 129.377 quilotoneladas em 2032 (OECD et al., 2023). A suinocultura está em constante expansão e busca por maior rentabilidade e sustentabilidade para atender a demanda de consumo populacional. O Brasil é considerado o quarto maior produtor e exportador de carne suína do mundo (ABPA, 2023; USDA, 2024).

No Brasil, a suinocultura industrial passou por um processo de intensificação que tornou o controle de doenças um desafio. O grande aumento dos sistemas de produção trouxe paralelamente uma maior densidade animal em determinadas áreas geográficas do país, aumentando a pressão de infecção (Sobestiansky, 2002; Zanella et al., 2016). No cenário da produção intensiva, a incidência de doenças respiratórias é maior em suínos do que em outras espécies de animais domésticos, devido à alta densidade animal favorecendo a transmissão e a persistência de patógenos nas granjas comerciais (Opriessnig et al., 2011). As doenças respiratórias ganharam maior espaço principalmente devido à queda na circulação e na qualidade do ar disponível nos galpões, mistura de animais de diferentes origens e estresse (Barcellos et al., 2008; Morés et al., 2015; Galdeano et al. 2019).

Neste contexto, *Mycoplasma* (*M.*) *hyopneumoniae* está distribuído mundialmente e é o agente bacteriano associado à pneumonia enzoótica suína (PES). A PES é

caracterizada por sua natureza crônica, alta morbidade, baixa mortalidade e redução do desempenho dos animais (Pieters & Maes, 2019), trazendo prejuízos na suinocultura intensiva devido a custos associados ao baixo rendimento zootécnico e as lesões de pneumonia e pleurisias associadas (Ferraz et al. 2020; Malcher et al. 2024). Dentro do complexo das doenças respiratórias dos suínos (CDRS), o *M. hyopneumoniae* é considerado patógeno bacteriano primário. A bactéria adere ao epitélio respiratório da traqueia, brônquios e bronquíolos e o coloniza, causando a destruição dos cílios e a supressão da imunidade do sistema respiratório, favorecendo a colonização por agentes secundários como *Pasteurella* (*P.*) *multocida*, *Streptococcus* (*S.*) *suis* e *Glaesserella* (*G.*) *parasuis* (Saade et al. 2020; Petri et al., 2023), agravando ainda mais a condição clínica e as perdas produtivas (Maes et al., 2018; Maes et al. 2023).

M. hyopneumoniae pertence à classe Mollicutes, ordem Mycoplasmoidales, família Metamycoplasmataceae e gênero *Mesomycoplasma* (Gupta et al., 2018). As bactérias dessa classe não possuem parede celular, necessitam de esteróis para crescer e podem ser aeróbias ou anaeróbias facultativas (Gupta et al., 2018; Mare & Switzer, 1965). Possuem genoma pequeno, baixo teor de G+C e são de crescimento fastidioso *in vitro*, devido à ausência de genes essenciais em vias biossintéticas, dependendo do hospedeiro para obtenção de ácidos graxos e colesterol (Razin et al., 1998). Apesar da existência de meio específico (Friis, 1975), o isolamento de *M. hyopneumoniae* não é utilizado comumente na rotina de diagnóstico, já que o crescimento é lento e demorado, podendo levar semanas (Pieters & Maes, 2019). Em suínos, destacam-se três espécies principais: *M. hyopneumoniae*, *M. hyorhinis* e *M. flocculare*. Outras espécies, como *M. hyosynoviae*, *M. suis*, *M. parvum* e *M. hyopharyngis*, também são encontradas (Petri et al. 2020; Nagy et al. 2025), mas pertencem a outros gêneros segundo a classificação proposta por Gupta et al. (2018) (Hoelzle, 2021; Rycroft, 2021; Sonalio et al., 2021).

A transmissão de *M. hyopneumoniae* pode acontecer pelo contato direto ou indireto com animais infectados através das secreções oro-nasais. Também é importante destacar o papel das matrizes, que transmitem o patógeno para suas leitegadas desde os primeiros dias de vida (Pieters & Maes, 2019). A infecção por *M. hyopneumoniae* pode acontecer em estágios iniciais da vida dos leitões, porém devido

a sua característica de causar uma doença crônica, é possível observar o quadro mais severo da infecção durante a fase de crescimento e terminação, período no qual as lesões pulmonares são mais evidentes (Sibila et al., 2009). A reinfecção em granjas livres do patógeno é comum, estudos mostram que a transmissão aerossol é favorável a dispersão de 4.7-9.2 km dentro de condições favoráveis de humidade, temperatura e velocidade do vento (Curtis et al. 1975; Dee et al. 2009; Otake et al. 2010). Além disso, os animais *backyard* também apresentam anticorpos para o patógeno, reforçando a possível transmissão cruzada com os animais de produção intensiva (Lima et al. 2024).

A detecção precoce da infecção por *M. hyopneumoniae* é fundamental para a adoção de medidas de controle. O teste sorológico por ELISA é o método mais comum, econômico e prático para monitoramento sanitário de rebanhos, pois avalia a resposta imunológica sistêmica pela quantificação de IgG em amostras de soro. No entanto, esse método não identifica infecções em estágios iniciais antes da soroconversão e não distingue respostas imunes decorrentes de infecção natural ou vacinação (Sorensen et al., 1993; Sitjar et al., 1996; Kurth et al., 2002).

Em contraste, a PCR em tempo real (qPCR) é cada vez mais considerada o teste de escolha por sua rapidez, alta precisão e capacidade de detectar baixos níveis de DNA bacteriano, inclusive em fases iniciais da infecção e antes do surgimento de sinais clínicos (Calsamiglia et al., 1999; Strait et al., 2008). O diagnóstico preciso da infecção pode ser alcançado tanto pela obtenção de amostras mais sensíveis quanto pelo desenvolvimento de ensaios com maior sensibilidade, como a qPCR (Garcia-Morante et al., 2022). Esse teste pode ser aplicado a diferentes tipos de amostras, mas apresenta maior acurácia em materiais oriundos das vias respiratórias inferiores, sendo os suabes laríngeos os mais sensíveis e práticos para detecção molecular de *M. hyopneumoniae* (Otagiri et al., 2005; Fablet et al., 2010; Pieters et al., 2017). Além disso, em rebanhos onde se discute erradicação, como nos Estados Unidos, já se dispõe de técnicas de qPCR baseadas em RNA capazes de detectar exclusivamente células viáveis do patógeno (Canturri et al., 2024).

O controle da PES e do CDRS baseia-se em medidas como biosseguridade, uso de antimicrobianos, monitoramento de patógenos e a vacinação dos rebanhos comerciais (Pieters & Maes, 2019). Embora antibióticos reduzam a carga infecciosa,

seu uso contínuo é limitado pelos riscos de resistência bacteriana e resíduos na carne (Vicca et al., 2004), levando a busca de novas moléculas e estratégias de controle a campo (Stingelin et al. 2022; Jozani et al. 2024). A vacinação, por outro lado, consolidou-se como medida central no controle de *M. hyopneumoniae* em escala global, com efeitos positivos sobre a saúde e o desempenho dos animais (Maes et al., 2021).

As vacinas comerciais contra *M. hyopneumoniae* consistem majoritariamente em bacterinas derivadas da cepa J, isolada na década de 1960 (Goodwin & Whittlestone, 1963) em associação com adjuvantes como hidróxido de alumínio, óleo mineral, parafina, Carbopol e esqualeno (Maes et al. 2018; Maes et al 2021). Embora amplamente utilizadas, oferecem proteção apenas parcial, reduzindo sinais clínicos, lesões pulmonares e excreção do agente, mas não prevenindo completamente a infecção ou a colonização (Maes et al., 2021).

Em geral, vacinas comerciais contra *M. hyopneumoniae* são aprovadas para uma ou duas doses, e podem ser combinadas com outras bacterinas, como *G. parasuis* ou Circovírus suínos tipo 2 (PCV-2). As vacinas comerciais possuem recomendações variadas, com a primeira dose indicada entre 3 e 21 dias de idade e a segunda dose administrada entre 2 e 4 semanas após a primeira vacinação (Maes et al., 2018). Estas vacinas normalmente induzem anticorpos sistêmicos contra *M. hyopneumoniae* detectáveis entre 2 e 4 semanas após imunização, e persistindo baseado na estratégia utilizada (uma ou duas doses). Entretanto, a concentração de anticorpos indica ativação do sistema imune, mas não está diretamente correlacionada com proteção contra a doença (Djordjevic et al., 1997; Thacker et al., 1998).

Fatores como a presença de anticorpos maternos, coinfecções concomitantes, diversidade de cepas circulantes e estresse no momento da vacinação influenciam sua eficácia (Simionatto et al., 2013). Além disso, os mecanismos imunológicos de proteção conferidos por essas vacinas ainda não estão totalmente esclarecidos, especialmente no que diz respeito à participação das respostas celulares (Th1 e Th17) e à produção de IgA secretora no trato respiratório (Matthijs et al., 2019a).

Estudos recentes avaliando vacinas experimentais em camundongos, e especialmente em suínos, sugerem que a construção, o tipo de antígeno e/ou carreador, e a rota e frequência de imunização pode induzir eficácia e resposta imune

variadas (Maes et al. 2021). A maioria das vacinas experimentais (baseadas em subunidade e bacterina) foram testadas em combinação com adjuvantes como hidróxido de alumínio, adjuvante de Freund, subunidade de toxinas bacterianas, adjuvantes a base de óleo ou polímero, vetores e uma combinação de diferentes ligantes de receptores Toll-like (TLR). Ainda, vacinas vetoriais foram administradas pelas vias oral ou intranasal, enquanto a maioria das vacinas de subunidades e bacterinas, administradas de maneira parenteral, pela via intramuscular (IM) ou intradérmica (ID), exceto por duas outras bacterinas, administradas pela via oral (Quadro 1).

Quadro 1: Vacinas experimentais contra *M. hyopneumoniae* avaliadas em suínos.

Tipo	Cepa / Antígeno	Plataforma / Adjuvante	Via	Desafio	Principais respostas	Referência
Inativada	F7.2C	Emulsões óleo-água (O/A) + receptores Toll-like 1/2, 7/8 e 9	IM/ID	Não	Células Th17, IgA, ↓ excreção	Matthijs et al. (2019a,b)
Inativada	232	Sílica nanoestruturada SBA-15	Oral	Sim, homólogo	↑IgA, ↑IL-4, ↓excreção	Mechler-Dreibi et al. (2021); Ferreira et al. (2023)
				Sim, heterólogo	Proteção frente a desafios de campo, dose *booster*	Storino et al. (2023)
Inativada			IM	Sim, homólogo	IgG, IgA, ↑IL-17, ↓excreção, ↓lesões	Petri et al. (2024); Aguiar et al. (2025)
Subunidade	P97R1, P46, P95 e P42	Proteínas recombinantes / bacterina vetorizada por *E. coli*	IM	Não	IgG	Oliveira et al. (2017)
Subunidade	P46 e P65	Proteína quimérica (células T)	IM, 2 doses	Não	Resposta celular Th1	Gao et al. (2022)
Subunidade	MHP418, MHP372, MHP199, P97, MHP0461	Saponina + $Al(OH)_3$	IM	Não	IgG	Santos et al. (2024)
Vetor	NrdF (R2)	*Salmonella* Typhimurium	Oral	Não	IgG, IgA pulmonar	Fagan et al. (2001)
Vetor	P97 (R1R2)	*Erysipelothrix rhusiopathiae* Koganei	Oral	Não	IgG, IgA sérico	Ogawa et al. (2009)
Vetor	P97R1, P46, GAPDH, PdhA, DnaK, EF-Tu	Adjuvante de Freund	IM	Não	IgG, IgA, Th17	Li et al. (2024)

IM: intramuscular; ID: intradérmica

Com o objetivo de superar essas limitações, pesquisas recentes têm explorado novos adjuvantes e plataformas vacinais. Formulações inovadoras como emulsões vegetais termoestáveis (Galliher-Beckley et al., 2015), fucoidana (SuYeon & HongGu, 2015), lipossomas catiônicos e ligantes de receptores do tipo Toll (Matthijs et al., 2019b) demonstraram capacidade de induzir respostas humorais e celulares mais robustas, incluindo maior indução de células Th17 e aumento da produção de IgA pulmonar. Tecnologias baseadas em nanopartículas vêm se destacando por possibilitarem liberação controlada de antígenos e direcionamento da resposta imune. A sílica mesoporosa nanoestruturada SBA-15, por exemplo, mostrou respostas imunes superiores em modelos murinos, quando comparadas a adjuvantes clássicos como o hidróxido de alumínio (Virginio et al., 2017).

Entre essas alternativas, destaca-se a utilização da sílica mesoporosa SBA-15 como plataforma para incorporação e liberação de proteínas de *M. hyopneumoniae*. Diferente de adjuvantes convencionais como emulsões oleosas e hidróxido de alumínio, a SBA-15 não apresenta toxicidade para células de defesa e promove respostas imunes equivalentes ou superiores a adjuvantes potentes como o Incomplete Freund's Adjuvant (Maes et al., 2021). Essa tecnologia, associada a formulações em pó seco de administração nasal, como a nanoemulsão revestida por quitosana desenvolvida por Canelli et al. (2023), abre novas perspectivas para o desenvolvimento de vacinas mucosais mais eficazes e capazes de induzir imunidade local e sistêmica.

A sílica mesoporosa SBA-15 tem sido amplamente investigada como plataforma para aplicações biomédicas e vacinais devido às suas propriedades físico-químicas favoráveis, como elevada área superficial, distribuição uniforme de poros e alta estabilidade estrutural (Zhao et al., 1998). Além de permitir eficiente adsorção e liberação controlada de antígenos, a SBA-15 apresenta vantagens econômicas relevantes, uma vez que pode ser sintetizada a partir de precursores amplamente disponíveis, utilizando protocolos relativamente simples, reprodutíveis e de baixo custo quando comparados a outros sistemas nanoestruturados (Vallet-Regí et al., 2007). Essas características tornam a SBA-15 uma alternativa atrativa para o desenvolvimento de vacinas aplicáveis em larga escala, especialmente na medicina

veterinária, onde o custo-benefício é um fator determinante para adoção em sistemas produtivos intensivos (Carvalho et al., 2010).

O estudo da microbiota em suínos tem revelado sua importância na resistência a infecções e na modulação da resposta imune. A diversidade microbiana no trato respiratório está associada à presença ou ausência de lesões pulmonares ocasionadas por *M. hyopneumoniae* e patógenos secundários (Huang et al., 2019; Sonalio et al., 2022), e microrganismos como *Lactobacillus* e *Prevotella* têm sido apontados como potenciais protetores contra infecções respiratórias (Blanco-Fuertes et al., 2021). Por outro lado, infecções por *M. hyopneumoniae* reduzem a diversidade bacteriana e aumentam a abundância de patógenos oportunistas, contribuindo para maior severidade clínica e favorecendo o desenvolvimento do complexo de doenças respiratórias dos suínos (CDRS) (Valeris-Chacin et al., 2021; Li et al., 2023). Estudos experimentais recentes demonstraram ainda que diferentes cepas de *M. hyopneumoniae* exercem efeitos distintos sobre a microbiota pulmonar, levando a perfis microbianos específicos associados à virulência e à dinâmica da infecção, com redução de diversidade e enriquecimento de gêneros como *Mycoplasma*, *Pseudomonas* e *Actinobacillus* (Toledo et al., 2026).

Siqueira et al. (2017) identificaram alterações na diversidade bacteriana e na composição do microbioma pulmonar em suínos com lesões pulmonares sugestivas de infecção por *M. hyopneumoniae* a campo. Sonalio et al. (2022) relataram que infecções naturais resultaram em forte correlação entre a carga bacteriana e a extensão das lesões pulmonares, indicando que maior densidade microbiana está associada a lesões mais graves. Em modelo experimental, Almeida et al. (2022) demonstraram que a inoculação endotraqueal de *M. hyopneumoniae* induz disbiose no trato respiratório inferior. De forma complementar, análises metagenômicas recentes revelaram que a microbiota do trato respiratório inferior em suínos com sinais respiratórios é composta por uma complexa rede de vírus e bactérias, incluindo PRRSV, vírus da influenza A suína, *M. hyopneumoniae*, *Glaesserella parasuis* e *Pasteurella multocida*, além de genes de virulência associados à adesão e citotoxicidade, o que reforça o papel da microbiota e de seus fatores funcionais na patogênese do CDRS (Panattoni et al., 2025). Em conjunto, esses achados evidenciam que tanto a composição quanto a funcionalidade da microbiota respiratória

influenciam a suscetibilidade à infecção e a gravidade das lesões pulmonares em suínos.

A relação entre microbiota e vacinação também vem sendo explorada. Estudos de Munyaka et al. (2019, 2020) identificaram que determinados perfis microbianos intestinais, especialmente a presença de *Prevotella*, *Anaerovibrio* e *Sutterella*, antes da vacinação, foram associados a melhores títulos de anticorpos pós-vacinais contra *M. hyopneumoniae*. Nair et al. (2019) relatou a suscetibilidade da infecção e lesões pulmonares causadas por *M. hyopneumoniae* relacionadas com a microbiota intestinal. Esses achados sugerem que a microbiota intestinal precoce pode atuar como preditor de resposta vacinal, destacando a interdependência entre imunidade entérica, mucosa respiratória e eficácia imuno protetora.

Apesar desses avanços, ainda existem lacunas significativas quanto à influência da vacinação na diversidade da microbiota respiratória e intestinal de suínos. Pouco se sabe sobre como diferentes protocolos ou vacinas inovadoras, incluindo as baseadas em nanotecnologia, impactam a composição bacteriana e, por consequência, a suscetibilidade ou resistência a infecções respiratórias.

Em síntese, *M. hyopneumoniae* permanece como agente-chave no desencadeamento da PES e do CDRS, com impactos relevantes na saúde e na produtividade da suinocultura mundial. Embora as vacinas comerciais atuais tenham reduzido consideravelmente a gravidade clínica da doença, sua eficácia parcial e a variabilidade frente a diferentes cepas reforçam a necessidade de estratégias inovadoras de imunização. Tecnologias emergentes, como vacinas baseadas em nanopartículas e adjuvantes alternativos, apresentam grande potencial para superar essas limitações, induzindo respostas imunes mais completas e duradouras.

Paralelamente, a crescente compreensão da microbiota respiratória e intestinal como moduladora da imunidade e da resposta vacinal abre novas perspectivas para o controle da PES. Investigar como a microbiota interage com a vacinação e vice-versa, pode possibilitar o desenvolvimento de estratégias integradas, menos dependentes de antimicrobianos e mais alinhadas ao conceito de saúde única, garantindo avanços sustentáveis na prevenção e no controle das doenças respiratórias suínas. Com isso, este trabalhou visou avaliar a eficácia de uma vacina experimental desenvolvida com um adjuvante nanotecnológico a sílica SBA-15 para

proteção contra o desafio por *M. hyopneumoniae* e, o papel da microbiota nasal frente a infecção e proteção clínica conferida pela vacinação na resposta imune e severidade de lesões pulmonares.

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[1]CAPÍTULO 2 – Shedding reduction and immunity modulation in piglets with an inactivated *Mycoplasma hyopneumoniae* vaccine encapsulated in nanostructured SBA-15 silica

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Abstract

Mycoplasma (*M.*) *hyopneumoniae* is a primary etiological agent of porcine enzootic pneumonia (PEP), a disease that causes significant economic losses to pig farming worldwide. Current commercial *M. hyopneumoniae* vaccines induce partial protection, decline in preventing transmission of this pathogen or inducing complete immunity, evidencing the need for improving vaccines against PEP. In our study, we aimed to test the effectiveness of the SBA-15 ordered mesoporous silica nanostructured particles as an immune adjuvant of a vaccine composed of *M. hyopneumoniae* strain 232 proteins encapsulated in SBA-15 and administered by intramuscular route in piglets to evaluate the immune responses and immune-protection against challenge. Forty-eight 24-day-old *M. hyopneumoniae*-free piglets were divided into four experimental groups with different protocols, encompassing a commercial vaccine against *M. hyopneumoniae*, SBA-15 vaccine, SBA-15 adjuvant without antigens and a non-immunized group. All piglets were challenged with the virulent strain 232 of *M. hyopneumoniae*. Piglets that received the SBA-15 and commercial vaccine presented marked immune responses characterized by anti-*M. hyopneumoniae* IgA and IgG antibodies in serum, anti-*M. hyopneumoniae* IgA antibodies in nasal mucosa and showed an upregulation of IL-17 and IL-4 cytokines and downregulation of IFN-γ in lungs 35 days post-infection. Piglets immunized with SBA-15 vaccine presented a reduction of bacterial shedding compared to piglets immunized with a commercial bacterin. In addition, piglets from SBA-15 adjuvant suspension group presented increased IL-17 gene expression in the lungs without involvement of Th1 and Th2 responses after challenge. These results indicated that SBA-15 vaccine induced both humoral and cell-mediated responses in the upper respiratory tract and lungs, first site of replication and provided protection against *M. hyopneumoniae* infection with a homologous strain with reduction of lung lesions and bacterial shedding. Finally, these results enhance the potential use of new technologies such as nanostructured particles applied in vaccines for the pig farming industry.

Keywords: Adjuvant; Nanotechnology; Porcine enzootic pneumonia; Th1; Th17; Upper respiratory tract.

1. Introduction

Mycoplasma (*M.*) *hyopneumoniae*, recently reclassified as *Mesomycoplasma hyopneumoniae*, is the primary cause of porcine enzootic pneumonia (PEP), and a key bacterial pathogen in the porcine respiratory diseases complex (PRDC) [1]. The chronic infection causes a mild and non-productive cough, delayed weight gain, high morbidity, low mortality, and cranio-ventral pulmonary consolidation (CVPC) lesions [2], leading to significant economic losses [3]. *M. hyopneumoniae* impairs lung clearance by affecting cilia and modulating the respiratory tract immune system [4]. Control strategies include pathogen monitoring, biosecurity, vaccination, and antimicrobials, with vaccination reducing clinical severity but not completely preventing colonization [5,6]. Inactivated, adjuvanted whole-cell vaccines are commonly used on commercial pig farms to control *M. hyopneumoniae* [7].

Adjuvants are crucial for enhancing vaccine immunogenicity, pro- longing immune response, and regulating immunity by promoting sustained antigen release, optimizing presentation and minimizing pathogen virulence [8]. While conventional *M. hyopneumoniae*-vaccine adjuvants like aluminum salts, carbopol, mineral oil, or biodegradable oil enhance dendritic cells (DC) activation [9], safety concerns limit the number of licensed options for human and veterinary vaccines [10,11]. The benefits and risks of different aluminum adjuvants and their concentrations remain uncertain [12]. Nanostructured systems, serving as innovative adjuvants, protect vaccine antigens and modulate immunity, serving as a pulmonary drug delivery [13]. They show an intrinsic activity that, after being internalized by antigen-presenting cells (APCs), allows the formation of a complex that induces and absorbs immune cells [14]. In this context, mesoporous silica nanoparticles (~20 pm), which are nanostructured internally, are particularly promising. They effectively enhance both cellular and humoral immunity in animal models [15,16], exhibit very low toxicity and have good biocompatibility [17], proving to be a promising nanotechnological candidate adjuvant against *M. hyopneumoniae* and veterinary vaccines.

SBA-15 nanostructured silica enhances immunogenicity and stimulate Th1 and Th2 immune activity via intramuscular or oral administration in humans and mice [8,15,18,19], and Th1, Th2 and Th17 immune responses via intraperitoneal injection [18] and the capacity to carry more than one type of antigen in its nanostructured pores,

as shown by a study testing a vaccine anti-diphtheria and anti-tetanus [19]. An experimental SBA-15 vaccine against *M. hyopneumoniae* showed efficacy comparable to aluminum adjuvant [20]. Previous studies by our group on SBA-15 via mucosal route in piglets provided protection against homologous and heterologous challenge, inducing specific immune responses in groups that received intramuscular vaccines plus any type of oral immunization with the SBA-15 vaccine [21,22], but did not perform as well as conventional vaccines in terms of immune parameters or protection from challenge after a single immunization without association with intramuscular injection [23]. Furthermore, field application in pig farms presents challenges related to product stability, accurate dosing, and efficacy at the herd level.

Finally, the aim of this study was to develop and evaluate the capacity of an inactivated vaccine formulated with encapsulation of protein cells of *M. hyopneumoniae* strain 232 in the SBA-15 nanostructured silica with one-dose administration by intramuscular route in piglets. The effectiveness based in lung lesions and shedding reduction after challenge and protective immunity elicited by antibody responses and cytokine gene expression were determined in vaccinated piglets challenged with a homologous strain of *M. hyopneumoniae*.

2. Materials and methods

2.1 Ethics statement

The experimental trial involving piglets adhered to ethical standards and guidelines for animal experimentation by the Brazilian College of Experimentation. Approval for all procedures was granted by the Animal Ethics Committee of the School of Agricultural and Veterinary Sciences, São Paulo State University, as registered by Protocol Number #003897/21. Various types of environmental enrichment were provided, and the pigs were regularly checked by veterinarians.

2.2 Mycoplasma hyopneumoniae cultivation and protein extraction

A pathogenic *M. hyopneumoniae* strain (232), certified pathogen-free, was purchased from Iowa State University (ISU, Ames, USA) [24]. Aliquots were cultivated in Friis medium, with fractions in 1× (liquid) and 2.8× (solid) Friis medium, as described

elsewhere [25]. Reactivation involves a 1:9 ratio of frozen inoculum to liquid medium, which is incubated at 37 ºC until a colour change indicates bacterial growth. After three passages, aliquots were frozen at − 80 ºC until inoculation and cellular proteins extraction. Quantification was performed by the Colour Changing Units (CCU) technique, and colony morphology was observed on 2.8× solid Friis medium incubated for seven days at 37 ºC [25].

Cellular proteins were extracted from bacterial culture via centrifugation at 13700g. The cell pellets were washed in 15 mL of phosphate-buffered saline (PBS) solution (1×, pH 7.4; Sigma-Aldrich, Germany) and centrifuged at 21000g until clean pellets formed. These pellets were resuspended in 10 mL of 1× PBS and stored in sterile Falcon tubes (Corning®, USA). The second bottle was sonicated on ice (three cycles, 20 Hz, 1 min each). A protease inhibitor (Cocktail Tablets, Sigma Aldrich, Germany) was added post-sonication. Protein concentration was determined using the Bradford method kit (Thermofisher Scientific, USA), and measured with a microplate reader (iMark™, Bio-Rad Laboratories Inc., USA). Flasks were stored at − 20 ºC until use.

2.3 Preparation and characterization of SBA-15-M. hyopneumoniae vaccine

The SBA-15 sample was synthesized following Zhao et al. [24] with modifications [25]. The structural and textural characterizations were performed using small angle X-ray scattering (SAXS) and nitrogen adsorption isotherm (NAI), respectively, at the Crystallography Laboratory of the Physics Institute of the University of São Paulo. Thermal characterization was conducted using thermogravimetric analysis (TG) and differential scanning calorimetry (DSC). Spectroscopic and morphological studies were carried out using Fourier transform infrared spectroscopy (FTIR) and scanning electron microscope (SEM), respectively (Supplementary data – Fig. S2). The synthesis of SBA-15, as well as all thermal, spectroscopic, and morphological characterizations, were conducted at the Hybrid Materials Laboratory of the Chemistry Department of the Federal University of São Paulo (UNIFESP/Diadema).

Vaccine preparation followed the Mechler-Dreibi et al. (2021) [21] with modifications. The sonicated *M. hyopneumoniae* protein extract was slowly incorporated into silica SBA-15, at a 1:35 mg antigen:SBA-15 ratio. The silica was weighed, macerated, heat-activated, and dried before adding the antigen. The mixture

was incubated for 5 days to form the SBA-15 + antigen complex. Finally, doses were prepared with the addition of PBS solution (1×, pH 7.4; Sigma-Aldrich, Germany) and the same process was made for SBA-15 without antigen doses and stored at 8 ◦C until administration.

2.4 In vitro characteristics of SBA-15-M. hyopneumoniae vaccine

SAXS experiments were performed with a Xeuss 2.0 (Xenocs) equipment, using a GeniX 3D X-ray source with a copper target (λ = 1.518 Å) and a FOX3D collimation optics in a multi-user Laboratory of the Physics Institute, University of São Paulo, Brazil. The SAXS analysis on dried samples (dried at 35 ºC for 24 h) of pristine SBA-15, SBA-15 with 4 mL of PBS and SBA-15 plus PBS plus Antigen (1:35 ratio), such that the amount of PBS in the final mixture was kept constant. The sample-to-detector distance was 1.20 m, giving a q-range of 0.00849–0.33197 Å − 1. The SAXS images were collected on a PILATUS 300 k 2D photon counting detector. The powder samples were spread between two Kapton foils. The scattering of these foils was used for background removal. The data were measured under mechanical pump vacuum at a temperature of 25 ºC. Azimuthal integration of the 2D images was performed with the software Fit2D [26]. Each 1D curve was processed using the SUPERSAXS software (Oliveira, C.L.P.; Pedersen, J. S., unpublished), including the estimated errors. The scattering intensity is displayed as a function of the scattering vector modulus $q = (4\pi/\lambda) \sin\theta$, where θ is half of the scattering angle. To gather more information on antigen incorporation in different pore scales (micro, meso and macro), NAI measurements were conducted on dried samples with a reduced antigen:SBA-15 ratio of 1:250 mg in to avoid antigen clustering.

2.5 Selection of piglets and experimental design

Forty-eight weaned piglets with 19 days of life weighing 6–7 kg from a *M. hyopneumoniae*-free farm were selected for the study. All piglets tested negative for *M. hyopneumoniae* via qPCR and ELISA in laryngeal and serum samples, respectively, prior to the inclusion in our study. They were housed in facilities at FCAV/Unesp, identified with individual ear tags and each group divided into separated

four pens until the end of the experiment period at 80 days of life (Day 56 of the study; D56). At 24 days old they were assigned to four groups: commercial vaccine (CV), prepared vaccine SBA-15 with antigen (SBA-15 + Ag), SBA-15 without antigen (SBA-15 + PBS), or sterile physiological solution (CONT). Vaccination was intramuscular at the ear based on D0. CV received M+PAC® (MSD Animal Health, NJ, USA), a worldwide utilized commercial product with a *M. hyopneumoniae* antigen adsorbed to an aluminum-hydroxide adjuvant in an oil emulsion, and SBA-15 + Ag received SBA-vaccine (0.5 mg of antigen/mL PBS, 1 mg dose in 2 mL). At 45 days of life (D21), all piglets were challenged with 5 mL of culture medium containing 10^6 CCU/mL of *M. hyopneumoniae* strain 232 via the endotracheal route (Fig. S1). After inoculation, from D14 to D56, between 9 and 10 a.m., the piglets underwent daily observations by the primary investigator for a minimum of 20 min. Each individual piglet's coughing severity was assessed using a respiratory disease score (RDS) [27] (Fig. S1 – Supplementary Material).

2.6 Sample collection (blood serum, nasal and laryngeal swabs)

Biological samples were taken weekly. Blood samples were collected from piglets via jugular vein puncture using sterile needles (25 ×8 mm) into clot-activating vacuum tubes (BD Vacutainer®, Franklin Lakes, NJ, USA). After centrifugation at 3000 rpm for 10 min, blood serum was aliquoted into 1.5 mL fractions, duplicated in 2 mL microtubes (Kasvi, Brazil), and stored at − 20 ºC. Nasal swabs were collected using sterile cotton swabs rubbed gently on the mucosa of both nostrils and stored in sterile microtubes containing 500 µL of PBS 1×(pH 7.4; Sigma-Aldrich, Germany) to quantification of IgA anti-*M. hyopneumoniae* in nasal secretions. Laryngeal swabs were taken using sterile cotton swabs and a laryngoscope with a light source, intending the detection of *p102-M. hyopneumoniae* gene by qPCR. Swabs were stored in sterile microtubes containing 500 µL of PBS and kept at − 80 ºC until use. All sampling materials were cleaned with sodium hypochlorite 1 % and sprayed with ethanol 70 % between sampling piglets.

2.7 Euthanasia of piglets and macroscopic and microscopic lung lesion evaluation

Five weeks post-challenge on D56, all piglets were humanely euthanized, with a protocol approved by the Federal Council of Veterinary Medicine, Brazil. Piglets were sedated with an intramuscular administration of acepromazine, ketamine and xylazine (0.05 mg/kg, 10 mg/kg and 0.05 mg/kg, respectively), followed by intravenous infusion of ketamine and propofol (3 mg/kg and 4 mg/kg, respectively). Euthanasia included a 10 mL of lidocaine 2 % puncture in the atlanto-occipital space. After evisceration, the respiratory set (trachea +lung) was isolated to assess macroscopic lung consolidated lesions (MLCL) according to the European Pharmacopeia methodology [28], summing the affected lobe areas [29]. Also, fragments were collected aiming bronchus-associated lymphoid tissue (BALT) scoring under a light microscope (Leica DM1000 LED, Leica Microsystems, Germany), with photomicrographs captured with a coupled camera (Leica MC190 HD, Leica Microsystems, Germany). Microscopic lung lesions were scored based on Hansen et al. (2010) methodology [30], where scores 3 and 4 are mainly considered inherent to *M. hyopneumoniae* infection.

2.8 Collection of lung fragments and BALF samples

During necropsy on D56, after evisceration and MLCL scoring, bronchoalveolar fluid (BALF) was collected by introducing 20 mL of PBS into the trachea, gently massaging the lung lobes and aspirating approximately 10 mL. BALF was aliquoted into sterile microtubes (Kasvi, Brazil) and stored at − 80 ºC. Then, portions of fresh lung tissue fragments were collected for DNA and RNA extraction using sterile tools, placed in DNAse and RNAse-free cryotubes (Corning, USA), and stored at − 80 ºC.

2.9 Quantification of M. hyopneumoniae-specific antibody responses in blood serum

A commercial ELISA kit (*M.hyo* Ab test, Idexx, USA) was used to measure the concentration of *M. hyopneumoniae*-specific Immunoglobulin (Ig) G in serum samples, following the manufacturer's instructions. Samples were considered positive when S/P ≥0.4. IgA in serum samples was measured utilizing the antigen-coated Nunc PolySorp® flat-bottom 96 well plates (eBioscience, USA) of the kit mentioned above,

with protocol modifications. Briefly, after blocking PBS containing 0.05 % Tween 20 and 1 % BSA for 1 h at 37 ºC, plates were washed three times with PBS +0.05 % Tween 20 (PBS-T20). Serum diluted in dilution solution (PBS-T20 containing 1 % BSA) was added, incubated for 60 min at 37 ºC, then washed. Peroxidase-labeled goat anti-porcine polyclonal IgA (Bethyl Laboratories, Montgomery, TX, USA) diluted 1:4000 was added, incubated for 60 min at 37 ºC, washed, and 3,3′5,5′-tetramethylbenzidine substrate (TMB, Sigma-Aldrich, Saint Louis, MO, USA) was added. After 10 min, the reaction was stopped with 2 N HCl solution (Sigma-Aldrich, Saint Louis, MO, USA), and optical densities (OD) were measured at 655 nm. To quantify the antibody levels a positive control (PC) was used from a pooled positive reference serum from animals on D35, and the negative control (NC) was from a serum from animals on D0 and set up as a cut off positivity.

2.10 Quantification of M. hyopneumoniae-specific antibody detection in nasal swabs and BALF samples

Nasal swabs and BALF samples collected on D56 were analyzed undiluted for *M. hyopneumoniae*-specific IgA antibodies using the antigen-coated Nunc PolySorp® flat-bottom 96 well plates (eBioscience, USA) from the commercial ELISA kit (*M.hyo* Ab test, Idexx, USA), as described in Section 2.9. The protocol was followed with a modification: peroxidase-labeled goat anti-porcine polyclonal IgA (Bethyl Laboratories, Montgomery, TX, USA) was diluted 1:4000 and 1:8000 for use in the indirect ELISA to measure IgA anti-*M. hyopneumoniae* in nasal secretions and BALF, respectively. Absorbance was measured at 655 nm. Cut-offs of positivity were established based on control OD means plus three times the standard deviation.

2.11 DNA extraction of lungs and laryngeal swabs

DNA extraction was conducted using the Tris-HCl protocol by Kuramae-Izioka et al. [31]. For the lung samples, 30 mg of lung tissue was used. For BALF samples, centrifugation was performed (Centrifuge 5804 R, Eppendorf®, Hamburger, Germany) at 13,000g at 4 ºC for 20 min before the DNA extraction. Regarding DNA extraction from liquid samples, 500µL of PBS from laryngeal swabs were used in Tris-HCl protocol. Measurement of the DNA concentration of the samples was performed by

spectrophotometry (NanoDrop™ One, Thermofisher Scientific, USA), having as an exclusion factor the samples that did not reach the purity of 1.8-2.0 in the 260/280 ratio.

2.12 Evaluation of bacterial loads by qPCR assays targeting p102 gene of M. hyopneumoniae

M. hyopneumoniae detection by qPCR was performed using DNA from samples and the primers used in the reaction were based on the bacterium *p102* adhesion protein gene sequence. All samples were tested in duplicates, using *M. hyopneumoniae*-specific primer pair and hydrolysis probe from a previously published protocol [32]. Quantification data were considered valid only when the efficiency fell within the range of 90–105 % [33]. If the efficiency was outside this range, the samples underwent retesting in triplicates. The results were only accepted with a ΔCq equal to or less than 0.5 between replicates. The standard curve is constructed using serial dilutions of synthetic dsDNA containing the target sequence (gBlock®, IDT, USA) with concentrations ranging from 10^7 copies/µL to 10^1 copies/µL, as a positive control.

2.13 RNA extraction and cDNA synthesis

Total RNA was extracted from 30 mg of lung tissue using the RNeasy Blood and Tissue Plus kit (Qiagen, USA). RNA was quantified at 260 nm using a NanoDrop™ One spectrophotometer (Thermo Fisher, USA) and evaluated for purity and integrity with a Bioanalyzer® (Thermo Fisher, USA). For cDNA synthesis, 500 ng of RNA per reaction was used with the High-Capacity cDNA Reverse Transcription kit (Thermo Fisher, USA), following the manufacturer's guidelines. Only samples with an RNA Integrity Number (RIN) above 7.0 were used. Samples of cDNA were stored at −20 ºC until qPCR analysis.

2.14 Evaluation of cytokine gene expression by RT-qPCR

On D56, the expression of Th1, Th2, Th17 and other cytokines were assessed in lung lesion fragments. Ribosomal protein L4 (*rpl-4*) was used as the reference gene for normalization by the $2^{-\Delta\Delta CT}$ method [34] based on the optimal results [21,32]. The study focused on transcript levels of inflammation (IL-6 and IL-8) and specific immunity

regulation where Th1 responses were marked by gene expression of IFN-γ, Th2 by IL-4, TGF-β gene expression and Th17 by IL-17 modulation in each immunization type compared to the control group. Specific primers for IL-4 and TGF-β were from Mechler-Dreibi et al. [21], while IL-6, IL-8 and IFN-γ from Almeida et al. [32] and IL-17 from Stepanova et al. [35].

2.15 Statistical analysis

The variables within each group and time point were assessed for normality and homoscedasticity using the Shapiro-Wilk and Bartlett tests, respectively. Mean differences were determined by Tukey's test ($p < 0.05$). Variables not meeting the assumptions underwent the non-parametric Kruskal-Wallis test ($p < 0.05$), and in cases of significance, the Dunn test (*Post-hoc*) was used. For parametric data, Pearson's correlation test ($p < 0.05$) was applied, while non-parametric data underwent Spearman's test. Cytokine gene expression was normalized, and graphs were plotted using GraphPad Prism 10.2.2 software (La Jolla, CA-USA).

3. Results

3.1. Silica-based M. hyopneumoniae vaccine characterization

The chemical parameters of SBA-15 and micrographs of the SBA-15 sample used in this study are shown in Fig. S2. The concentration of the protein extract was 810 µg/mL in PBS 1×. Then they were incorporated into SBA-15 samples (SBA-15:antigen equal to 35:1 mg). Post-incorporation, *M. hyopneumoniae* proteins in the vaccine tested positive by qPCR, showing estimated quantification of 1.13 $\times 10^7$ copies/uL of the *p102* adhesin protein gene. Analysis of silica revealed five diffraction peaks, indicating a well-ordered mesoporous structure with a two-dimensional hexagonal arrangement (Fig. S3). Differences in SAXS curves indicate a successful protein encapsulation within the silica pores.

NAI results showed a 14 % decrease in the surface area of SBA-15 with PBS compared to pure SBA-15. With antigen incorporation, assuming a constant PBS volume, the surface area decreased by 74 %. PBS fills 14 % of the pore volume of pure SBA-15, and antigen presence reduced mesopore volume by 68 %, likely due to

encapsulation blockage. Table S1 presents relevant NAI parameters, and pore size distributions are shown in Fig. S4.

3.2. Macroscopic and microscopic lung lesion score

The macroscopic lung consolidation lesions (MLCL) and microscopic lung lesions (MLL) were evaluated at 35 days post-infection (D56). MLCL were observed in 8/12 and 10/12 piglets from vaccinated groups (CV and SBA-15 + Ag) and 11/12 and 10/12 piglets from non-immunized groups (SBA-15 + PBS and CONT). Vaccinated groups had significantly lower MLCL scores compared to the non-immunized groups ($p < 0.05$) (Fig. 1A). In contrast, no statistical differences within the immunized groups were observed (Fig. 1B). Mean MLCL scores (±sd) were CV: 4.265 % (±5.53), SBA-15 + Ag: 6.49 % (±4.67), SBA-15 + PBS: 9.67 % (±7.33) and CONT: 25.26 % (±15.66). Additionally, we observed expressive MLCL reduction between immunized groups and control groups. Lung lesion extent was reduced to 83.1 % and 74.31 % in piglets from CV and SBA-15 + Ag groups, respectively. Also, the SBA-15 + PBS group had a reduction of 61.72 % when compared to CONT.

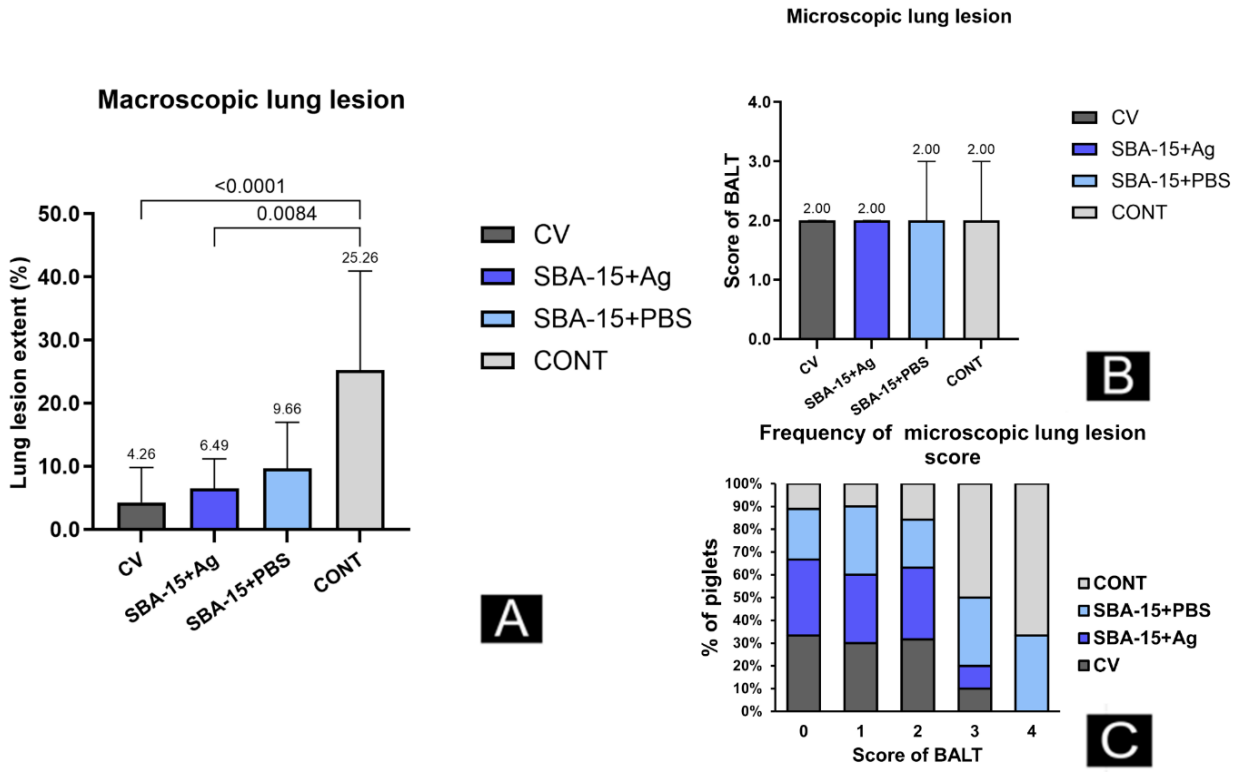


Figure 1: Macroscopic and microscopic lung lesions were assessed at euthanasia on D56 in piglets infected with *M. hyopneumoniae* strain 232. (A) Mean macroscopic lung lesion area (±sd) and (B) median microscopic lung lesion score (with interquartile range) was compared across four vaccination protocols and (C) the frequency of microscopic lung lesion scores (0-4) related to BALT hyperplasia was assessed. *Statistical differences between groups were checked by Tukey test (p<0.05).

Median MLL scores (interquartile range) were CV: 1 (1), SBA-15 + Ag: 2 (1), SBA-15 + PBS: 2 (1.75) and CONT: 2 (2) (Fig. 1B). About 60 %–70 % of piglets from vaccinated groups had BALT hyperplasia scores of 0 and 1, whereas all lung tissues with a BALT score of 4 were from the SBA-15 + PBS and CONT group (Fig. 1C). No significant differences were found between groups for MLL scores (p > 0.05) (Fig. 1B). Representative MLCL and major MLL, including inflammatory infiltrate and lymphoid follicle hyperplasia are shown in Figs. 2 and 3.

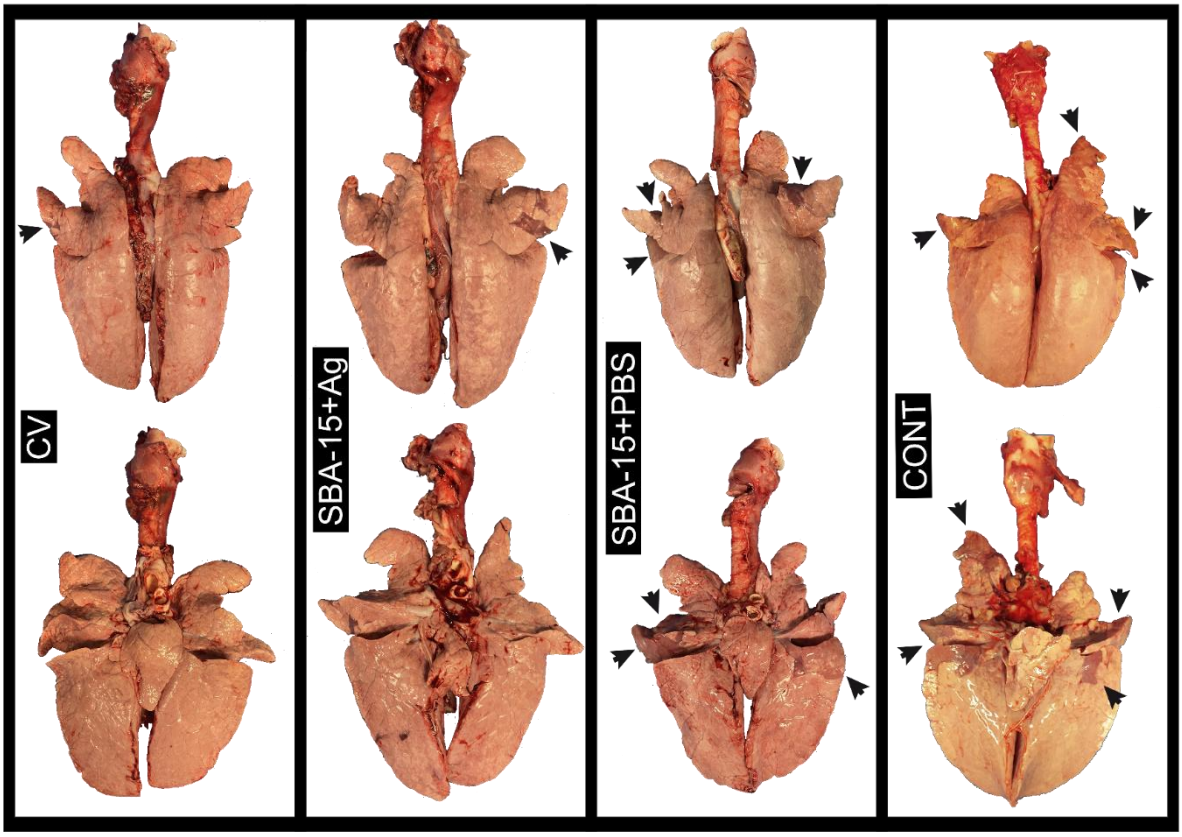


Figure 2: Macroscopical assessment of lung lesions. Representative photographs of cranioventral consolidation pulmonary from piglets of experimental groups; macroscopic lesion compatible with *M. hyopneumoniae* infection (black arrows). CV: commercial vaccine; SBA-15+Ag: SBA-15 vaccine; SBA-15+PBS: SBA-15 suspension and CONT: physiological saline solution.

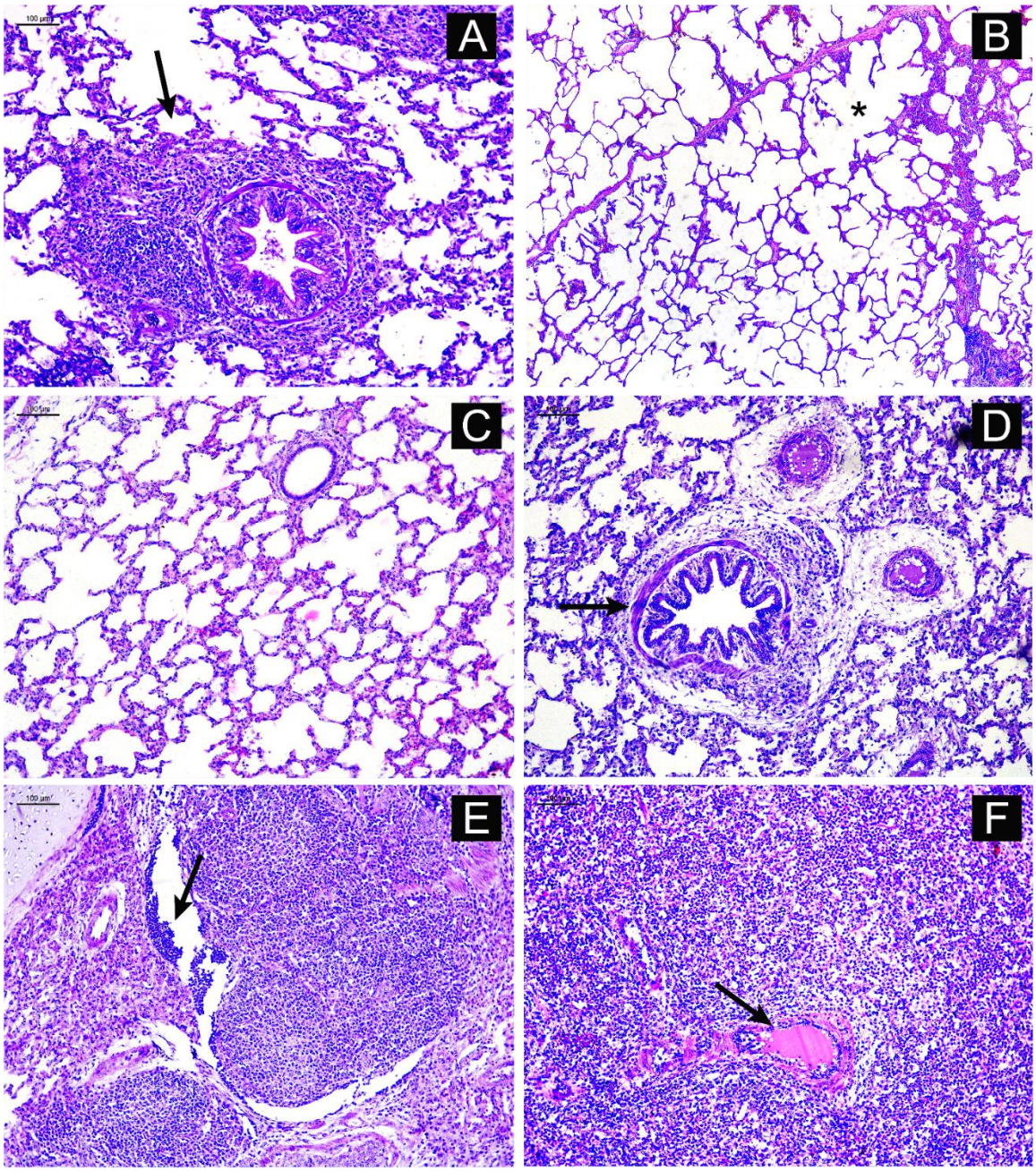


Figure 3: Microscopic lung lesions observed after challenge with *Mycoplasma hyopneumoniae* including: (A) intense inflammatory infiltrate of degenerate neutrophils, lymphocytes, and macrophages around bronchioles (arrow); (B) no alterations in alveoli lumen (asterisk); (C) thickened alveolar septa with interstitial infiltrate; (D) bronchiole infiltration by degenerate neutrophils and macrophages (arrow); (E) lymphoid follicle hyperplasia (arrow); and (F) edema with amorphous, acidophilic material surrounded by inflammatory infiltrate. Hematoxylin-Eosin, 100x.

3.3. Serum IgG and IgA antibody responses against M. hyopneumoniae

At systemic level, all piglets were negative for anti-*M. hyopneumoniae* IgG and IgA antibodies at the beginning of the study (S/P ratio <0.4 and OD value<0.25). The cut-off for indirect IgA ELISA was set up at an OD value of 0.25. Both immunized groups (CV and SBA-15 + Ag) first presented piglets with *M. hyopneumoniae* IgG and IgA levels at 21- and 28-days post-vaccination, respectively, significantly higher than non-immunized groups (SBA-15 + PBS and CONT) ($p < 0.05$). On 35 days post-vaccination, immunized groups showed significantly higher anti-*M. hyopneumoniae* IgG levels than SBA-15 and CONT groups ($p <0.05$). At 35- and 42-days post-infection (D49 and D56), IgG levels further increased in immunized groups, distinct from SBA-15 + PBS and CONT groups ($p <0.05$), as shown in Fig. 4A. All challenged groups displayed elevated IgG levels at 35 days post-infection (D56), differing from the negative group ($p < 0.05$) (Table S2). For the response to *M. hyopneumoniae* expressed by the production of IgA in serum was evaluated in all piglets (Fig. 4B). Overall, immunized piglets (CV and SBA-15 + Ag groups) had higher levels than non-immunized groups at D35 ($p <0.05$) (Table S3). The SBA-15 + Ag group showed increased IgA levels at D49 and D56, while the CV group had a slight decline, with no significant difference between them ($p >0.05$) (Fig. 4B).

Additionally, significant positive correlations were observed between IgG and IgA anti-*M. hyopneumoniae* antibody levels in serum samples from vaccinated piglets at D56 ($p < 0.05$). Correlation coefficients were r =0.736 for CV and r =0.677 for SBA-15 + Ag.

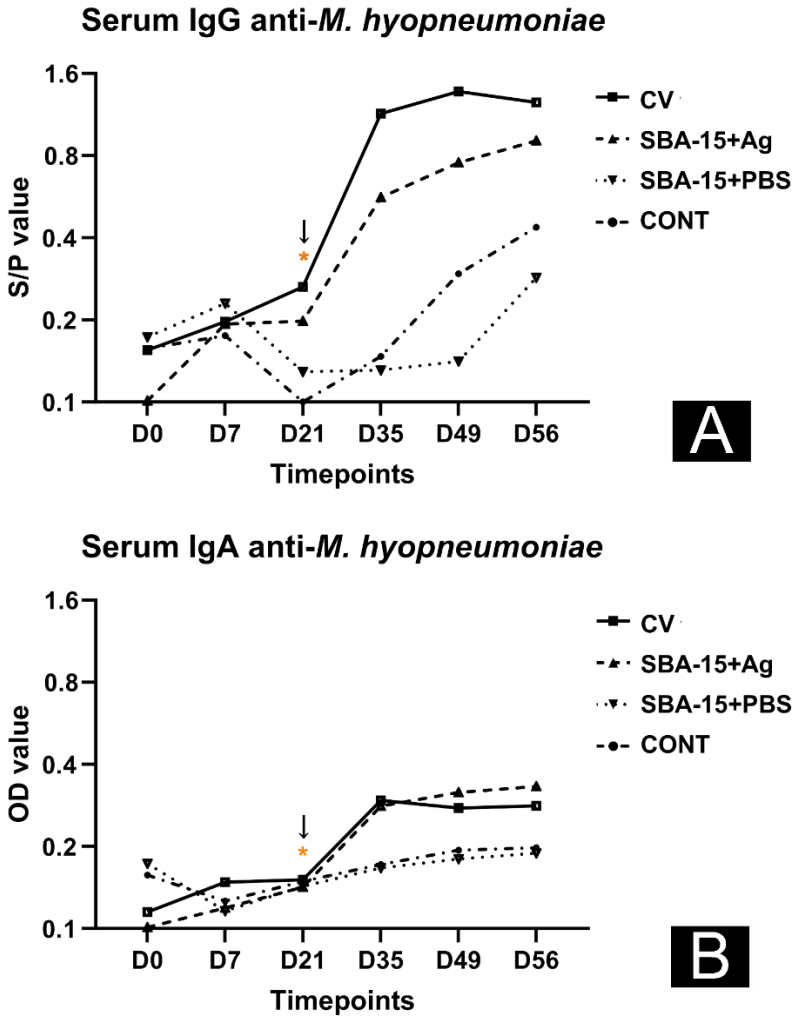


Figure 4: Specific IgA and IgG anti-*M. hyopneumoniae* antibody responses in serum. On Day 21, all piglets were challenged with *M. hyopneumoniae* (red asterisk). Data points represent group mean values on each study day. Absorbance was measured at 655 nm, with positivity thresholds set at (A) S/P values>0.4 for IgG anti-M. hyopneumoniae and (B) OD values>0.2 for IgA anti-*M. hyopneumoniae*.

3.4. Mucosal responses against M. hyopneumoniae

The mucosal immune response to *M. hyopneumoniae*-specific IgA antibodies in respiratory mucosa were quantified in all piglets (Fig. 5). The cut-off value of the indirect IgA ELISA was set up at an OD value of 0.2 and 0.3 for nasal secretions and BALF, respectively. Immunized piglets (CV and SBA-15 + Ag) showed elevated anti-*M. hyopneumoniae* IgA levels of antibodies in nasal swabs at 28-days post-

vaccination, significantly higher than non-immunized groups (SBA-15 + PBS and CONT) ($p < 0.05$). At 14 days post-infection (D35), higher anti-*M. hyopneumoniae* IgA levels were detected in nasal mucosa of immunized groups (CV and SBA-15 + Ag), differing statistically from non- immunized groups ($p < 0.05$) (Fig. 5A; Table S4). Further elevation in anti-*M. hyopneumoniae* IgA levels was first detected at 21 days post-infection (D42) in the SBA-15 + PBS group. At necropsy, all BALF samples from piglets immunized or non-immunized groups showed higher IgA anti-*M. hyopneumoniae* levels, with no statistical differences between groups ($p >0.05$) (Fig. 5B; Table S4).

Significant negative correlations were found between IgA levels in mucosal sites and MLCL ($p < 0.05$; Table S3), with a correlation coefficient of $r =− 0.57$ for MLCL vs. IgA in BALF ($p = 0.02$) in the commercial vaccine group. Positive correlations were observed between IgA levels in nasal swabs and BALF ($r =0.726$, $p = 0.006$) and between IgA in nasal swabs and IgG in serum for the SBA-15 group on D56 ($r =0.67$; $p = 0.01$).

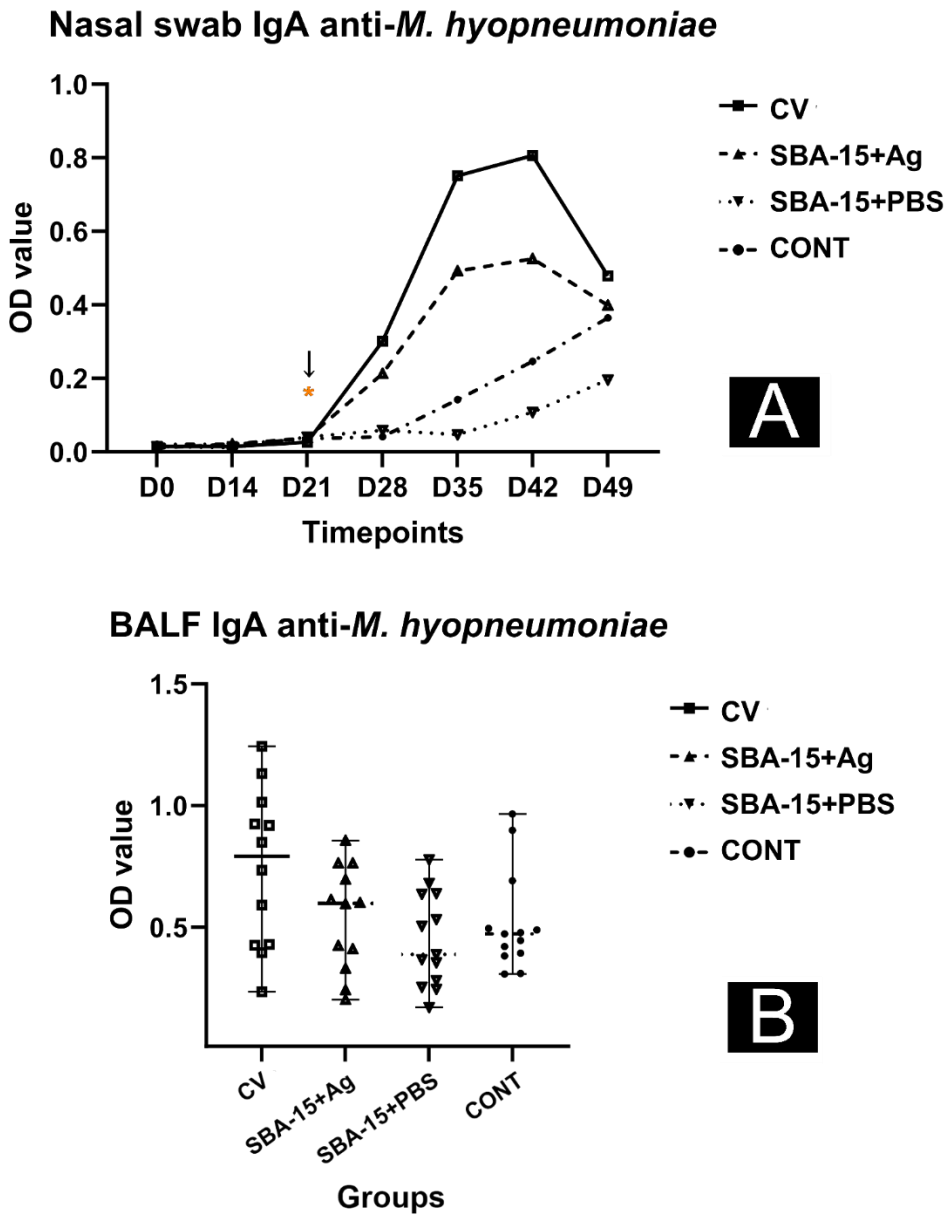


Figure 5: Specific IgA anti-*M. hyopneumoniae* antibody responses in nasal and lung mucosa. On Day 21, all piglets were challenged with *M. hyopneumoniae* (red asterisk). Mean values for each group were plotted by nasal swabs on each study day and in BALF samples were measured on D56. Absorbance was read at 655 nm with positivity thresholds of (A) OD > 0.2 for nasal swabs and (B) OD > 0.3 for BALF.

3.5. Clinical signs, shedding and colonization of bacteria in lung tissue

The beginning of clinical signs post-infection was reported in piglets from all groups 9 days post-infection on D31 and the mean increased progressively by group until D49 (Fig. 6). In groups CV, SBA-15 + Ag and CONT, coughing peaked between D50-D56 (29- and 35-days post-inoculation), whereas in group SBA-15 + PBS a peak of coughing events was observed between D43-D49 (22- and 28-days post- inoculation). Both vaccinated groups had the highest RDS mean recorded in the end of study. The highest RDS mean was registered in CONT group.

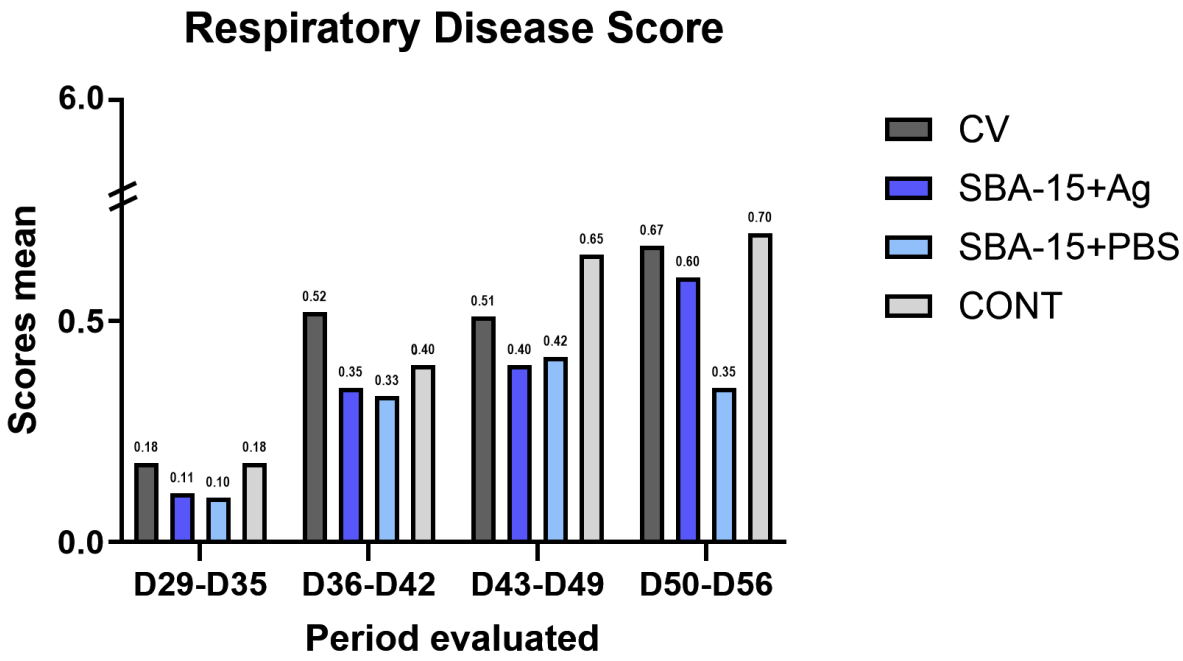


Figure 6: Mean respiratory disease score (RDS) per experimental group. Coughing events were daily counted for 20 minutes from D14 (data not shown) until D56. The mean RDS was the calculated and expressed by experimental group and week.

M. hyopneumoniae shedding was first detected 7 days post-infection (D28) in laryngeal swabs (Fig. 7A). Immunized piglets showed lower bacterial shedding compared to non-immunized groups, with SBA-vaccinated piglets showing less shedding than those with the commercial vaccine on D35. By D49, all immunized piglets tested negative for *M. hyopneumoniae* in qPCR in laryngeal swabs. All piglets

had *M. hyopneumoniae-p102* gene in tissue fragments and non-vaccinated groups showed 1–2 log higher mean bacterial loads in lung lesions than vaccinated ones with statistical differences between them (Fig. 7B).

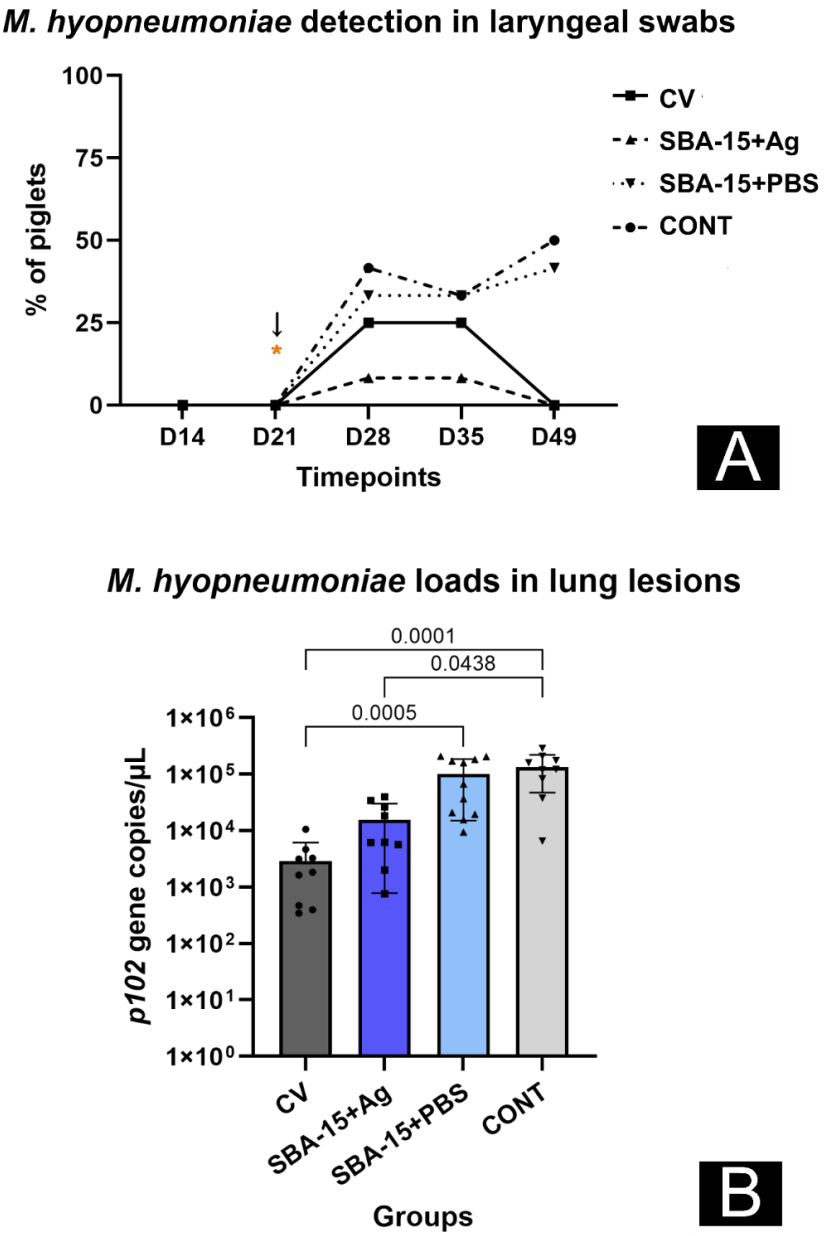


Figure 7: Dynamic shedding and colonization of *M. hyopneumoniae* in piglets: (A) Percentage of piglets with detectable bacterial loads in laryngeal swabs over time post-infection with *M. hyopneumoniae* strain 232 (red asterisk). (B) Bacterial loads in lung fragments measured as log10 of *M. hyopneumoniae p102* gene copies by qPCR, shown as mean (±sd). Group comparisons used the Kruskal-Wallis test ($p>0.05$).

In addition, a negative moderate correlation was found between lung bacterial loads and IgA anti-*M. hyopneumoniae* antibodies in BALF of piglets from CV (r = −0.632, p = 0.024). Colonization and shedding are shown in Fig. 7, and qPCR parameters are in Table S5.

3.6. Gene expression of cytokines in lung tissue

The in vivo expression of the Th1, Th2, Th17 and other immune response cytokines were studied on D56 encompassing two events: 56 days post-vaccination and 35 days post-infection with *M. hyopneumoniae* strain 232. We found that a significant modulation of IL-4, IL-6, IL-8, IL-17, IFN-γ and TGF-β gene expression in lung lesions was observed in both immunized and non-immunized piglets (Fig. 8). In comparison among groups, CV and SBA-15+Ag presented and upregulation of IL-4 gene expression, while SBA-15+PBS and CONT showed a downregulation, with significant statistical difference between them (p=0.002). For IFN-γ we found a tendency for a downregulation in CV and SBA-15+Ag, non-variation on the expression for SBA-15+ PBS, and a tendency for an upregulation in CONT, with difference between them (p< 0.05). In addition, an upregulation in the expression of IL-17 occurred in CV, SBA-15 +Ag, and SBA-15+PBS, while in CONT occurred a downregulation (p=0.002). A tendency for downregulation of the expression of IL-6 gene in CV and SBA-15+Ag groups, differing from the animals from SBA-15+PBS and CONT groups, though no significant statistical differences were found between these groups (p>0.05). Regarding the expression of IL-8 gene, CV group presented a tendency for downregulation, and SBA-15+Ag presented no modulation, while SBA-15+PBS and CONT groups presented a tendency for an upregulation, though no significant statistical differences between them (p>0.05). Finally, the expression of TGF-β gene showed a tendency for slight upregulation in CV, SBA-15+Ag, and CONT groups and no modulation in SBA-15+PBS group no statistical differences were observed (p> 0.05).

A very strong negative correlation was found between IFN-γ gene expression in lungs and nasal IgA anti-*M. hyopneumoniae* antibody levels (r =− 0.741, p < 0.01) and between IL-17 and IL-6 gene expression levels (r =− 0.713, p <0.02) in vaccinated piglets from CV on D56. In SBA-15 + Ag piglets, a strong negative correlation was

found between IL-17 gene expression and *M. hyopneumoniae* bacterial loads in lungs (r = −0.709, $p < 0.02$). qPCR parameters for cytokine gene expression assays are in Supplementary Table S6.

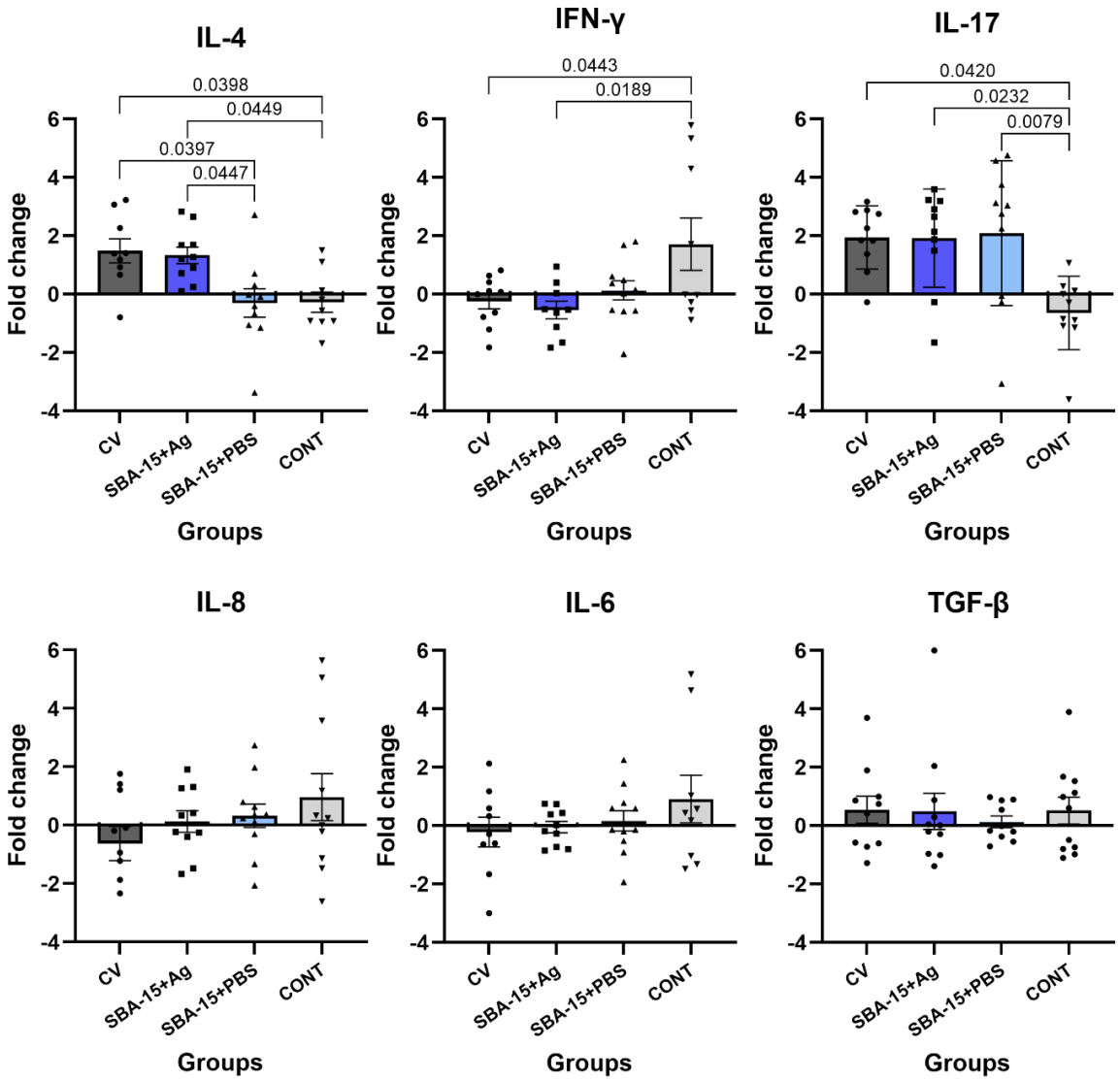


Figure 8: Relative quantification of cytokine mRNA by RT-qPCR. Mean fold change levels ± sd in modulation of gene expression of IL-4, IL-6, IL-8, IL-17, IFN-γ and TGF-β in lung lesions of piglets relative to 56 days after immunization protocols and 35 days after experimental infection with *M. hyopneumoniae* (Strain 232). Target gene expression was normalized based on rpl-4 gene expression. Group comparisons used the Kruskal-Wallis test ($p<0.05$).

4. Discussion

In intensive pig farming, vaccination is one of the most effective approaches for protection against porcine enzootic pneumonia (PEP) caused by *M. hyopneumoniae*. Various inactivated, whole-cell vaccines are available and widely used worldwide. The nanostructured adjuvant-*M. hyopneumoniae* vaccine formulated in this study offers advantages such as easy formulation, low cost, non-toxicity, and specific mucosal responses, making it an effective model for controlling respiratory diseases like PEP [8]. Vaccination of piglets, administered once or twice, is commonly practiced. A single vaccination at either 7 or 21 days of age proved effective in a pig herd experiencing clinical respiratory disease during the latter half of the fattening period [36].

This study investigated one mesoporous silica nanoparticle (MSN) formulation, demonstrating effective protection against homologous and pathogenic *M. hyopneumoniae* strain 232. The analysis of pure silica and silica containing antigen in our experimental vaccine formulation demonstrates that the SBA-15 is an efficient matrix for encapsulation and delivery of macromolecules. This reinforces its potential as a versatile vehicle and adjuvant for vaccination strategies, aligning with previous reports [15,19]. These findings were based on the induction of specific systemic and mucosal antibodies, reduction in shedding of bacteria in challenged piglets, and a statistically significant 74 % reduction in MLCL scores in these animals compared to a non-immunized control group. The intrinsic physicochemical properties of SBA-15 and its interaction with antigens presented by antigen-presenting cells (APCs) can prompt CD4+T cells to differentiate into a variety of effector cells to mediate different immune mechanisms, including classical T helper (Th) 1, Th2 and Th17 cells. Substantial recent evidence demonstrates a pivotal role for Th17 cells and IL-17 in vaccine-mediated immunity against PEP [37,38].

Due to the clinical protective effect of the vaccine, the MLCL score was reduced in piglets from both immunized groups, with statistically significant difference when compared to non-immunized groups. On the other hand, MLL scores of BALT hyperplasia had no statistical difference between groups, commonly found in other studies testing *M. hyopneumoniae* vaccines, since conventional immunization does not prevent colonization and shedding of this microorganism [7,39]. Regarding humoral responses, the systemic anti-*M. hyopneumoniae* IgA and IgG antibodies were detected

in both immunized groups. In piglets from SBA-15 vaccine group, IgG and IgA were first detected in serum at 21- and 28-days post-vaccination, respectively, as reported elsewhere [6]. The lymphoid associated tissues of upper respiratory tract mucosa were stimulated after vaccination as demonstrated by the anti-*M. hyopneumoniae* IgA antibodies in nasal swabs at 28 days post-vaccination. Likewise, a study conducted in rats reported the depositions of NPs compounds at mucosal sites such as lungs after administration of silica adjuvanted vaccine [40], emphasizing the potential mechanisms underlying the vaccine's efficacy prepared with this type of carrier-adjuvant. Overall, these results highlight the promising role of vaccination in mitigating *M. hyopneumoniae* infections utilizing nanostructure systems, which have a greater potential for antigen carriage to mucosal sites due to their deposition capability.

In field conditions, clinical respiratory disease may occur in the fattening stage [36], making a prolonged protective immune response desirable. Our findings show that vaccinated piglets exhibited a modulation in antibody responses throughout experimental period where piglets immunized with SBA-15 vaccine had a production of antibodies with an increase in the latter stages of sampling. Additionally, it is important to note that cellular immunity also plays a crucial role in protection, complementing the antibody-mediated responses. While serum IgG and IgA levels started to decrease in piglets vaccinated with the commercial vaccine, they increased in those that received our experimental vaccine and this may be attributed to the capacity of slow release of antigen from the pores of silica [8,41], which may prolong the protective immune response during the later stages of production. Also, it is known that the duration and dynamics of bacterial shedding after a challenge can vary, as M*. hyopneumoniae* often exists at low concentrations in the upper respiratory tract of pigs [42]. Even employing sensitive biomolecular techniques, this makes diagnosis based on nasal swab samples irregular and inconsistent [43].

Since shedding of *M. hyopneumoniae* strain 232 occurs and its detectable in laryngeal swabs in early stages of infection [44,45], in our study, only one piglet from the SBA-15 vaccine was positive in laryngeal swab detection by qPCR 7- and 14-days post-infection, while the other commercial bacterin vaccinated group had 3 positive animals on the same time-points and both groups became negative for *M. hyopneumoniae* on D49 (28 days post-infection) in laryngeal swabs. In contrast,

Ferreira et al. [23] reported a high prevalence of bacterial loads in laryngeal swabs after challenge in piglets using SBA-15 as an oral immunization platform against *M. hyopneumoniae*. This finding suggests that the bacteria might be present at low concentrations in the upper respiratory tract as reported elsewhere [42]. Additionally, both vaccinated groups showed decreased bacterial loads in lung tissue compared to non-immunized group.

SBA-15 particles have been used as adjuvants for mucosal vaccines due to their ability to increase both cellular and humoral response, especially at mucosal sites due to stimulation of Th cells [46] and induction of NLRP3 inflammasome activation as aluminum [47,48]. Indeed, our study demonstrated that piglets from an experimental vaccine group, even if stimulated parenterally, developed at mucosal sites of the upper respiratory tract a marked memory of anti-*M. hyopneumoniae* IgA antibodies, as well as an increase of these antibodies in the lower respiratory tract. An immunomodulation of Th1, Th2 and Th17 response characterized by an increase in IL-4 and IL-17 and a decrease in IFN-γ gene expression in the lung, when compared with piglets from non-immunized groups and similar with piglets that received the commercial bacterin vaccine. In our study, the vaccine formulation successfully induced both IgA and IgG anti-*M. hyopneumoniae* levels in serum of immunized piglets, which was not observed with other formulations using SBA-15 administered orally [21,23]. Beyond that, piglets receiving the SBA-15 formulation via the intramuscular route exhibited a reduction in bacterial shedding, as detected by laryngeal swabs. Moreover, we observed modulation of IL-17 gene expression in the lung lesions of piglets immunized with the silica-based vaccine.

Additionally, piglets from the group that received only the SBA-15 adjuvant suspension also presented an increase in IL-17 gene expression in the lungs. Also, the inflammatory activity measured by modulation of IL-6 and IL-8 expression was reduced in immunized piglets with commercial and SBA-15 vaccine. In addition, mucosal immune responses at upper respiratory tract mediated by IgA and IgG antibodies and T effector cells responses were elicited by this experimental vaccine. These responses are crucial to prevent adhesion and neutralize *M. hyopneumoniae* infection at these first mucosal barriers. As mycoplasma is mostly transmitted among pigs via nose-to-nose contact, the effectiveness of the vaccine can be also assessed

by monitoring the frequency of shedding [43]. Although frequently associated with surface mucosal protection, IgA anti-*M. hyopneumoniae* alone does not confer effective protection alone, so that other immune mechanisms play important roles in the protection against PEP [49].

The higher gene expression of IL-4 observed in the lungs of immunized piglets may be linked to the positive regulation of a Th2-mediated immune response [50], produced by B cells, and potentially modulating the chronic inflammatory response in *M. hyopneumoniae* infection [51]. Activation of Th lymphocytes represents an important pathway in eliciting a protective immune response against *M. hyopneumoniae* [52]. IL-17 is a pro-inflammatory cytokine that has been described to be involved in immunity against extracellular bacteria, viruses and intracellular bacteria [53]. Th17 lymphocytes play a key role in adaptive immunity and protection of the lung mucosa against respiratory pathogens by recruiting other immune cells to the inflamed mucosa for pathogen clearance [54]. After a bacterial infection, IL-17 has a significant role in neutrophil-mediated protection, especially in the lung mucosa during a mycoplasma infection [54,55]. The cytokines, produced by Th1, Th2, and Th17 cells, support earlier findings that Th1 and Th17 responses are vital for protecting against *Mycoplasma*-related disease [38]. Likewise, part of our results and another study reported a development of a microparticle-based vaccine that induced no antibodies, and no Th1 response, but elicited Th17 responses and thus provided a good level of protection, supporting that targeting Th17 responses for *M. hyopneumoniae* represents a promising approach for vaccine protection [49].

In our experimental model, the immunized animals, both with the commercial vaccine and the SBA-15 vaccine, exhibited a tendency for downregulation of the IFN-γ gene, whereas the control animals showed a tendency for upregulation of this same gene. Beyond that, the SBA-15 vaccine seems to modulate the expression of IL-6 and IL-8, contributing to a reduction in lung lesion levels of IFN-γ gene expression. This modulation of IL-6, IL-8 and IFN-γ cytokines may be caused by the immunization, preventing intense inflammatory and immune responses, lesion formation and intense inflammatory response at lung lesion sites commonly found in infections with *M. hyopneumoniae* [32]. Since IFN-γ is stimulating a strong cellular immune response, this could be associated with a reduction in IgA production [21], and negatively

correlated with bacterial loads in lung lesions, the seroconversion time and the macroscopic lung scores [32], mainly in piglets non-immunized and infected with *M. hyopneumoniae*. These findings are like those found in other studies of vaccines against *M. hyopneumoniae* [21,38]. Together, these data suggest that vaccination with SBA-15 induces robust Th17 recall responses, enhances lymphocytic infiltration into lung parenchyma, and mediates vaccine-induced protection in synergism with modulation of other cytokines such as IFN-γ and IL-4, conferring protection against *M. hyopneumoniae* challenge.

However, it is noteworthy that the silica-based vaccine induced IL-17-producing Th cells in both the vaccinated group and the group that received only the adjuvant. In addition, SBA-15 suspension without antigen immunization induced a Th17-type immune response, with no involvement of Th1 and Th2 immune responses without antigen immunization. In contrast to the Th1-cell-mediated anti-intracellular bacterial responses, Th17 cells produce IL-17, IL-17F and IL-22 and play important roles in the expression of chemokines, antibacterial peptides and clearance of extracellular bacteria and fungi, especially at mucosal surfaces, and in autoimmune processes protecting the host from pathogenic infections [56,57]. While the production of these cytokines alone may not provide effective protection against *M. hyopneumoniae* infection, it should be kept in mind for the need of future investigations because it might indicate desirable intrinsic immunogenicity of the SBA-15 adjuvant required in *M. hyopneumoniae* protection.

In summary, we demonstrated that the encapsulated SBA-15 mesoporous silica vaccine administered intramuscularly induced systemic and local immune-specific responses against *M. hyopneumoniae* without inferiority in relation to other more classical adjuvant type. Immunized piglets had systemic immune responses with the production of IgA and IgG anti-*M. hyopneumoniae* antibodies in serum. Additionally, the immunized piglets showed modulated cytokine gene expression in the lung, which is likely influenced by both the vaccine and the experimental challenge. This cytokine modulation, alongside the presence of anti-*M. hyopneumoniae* IgA antibodies in the upper respiratory tract, contributed to a reduction in the shedding of *M. hyopneumoniae* in laryngeal swabs and reduced macroscopic pulmonary lesions after challenge with a pathogenic strain of *M. hyopneumoniae*, thereby providing effective protection against

PEP compared to non-immunized piglets. Interestingly, piglets that received SBA-15 vaccine presented a reduction in bacterial shedding compared to those in the commercial vaccine group. However, it is important to note that since an exclusively vaccinated and uninfected group was not included in our study, the specific contribution of the vaccine to cytokine modulation cannot be definitively isolated from the effects of the experimental inoculation.

Finally, our study has also provided promising results for the potential use of immunogenic complexes with SBA-15 in the veterinary vaccine field, particularly for a single-dose *M. hyopneumoniae* vaccine. Beyond that, further studies aimed at improving the SBA-15 vaccine, potentially using other *M. hyopneumoniae* strains, can be implemented. It is also interesting to explore the use of proteomics in antigen formulation using the SBA-15, or even to employ the nanostructured platform against other bacterial or viral pathogens, leading to innovation and protection utilizing ordered mesoporous silica in swine vaccines.

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CRediT authorship contribution statement

Fernando Antonio Moreira Petri: Writing – review & editing, Writing – original draft, Validation, Project administration, Methodology, Investigation, Formal analysis, Conceptualization. **Clarisse Sena Malcher**: Investigation, Formal analysis. **Marina Lopes Mechler-Dreibi:** Writing – review & editing, Validation, Methodology, Investigation, Formal analysis, Conceptualization. **Ana Karolina Panneitz**: Investigation, Formal analysis. **Eduarda Ribeiro Braga**: Investigation, Formal analysis. **Gabriel Alexandre de Aguiar**: Investigation, Formal analysis. **Leonardo Teófilo Toledo**: Investigation, Formal analysis. **Tereza Silva Martins**: Writing – review & editing, Resources, Methodology. **Luis Carlos Cides-da-Silva**: Formal analysis.

Márcia C.A. Fantini: Writing – review & editing, Writing – original draft, Validation, Methodology. **Osvaldo A. Sant'Anna**: Writing – review & editing, Resources, Methodology. **Hélio J. Montassier**: Writing – review & editing, Writing – original draft, Validation, Methodology, Data curation. **Luís Guilherme de Oliveira**: Writing – review & editing, Writing – original draft, Validation, Supervision, Project administration, Methodology, Funding acquisition, Data curation, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2024.126268.

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Supplementary material

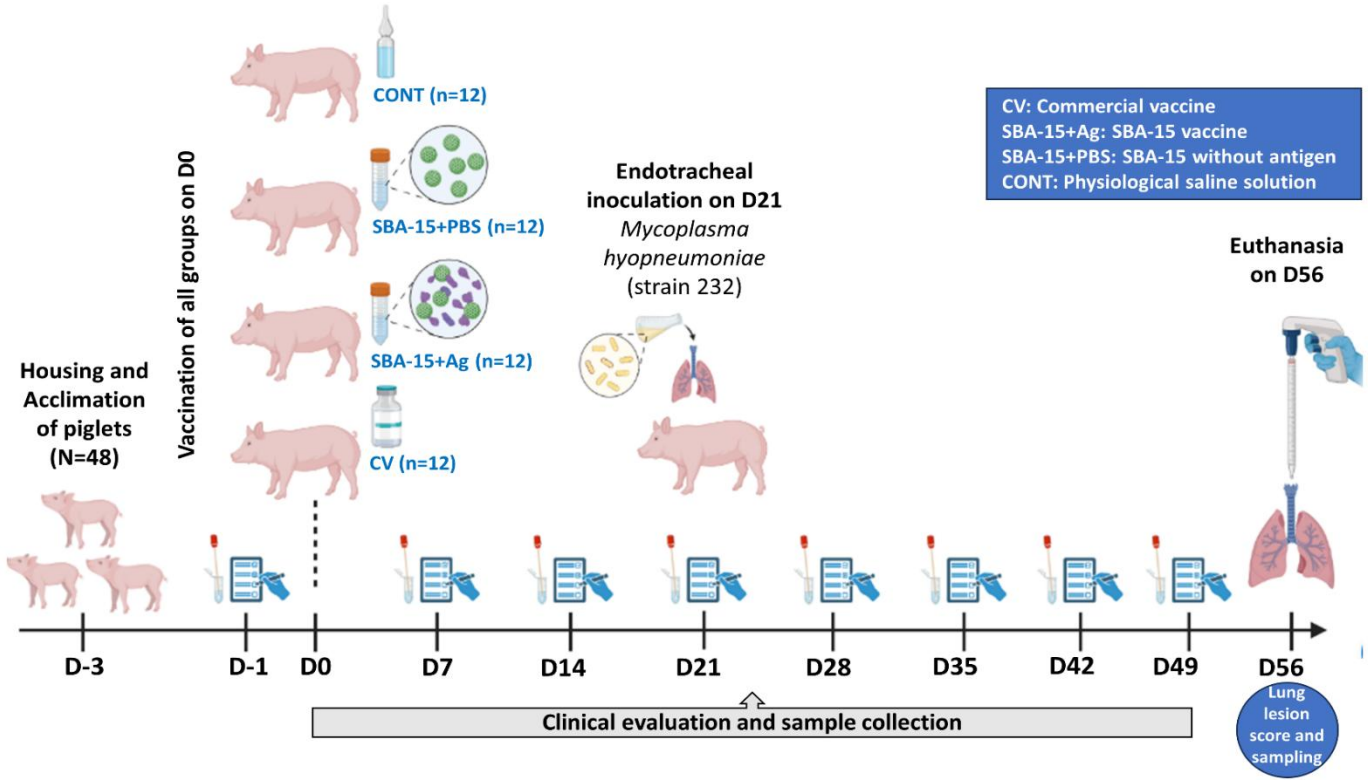


Figure S1: Study design. Timeline of scheduled procedures for the experimental period, including animal selection, administration of the vaccination protocols, challenge with *M. hyopneumoniae*, animal slaughter, and collection of biological samples. CV: group of piglets vaccinated with commercial vaccine; SBA-15+Ag: group of piglets vaccinated with experimental vaccine formulated with SBA-15; SBA-15+PBS: group of piglets that received SBA-15 suspension and CONT: group of piglets that received an injection of physiological saline solution; BALF: Broncho-alveolar lavage fluid, D: day.

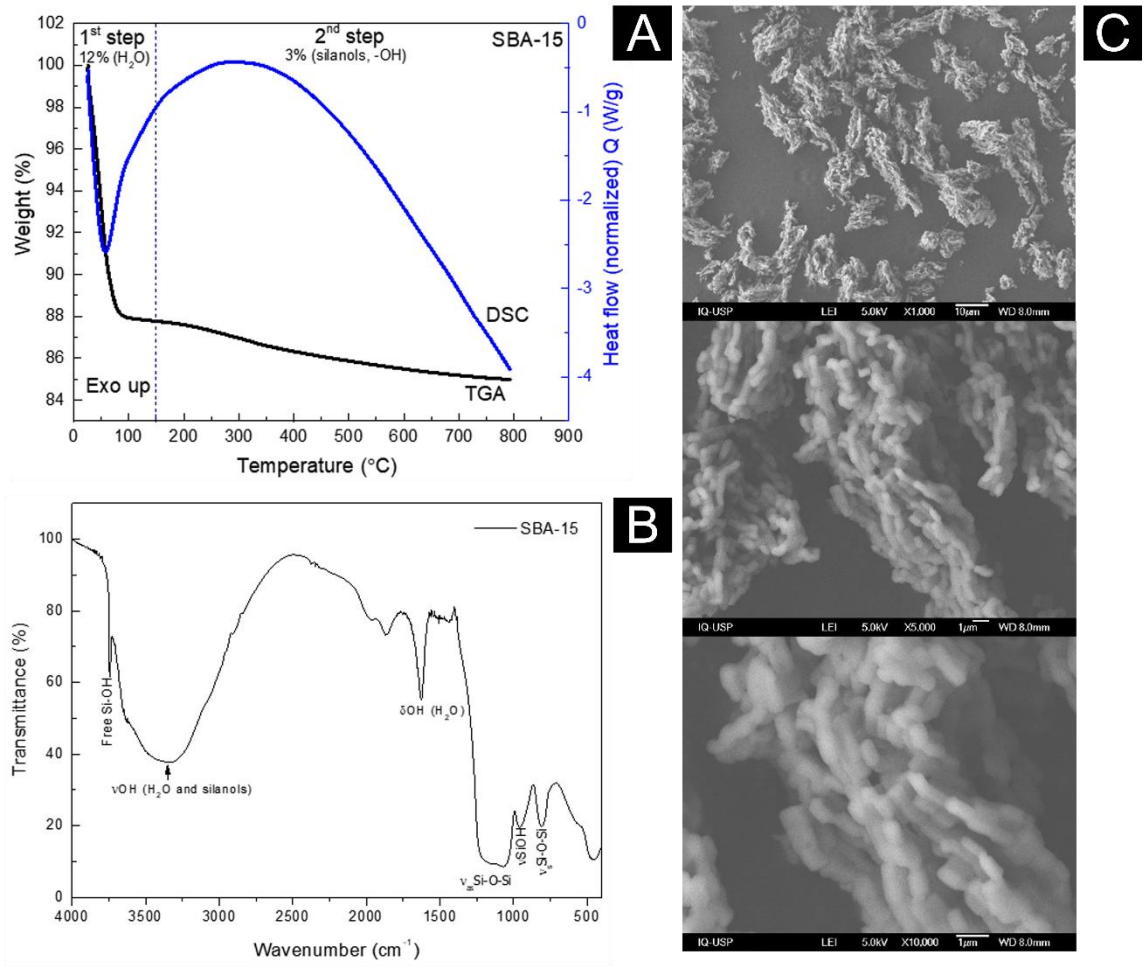


Figure S2: Analysis of SBA-15 sample utilized in the study. (A) Thermogravimetry and DSC curves of SBA-15 sample; (B) The Fourier-transform infrared (FTIR) spectra of SBA-15 sample and (C) micrographs of SBA-15 sample obtained in scanning electron microscopy (SEM): 1000x, 5000x and 10000x magnifications.

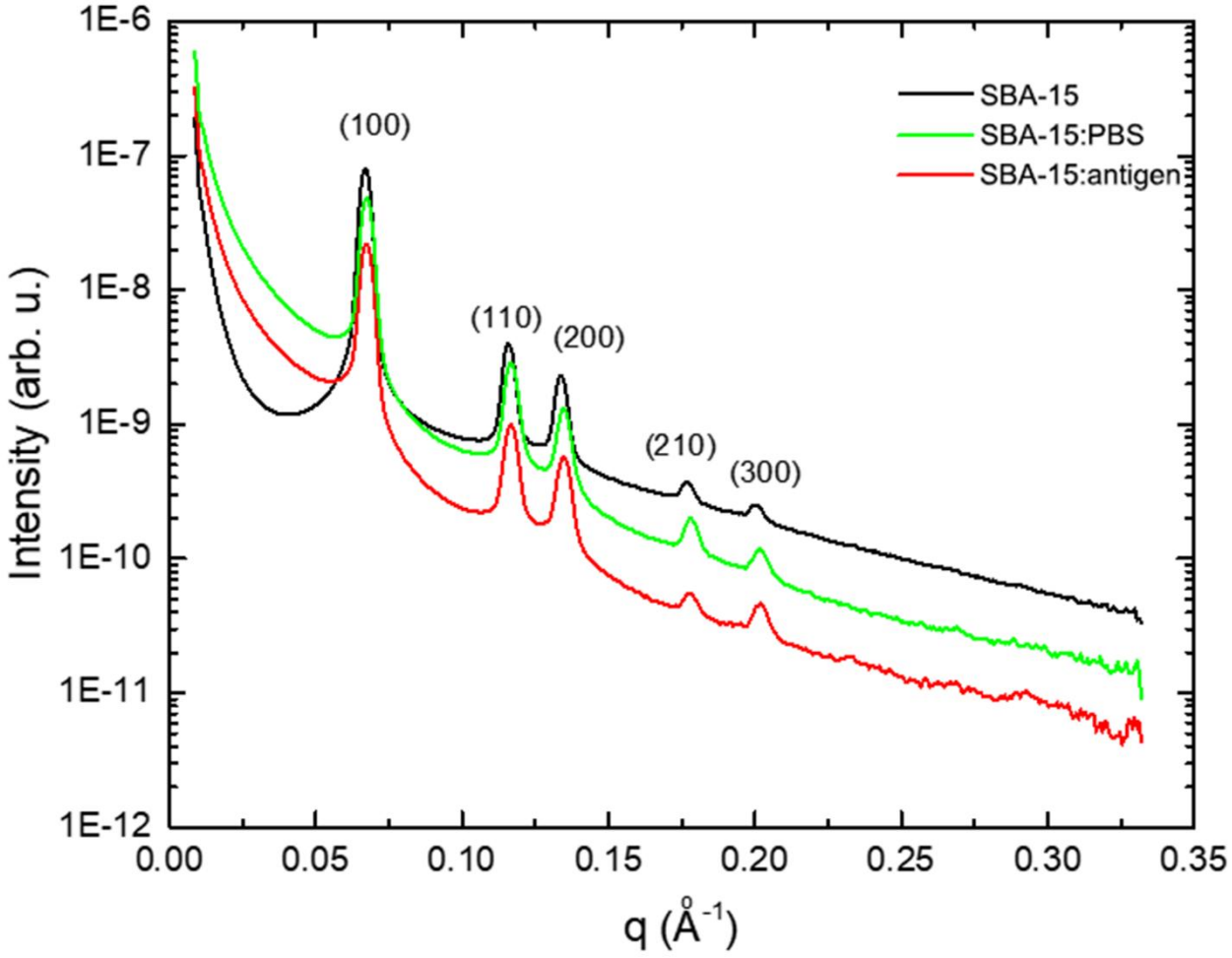


Figure S3: SAXS curves of samples. SBA-15, SBA-15:PBS and SBA-15:antigen composites.

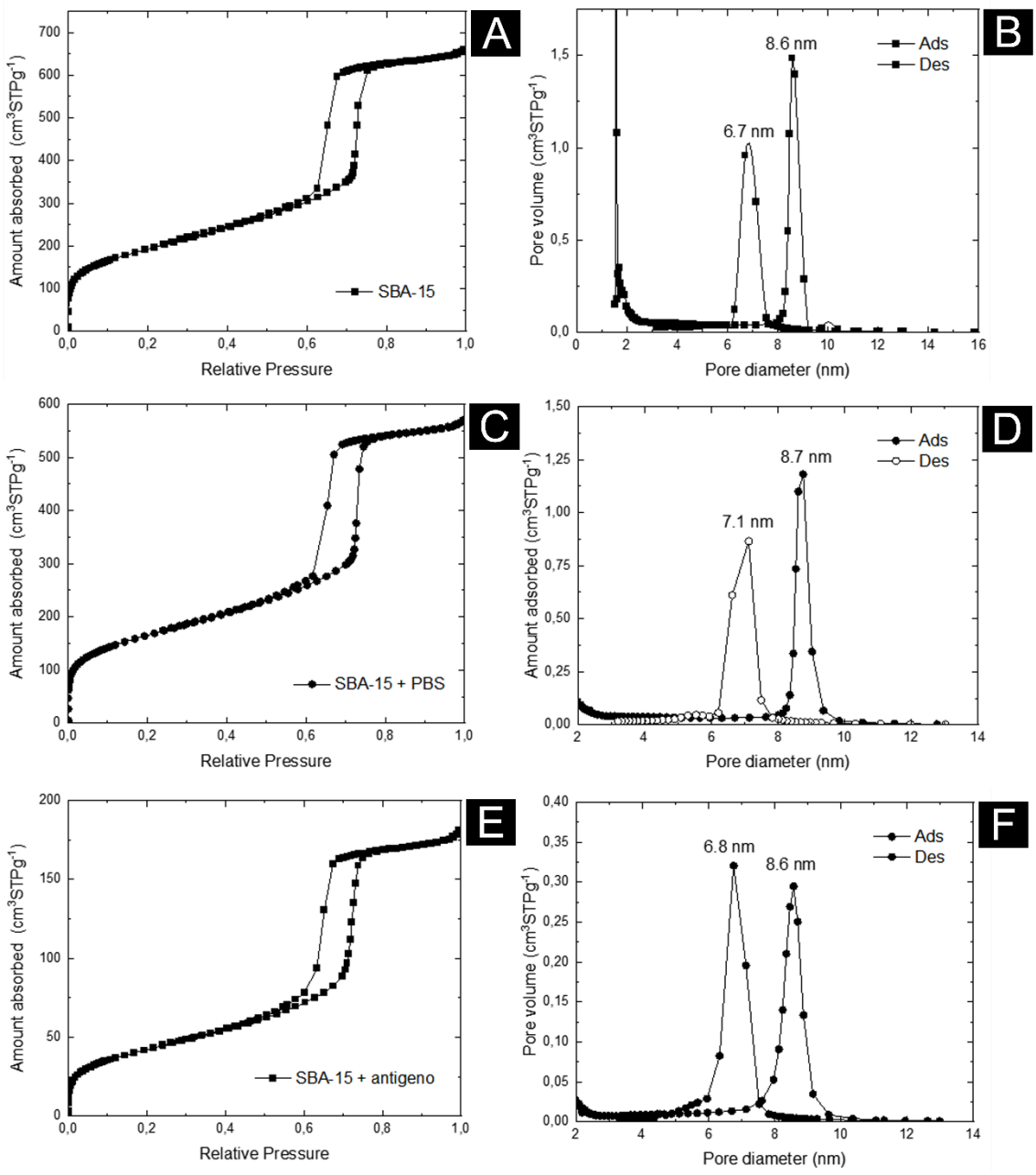


Figure S4: Nitrogen adsorption-desorption isotherms (NAI) and pore size distribution were analyzed for various silica SBA-15 samples: pure SBA-15 (A, B), SBA-15 with PBS (C, D), and SBA-15 with PBS and antigen incorporation (E, F). Ads: adsorption; Des: desorption.

Table S1: Results from evaluated parameters obtained in NAI analysis.

Sample	BET Surface Area (m^2/g)	Micropore Area (m^2/g)	External Surface Area (m^2/g)	Single point total volume of pore (cm^3/g)	Micropore volume (cm^3/g)	Mesopore volume (Total-Micro) (cm^3/g)	Mean Pore diameter (nm) Adsorption	Mean Pore entrance (nm) Desorption
SBA-15 pure	691	130	561	1.02	0	1.02	8.6	6.7
SBA-15+PBS	593	120	473	0.88	0	0.88	8.7	7.1
SBA-15+PBS+Ag	152	0.38	152	0.28	0	0.28	8.6	6.8

BET=Brunauer-Emmett-Teller

Table S2: Levels of IgG anti-*M. hyopneumoniae* in serum samples. Means ± standard error of mean of S/P of IgG obtained for groups 1 to 4 over the study period.

Timepoint	Group	Positives	Mean[1]	Standard Error of Mean	*p*-value
D0 (Vaccination)	CV	0/12	0.155[a]	0.078	0.250
	SBA-15+Ag	0/12	0.101[a]	0.063	
	SBA-15+PBS	0/12	0.173[a]	0.101	
	CONT	0/12	0.157[a]	0.047	
D7	CV	0/12	0.197[a]	0.025	0.463
	SBA-15+Ag	0/12	0.193[a]	0.029	
	SBA-15+PBS	0/12	0.230[a]	0.030	
	CONT	0/12	0.175[a]	0.017	
D21 (Challenge)	CV	3/12*	0.264[a]	0.043	0.001
	SBA-15+Ag	2/12*	0.206[a, b]	0.031	
	SBA-15+PBS	0/12	0.129[b,c]	0.022	
	CONT	0/12	0.093[c]	0.015	
D35	CV	11/12	1.140[a]	0.148	0.001
	SBA-15+Ag	9/12	0.563[b]	0.114	
	SBA-15+PBS	0/12	0.131[c]	0.017	

	CONT	0/12	0.147[c]	0.025	
D49	CV	11/12	1.374[a]	0.178	0.001
	SBA-15+Ag	10/12	0.754[a,b]	0.132	
	SBA-15+PBS	0/12	0.141[b,c]	0.024	
	CONT	2/12[*]	0.295[c]	0.046	
D56 **Necropsy**	CV	12/12	1.254[a]	0.14	0.001
	SBA-15+Ag	12/12	0.908[a,b]	0.133	
	SBA-15+PBS	4/12[*]	0.286[b,c]	0.07	
	CONT	8/12	0.437[c]	0.065	

[1] For each time point, means followed by the same letter are not significantly different by Tukey's parametric test ($p< 0.05$) and Kruskal Wallis' non-parametric test ($p< 0.05$).
[*] Indicates the first time the group presented an individual with a positive result at ELISA (S/P>0.3).

Table S3: Levels of IgA anti-*M. hyopneumoniae* in serum samples. Means ± standard error of mean of OD of IgA obtained for groups 1 to 4 over the study period.

Timepoint	Group	Positives	Mean[1]	Standard Error of Mean	*p*-value
D7	CV	0/12	0.148[a]	0.005	0.1
	SBA-15+Ag	0/12	0.119[a]	0.015	
	SBA-15+PBS	0/12	0.116[a]	0.014	
	CONT	0/12	0.126[a]	0.015	
D21 **(Challenge)**	CV	0/12	0.151[a]	0.003	0.148
	SBA-15+Ag	0/12	0.142[a]	0.002	
	SBA-15+PBS	0/12	0.143[a]	0.004	
	CONT	0/12	0.149[a]	0.003	
D28	CV	2/12[*]	0.151[a]	0.005	0.537
	SBA-15+Ag	2/12[*]	0.155[a]	0.009	
	SBA-15+PBS	0/12	0.142[a]	0.002	
	CONT	0/12	0.147[a]	0.003	
D35	CV	9/12	0.295[a]	0.036	0.004
	SBA-15+Ag	10/12	0.281[a]	0.033	
	SBA-15+PBS	5/12[*]	0.167[b]	0.007	

	CONT	8/12*	0.172[b]	0.005	
D49	CV	9/12	0.277[a]	0.037	0.018
	SBA-15+Ag	10/12	0.316[a,b]	0.039	
	SBA-15+PBS	7/12	0.180[a, b]	0.008	
	CONT	10/12	0.194[b]	0.007	
D56 (Necropsy)	CV	11/12	0.282[a]	0.033	0.001
	SBA-15+Ag	11/12	0.332[a,b]	0.034	
	SBA-15+PBS	7/12	0.189[b]	0.016	
	CONT	8/12	0.198[b]	0.012	

[1] For each time point, means followed by the same letter are not significantly different by Tukey's parametric test ($p < 0.05$) and Kruskal Wallis' non-parametric test ($p < 0.05$).

* Indicates the first time the group presented an individual with a positive result at ELISA (OD > 0.2).

Table S4: IgA anti-*M. hyopneumoniae* antibody levels in nasal swab and BALF samples. Means ± standard error of mean of OD of IgA obtained for groups 1 to 4 over the study period.

Timepoint	Group	Positives	Mean[1]	Standard Error of Mean	*p*-value
D14	CV	0/12	0.015[a]	0.023	0.733
	SBA-15+Ag	0/12	0.021[a]	0.033	
	SBA-15+PBS	0/12	0.011[a]	0.029	
	CONT	0/12	0.02[a]	0.032	
D21 (Challenge)	CV	0/12	0.026[a]	0.013	0.633
	SBA-15+Ag	0/12	0.038[a]	0.011	
	SBA-15+PBS	0/12	0.040[a]	0.012	
	CONT	0/12	0.035[a]	0.007	
D28	CV	7/12*	0,301[a]	0.07	0.001
	SBA-15+Ag	6/12*	0,213[a]	0.013	
	SBA-15+PBS	0/12	0,059[b]	0.001	
	CONT	0/12	0,041[b]	0.002	
D35	CV	8/12	0.751[a]	0.176	0.001
	SBA-15+Ag	8/12	0.492[a]	0.129	

	SBA-15+PBS	0/12	0.047[a,b]	0.013	
	CONT	4/12*	0.142[b]	0.031	
D42	CV	8/12	0.806[a]	0.213	0.008
	SBA-15+Ag	5/12	0.525[a]	0.096	
	SBA-15+PBS	2/12*	0.108[a,b]	0.197	
	CONT	5/12	0.246[b]	0.045	
D49	CV	8/12	0.479[a]	0.118	0.075
	SBA-15+Ag	10/12	0.399[a]	0.081	
	SBA-15+PBS	8/12	0.196[a]	0.023	
	CONT	11/12	0.365[a]	0.039	
D56 (BALF)	CV	12/12	0.697 [a]	0.096	0.242
	SBA-15+Ag	12/12	0.514 [a]	0.063	
(Necropsy)	SBA-15+PBS	12/12	0.449 [a]	0.053	
	CONT	12/12	0.519 [a]	0.057	

[1] For each time point, means followed by the same letter are not significantly different by Tukey's parametric test (p< 0.05) and Kruskal Wallis' non-parametric test (p< 0.05).

* Indicates the first time the group presented an individual with a positive result at ELISA (OD > 0.3).

Table S5: Details of the qPCR assays targeting the *p102* gene of *M. hyopneumoniae*.

Plate	qPCR Efficiency (%)	R^2	Slope	y-intercept
1	94.7	0.999	3.456	37.645
2	101.6	0.993	3.284	37.986
3	90.0	0.996	3.589	38.926
4	103.8	0.999	3.234	37.012
5	104.7	0.999	3.214	36.833
6	102.9	0.999	3.255	36.858
7	100.0	0.997	3.323	38.241
8	101.5	0.993	3.286	37.188
9	102.6	0.999	3.261	37.546
10	99.0	0.997	3.346	38.178
11	101.8	0.996	3.279	35.704

Table S6: The nucleotide sequences of the RT-qPCR primers were used to study the expression of inflammatory cytokine genes. The annealing temperature of the primers was optimized by a gradient PCR and the optimal amplification was observed.

Gene	Sequence (5' 3')	Acess number (GenBank)	Amplicon size (bp)	Melting temperature (°C)	Plate no	qPCR Efficiency (%)	R^2	Slope	y-int
IL-4	(F) AGAGCTCTATTCATGGGTCT	NM_214123.1	209	82.0	1	99.6	0.994	3.332	37.221
	(R) CTTCTCCGTCGTGTCTCT				2	92.3	0.996	3.422	38.288
IL-6	(F) CTGAGGCAAAAGGGAAAGA	NM_214399.1	185	81.5	1	103.9	0.995	3.211	36.327
	(R) CCCAGCTACATTATCCGAAT				2	93.3	0.989	3.494	38.408
IL-8	(F) AGGAAAAGTGGGTGCAGAAG	NM_213867.1	190	78.0	1	99.1	0.993	3.344	39.121
	(R) CAACCCTATGTCTGACCAGC				2	97.8	0.996	3.376	38.097
IL-17	(F) ATCCTCGTCCCTGTCACTGC	NM_001005729.1	120	82.0	1	101.2	0.997	3.294	37.192
	(R) ACATGCTGAGGGAAGTTCTTGTC				2	100.0	0.997	3.322	37.104
IFN-γ	(F) ATTGGAAAGAGGAGAGTGAC	NM_213948.1	168	76.5	1	104.7	0.996	3.213	36.264
	(R) CATTCAGTTTCCCAGAGCTA				2	91.9	0.996	3.431	37.516
TGF-β	(F) GGATACCAACTACTGCTTCA	NM_214015.2	228	83.0	1	101.4	0.992	3.289	36.832
	(R) TTGTACAGAGCCAGGACTT				2	100.2	0.996	3.316	37.476
*rpl-4**	(F)CAAGAGTAACTACAACCTTC	NC_010443.5	122	75.0	1	98.9	0.996	3.348	36.480
	(R)GAACTCTACGATGAATCTTC				2	104.2	0.996	3.226	36.355

**rpl-4* gene was used as housekeeping gene for gene expression analysis

[1]CAPÍTULO 3 – Early-life nasal microbiota composition predicts *Mycoplasma hyopneumoniae* respiratory disease outcomes and is preserved by vaccination in piglets

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Highlights

1. Higher nasal microbiota diversity at weaning was associated with stronger systemic IgG responses to *Mycoplasma hyopneumoniae*.
2. Distinct microbial profiles at weaning predicted both antibody responses and severity of lung lesions after challenge.
3. Incomplete seroconversion in vaccinated animals suggests nasal microbiota composition may modulate vaccine-induced antibody immunity.
4. Non-vaccinated animals showed lower nasal microbiota diversity than vaccinated animals 2-week post-challenge
5. The abundance of *Alloprevotella* spp. at weaning correlated negatively with lung lesions, indicating potential as a protective biomarker.

Abstract

The nasal microbiota plays a key role in shaping immune responses and susceptibility to respiratory diseases in swine. This study evaluated whether nasal microbiota composition at weaning can predict the immune response and clinical protection induced by two intramuscular vaccines against *Mycoplasma (M.) hyopneumoniae* - a commercial vaccine and an experimental formulation using mesoporous silica (SBA-15) as a carrier-adjuvant. Additionally, we assessed how early-life microbial profiles influenced immune responses and disease outcome. Thirty-two piglets were divided into three groups: commercial vaccine (CV) group, SBA-15 vaccine (SBA-15) group and non-vaccinated control (CONT) group. Animals were vaccinated at 24 days of age (D0) and challenged with a virulent *M. hyopneumoniae* strain at day 45 of life (D21). Nasal swabs and serum were collected for microbiota profiling and quantification of anti-*M. hyopneumoniae* systemic IgG responses, respectively. Lung lesions were evaluated at necropsy (D56). Longitudinal microbiota analysis revealed that vaccinated animals preserved nasal microbial diversity post-challenge, while CONT group exhibited a marked decrease in alpha diversity. Notably, higher nasal microbiota diversity at weaning was associated stronger IgG responses and milder lung lesions in non-vaccinated animals. Beta diversity analysis also revealed a distinct microbial community structure between high and low immune responders. In particular, the presence of *Alloprevotella* spp. at weaning was negatively correlated with lung lesion severity, suggesting a potential protective role. These results highlight a bidirectional interaction between nasal microbiota, vaccination, and immune response, and suggest that microbiota profiling may help predict vaccine efficacy and disease outcome in pigs.

Keywords: immune response, lung lesions, metagenomics, nasal microbiota, SBA-15 silica, vaccine efficacy.

1. Introduction

The nasal microbiota plays a key role as a primary defense barrier in the swine respiratory system. As the initial interface between the host and environmental microorganisms, the nasal microbiota significantly influences susceptibility to respiratory diseases, including viral infections (Abt et al., 2012) such as Porcine Reproductive and Respiratory Syndrome Virus type 1 (PRRSV-1) (Obregon-Gutierrez et al., 2025) and Influenza A virus (Hau et al., 2023), as well as bacterial pathogens like *Glaesserella* (*G.*) *parasuis* (Correa-Fiz et al., 2016), *Mycoplasma* (*M.*) *hyorhinis* (Blanco-Fuertes et al., 2021) and *Bordetella* (*B.*) *bronchiseptica* (Nielsen et al., 2023). Commensal bacteria not only help excluding pathogens through competitive inhibition but also contribute to the development and modulation of mucosal immunity (Li et al., 2023; Vlasblom et al., 2024). Studies have demonstrated that beneficial genera such as *Lactobacillus*, *Lactococcus*, *Staphylococcus*, and *Enterococcus* are more prevalent in healthy pigs (Correa-Fiz et al., 2016; Ramsey et al., 2016; Correa-Fiz et al., 2019), while genera associated with respiratory diseases—such as *Streptococcus*, *Glaesserella*, *Pasteurella*, *Moraxella*, and *Bordetella*—are commonly found in animals with compromised respiratory health (Mahmmod et al., 2020; Li et al., 2021). In addition, changes in nasal microbial composition caused by indiscriminate use of antibiotics can predispose animals to respiratory infections or even exacerbate ongoing disease conditions (Correa-Fiz et al., 2019). Moreover, environmental factors—including ammonia exposure, stress, antimicrobial usage, and diet—have been reported to influence respiratory microbial communities in pigs (Pirolo et al., 2021).

Mycoplasma (*M.*) *hyopneumoniae* is the etiological agent of the porcine enzootic pneumonia (PEP). The infection causes a chronic disease, known for the impact in the respiratory health as a primary bacterial pathogen of the porcine respiratory diseases complex (PRDC), mainly observed by pneumonia, and pleurisy lesions when associated with secondary respiratory pathogens (Maes et al. 2023; Petri et al. 2023; Arruda et al., 2024) and zootechnical impact associated with higher production costs as the use of antibiotics, vaccination strategies, diagnostic and reduced daily weight gain (Ferraz et al. 2020; Malcher et al 2024). Gross lung lesions in pigs affected by PEP consist of cranioventral pulmonary consolidation (CVPC) and are the most

significant pathological disease outcome in the slaughterhouse. For instance, *M. hyopneumoniae* infection is known to be associated with secondary bacterial pathogens in lungs such as *Pasteurella* (*P.*) *multocida*, *Glaesserella* (*G.*) *parasuis,* and *M. hyorhinis*, further compromising respiratory health (Saade et al. 2020; Maes et al., 2023).

Vaccination not only targets specific pathogens like *M. hyopneumoniae* but also has the potential to modulate microbial communities and influence the immune system's development (Ciabattini et al. 2019; Lynn et al. 2022). By reducing pathogen load and altering the respiratory environment, vaccines may indirectly support the stabilization of beneficial microbial populations in the respiratory or intestinal tract. Most evidence to date points to interactions with the gut microbiota (Munyaka et al., 2019; Munyaka et al., 2020), although the role of nasal microbiota in the modulating responses to parenteral vaccines remains less well established. Recent studies in pigs have shown associations between the nasal and gut microbiota and antibody responses following *G. parasuis* vaccination (Obregon-Gutierrez et al., 2025), as well as shifts in the nasal microbiota of offspring from vaccinated sows (Blanco-Fuertes et al., 2022). Research into mucosal immunity, particularly the production of secretory IgA, has highlighted the significance of local immune responses in mediating protection against respiratory pathogens (Canelli et al., 2023) but the response to the vaccination into the immune system can be checked by the systemic IgG anti-*M. hyopneumoniae*. Furthermore, studies have shown that certain gut microbial communities, particularly members of the *Prevotella* genus, may serve as predictors of stronger immune responses post-vaccination against Influenza A (Borey et al., 2021) and *G. parasuis* (Obregon-Gutierrez et al., 2025), reinforcing the connection between microbiota composition and immunological outcomes.

The use of bacterin vaccines, mostly based on inactivated whole-cell *M. hyopneumoniae* has been essential in controlling porcine enzootic pneumonia (PEP) (Maes et al. 2021). Although commercial vaccines reduce clinical signs and lung lesions, they do not confer full protection, particularly due to factors such as types of antigens employed, choice of adjuvant and/or carrier, route and frequency of vaccination (Maes et al., 2021). Also, the antigenic and virulence diversity and variability among *M. hyopneumoniae* strains, as well as the presence of coinfections

may be involved in the vaccine efficacy (Del Pozo et al. 2014; Bourry et al. 2015; Betlach et al. 2019). The limitation in protection conferred by commercial vaccines has prompted growing interest in novel vaccines, testing new technologies of adjuvants that allow for controlled and targeted release of antigens. Among the newly developed technologies, the use of SBA-15 mesoporous silica as a vaccine carrier and adjuvant. Compared to conventional adjuvants like aluminum hydroxide or oil-in-water emulsions, this nanostructured silica platform demonstrates immunogenic modulation and mucosal stimulation, with a sustained liberation of antigen suggesting this versatile and low cost adjuvant that could be used as intramuscular single dose (Petri et al., 2024; Aguiar et al., 2025) or by oral route combined with other traditional commercial vaccines (Storino et al., 2023). SBA-15 enables the encapsulation and sustained release of *M. hyopneumoniae* proteins, inducing both humoral and cellular immune responses, with reduction in macroscopic lung lesions at slaughter, in both oral and/or intramuscular administration (Mechler-Dreibi et al., 2021; Petri et al. 2024).

In this context, microbial diversity is not merely a marker of health but may be directly involved in mediating disease resistance. Experimental and field studies have revealed associations between specific microbial profiles and the severity or absence of lung lesions (Huang et al., 2019; Valeris-Chacin et al., 2021). Understanding these relationships, particularly in vaccinated animals, opens new avenues for antimicrobial-independent prevention strategies. It also highlights the potential of microbiota-focused interventions, such as probiotics or microbiome-informed vaccine protocols, in improving respiratory health outcomes in swine populations.

Finally, in this study we investigate whether the composition of the nasal microbiota at weaning can predict the immune response and clinical protection conferred by challenge associated with vaccines against *M. hyopneumoniae*. To address this, we used a controlled pig model comparing two intramuscular vaccines – a commercial vaccine aluminum hydroxide based and an experimental SBA-15 silica as a carrier-adjuvant following a challenge with *M. hyopneumoniae* post-vaccination. We evaluated the nasal microbiota diversity, systemic immune responses (IgG anti-*M. hyopneumoniae*), and lung lesions outcomes to determine whether microbial diversity and composition at baseline influence disease progression. Additionally, we assessed

how vaccination may reshape the nasal microbial community in relation to respiratory health.

2. Materials and methods

2.1 Study design, sampling and clinical evaluation

The experimental study was described previously by Petri et al. (2024) and the scheme is presented in Figure 1. All procedures with experimental piglets were approved by the Animal Ethics Committee of School of Agricultural and Veterinary Sciences from São Paulo State University (Protocol Number: 003897/21) in accordance with ethical principles and guidelines adopted by Brazilian College of Experimentation.

Briefly, 19 days-old, weaned piglets (N=32) were randomly selected from a *M. hyopneumoniae*-free farm, based on the absence of antibodies in serum and bacterial DNA in laryngeal swabs checked by ELISA and qPCR, respectively. Piglets with 24 days old were divided in 3 groups: CV (commercial vaccine, n=10), SBA-15 (experimental silica-based vaccine, n=11) and CONT (non-immunized control group, n=11). Piglets from CV group were vaccinated intramuscularly with the vaccine M+PAC (MSD Animal Health, USA) following manufacturer guidelines, and SBA-15 group received 2 mL of 0.5 mg/mL antigen (protein extract of virulent *M. hyopneumoniae* strain 232) encapsulated in SBA-15 nanoparticles adjuvant, while the piglets from CONT group remained without immunization receiving a sterile phosphate buffered saline solution (PBS 1×, pH 7.4; Sigma-Aldrich, Germany), and used as control. Vaccination was done at 24 days of life (D0). After three weeks on D21, all piglets were challenged by the endotracheal route with 5 mL of Friis medium containing 10^6 CCU/mL (color changing units/mL) of the pathogenic strain 232 of *M. hyopneumoniae*.

Nasal swabs were taken from all piglets at the moment of inclusion in the study on D0 (weaning), in the challenge day on D21 and two weeks after challenge on D35 and placed in 500 µl of PBS (1X, pH 7.4; Sigma-Aldrich, Germany) in DNAse and RNAse-free criovyals (Corning, NY, USA), vortexed for 30 s and stored at -80°C until used for microbiota analysis. After challenge, the animals were individually observed regarding the severity and presence of clinical signs using the respiratory disease score (Halbur

et al. 1996). Then, five weeks' post-challenge, all piglets were humanely euthanized with anesthetic protocol approved by the Federal Council of Veterinary Medicine, Brazil. After evisceration, the respiratory set (lung and trachea) was isolated to assess macroscopic lung consolidated lesions using the methodology described by Straw et al. (1986). The classification was based on the portion of each affected lobe, which the summing can vary from 0% to 100%, or from no lesions to entire lung affected, respectively. Since the macroscopic lung lesions on D56 (necropsy) and IgG anti-*M. hyopneumoniae* on D35 from sampled animals of the original study presented statistical differences (Petri et al. 2024), nasal swabs of D0 (vaccination), D21 (challenge) and D35 (two weeks post-challenge) of the clinical trial (Figure 1) were included for microbiota analysis.

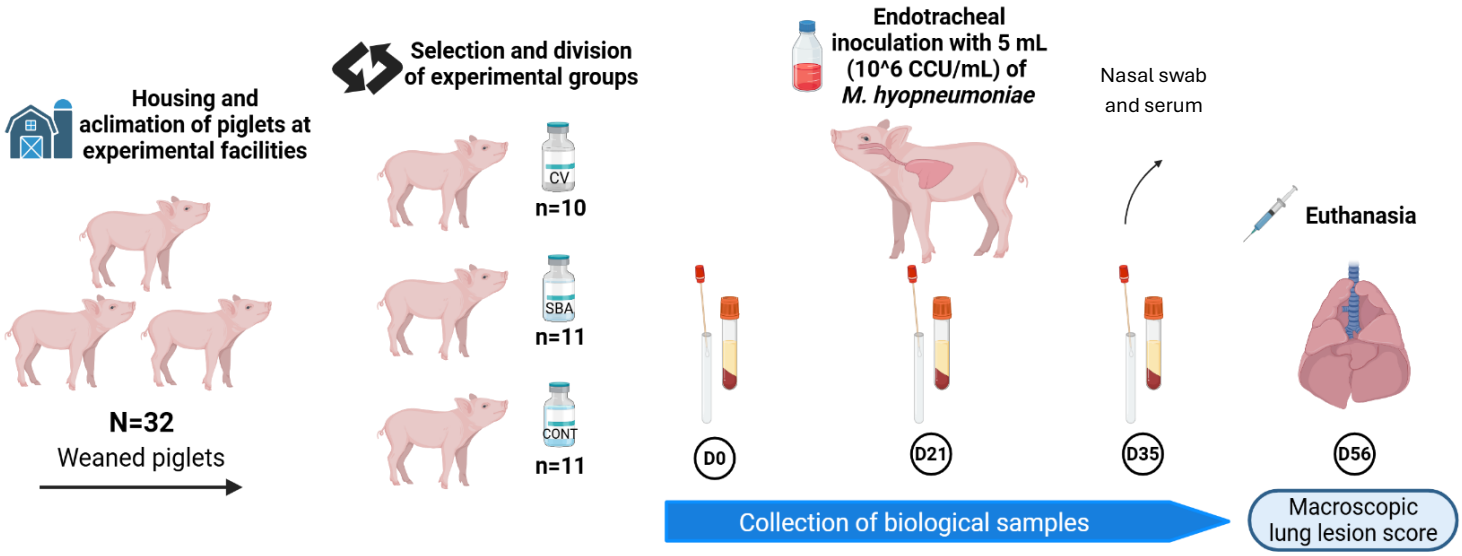


Figure 1: Study design. Timeline of scheduled procedures for the experimental period, including animal selection, administration of the vaccination protocols, challenge with *M. hyopneumoniae*, animal necropsy, lung lesion assessment and collection of biological samples in CV, SBA-15 and CONT groups. D: day.

2.2 Quantification of IgG anti-M. hyopneumoniae levels in serum

A commercial ELISA kit (*M.hyo* Ab test, Idexx, USA) was employed to measure the concentration of *M. hyopneumoniae*-specific Immunoglobulin (Ig) G using 100 µL of

serum samples from the piglets, following the manufacturer's instructions. Samples were considered positive when S/P ≥ 0.4.

2.3 Clinical parameters and metadata determination of immune responsiveness and disease susceptibility in pigles

Animals were individually classified for each clinical parameter within their respective experimental group, using the group-specific median as cut-off. For macroscopic lung lesions, animals with values (%) below the group median were considered low-susceptibility (LS), while those above the median were classified as high-susceptibility (HS). Conversely, for systemic IgG (S/P ratio) against *M. hyopneumoniae*, animals with antibody levels above the group median were considered high immune responders (HI), and those below the median as low immune responders (LI). This approach allowed a balanced intra-group comparison and better correlation with microbiota profiles.

Additionally, for IgG antibody responses, a delta (Δ) calculation was used to control baseline variability and nonspecific immune activation. In the vaccinated groups (CV and SBA-15), antibody responses were calculated as Δ = D21 – D0, reducing the effect of distinct initial levels at weaning. In the CONT group, where animals did not receive vaccination at D0, the antibody response was defined as Δ = D35 – D21, reflecting the specific response to infection following challenge.

For integrative analyses, animals were further stratified into high-protection and low-protection phenotypes based on the combined immunological and pathological criteria. Animals presenting low antibody responses and high lung lesion burden were classified as low-protection, whereas those showing high antibody responses and low lung lesion burden were classified as high-protection.

2.4 DNA extraction and 16S gene amplicon sequencing

DNA was extracted from 250 µL of resuspended nasal swab samples using the DNeasy Blood and Tissue (Qiagen, Germantown, MD, USA) commercial extraction kit, according to the manufacturer’s instructions. DNA concentration was assessed through NanoDrop™ One Spectrophotometer (ThermoFisher Scientific®, Wilming,

USA), and, to acceptance, all samples had to reach the purity of 1.8 to 2.0 in the 260/280 ratio. The DNA samples were then immediately frozen in a freezer at −20°C until molecular analysis.

The library was prepared using primers for cover the V3-V4 hypervariable region of 16S rRNA (Klindworth et al. 2013) (~470 bp, amplified with primers 341F x 806R), and bacteria amplicons were sequenced by Illumina Novaseq 6000 platform (PE 250, 50-100 mil reads raw data per sample). Primers' sequence 341F (5'-CCTAYGGGRBGCASCAG-3') and 806R (5'- GGACTACNNGGGTATCTAAT-3'). After sorting reads into samples (demultiplexing), samples were further processed through bioinformatics analyses.

2.5 Microbiota analysis

Bioinformatics analysis and feature classification of raw reads was performed using the Quantitative insights into microbial ecology (Qiime2, version 2023.9) platform (Caporaso et al. 2010; Bolyen et al. 2019). Briefly, primers were removed with *q2 cutadapt* (Martin et al. 2011), and DADA2 (integrated QIIME2 plugin) was used for quality filtering, merging, chimera removal, and amplicon sequence variants (ASV) classification (Callahan et al. 2016). Unlike traditional OTU-based clustering methods, DADA2 infers exact amplicon sequence variants (ASVs), providing higher resolution by distinguishing sequences differing by as little as one nucleotide. Additional filtering steps removed non-prokaryotic sequences and non-bacterial ASVs (*Archaea*, *Chloroplast*, *Mitochondria*) using the Greengenes 13.8 database (McDonald et al., 2012) and VSEARCH (Rognes et al., 2016), as described by Obregon-Gutierrez et al. (2024).

The Chao1 index (Chao, 1984) was used to assess bacterial estimated alpha diversity richness and the Shannon index (Shannon, 1948) to evaluate evenness in addition to richness. The significance of the differences between the study groups were tested with pairwise t-tests (999 random permutations), included in *alpha-group-significance* (Kruskal-Wallis, 1952). The Jaccard similarity coefficient (Jaccard, 1908) and Bray-Curtis dissimilarity index (Bray and Curtis, 1957) was used to assess qualitative and quantitative beta diversity metrics, respectively. Additionally, weighted and unweighted Unifrac phylogenetic metrics (Lozupone et al., 2007) were calculated.

Statistical differences in beta diversity between experimental groups were assessed using PERMANOVA (Permutational Multivariate Analysis of Variance) with 999 permutations, implemented in the beta-group-significance method within Qiime2.

To evaluate the temporal dynamics of the nasal microbiota in response to vaccination and pathogen challenge, a longitudinal analysis was conducted by calculating the delta (Δ) of alpha diversity metrics between post-challenge (D35) and baseline (D0) for everyone. The indices included Shannon diversity, Chao1 richness, Faith's phylogenetic diversity (pd), observed features, and Pielou's evenness. These differences (D35–D0) were computed per animal and compared across groups (CV, SBA-15 and CONT) using pairwise Wilcoxon rank-sum tests to assess whether microbial diversity was preserved, lost, or enriched over time.

The feature table was processed using QIIME 2 using *q2 feature-table filtering*. Taxa were filtered prior to downstream analysis to include only those present in at least 80% of samples per time point (D0, D21 and D35) in each group and exhibiting a minimum relative frequency ≥1%. This filtering approach aimed to highlight core microbiota components by excluding rare and inconsistently detected taxa.

The microbiota indexes were subjected to correlation analysis using the parametric Pearson and non-parametric Spearman tests, to measure the association between the clinical variables and microbiota indexes over time. Microbiome analysis output data was further processed and used to create plots and tables in RStudio (version 2024.04.2) software (Posit team, 2024) with the packages *qiime2r* (Bisanz, 2021), *reshape2* (Wickham, 2007), *ggplot2* (Wickham, 2016), *ggrepel* (Slowikowski, 2024), *vegan* (Oksanen et al. 2024) and *tidyverse* (Wickham et al. 2019).

2.6 Correlation analysis between microbiota diversity and immune outcomes

Spearman correlation analyses were performed in CV, SBA-15 and CONT groups to assess associations between nasal microbiota alpha diversity indices (Chao1, Shannon entropy, and Evenness) and two outcome variables: (i) systemic IgG levels against *M. hyopneumoniae* (S/P values ΔIgG D35) and the (ii) extent of macroscopic lung lesions (%). For immune response evaluation, correlations were calculated using alpha diversity values and serum IgG levels measured at day 35 post-vaccination. For disease severity assessment, correlations were calculated between alpha diversity

indices at weaning (D0) and lung lesion scores assessed at necropsy (D56). All analyses were conducted using R software and graphs were plotted using GraphPad Prism 10.2.2 software (La Jolla, CA-USA).

3. Results

3.1. Sequencing throughput stats

Out of the 96 samples submitted to *16S* gene amplification and Illumina sequencing, 3 were excluded because of below-threshold control quality parameters. From the 93 samples sequenced, 32 belonged to nasal swab at D0 (50,345 ASVs); 32 from D21 (19,170 ASVs) and 29 from D35 (13,730 ASVs). After the denoising and quality control steps, a total of 9,875 features (ASVs) were found in the 93 samples of the study analyzed. A total of 7,666,319 sequences were analyzed with a minimum ASVs frequency per sample of 13,730 and maximum of 133,683 per sample and 898,029 per feature, being the mean 82,433 and 776.33 respectively. After processing, 82.8% of the raw sequences passed the quality-control steps and were included in the microbiota analyses.

3.2. Alfa and beta diversity description in D0, D21 and D35 by each group

At baseline (D0), alpha diversity revealed a trend toward higher richness in SBA-15 compared to CONT (Observed features, $p = 0.057$) and significantly greater phylogenetic diversity in CV compared to CONT (Faith's PD, $p = 0.014$). In terms of beta diversity, almost all pairwise comparisons among CV, SBA-15, and CONT showed significant differences (Supplementary Table S1), indicating distinct microbial community structures across groups prior to vaccination.

At D21 (challenge day), alpha diversity analysis showed reduced evenness in CONT when compared with CV ($p = 0.03$). Beta diversity analysis showed that vaccinated groups (CV and SBA-15) clustered apart from non-vaccinated animals (CONT), consistently across Bray–Curtis, unweighted UniFrac, and weighted UniFrac metrics, while vaccinated groups did not differ from each other. In contrast, the Jaccard index distinguished all groups from one another (PERMANOVA, $p < 0.001$; Supplementary Table S2).

By D35 (two weeks post-challenge), alpha diversity again revealed reduced evenness in CONT compared to CV ($p = 0.04$). Beta diversity analyses confirmed differences among all groups with the Jaccard metric, with clear separation between vaccinated and non-vaccinated animals ($p = 0.004$ vs CV; $p = 0.011$ vs SBA-15), while CV and SBA-15 remained similar ($p = 0.286$). Unweighted UniFrac indicated a trend for separation between CV and CONT ($p = 0.054$), whereas weighted UniFrac revealed both a significant difference between CV and CONT ($p = 0.01$) and a tendency toward separation between SBA-15 and CONT ($p = 0.065$; Supplementary Table S3).

3.3. Nasal microbiota at weaning and the longitudinal modulation after vaccination and challenge

To evaluate the nasal microbiota across the study in the vaccinated groups versus the non-vaccinated one, we performed a longitudinal analysis of alpha diversity indices by the difference in the microbiota diversity between D35 and D0 (ΔD35–D0) for each animal per group. Across all metrics evaluated — including Shannon diversity, Evenness, Chao1, Observed Features, and Faith's PD — non-vaccinated animals in the CONT group exhibited a significantly greater reduction in microbial diversity and richness compared to vaccinated groups. This was most evident in the number of observed features (OTUs; $p = 0.011$ vs CV; $p= 0.0021$ vs SBA-15) and in the Shannon index ($p = 0.0015$ vs CV; $p = 0.0068$ vs SBA-15), highlighting a clear loss in microbial diversity and balance (Figure 2). Consistently, other richness-related indices, including Chao1 ($p = 0.01$ vs CV; $p = 0.0076$ vs SBA-15) and Faith's PD ($p = 0.0014$ vs CV), as well as Evenness ($p = 0.0008$ vs CV; $p = 0.0062$ vs SBA-15), also confirmed the reduction observed in the CONT group.

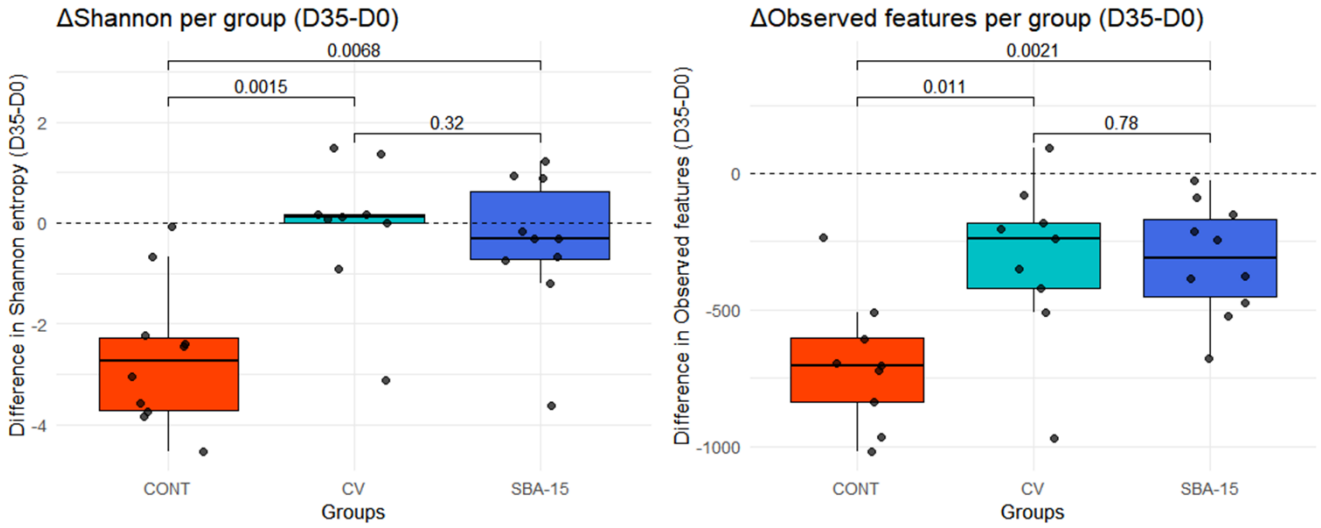


Figure 2: Longitudinal analysis of nasal microbiota alpha diversity before (D0) and after (D35) *M. hyopneumoniae* challenge. Boxplots represent the difference (D35–D0) in five alpha diversity metrics—Shannon index and Observed Features for each experimental group: CV, SBA-15, and CONT. Statistical comparisons were performed using pairwise Wilcoxon tests between groups; p-values are shown above the brackets.

3.4. Taxonomy identification between timepoints and the core microbiota

The raw feature table generated from QIIME 2 contained a total of 545, 405, and 444 taxa at D0, D21, and D35, respectively, in the CV group; 550, 481, and 523 taxa in the SBA-15 group; and 643, 563, and 455 taxa in the CONT group at the corresponding time points (Supplementary material - Tables).

To assess the composition and persistence of core nasal microbiota, we analyzed the shared and exclusive bacterial genera among the experimental groups at different timepoints. After filtering for taxa present in ≥80% of samples per time point and with a minimum relative abundance of ≥1%), the CV group, 11 taxa were retained at D0, 11 at D21, and 11 at D35. For the SBA-15 group, 13 taxa passed the filtering threshold at D0, 14 at D21, and 12 at D35. In the CONT group, 13 taxa were retained at D0, 10 at D21, and 6 at D35 (Table S4). The relative abundance was calculated by averaging the proportions of each genus across all samples within each group and time point (Figure 3).

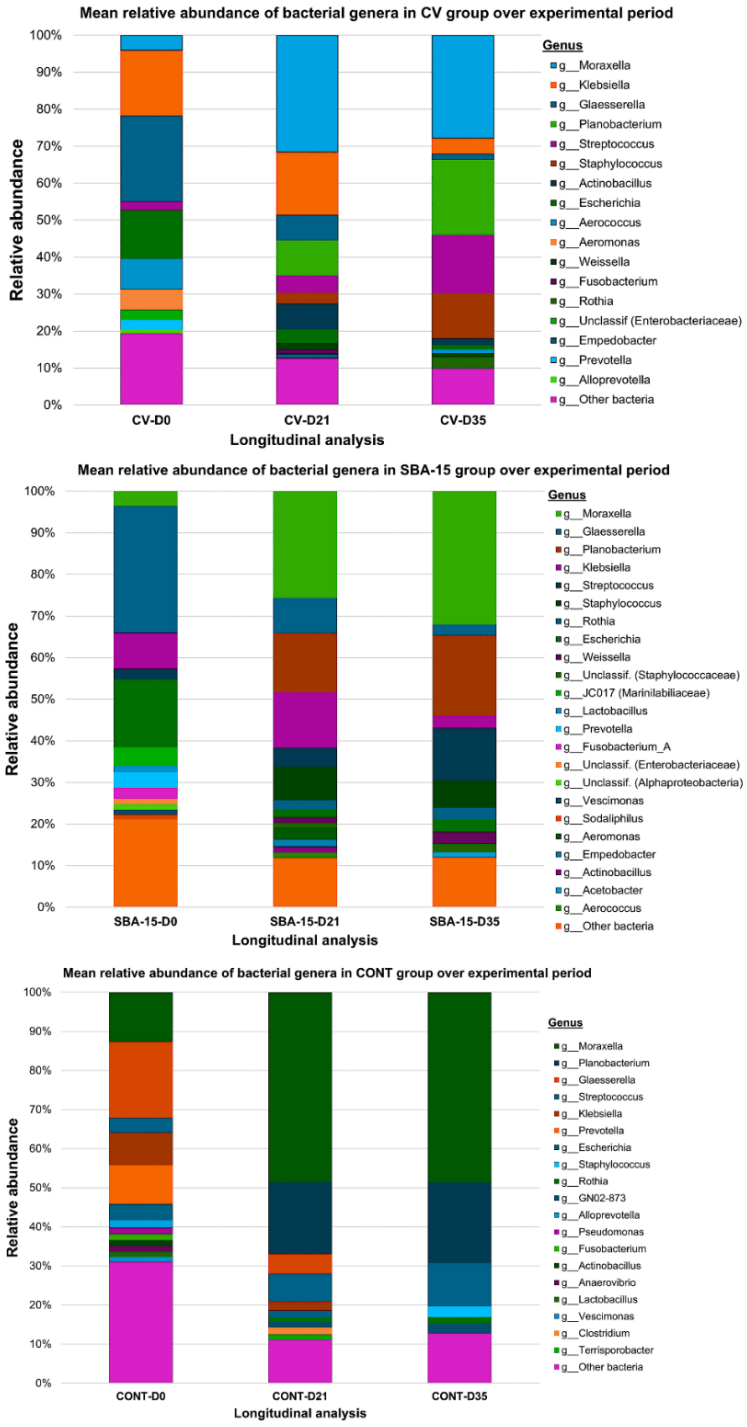


Figure 3: Mean relative abundance of bacterial genera in the CV, SBA-15, and CONT groups over the experimental period (D0, D21, and D35). Stacked bar plots represent the average relative abundance of bacterial genera across all samples within each group and time point. Only genera with ≥1% relative abundance in at least one group and time point are shown individually; all others are grouped under "Other bacteria".

At weaning (D0), the three groups showed a considerable overlap in core nasal genera, including *Glasserella*, *Escherichia*, *Rothia*, and *Streptococcus*. However, divergence increased following vaccination and challenge. By D35, the vaccinated groups (CV and SBA-15) maintained a broader and more stable set of shared genera, whereas the CONT group exhibited marked loss of core taxa, reflecting greater instability in nasal microbial composition (Figure 4A).

Temporal analysis within each group revealed further differences in microbial dynamics (Figure 4B). The CV and SBA-15 groups retained a more consistent microbiota composition across timepoints, with a substantial number of genera shared from D0 to D35. In contrast, the CONT group displayed greater temporal variation, with only two genera consistently maintained across all three timepoints. These results suggest that vaccination—especially with SBA-15—may help preserve microbial stability and diversity in the nasal cavity during the post-weaning and post-challenge period.



Figure 4: Shared and exclusive nasal bacterial genera among experimental groups and overtime. (A) Venn diagrams showing the distribution of core bacterial genera in the nasal microbiota at weaning (D0), post-vaccination (D21), and post-challenge (D35) across the three experimental groups: CV, SBA-15, and CONT. (B) Temporal comparison of core nasal microbiota composition within each group (CV, SBA-15, and CONT) at D0, D21, and D35. Shared and exclusive genera are listed alongside each

panel, with percentages indicating the proportion of genera shared between groups or timepoints.

In low-protection (Figure 5A), *Glaesserella*, *Streptococcus*, and *Moraxella* were common among the four overlapping piglets. In high-protection (Figure 5B), shared genera included *Prevotella*, *Alloprevotella*, *Streptococcus*, *Fusobacterium*, and *Lactobacillus* among four piglets. Also, distinct genus was found exclusively in individual piglets and are also detailed by family in Figure 8.

Some variabilities were found: two piglets (#1342 and #1346), classified as low immune responders based on serological response (low IgG anti-*Mycoplasma hyopneumoniae* levels on D35), were concurrently classified as low-susceptibility based on lung lesion scores at necropsy (9.5% and 3%, respectively). Conversely, one piglet (#1340), categorized as a high immune responder based on serological criteria, exhibited pronounced lung lesions (19.3%) and was therefore classified as high-susceptibility. Common taxa among all these three piglets (#1340, #1342 and #1346) include *Glaesserella* and *Prevotella* (Figure 5C).

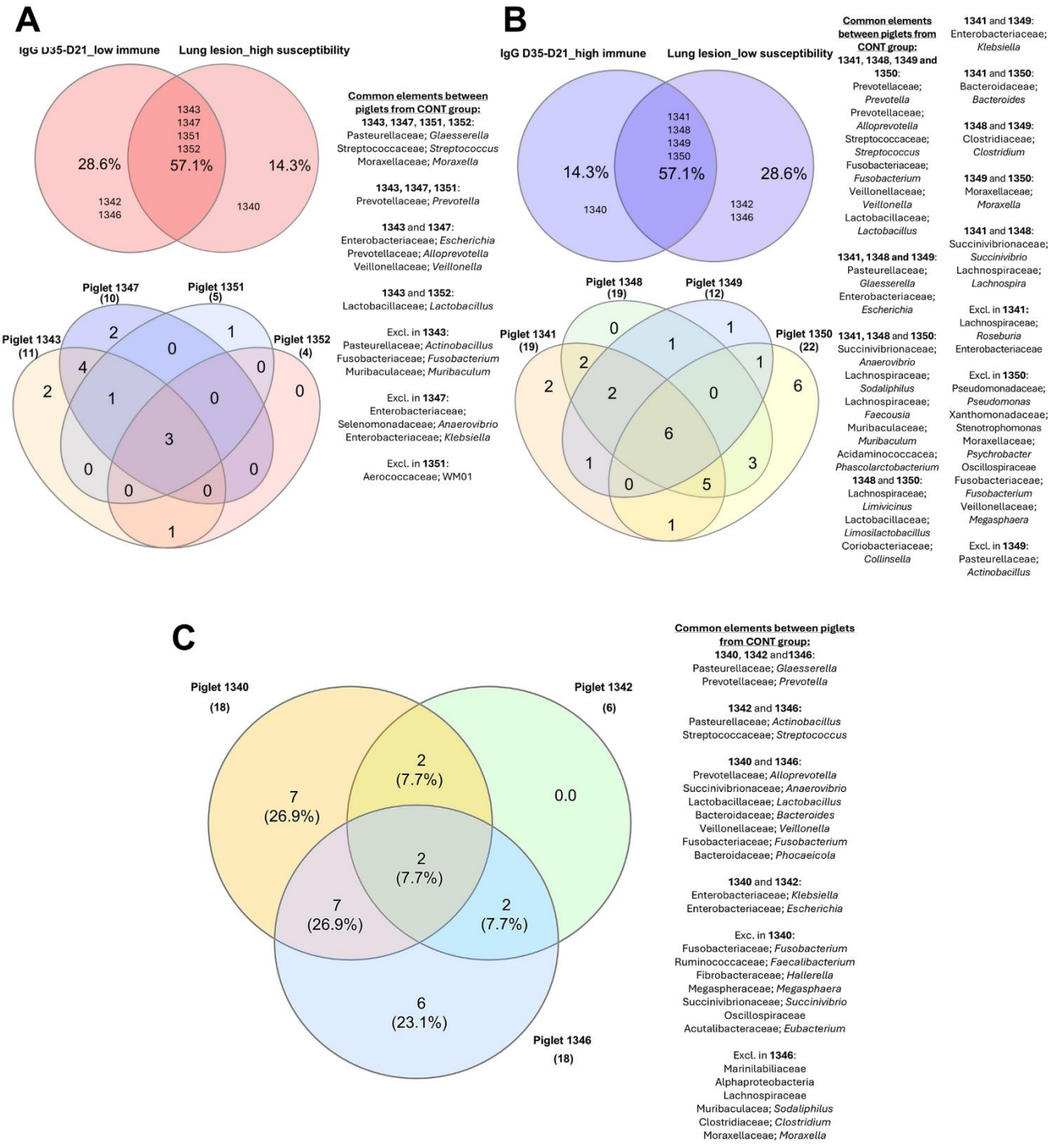


Figure 5: Nasal microbiota composition at D0 in piglets from the CONT group classified according to respiratory outcomes. Venn diagrams illustrate the overlap of bacterial genera identified in nasal microbiota from piglets retrospectively classified based on two independent clinical parameters: the variation in serum IgG levels (ΔIgG D35–D21) and macroscopic lung lesion scores at D56. (A) low-protection, (B) high-protection, and (C) discordant responders. Numbers within the circles indicate the total

number and percentage of genera detected in each category. Bacterial genera shared across classifications and their respective families are annotated to the right of each panel.

However, despite a 4-day acclimation period in the study facilities, we observed significant differences in beta diversity indexes of the nasal microbiota in piglets from the CV, SBA-15, and CONT groups at D0 (weaning, pre-vaccination) (Figure 6). Bray–Curtis and Jaccard distances revealed distinct microbial community compositions among groups, with 15% of the variance explained by treatment group (PERMANOVA, $p = 0.001$; $R^2 = 15\%$). Unweighted UniFrac analysis also confirmed group differences in phylogenetic composition ($p = 0.001$), albeit with slightly lower explanatory power ($R^2 = 11\%$). Clustering patterns suggest that the SBA-15 group presented the most distinct microbial profile relative to the CV and CONT groups.

The corresponding biplots (Figure 6) illustrate the contribution of specific main bacterial genera to the observed group-level variation. While directional trends were observed for taxa such as *Moraxella*, *Prevotella*, and *Pseudomonas*, no single genus dominated the separation among groups.

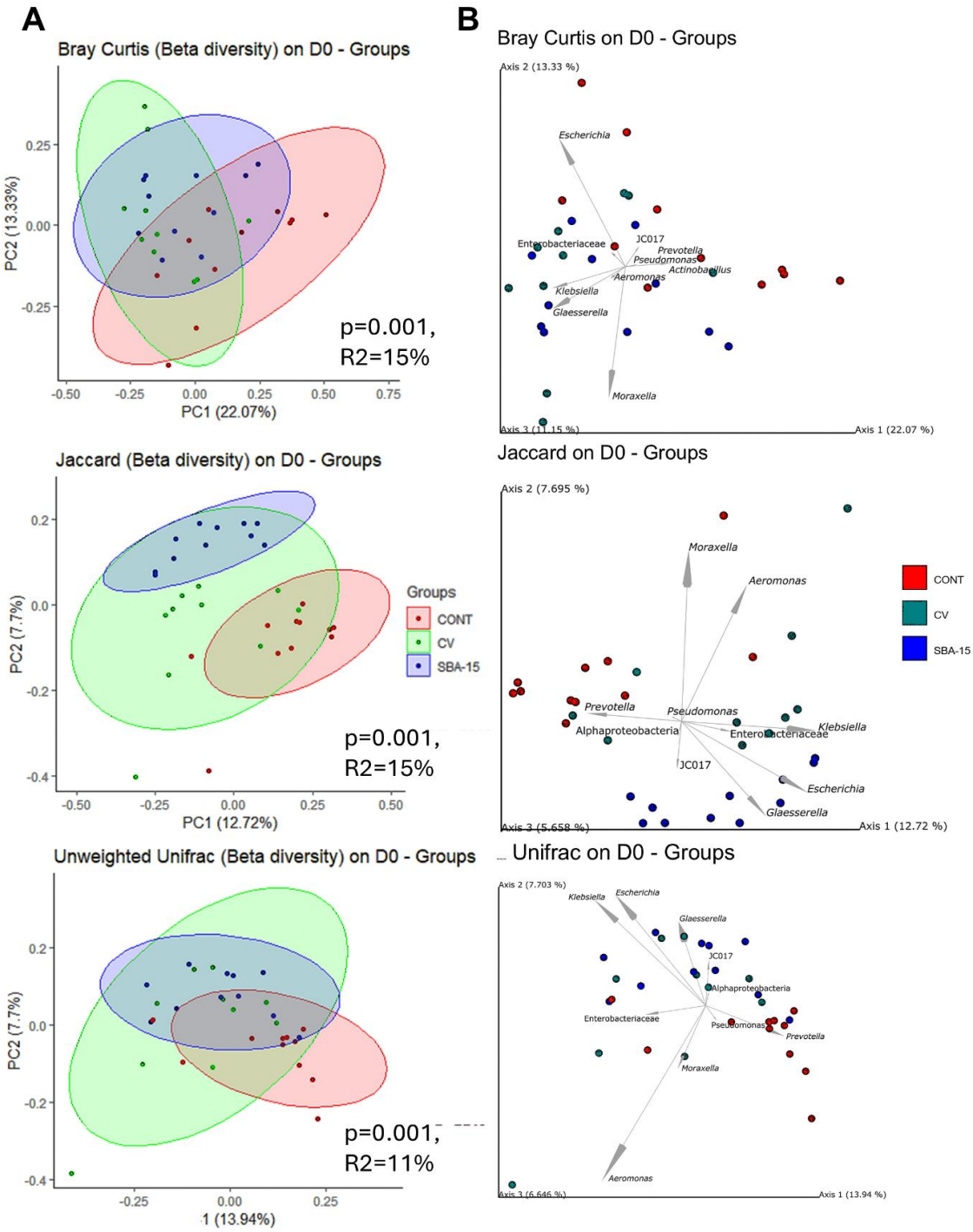


Figure 6: Beta diversity of the nasal microbiota at weaning (D0) and associated bacterial taxa across treatment groups. Principal coordinate analysis (PCoA) plots (A) show differences in nasal microbial community composition among piglets in the CONT, CV, and SBA-15 groups at D0, based on Bray–Curtis, Jaccard, and Unweighted UniFrac distance metrics. Statistical significance was evaluated using

PERMANOVA (p = 0.001). Biplots (B) illustrate the contribution of specific bacterial genera to group-level separation.

Despite the statistical differences observed in beta diversity metrics, the core microbiota analysis (genera present in at least 80% of the samples with a minimum relative abundance of 1%. At weaning (D0) prior to vaccination, the nasal microbiota composition was highly similar across all experimental groups. Overall, the core microbiota composition seems consistent among piglets, with no genus clearly dominating or distinguishing one group from another at this timepoint (Figure 7A). Three weeks after vaccination (D21), prior to challenge, the core nasal microbiota composition had diverged across groups. Vaccinated groups (CV and SBA-15) maintained stable and diverse microbial profile, while the CONT group exhibited an increased relative abundance of potentially dysbiotic genera, such as *Moraxella*, *Glaesserella* and *Streptococcus* (Figure 7B).

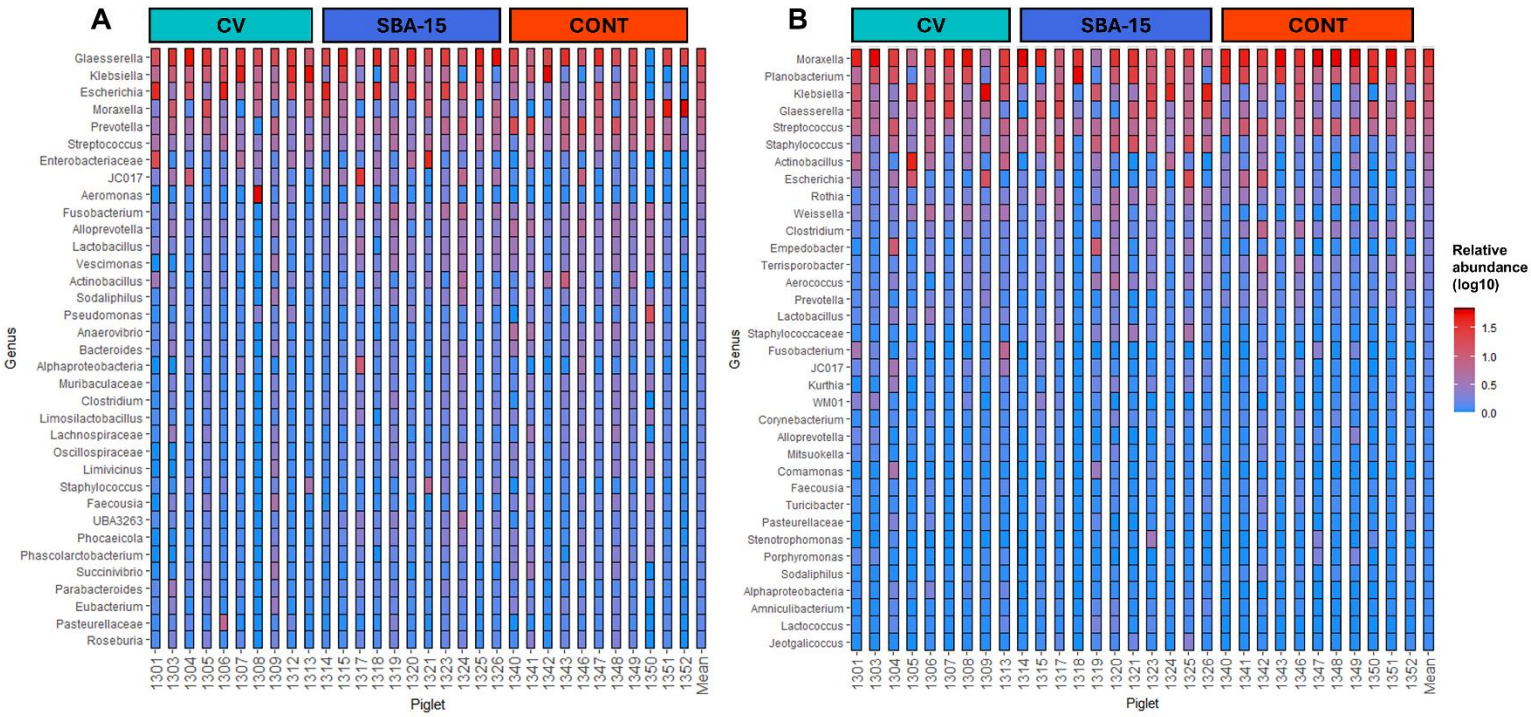


Figure 7: Nasal microbiota composition at weaning and post-vaccination across experimental groups. (A) Heatmap showing the log10-transformed relative abundance of the most common bacterial genera in nasal swabs from piglets at weaning (D0), prior to vaccination. (B) Heatmap of the same genera three weeks post-vaccination (D21), prior to *M. hyopneumoniae* challenge.

3.5. Macroscopic lung lesion outcomes and IgG anti-M. hyopneumoniae quantification in serum

The results are described elsewhere (Petri et al. 2024). Briefly, macroscopic lung lesion analysis showed significant differences between groups. The CV group had the lowest median lung lesion score (1.7%), followed by the SBA-15 group (3.25%), while the non-vaccinated CONT group exhibited the highest lesion score (9.5%) ($p<0.05$). Additionally, we found correlations between alpha diversity indexes at D0 (weaning) and lung lesions extension in the CONT group. Spearman correlation coefficients were as follows: Chao1 ($r = -0.718$, $p = 0.016$), Shannon entropy ($r = -0.645$, $p = 0.037$), Evenness ($r = -0.645$, $p = 0.037$) and Observed features ($r = -0.700$, $p = 0.02$).

Humoral immune responses were assessed by measuring levels of anti-*M. hyopneumoniae* IgG in serum. The CV group presented the highest serum IgG levels, with a mean of 0.876 (range 0–1.67), followed by SBA-15 (median 0.363; range 0–1.19), and CONT with lower levels (median 0.03; range 0–0.275). Similarly, nasal IgA levels were highest in the CV group (mean 1.02; range 0–1.63), followed by SBA-15 (median 0.3; range 0–1.27), and lowest in CONT (mean 0.07; range 0–0.29). The dynamics of anti-*M. hyopneumoniae* IgG and IgA antibody responses over time, including mean levels and seroconversion rates for each group, are detailed in Table 1.

Table 1: Serum specific IgG anti-*M. hyopneumoniae* levels. Quantification of systemic IgG (ELISA from serum) of piglets from CV, SBA-15 and CONT groups during the experimental period. Values expressed as S/P ratio mean.

	Dynamics of IgG anti-*M. hyopneumoniae*						
	CV		**SBA-15**		**CONT**		
	Levels (mean ±sd)	**Seroconversion (%)**	**Levels (mean ±sd)**	**Seroconversion (%)**	**Levels (mean ±sd)**	**Seroconversion (%)**	***p*-value***
D0 Vaccination	0.138 (± 0.08)^{A}	0/10 (0%)	0.101 (± 0.1)^{A}	0/11 (0%)	0.158 (±0.05) ^{A}	0/11 (0%)	0.225
D21 Challenge	0.271 (± 0.17)^{A}	3/10 (30%)	0.214 (±0.12)^{A}	2/11 (18.2%)	0.111 (±0.05) ^{B}	0/11 (0%)	0.007
D35	1.147 (± 0.59)^{A}	9/10 (90%)	0.610 (± 0.41)^{B}	8/11 (72.7%)	0.159 (±0.09) ^{C}	0/11 (0%)	0.001
D56	1.283 (±0.52)^{A}	9/10 (90%)	0.89 (±0.5)^{A}	9/11 (81.8%)	0.471 (±0.22) ^{B}	8/11 (72.7%)	0.001

*Means followed by different letters on the same row differ significantly by Tukey test (p<0.05).

3.6. Classification of piglets according to IgG anti-M. hyopneumoniae and macroscopic lung lesions

Based on combined immunological and pathological outcomes, animals were classified into high-protection and low-protection phenotypes. In the CV group, the high-protection phenotype showed a mean lung lesion score of 0.3% and ΔD35 IgG values of 1.45, whereas the low-protection phenotype presented 7.0% lung lesions with ΔD35 IgG of 0.18. In the SBA-15 group, the high-protection phenotype exhibited 1.5% lung lesions and ΔD35 IgG of 0.52, while the low-protection phenotype showed 5.0% lung lesions with ΔD35 IgG of 0.18. In the CONT group, the high-protection phenotype had lower lung lesion scores (6.6%) and modest antibody responses (ΔD35

IgG: 0.13), whereas the low-protection phenotype displayed the most severe lung lesions (19.1%) and minimal or absent antibody responses (ΔD35 IgG: 0) (Table 2).

Table 2: Median values (range) for the evaluated parameters, along with mean values according to immune responsiveness and diseases susceptibility within each group (CV, SBA-15 and CONT). Parameters included macroscopic lung lesions (%) and serum levels of IgG and IgA specific to *M. hyopneumoniae*.

Groups	Parameter	Median (min-max)	Low-susceptibility/ High immune response* (mean)	High-susceptibility/ Low immune response** (mean)
CV (n=10)	**Macroscopic lung lesion (D56)**	1.7% (0-24.2)	n=5 (0.3%)	n=5 (7%)
SBA-15 (n=11)		3.25% (0-11.3)	n=5 (1.5%)	n=6 (5%)
CONT (n=11)		9.5% (3-35.1)	n=6 (6.6%)	n=5 (19.1%)
CV (n=10)	**IgG anti-*M. hyopneumoniae* (ΔD35 values)**	0.876 (0-1.67)	n=5 (1.45)	n=5 (0.18)
SBA-15 (n=11)		0.363 (0-1.19)	n=5 (0.52)	n=6 (0.18)
CONT (n=11)		0.03 (0-0.275)	n=5 (0.13)	n=6 (0)

*For lung lesions: values below the group median; For IgG: values above the group median.

**For lung lesions: values above the group median; For IgG: values below the group median.

3.7. Effect of the weaning-associated microbiota on susceptibility to M. hyopneumoniae-induced lung lesions

Analysis were conducted in the non-vaccinated CONT group based on disease susceptibility outcomes defined in Section 3.6. Alpha diversity metrics, including Observed features and Chao1 index, showed that low-susceptibility piglets exhibited significantly higher bacterial richness at weaning compared with high-susceptibility piglets within the same group (Observed features: $p = 0.04$; Chao1: $p = 0.04$). No significant differences in richness were observed between susceptibility phenotypes in the vaccinated groups (CV and SBA-15) (Figure 8A–C).

Regarding the beta diversity analysis (Bray–Curtis dissimilarity) revealed that, within the CONT group, low- and high-susceptibility phenotypes had significantly distinct nasal microbial community structures (p = 0.04, R^2 = 20%). In contrast, no significant differences in microbial composition were observed between susceptibility phenotypes in the vaccinated groups (CV and SBA-15) (Figure 8D–F).

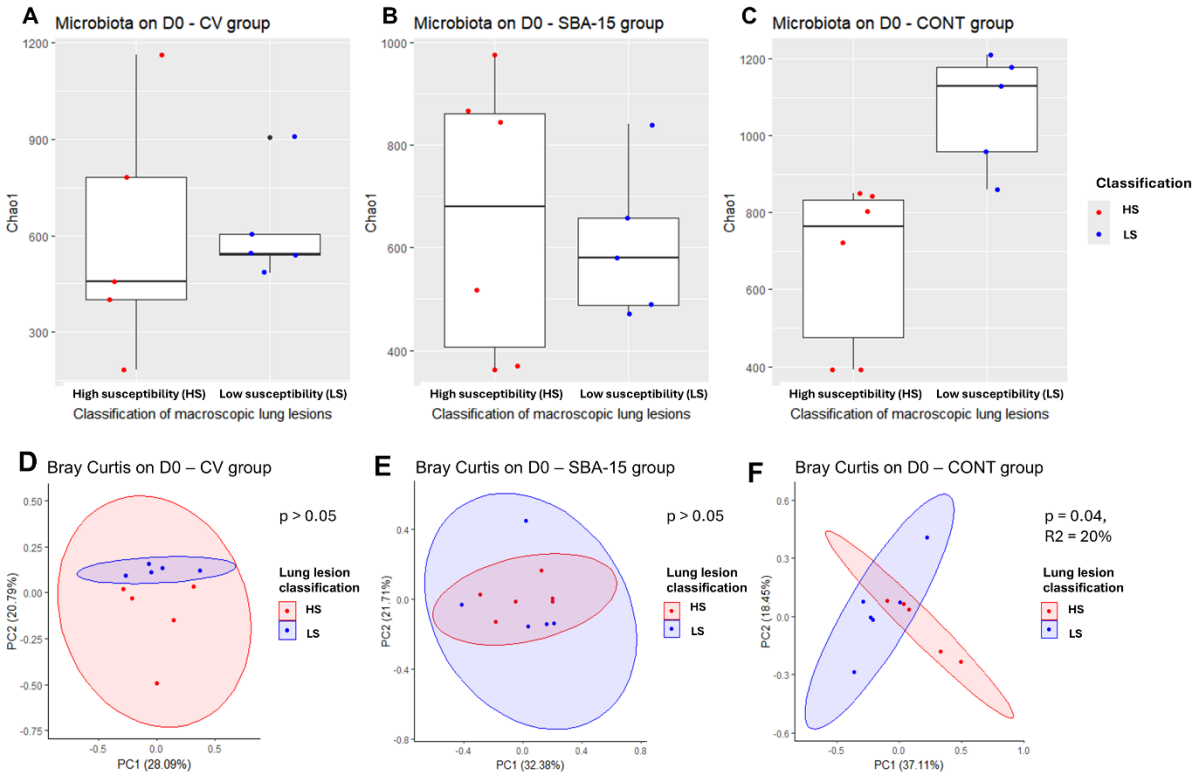


Figure 8: Nasal microbiota diversity at weaning (D0) according to disease susceptibility at necropsy (D56). Piglets were retrospectively classified as low-susceptibility (blue) or high-susceptibility (red) based on *M. hyopneumoniae*-induced lung lesion scores. (A–C) Alpha diversity (Chao1 index) in CV, SBA-15 and CONT groups (Kruskal–Wallis test). (D–F) Beta diversity (Bray–Curtis) visualized by PCoA for each group (PERMANOVA).

3.8. Effect of the weaning-associated microbiota on immune responsiveness after challenge

Analysis was performed in the non-vaccinated CONT group based on immune responsiveness phenotypes defined by ΔIgG values (Section 3.6). At weaning (D0),

alpha diversity metrics differ significantly between high immune responders and low immune responders. High immune responders exhibited significantly greater microbial diversity, as indicated by Shannon entropy (p = 0.028), Observed features (p = 0.04), and Pielou's Evenness (p = 0.04) (Figure 9A–C).

Beta diversity analysis also demonstrated distinct nasal microbial community structures between high and low immune responders. PCoA plots showed clear separation based on Bray–Curtis (p = 0.028, R^2 = 20%), Unweighted UniFrac (p = 0.022, R^2 = 14%), and Weighted UniFrac (p = 0.046, R^2 = 30%) (Figure 9D–F).

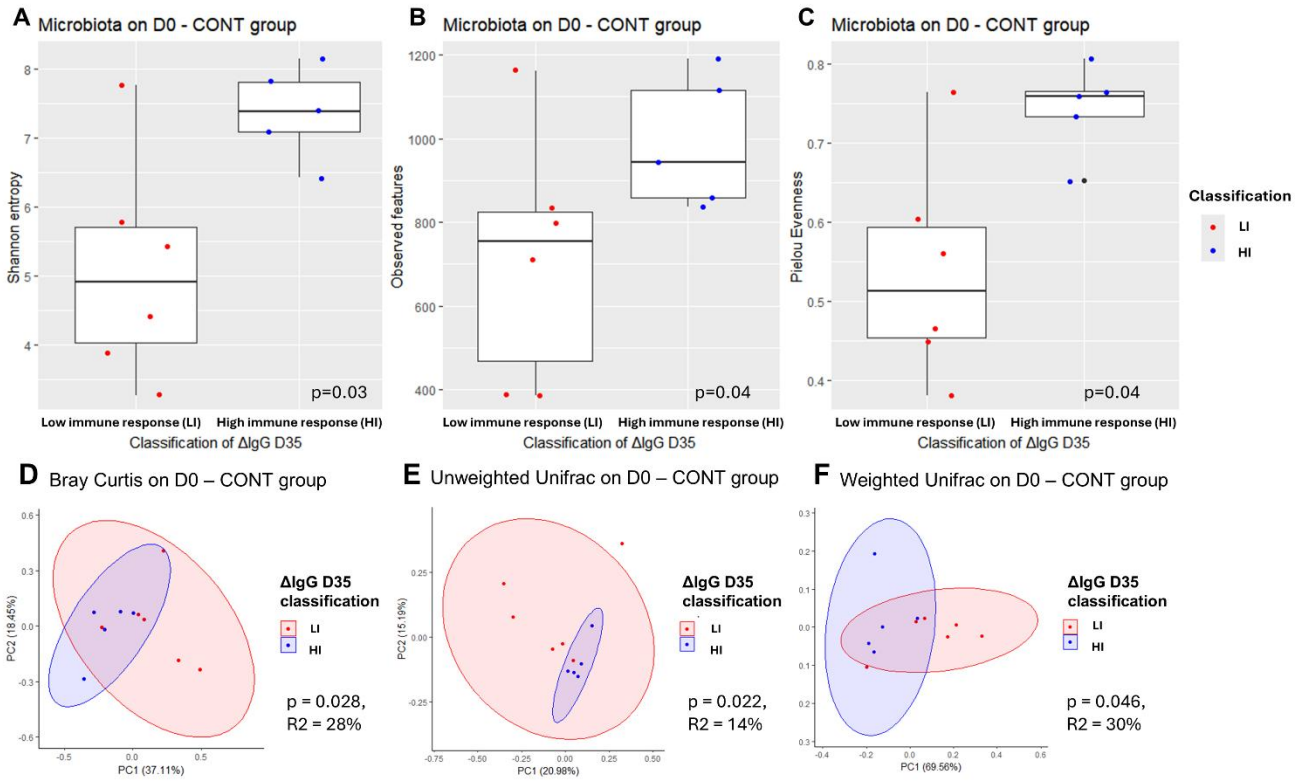


Figure 9: Nasal microbiota diversity at weaning (D0) in relation to immune responsiveness to *M. hyopneumoniae* on ΔD35-D21 (CONT group). Piglets were retrospectively classified as high immune responders (blue) or low immune responders (red) based on their anti-*M. hyopneumoniae* IgG levels. (A–C) Alpha diversity metrics (Shannon, Observed Features, and Pielou's Evenness); Kruskal–Wallis test. (D–F) Beta diversity based on Bray–Curtis, Unweighted UniFrac, and Weighted UniFrac distances using PCoA; PERMANOVA test ($p < 0.05$).

3.9. Effect of intramuscular vaccination at D0 and challenge at D21 on the nasal microbiota

To investigate vaccination-associated shifts in the nasal microbiota, differences in microbial composition were assessed longitudinally. At weaning (D0), both vaccinated groups already exhibited distinct microbial profiles compared with the CONT group, with several taxa being differentially enriched or depleted (Supplementary Figures S1A and S1B). These differences became more pronounced by D21, when marked shifts in the relative abundance of several bacterial families and genera were observed in both CV and SBA-15 groups (Supplementary Figures S2A and S2B).

Additionally, two weeks post-challenge (D35), differential abundance analysis using ANCOM-BC revealed a consistent depletion of the family *Metamycoplasmataceae* in both vaccinated groups compared with the non-vaccinated CONT group. In the CV group, this family showed a log-fold change (LFC) of approximately −3.8, whereas in the SBA-15 group the LFC was approximately −4.2, indicating a substantial post-vaccination reduction (Figure 10). Importantly, this family was not detected in the nasal cavity at D0 or D21, supporting its association with the experimental challenge. In both vaccinated groups, the genus *Brachymonas* was also depleted (LFC ≈ −2), whereas Lactococcus was enriched in the SBA-15 group (LFC ≈ 3.1) and *Weissella* in the CV group (LFC ≈ 2.5).

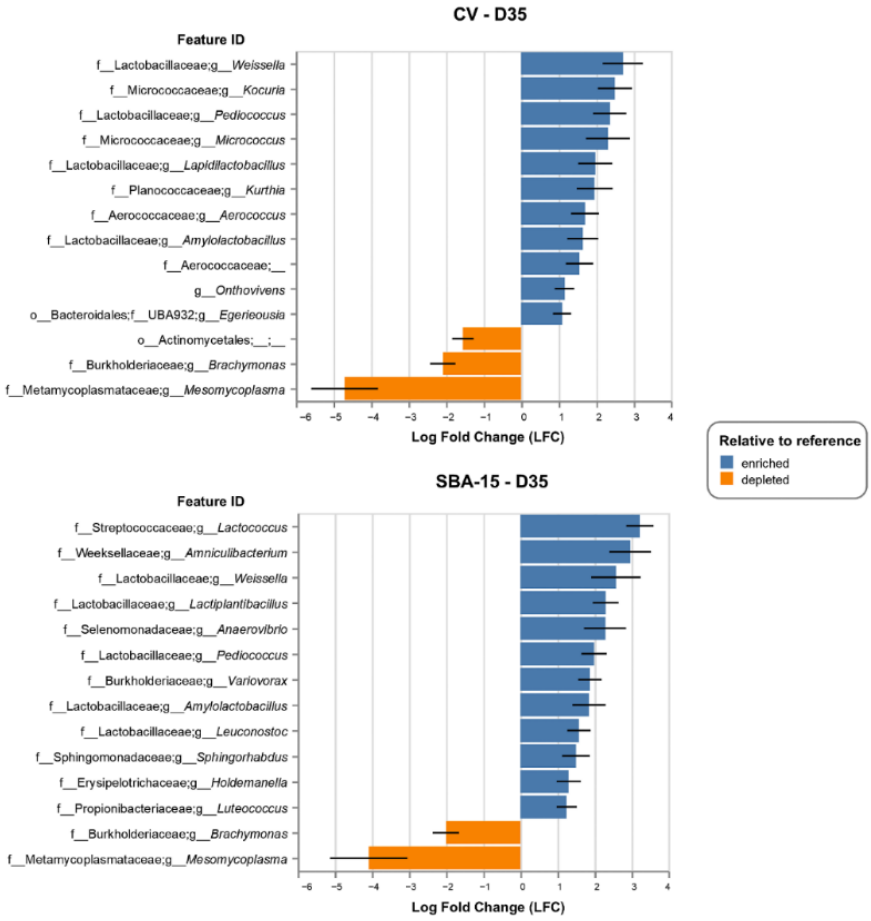


Figure 10: Differential abundance analysis of nasal microbiota at D35 post-challenge using ANCOM-BC, comparing vaccinated groups (CV and SBA-15) to the CONT group. Bar plots represent log fold change (LFC) of bacterial families significantly enriched or depleted in vaccinated groups relative to the control.

Despite an observed increase in ASVs assigned to the family Metamycoplasmataceae in non-vaccinated animals post-challenge, representative ASVs obtained from the nasal swab sequencing at D35 were aligned against reference genomes. The sequences matched closely with *M. flocculare*, a commensal species, rather than the pathogenic *M. hyopneumoniae*. To confirm this taxonomic classification, a phylogenetic tree was constructed using representative strains of *M. hyopneumoniae* and *M. flocculare*. The ASVs clustered within the *M. flocculare* clade, showing higher similarity to strains Ms42 and MF29, distinct from pathogenic strains such as *M. hyopneumoniae* 232, J, and 7448 (Supplementary Figure S3).

3.10. Microbiota correlations with vaccination and challenge responses

Correlation analyses revealed significant associations between nasal microbiota diversity indices, humoral immune response and lung lesion severity. In the CV group, a strong negative correlation was found between Chao1 richness at D35 and IgG levels against *M. hyopneumoniae* (r=-0.867, p=0.005) (Figure S4A). When analyzing all vaccinated animals (CV and SBA-15), Shannon entropy and Evenness at D35 showed moderate positive correlations with serum IgG levels (r = 0.468, p = 0.032 and r = 0.492, p = 0.023, respectively) (Figures S4B–C).

In the non-vaccinated animals, alpha diversity indices at weaning (D0) were negatively correlated with disease susceptibility assessed at necropsy on D56 Specifically, Chao1 richness (r = –0.727, p = 0.014), Shannon entropy (r = –0.645, p = 0.037), and Evenness (r=-0.645, p = 0.037) were all inversely associated with lesion extent (Figures S4D-F). Additionally, a significant negative correlation was observed between Chao1 index at D35 and the humoral immune response (IgG) against *M. hyopneumoniae* (r = –0.779, p = 0.007).

At the genus level, analyses within the high-protection phenotype of the CONT group revealed further associations of potential biological relevance. A strong negative correlation was observed between the relative abundance of *Alloprevotella* spp. at D0 and lung lesion severity at D56 (r = –0.858, p = 0.029). In contrast, *Glaesserella* spp. abundance at D0 was negatively correlated with IgG levels (r = –0.777, p = 0.041) (Supplementary Figure S4G-H).

4. Discussion and conclusion

Vaccination against *M. hyopneumoniae* remains a key measure of respiratory disease control in swine production systems worldwide. By reducing the severity and extent of lung lesions, vaccination plays a critical role in improving animal health, growth performance, and overall farm productivity. However, the mechanisms underlying vaccine-induced protection extend beyond direct immune responses and may involve broader interactions with the host’s mucosal environment, mainly the intestinal mucosa and the fecal microbiota (Munyaka et al. 2020; Borey et al. 2021). The nasal cavity, as the primary site of defense and harbors a dynamic microbial community that contributes to mucosal immunity and pathogen exclusion.

Understanding how vaccination strategies can influence nasal microbiota and how the nasal microbiota can modulate the outcomes in the vaccination-challenge context is essential for a more comprehensive view of disease prevention.

Weaning is recognized as a major stressor in piglets, associated with abrupt dietary, social, and environmental changes that can disrupt mucosal homeostasis and microbial balance (Correa-Fiz et al. 2020; Tang et al. 2022). Several studies have shown that stress during this period can induce transient dysbiosis, particularly in the gut, but also in the respiratory tract (Heo et al., 2013; Gresse et al., 2017). The early life microbiota plays a key role in the protection against pathogens in the intensive pig farming. Obregon-Gutierrez et al. (2025) found that the pig nasal and rectal microbiotas are involved to response against *G. parasuis* in weaned piglets. In our study, animals presenting lower richness and diversity indices early in life tended to develop more severe lung lesions following *M. hyopneumoniae* challenge. Furthermore, differences in beta diversity between animals classified by lung lesion severity support the concept that microbial community structure, beyond mere diversity metrics, plays a critical role in shaping host-pathogen interactions and immune outcomes.

The longitudinal and comparative analysis of the nasal microbiota – from weaning at the time of vaccination to five weeks later (two weeks post-*M. hyopneumoniae* challenge) – revealed distinct microbial dynamics influenced by the vaccination protocols and subsequent pathogen exposure. Vaccinated animals maintained greater stability in richness and diversity compared to non-vaccinated piglets, suggesting enhanced resilience to both weaning-associated stress and disease progression. Despite the lack of significant differences in beta diversity at weaning among the groups, the core microbiota composition appeared highly uniform at this time point. No dominant genus was identified in any group, suggesting a shared early microbial signature across treatment groups at beginning of the study. This observation strengthens the interpretation that the changes in microbial composition and clinical outcomes observed at later time points are likely driven by the specific vaccination protocols and pathogen exposure, rather than inherent differences in baseline microbiota. Previous studies have highlighted the significant role sows play in shaping the microbiota composition of piglets, with sows being one of the primary sources of colonizers for the upper respiratory tract in early life. As a result, the microbiota of

piglets can closely resemble that of their sows (Nowland et al. 2019; Obregon-Gutierrez et al. 2021; Blanco-Fuertes et al. 2022).

In the non-vaccinated control group, both alpha and beta diversity analyses of the nasal microbiota at weaning revealed significant associations with the systemic IgG response measured two weeks post-challenge. Animals classified as high immune responders by systemic antibodies exhibited significantly higher microbial diversity and richness at weaning, as reflected by increased alfa diversity metrics. Our findings align with previous reports indicating that a more diverse microbiota early in life may prime the host for more effective immune responses to respiratory pathogens (Gensollen et al. 2016; Munyaka et al. 2020).

Although our experimental model for *M. hyopneumoniae* infection in this study was induced via the endotracheal route, our findings suggest that the composition of the nasal microbiota may still influence systemic immune activation, particularly IgG responses specially in non-vaccinated animals. The upper respiratory tract of piglets' harbors immunologically active mucosal tissues that interact with regional lymphoid structures, including mandibular and retropharyngeal lymph nodes, which contribute to the development of systemic immunity (Pabst, 2015; Abt et al., 2012). A diverse and balanced nasal microbiota in early life may promote immune tolerance, modulate antigen-presenting cell function, and maintain mucosal homeostasis, thereby activating systemic immune responsiveness (Belkaid & Hand, 2014; Correa-Fiz et al., 2016). In contrast, low microbial richness may result in impaired mucosal regulation, leading to exaggerated systemic responses upon pathogen exposure. Furthermore, indirect mechanisms—such as mucosal communication between upper and lower respiratory tracts (Man et al., 2017) or modulation of pathogen load and persistence (Valeris-Chacin et al., 2021)—may also contribute to the observed association between nasal microbiota richness and IgG levels.

On the other hand, not all vaccinated and challenged piglets seroconverted for anti-*M. hyopneumoniae* IgG by the end of the study, eight weeks post-vaccination and five weeks after challenge. While individual variability in vaccine response and potential vaccine failure for *M. hyopneumoniae* are commonly cited explanations as the presence of maternally derived antibodies (Augustyniak et al. 2023), our data suggest that the nasal microbiota may also play a modulatory role in shaping the humoral

immune *M. hyopneumoniae* outcome. Piglets with lower microbial richness and diversity at weaning tended to exhibit weaker IgG responses, highlighting the role of early-life microbial composition in shaping vaccine-induced immunity. Evidence in the literature supports the concept that the microbiota can modulate both innate and adaptive immune pathways, thereby affecting vaccine responsiveness (Niederwerder et al. 2017; Lynn & Pulendran et al. 2017). In the context of respiratory pathogens vaccination, the upper airway microbiota may be a key determinant of mucosal and systemic immunogenicity.

In support of this hypothesis, correlation analyses revealed distinct microbiota–immunity relationships depending on vaccination status. Among vaccinated piglets, lower nasal microbial richness at D35 was significantly associated with higher anti-*M. hyopneumoniae* IgG levels, suggesting that even in immunized animals, post-challenge mucosal disturbing may influence systemic humoral responses. Conversely, in the non-vaccinated control group, higher alpha diversity at weaning (D0) was negatively correlated with lung lesion severity and positively correlated with IgG response, indicating that early-life microbiota composition may confer protective effects against disease progression. Our findings emphasize that the nasal microbiota acts both as a modulator of vaccine-induced immunity and as a determinant of clinical outcome in the absence of vaccination (Belkaid & Hand, 2014; de Jong et al. 2023).

Although both infection and vaccination against *M. hyopneumoniae* activate the immune system through pattern recognition receptors (such as TLR2 and TLR4), the site and magnitude of immune stimulation differ substantially. Natural infection occurs at the mucosal surface of the respiratory tract, promoting strong local innate and adaptive responses, including mucosal IgA and inflammatory cell recruitment. In contrast, intramuscular vaccination primarily elicits systemic IgG responses, with limited engagement of the mucosal immune system (Canelli et al., 2023). This distinction may explain why baseline nasal microbiota diversity - as a key modulator of mucosal immunity—was more strongly associated with immune response and disease severity in non-vaccinated animals in our study. Our data showed that animals with higher nasal microbial diversity at weaning exhibited both reduced lung lesion scores and enhanced IgG responses, even in the absence of vaccination. This finding supports the hypothesis that a diverse and balanced microbial community can

contribute to a primed immune environment, improving resilience to respiratory pathogens. Similar results were reported by Obregón-Gutiérrez et al. (2025), who demonstrated that higher nasal and rectal microbial diversity in piglets correlated with better antibody responses to *G. parasuis* vaccination.

The observed relationship between nasal microbiota diversity and the immune response to *M. hyopneumoniae* further reinforces the role of this pathogen as a key bacterial pathogen in the porcine respiratory disease complex (PRDC), facilitating secondary infections by pathogens such as *Pasteurella (P.) multocida*, *Actinobacillus (A.) pleuropneumoniae*, and *G. parasuis* (Maes et al. 2019; Saade et al. 2020; Sonalio et al. 2022). These pathogens typically enter the upper respiratory tract before reaching the lungs, where they contribute to the clinical manifestation of PRDC. In our study we observed not only the reduction of lung lesions in vaccinated piglets with both vaccines (the conventional hydroxide aluminum and the silica-based adjuvants) but also the absence of *Mycoplasma* spp. in the nasal cavity associated with the reduction of lung colonization and the shedding reduction in the SBA-15 vaccine. The present findings indicate that effective vaccination not only reduces lung lesions but also supports nasal microbial stability, underscoring the central role of *M. hyopneumoniae* in the cascade of respiratory disease. Therefore, integrated disease prevention strategies—combining immunization with improved environmental management (air quality, temperature, humidity) and microbiota-targeted interventions—may offer a highly effective approach to enhancing respiratory health and resilience in swine production systems (Opriessnig et al., 2011; Van Dixhoorn et al., 2016; Pessoa et al. 2022).

Among the nasal bacterial genera identified in this study, *Alloprevotella* spp. stood out for its consistent association with protective immune outcomes. In our study, vaccinated groups presented genera such as *Alloprevotella* and *Prevotella* were more frequently detected in the core nasal microbiota at D21 and have previously been associated with mucosal health and anti-inflammatory properties (Correa-Fiz et al., 2016; Munyaka et al. 2020; de Jong et al., 2023). These genera may contribute to immune regulation and microbial homeostasis, potentially supporting a more controlled immune response following challenge.

In contrast, the non-vaccinated control group exhibited higher relative abundance of genera commonly linked to respiratory dysbiosis and opportunistic infections, including *Moraxella*, *Glaesserella*, *Streptococcus*, and *Klebsiella*. These taxa have been implicated in the development or exacerbation of PRDC components and may reflect a destabilized mucosal environment more permissive to secondary infections (Lowe et al., 2011; Niederwerder et al., 2017). At the time of weaning and vaccination, piglets from all experimental groups exhibited a uniform core nasal microbiota, with no genus clearly dominating or differentiating the groups, these findings reinforce the role of immunization not only in modulating host immune responses but also in contributing to the ecological stability of the nasal microbiota in the early post-weaning period. Also, the genus *Planobacterium* was detected in the nasal cavity of pigs as part of the core microbiota. *Planobacterium* is a genus described in the family Flavobacteriaceae, with species isolated from natural environments such as desert soil (Peng et al. 2009). It likely originates from environmental contamination (feed or water).

Additionally, we observed a strong negative correlation between the relative abundance of *Alloprevotella* spp. in nasal swabs at weaning (D0) and lung lesion severity at necropsy (D56) among non-vaccinated piglets classified as high-protection phenotype ($p = 0.029$, $r = –0.858$). While *Alloprevotella* is traditionally considered a gut-associated genus, its selective presence in nasal samples from animals with milder macroscopic lung lesion and stronger immune responses suggests a potential protective role in the upper respiratory tract. Similar associations have been reported in other host systems, where *Alloprevotella* has been linked to mucosal homeostasis, short-chain fatty acid production, and anti-inflammatory signaling (Han et al. 2024). Importantly, the genus was not uniformly present across all animals or groups, and other gut-typical genera were not enriched in nasal samples, making fecal or environmental contamination unlikely. In the other hand, other study showed that the *Ruminococcus* spp. relative abundance in the gut microbiota is strongly correlated with piglets showing lowest *M. hyopneumoniae*-induced lung lesions (Nair et al. 2019).

The identification of *M. flocculare* rather than *M. hyopneumoniae* in nasal microbiota sequences at two weeks post-challenge highlights the site-specific colonization pattern within the respiratory tract. Although *M. hyopneumoniae* was not detected in nasal samples via 16S, its presence was confirmed by qPCR in lung and

BALF samples, validating the challenge model. While *M. hyopneumoniae* predominantly colonizes the lower respiratory tract, particularly the bronchial epithelium, *M. flocculare* is more commonly found in the upper tract and is considered a non-pathogenic commensal (Maes et al., 2018; Almeida et al., 2022). This anatomical distinction may explain the absence of *M. hyopneumoniae* in our nasal samples despite confirmed lung colonization post-challenge, especially in the control group. Interestingly, the expansion of *M. flocculare* (as inferred by the increase in Metamycoplasmataceae ASVs) in non-vaccinated animals could reflect niche competition, mucosal dysbiosis, or even a microbial succession process following challenge with *M. hyopneumoniae*. Prior work by Almeida et al. (2022), using the same strain (232), also detected *M. hyorhinis*—another opportunistic *Mycoplasma* species—in the upper respiratory tract of non-vaccinated and inoculated pigs, reinforcing the notion that potential cross-reactivity of *M. hyopneumoniae* vaccination and its effects on commensal *Mycoplasma* species.

Early-life nasal microbiota diversity and composition were associated with the magnitude of the immune response and the severity of lung lesions following *M. hyopneumoniae* challenge, particularly in non-vaccinated animals. Although no statistically significant differences in microbial diversity were detected between the vaccinated groups, the preservation of microbial diversity post-challenge, combined with the correlation between baseline microbial profiles and clinical-immunological outcomes, suggests that the nasal microbiota may play a modulatory role in infection response and potentially in vaccine efficacy. In line with these observations, nasal microbiota diversity was associated with immune response and disease outcome. In the vaccinated group, higher alpha diversity at weaning showed a moderate positive correlation with mucosal IgA levels two weeks post-challenge, suggesting a potential role in priming mucosal immunity. In the non-vaccinated group, diversity indices at weaning were negatively correlated with lung lesion severity, indicating that greater microbial richness early in life may protect against disease progression in the absence of vaccination.

Finally, our study demonstrates the complex interplay between host microbial ecology, immune responses, and disease outcomes in swine production in controlling the PEP caused by *M. hyopneumoniae* challenge, shedding light into microbiota-based

strategies for improving animal health and productivity. In conclusion, our findings demonstrate that nasal microbiota composition at weaning is a significant predictor of immune responsiveness and clinical protection against *M. hyopneumoniae* infection in pigs. Higher alpha diversity and the presence of specific taxa such as *Alloprevotella* spp. were associated with milder lung lesions and enhanced antibody responses, particularly in non-vaccinated animals. Both the commercial and SBA-15-based vaccines reduced lesion severity and helped preserve microbial stability, but early-life microbiota signatures remained strongly associated with outcomes. These results underscore the relevance of the nasal microbiota as both a modulator and biomarker of respiratory vaccine efficacy. Future strategies aiming to improve vaccine responses may benefit from integrating microbiota-informed approaches to promote mucosal resilience in swine production systems.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary material

Table S1: Alpha and beta diversity indices of the nasal microbiota in piglets on day 0 (D0), at weaning and at the time of intramuscular vaccination with a commercial vaccine (CV), an SBA-15-based experimental formulation, or no vaccine (CONT). Alpha diversity was assessed using Chao1, Shannon, Evenness, Observed Features, and Faith's phylogenetic diversity indices. Statistical differences among groups were evaluated using the Kruskal–Wallis test ($p < 0.05$). Beta diversity was assessed with Jaccard, Bray–Curtis, Unweighted UniFrac, and Weighted UniFrac distances and analyzed using PERMANOVA ($p < 0.05$).

Microbiota on D0 (weaning)																
Alpha diversity								**Beta diversity**								
Chao1				**Shannon**				**Jaccard**				**Bray Curtis**				
Groups		p-value*		**Groups**		p-value		**Groups**		p-value**		**Groups**		p-value		
CV	**CONT**	0,091	p = 0.132	**CV**	**CONT**	0,105	p = 0.145	**CV**	**CONT**	0,001	p=0.001, R2=0.15	**CV**	**CONT**	0,003	p=0.001, R2=0.15	
	SBA-15	0,778			**SBA-15**	0,944			**SBA-15**	0,001			**SBA-15**	0,097		
SBA-15	**CONT**	0,082		**SBA-15**	**CONT**	0,082		**SBA-15**	**CONT**	0,001		**SBA-15**	**CONT**	0,008		
Evenness				**Observed features**				**Unweighted Unifrac**				**Weighted Unifrac**				
Groups		p-value		**Groups**		p-value		**Groups**		p-value		**Groups**		p-value		
CV	**CONT**	0,159	p = 0.189	**CV**	**CONT**	0,091	p = 0.109	**CV**	**CONT**	0,002	p=0.001, R2=0.11	**CV**	**CONT**	0,026	p=0.02, R2=0.15	
	SBA-15	0,778			**SBA-15**	0,832			**SBA-15**	0,047			**SBA-15**	0,067		
SBA-15	**CONT**	0,095		**SBA-15**	**CONT**	0,057		**SBA-15**	**CONT**	0,003		**SBA-15**	**CONT**	0,173		
Faith pd																
Groups		p-value														
CV	**CONT**	0,014	p = 0.059													
	SBA-15	0,526														
SBA-15	**CONT**	0,158														

*Kruskal-Wallis test was used ($p<0.05$)

**PERMANOVA test was used ($p<0.05$)

Table S2: Alpha and beta diversity indices of the nasal microbiota in piglets at day 21 (D21), three weeks after intramuscular vaccination with a commercial vaccine (CV), an SBA-15-based experimental formulation, or no vaccine (CONT). Alpha diversity was measured using Chao1, Shannon, Evenness, Observed Features, and Faith's phylogenetic diversity, with statistical comparisons performed using the Kruskal–Wallis test ($p < 0.05$). Beta diversity was assessed using Jaccard, Bray–Curtis, Unweighted UniFrac, and Weighted UniFrac distances, and tested with PERMANOVA ($p < 0.05$).

Microbiota on D21 (three weeks post-vaccination)															
Alpha diversity								**Beta diversity**							
Chao1				**Shannon**				**Jaccard**				**Bray Curtis**			
Groups		p-value*		**Groups**		p-value		**Groups**		p-value		**Groups**		p-value	
CV	**CONT**	0,087	p = 0.263	**CV**	**CONT**	0,087	p = 0.222	**CV**	**CONT**	0,001	p=0.001, R2=0.12	**CV**	**CONT**	0,001	p=0.002, R2=0.17
	SBA-15	0,278			**SBA-15**	0,732			**SBA-15**	0,005			**SBA-15**	0,21	
SBA-15	**CONT**	0,969		**SBA-15**	**CONT**	0,2		**SBA-15**	**CONT**	0,001		**SBA-15**	**CONT**	0,002	
Evenness				**Observed features**			Kruskal Wallis	**Unweighted Unifrac**				**Weighted Unifrac**			
Groups		p-value		**Groups**		p-value		**Groups**		p-value		**Groups**		p-value	
CV	**CONT**	0,03	p = 0.083	**CV**	**CONT**	0,47	p = 0.751	**CV**	**CONT**	0,001	p=0.001, R2=0.11	**CV**	**CONT**	0,022	p=0.041, R2=0.14
	SBA-15	0,732			**SBA-15**	0,909			**SBA-15**	0,122			**SBA-15**	0,504	
SBA-15	**CONT**	0,094		**SBA-15**	**CONT**	0,576		**SBA-15**	**CONT**	0,004		**SBA-15**	**CONT**	0,045	
Faith pd															
Groups		p-value													
CV	**CONT**	0,621	p = 0.685												
	SBA-15	0,676													
SBA-15	**CONT**	0,412													

*Kruskal Wallis test was used ($p<0.05$)

**PERMANOVA test was used ($p<0.05$)

Table S3: Alpha and beta diversity indices of the nasal microbiota in piglets at day 35 (D35), two weeks after challenge with *M. hyopneumoniae*, between commercial vaccine (CV), an SBA-15-based experimental formulation, or no vaccine (CONT) groups. Alpha diversity was measured using Chao1, Shannon, Evenness, Observed Features, and Faith's phylogenetic diversity, with statistical comparisons performed using the Kruskal–Wallis test ($p < 0.05$). Beta diversity was assessed using Jaccard, Bray–Curtis, Unweighted UniFrac, and Weighted UniFrac distances, and tested with PERMANOVA ($p < 0.05$).

Microbiota on D35 (two weeks post-challenge)															
Alpha diversity								**Beta diversity**							
Chao1				**Shannon**				**Jaccard**				**Bray Curtis**			
Groups		p-value*		**Groups**		p-value		**Groups**		p-value		**Groups**		p-value	
CV	**CONT**	0,233		**CV**	**CONT**	0,058		**CV**	**CONT**	0,001		**CV**	**CONT**	0,004	
	SBA-15	0,744	p = 0.315		**SBA-15**	0,806	p = 0.184		**SBA-15**	0,024	p=0.001, R2=0.11		**SBA-15**	0,286	p=0.002, R2=0.15
SBA-15	**CONT**	0,165		**SBA-15**	**CONT**	0,221		**SBA-15**	**CONT**	0,002		**SBA-15**	**CONT**	0,011	
Evenness				**Observed features**				**Unweighted Unifrac**				**Weighted Unifrac**			
Groups		p-value		**Groups**		p-value	Kruskal Wallis	**Groups**		p-value		**Groups**		p-value	
CV	**CONT**	0,145		**CV**	**CONT**	0,063		**CV**	**CONT**	0,054		**CV**	**CONT**	0,01	
	SBA-15	0,414	p = 0.315		**SBA-15**	0,744	p = 0.174		**SBA-15**	0,472	p=0.13, R2=0.08		**SBA-15**	0,69	p=0.046, R2=0.14
SBA-15	**CONT**	0,414		**SBA-15**	**CONT**	0,165		**SBA-15**	**CONT**	0,155		**SBA-15**	**CONT**	0,065	
Faith pd															
Groups		p-value													
CV	**CONT**	0,894													
	SBA-15	0,87	p = 0.949												
SBA-15	**CONT**	0,744													

*Kruskal Wallis test was used ($p<0.05$)

**PERMANOVA test was used ($p<0.05$)

Table S4: Relative abundance (%) of bacterial genera (and respective families) detected in nasal secretions from piglets across treatments (CV, SBA-15 and CONT) and timepoints (D0, D21 and D35). Taxa are listed by genus name, followed by the corresponding family in parentheses. D0: vaccination; D21: challenge with *M. hyopneumoniae*.

***Genus* name (Family)**	**D0 (%)**			**D21 (%)**			**D35 (%)**		
	CV	**SBA-15**	**CONT**	**CV**	**SBA-15**	**CONT**	**CV**	**SBA-15**	**CONT**
Glaesserella (Pasteurellaceae)	24.98	29.5	19.47	6.95	8.46	5.03	1.66	2.65	0
Klebsiella (Enterobacteriaceae)	18.84	8.29	8.28	16.81	13.31	2.2	4.1	2.99	0
Escherichia (Enterobacteriaceae)	14.22	15.61	4.18	3.51	1.98	1.8	1.1	2.95	0
Moraxella (Moracellaceae)	4.48	3.44	12.69	31.74	25.65	48.59	28.0	31.6	48.64
Streptococcus (Streptococcaceae)	2.3	2.57	3.79	4.64	4.62	7.12	15.83	12.45	11.04
Planobacterium (Weeksellaceae)	0	0	0	9.54	14.2	18.4	20.2	18.97	20.61
Actinobacillus (Pasteurellaceae)	0	0	1.51	7.1	1.41	0	1.94	0	0
Staphylococcus (Staphylococcaceae)	0	0	0	2.94	8.01	0	12.2	6.53	2.76
Rothia (Micrococcaceae)	0	0	0	0	2.19	1.16	3.1	2.78	1.74
Prevotella (Prevotellaceae)	2.9	3.68	9.86	0	0	0	0	0	0
Weissella (Lactobacillaceae)	0	0	0	1.95	1.32	0	1.16	2.79	0
Empedobacter (Weeksellaceae)	0	0	0	1.3	1.70	0	0	0	0
Aerococcus (Aerococcaceae)	9	0	0	0	1.26	0	1	0	0
Aeromonas (Aeromonadaceae)	5.8	0	0	0	2.97	0	0	0	0
Enterobacteriaceae	2.9	1.3	0	0	0	0	0	0	0
Marinilabiliaceae JC017	1.9	4.45	0	0	0	0	0	0	0
Lactobacillus (Lactobacillaceae)	0	1.43	1.36	0	0	0	0	0	0
Fusobacterium (Fusobacteriaceae)	0	2.49	1.52	0	0	0	0	0	0
Vescimonas (Oscillospiraceae)	0	1.14	1.29	0	0	0	0	0	0
Alloprevotella (Prevotellaceae)	1	0	2.03	0	0	0	0	0	0
Staphylococcaceae	0	0	0	0	1.07	0	0	2.02	0
Alphaproteobacteria	0	1.3	0	0	0	0	0	0	0
Sodaliphilus (Lachnospiraceae)	0	1.06	0	0	0	0	0	0	0
Acetobacter (Acetobacteraceae)	0	0	0	0	0	0	0	1.22	0
Fusobacterium (Fusobacteriaceae)	0	0	0	1	1	0	0	0	0

Gracilibacteria GN02-873	0	0	0	0	0	1.39	0	0	2.43
Pseudomonas (Pseudomonadaceae)	0	0	1.59	0	0	0	0	0	0
Anaerovibrio (Succinivibrionaceae)	0	0	1.43	0	0	0	0	0	0
Clostridium (Clostridiaceae)	0	0	0	0	0	1.82	0	0	0
Terrisporobacter (Peptostreptococcaceae)	0	0	0	0	0	1.38	0	0	0
Other bacteria*	20.8	20.4	31.01	12.6	11.85	11.1	9.8	11.82	12.79

* The "Other bacteria" row includes the sum of all remaining low-abundance taxa below the threshold.

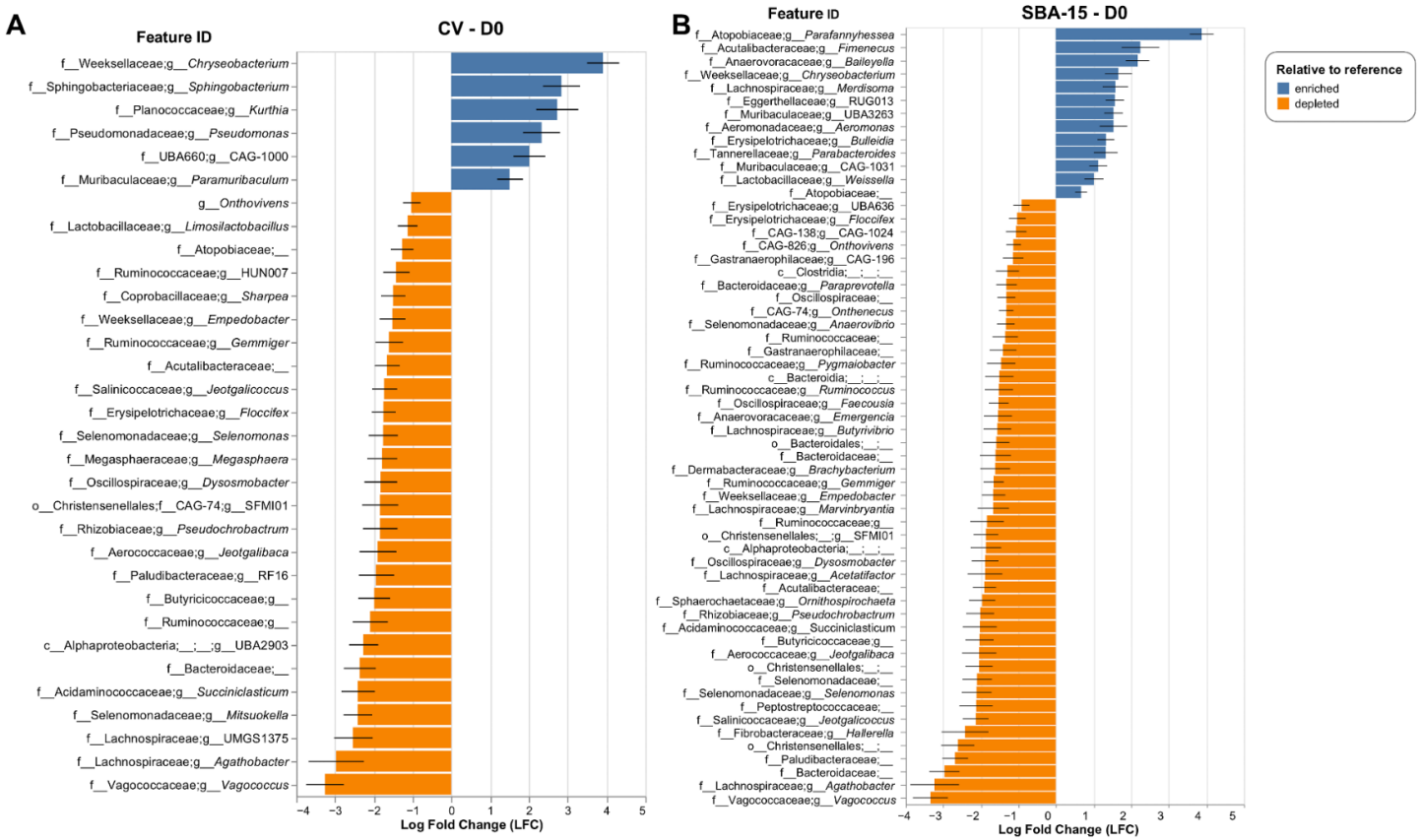


Figure S1: Differential abundance analysis of the nasal microbiota in vaccinated groups (CV and SBA-15) compared to the control group (CONT) on D0 (vaccination; weaning), using the ANCOM-BC method. (A) Comparison between CV and CONT. (B) Comparison between SBA-15 and CONT. Bars represent the log fold change (LFC) of differentially abundant taxa, with confidence intervals. Taxa enriched relative to the CONT group are shown in blue, while depleted taxa are shown in orange. Taxonomic identification is presented in the format family;genus.

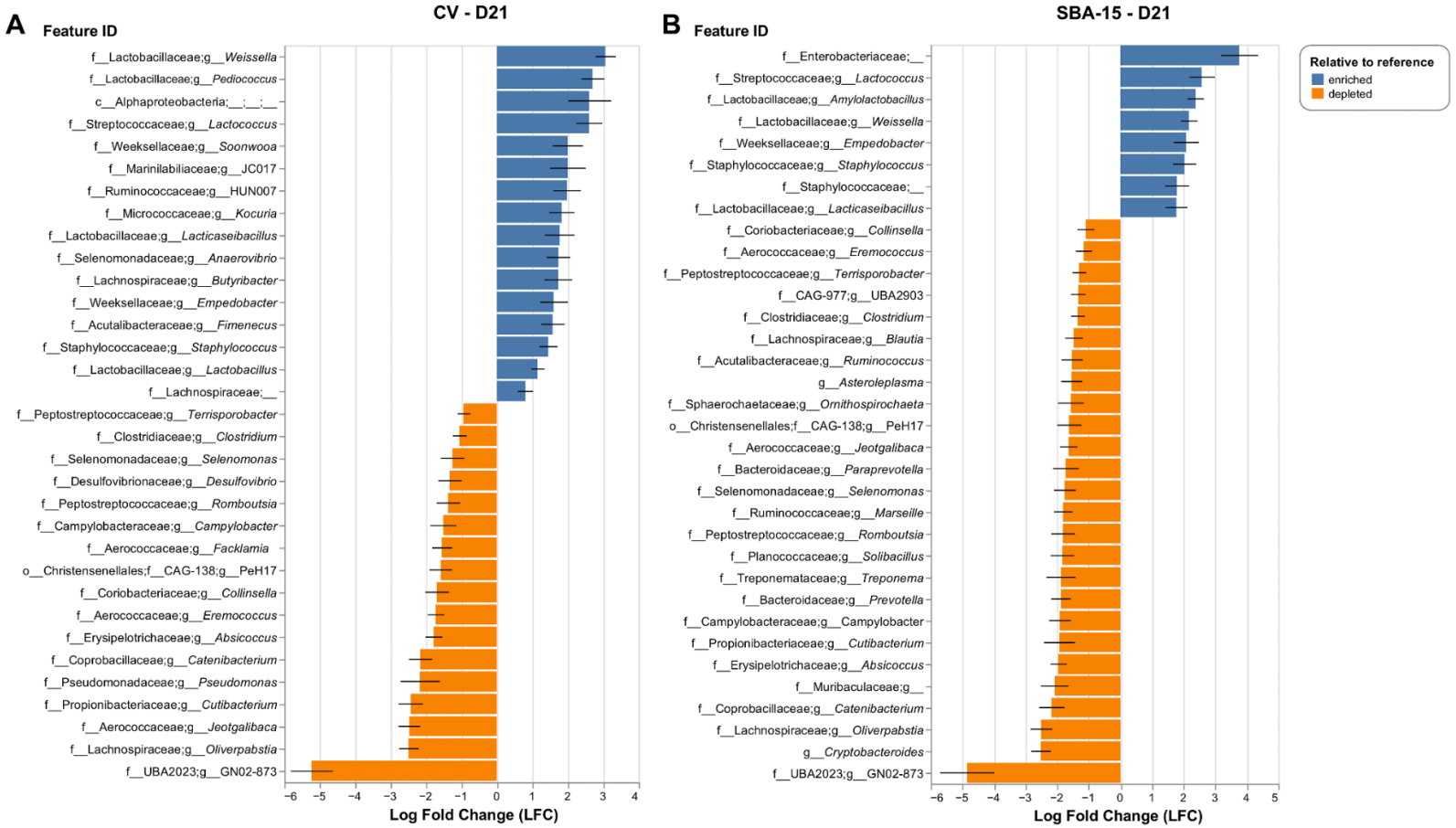


Figure S2: Differential abundance analysis of the nasal microbiota in vaccinated groups (CV and SBA-15) compared to the control group (CONT) on D21, using the ANCOM-BC method. (A) Comparison between CV and CONT. (B) Comparison between SBA-15 and CONT. Colors follow the same scheme as in Figure S1: blue indicates taxa enriched and orange indicates taxa depleted relative to the CONT group.

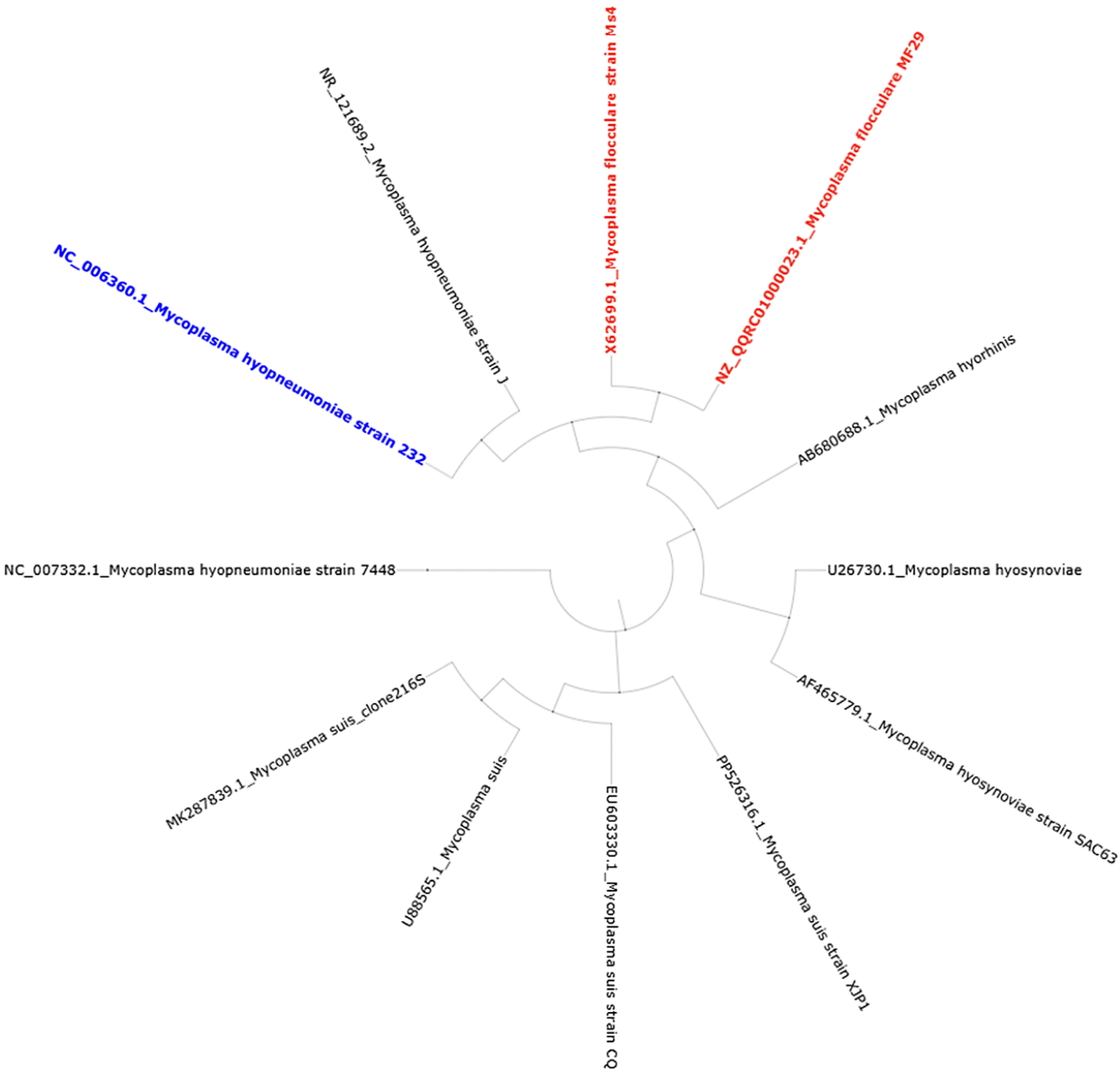


Figure S3: Phylogenetic tree of *Mycoplasma* amplicon sequence variants (ASVs) detected in nasal samples. A circular phylogenetic tree was constructed based on 16S rRNA sequences assigned to the family *Mesomycoplasmataceae*. ASVs identified in the nasal cavity of piglets clustered most closely with *M. flocculare* (red), rather than with *M. hyopneumoniae* strain 232 (blue), the strain used for challenge and SBA-15 vaccine formulation. Other *Mycoplasma* species included for comparison are shown in black.

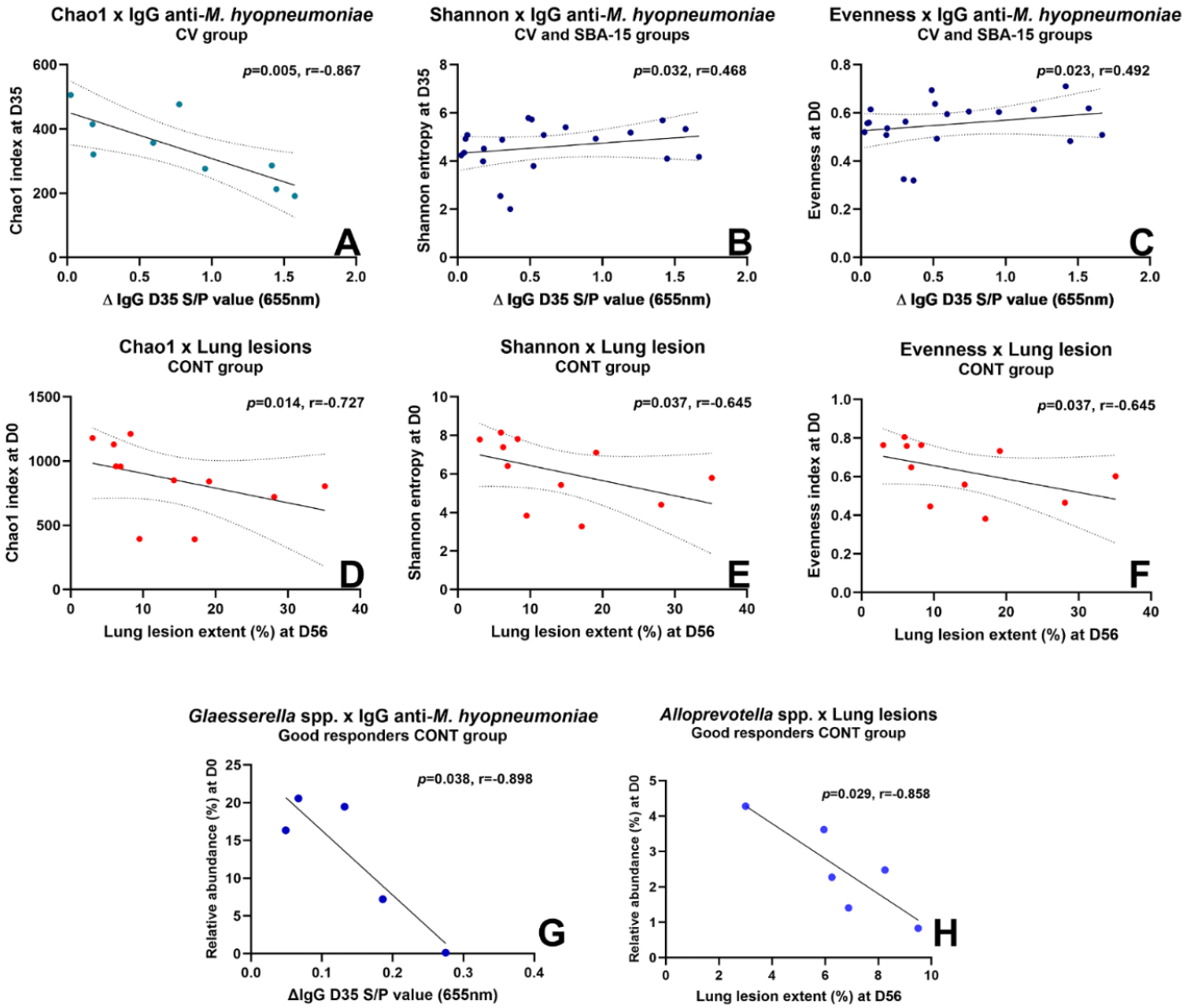


Figure S4: Correlations between nasal microbiota diversity, taxonomic composition, antibody levels, and lung lesion severity. (A–C): correlations in vaccinated animals; (D–F): diversity vs. lesion severity in CONT group; (G–H): genus-level correlations in high-protection phenotype (CONT group).