UNIVERSIDADE ESTADUAL PAULISTA – UNESP

CÂMPUS DE JABOTICABAL

USO DA SÍLICA NANOESTRUTURADA SBA-15 COMO ADJUVANTE DE VACINA ORAL PARA CONTROLE DE *Mycoplasma hyopneumoniae* NA PRODUÇÃO DE SUÍNOS

Gabriel Yuri Storino

Médico Veterinário

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UNIVERSIDADE ESTADUAL PAULISTA – UNESP

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Uso da sílica nanoestruturada SBA-15 como adjuvante de vacina oral para controle de *Mycoplasma hyopneumoniae* na produção de suínos

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Orientador: Prof. Dr. Luís Guilherme de Oliveira

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

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O trabalho apresentado nesta tese tem um impacto significativo na sociedade, abordando a infecção pelo *Mycoplasma hyopneumoniae* em suínos, um dos principais patógenos respiratórios que afeta a suinocultura em todo o mundo. Ao fornecer atualizações e informações sobre esse patógeno, a tese serve como um recurso fundamental para compreender a problemática causada por essa infecção e seu impacto na cadeia de produção de suínos.

Um dos destaques deste trabalho é desenvolvimento de uma vacina oral contra o *M. hyopneumoniae*, o que representa uma abordagem inovadora para o controle desse patógeno. Atualmente, não existem produtos comerciais disponíveis para administração oral com essa finalidade, tornando essa vacina uma solução pioneira. Além disso, o fato de ser administrada oralmente está alinhado com questões de bem-estar animal, uma vez que não requer o uso de agulhas, reduzindo o estresse e o desconforto dos suínos durante a imunização.

A vacina oral desenvolvida apresenta resultados positivos no controle do *M. hyopneumoniae* o que é de grande importância considerando a crescente preocupação com a resistência aos antimicrobianos e a necessidade de encontrar alternativas eficazes para reduzir os efeitos sanitários e econômicos da infecção.

Além disso, as informações apresentadas nesta tese servem como base para o desenvolvimento de novas pesquisas e no controle de patógenos respiratórios com impacto direto na tomada de decisões de produtores e profissionais da área, fornecendo orientações valiosas para melhorar e fortalecer a cadeia de produção de suínos.

Portanto, este trabalho tem um impacto amplo na sociedade, fornecendo informações atualizadas sobre a infecção pelo M*. hyopneumoniae*, introduzindo uma vacina oral inovadora e sustentável, e orientando ações futuras no campo da suinocultura na promoção da saúde animal e no desenvolvimento sustentável da produção de suínos.

Impact Record

According to Unesp Ordinance No. 117, dated December 21, 2022.

The work presented in this thesis has a significant impact on society, addressing *Mycoplasma hyopneumoniae* infection in pigs, one of the major respiratory pathogens that affect the global swine industry. By providing updates and information about this pathogen, the thesis serves as a fundamental resource for understanding the issues caused by this infection and its impact on the swine production chain.

One of the highlights of this work is the development of an oral vaccine against *M. hyopneumoniae*, which represents an innovative approach to control this pathogen. Currently, there are no commercially available products for oral administration for this purpose, making this vaccine a pioneering solution. Furthermore, the fact that it is administered orally aligns with animal welfare concerns as it does not require the use of needles, reducing stress and discomfort for pigs during immunization.

The developed oral vaccine demonstrates positive results in controlling M. hyopneumoniae, which is of great importance considering the growing concern about antimicrobial resistance and the need to find effective alternatives to reduce the sanitary and economic effects of the infection.

Additionally, the information presented in this thesis serves as a foundation for the development of new research and the control of respiratory pathogens, directly influencing the decision-making of producers and professionals in the field. It provides valuable guidance to improve and strengthen the swine production chain.

Therefore, this work has a broad impact on society by providing updated information on *M. hyopneumoniae* infection, introducing an innovative and sustainable oral vaccine, and guiding future actions in the field of swine production, promoting animal health and sustainable development in swine production.

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CERTIFICADO DE APROVAÇÃO

TÍTULO DA TESE: USO DA SÍLICA NANOESTRUTURADA SBA-15 COMO ADJUVANTE DE VACINA ORAL PARA CONTROLE DE *Mycoplasma hyopneumoniae* NA PRODUÇÃO DE SUÍNOS

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Jaboticabal, 10 de abril de 2023

DADOS CURRICULARES DO AUTOR

Nascido em Porto Ferreira - SP no ano de 1994, ingressou no curso de Medicina Veterinária da Faculdade de Ciência Agrárias e Veterinárias - Universidade Estadual Paulista “Júlio de Mesquita Filho” (FCAV/Unesp) em 2012. Obteve grau de bacharel em Medicina Veterinária em 2016 e no ano seguinte deu início ao curso mestrado em Medicina Veterinária pela FCAV/Unesp. Durante parte do período do mestrado foi bolsista CAPES tento obtido o título de Mestre em Medicina Veterinária em 2018, concluindo o projeto como bolsista FAPESP (2017/00950-0). Candidato ao título de Doutor em Medicina Veterinária, área: Clínica Médica Veterinária, pelo programa de Medicina Veterinária da FCAV/Unesp, inicialmente foi bolsista CNPq e posteriormente bolsista FAPESP (2020/05198-7). Atualmente é servidor público efetivo exercendo o cargo de Assistente Técnico de Defesa Agropecuária “B” na Coordenadoria de Defesa Agropecuária do Estado de São Paulo, CDA Regional de Votuporanga, SP e faz parte do Grupo Técnico de Trabalho do Programa Estadual de Sanidade dos Suídeos. Durante sua trajetória acadêmica foi bolsista de Iniciação Científica PROPe (2014 - 2015), coordenador do Grupo de Estudos em Suinocultura (SUINESP) - Unesp/FCAV (2016 - 2018) e pesquisador visitante pela Iowa State University - Veterinary Diagnostic Laboratory (2019).

“Tens de permanecer vigilante, para que os teus êxitos profissionais ou os teus fracassos - que virão! - não te façam esquecer, nem por um instante, qual é o verdadeiro fim do teu trabalho: a glória de Deus!”

Forja, 704

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Ao Laboratório de Medicina de Suínos, local onde desenvolvi tantas atividades ao passo que pude participar de sua estruturação e desenvolvimento, sendo hoje uma referência em pesquisas com suínos. No LMS tive o prazer de conhecer excelentes profissionais, que foram em algum momento meus colegas de pós-graduação ou estudantes de iniciação científica, que tanto me auxiliaram e com quem muito aprendi; em especial agradeço a Marina Lopes Mechler-Dreibi e ao Henrique Meiroz de Souza Almeida pela amizade e toda a ajuda com a idealização e execução dos meus projetos de pesquisa.

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

CEUA – COMISSÃO DE ÉTICA NO USO DE ANIMAIS

CERTIFICADO

Certificamos que o projeto de pesquisa intitulado **"Controle da pneumonia enzoótica suína por meio do uso de imunoestimulante oral específico para *Mycoplasma hyopneumoniae* na produção comercial de suínos"**, protocolo nº 00915/20, 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 13 de fevereiro de 2020.

Vigência do Projeto	01/03/2020 a 01/07/2021
Espécie / Linhagem	*Sus scrofa domesticus* / Linhagem comercial (Landrace x Large White)
Nº de animais	50
Peso / Idade	Entre 5 e 120 kg / entre 18 e 140 dias
Sexo	Machos e fêmeas
Origem	Unidade de criação comercial

Jaboticabal, 13 de fevereiro de 2020.

Fabiana Pilarski

Profª Drª Fabiana Pilarski
Coordenadora – CEUA

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Uso da sílica nanoestruturada SBA-15 como adjuvante de vacina oral para controle de *Mycoplasma hyopneumoniae* na produção de suínos

RESUMO – *Mycoplasma hyopneumoniae* é uma bactéria de difícil controle, uma vez que vacinas comerciais não impedem a colonização e excreção do agente. O presente estudo teve como objetivo avaliar o desempenho de uma vacina de administração oral, composta por antígenos extraídos de *Mycoplasma hyopneumoniae* e incorporados à sílica mesoporosa ordenada tipo SBA-15, que possui função adjuvante-carreador, visando potencializar a ação da vacina intramuscular comercial. A vacina oral foi administrada em leitões nascidos de fêmeas nulíparas desafiadas com *M. hyopneumoniae*, com o propósito de promover um incremento na resposta imune contra o patógeno a partir do estímulo do tecido linfoide associado às mucosas (MALT), com início na mucosa intestinal. No total 60 leitões foram divididos em 4 grupos (n=15) submetidos a diferentes protocolos vacinais da seguinte forma, Grupo 1: SBA15 pela via oral + vacina comercial aos 24 dias no desmame, G2: vacina oral ao terceiro dia de vida + vacina comercial aos 24 dias, G3: vacina comercial aos 24 dias e G4: vacina comercial + vacina oral aos 24 dias. No primeiro dia, os leitões foram pesados e a partir do 3º dia, submetidos a colheitas de sangue, para detecção e quantificação de IgG anti-*Mycoplasma hyopneumoniae*, bem como suabes nasais, para o monitoramento de IgA por ELISA, e de suabe orofaríngeo para avaliação da carga bacteriana por qPCR. As colheitas de amostras biológicas foram realizadas periodicamente até o 73º dia a partir do terceiro dia de vida. Aos 41 dias de vida, 15 indivíduos de mesma idade, experimentalmente desafiados com inóculo contendo *M. hyopenumoniae* foram inseridos na baia dos animais dos grupos (1 a 4) de forma a elevar a pressão de infecção durante período de creche. Ao final do estudo, aos 73 dias, todos os leitões foram eutanasiados e necropsiados para avaliação dos pulmões e colheita de amostras para estimativa de carga bacteriana por qPCR, e para a avaliação histopatológica. Também foram colhidas amostras de lavado traqueobrônquico, submetidas ao teste de qPCR. Os dados quantitativos obtidos nos exames clínicos, físicos e laboratoriais foram submetidos a avaliação dos pressupostos de normalidade e homocedasticidade pelos testes de Shapiro-Wilk e Bartlett, previamente à realização de testes estatísticos paramétricos ou não paramétricos.

Palavras-chave: adjuvante, doenças infecciosas, doenças respiratórias, imunologia, vacinas orais

Use of oral vaccine against *Mycoplasma hyopeumoniae* combined with a commercial vaccine to control swine enzootic pneumonia

RESUMO – *Mycoplasma hyopneumoniae* is a difficult-to-control bacterium since commercial vaccines do not prevent colonization and excretion. The present study aimed to evaluate the performance of an orally administered vaccine, composed of antigens extracted from *Mycoplasma hyopneumoniae* and incorporated into mesoporous silica (SBA-15), which has an adjuvant-carrier function, aiming to potentiate the action of the commercial intramuscular vaccine. The oral vaccine was administered to piglets born to nulliparous females challenged with *M. hyopneumoniae*, to promote an increase in the immune response against the pathogen from the stimulation of the mucosa-associated lymphoid tissue (MALT), starting in the intestinal mucosa. A total of 60 piglets were divided into four groups (n=15) submitted to different vaccination protocols as follows, Group 1: oral SBA15 + commercial vaccine at 24 days after weaning, G2: oral vaccine on the third day of life + vaccine commercial vaccine at 24 days, G3: commercial vaccine at 24 days and G4: commercial vaccine + oral vaccine at 24 days. On the first day, the piglets were weighed and, from the 3rd day onwards, submitted to blood collections for the detection and quantification of anti-*Mycoplasma hyopneumoniae* IgG. Nasal swabs were collected for monitoring IgA by ELISA, and oropharyngeal swabs were for assessing the bacterial load by qPCR. Biological samples were collected periodically from the third day of life until the 73rd day. At 41 days of life, 15 individuals of the same age, experimentally challenged with an inoculum containing *M. hyopneumoniae*, were inserted into the pen with the animals from groups (1 to 4) to increase the infection pressure during the nursery period. At the end of the study, at 73 days, all piglets were euthanized and necropsied for evaluation of the lungs and collection of lung samples for estimation of bacterial load by qPCR and histopathological evaluation. Tracheobronchial lavage samples were also collected and submitted to the qPCR test. Quantitative data obtained from physical parameters and laboratory investigation were analyzed regarding assumptions of normality and homoscedasticity using the Shapiro-Wilk and Bartlett tests, before performing parametric or non-parametric statistical tests.

Keywords: adjuvant, immunology, infectious diseases, oral vaccines, respiratory diseases

CHAPTER 1 – General Considerations

1. Introduction

Mycoplasma hyopneumoniae is a major respiratory pathogen of swine, causing enzootic pneumonia, a chronic and debilitating respiratory disease that results in significant economic losses to the swine industry worldwide. This bacterium is difficult to control due to its slow growth and the lack of effective vaccines, and therefore, it remains a major concern for swine producers. In recent years, there has been a growing interest in understanding the host-pathogen interactions of *M. hyopneumoniae* and developing new strategies to control the disease.

This has led to significant advances in our understanding of the pathogenesis of the disease, the immune response of the host, and the molecular mechanisms underlying virulence and pathogenicity. However, there is still a significant knowledge gap in scientific information regarding *M. hyopneumoniae* infection in swine herds. Studies conducted by the Swine Medicine Laboratory - FCAV/Unesp have generated valuable information that has helped to clarify the disease's mechanism of action and its impact on pig herds in Brazil. Moreover, these studies aim to develop new technologies and optimize practices that are carried out in the field, with the aim of achieving better outcomes concerning the control of respiratory diseases involving *M. hyopneumoniae*.

Preventive measures, such as biosecurity, antimicrobial and vaccination protocols are currently used by producers to control bacterial infections. However, these strategies do not offer complete protection or allow for the permanent eradication of the pathogen from the herd. Commercial vaccines for *M. hyopneumoniae* have limitations in inducing the mucosal immunity needed to prevent or limit *M. hyopneumoniae* adhesion. An experimental oral vaccine based on SBA-15 has shown promising results in controlling *M. hyopneumoniae* and reducing Swine Enzootic Pneumonia (PES) when administered to animals challenged with a homologous strain of *M. hyopneumoniae*. This oral vaccine can be absorbed by the intestinal mucosa and distributed to several organs, including the respiratory tract, which is the main gateway for *M. hyopneumoniae*.

The development and improvement of this oral vaccine are crucial for the control of pathogens like *M. hyopneumoniae* and the optimization of existing vaccines. The evaluation of the oral vaccine against *M. hyopneumoniae* produced with SBA-15 under field conditions against a heterologous strain of *M. hyopneumoniae* is a crucial step in its development and a solution to minimize the impacts of PES in commercial pig farming. The oral vaccine is a less invasive alternative as it does not require needles and can be easily integrated with other farm management practices. Moreover, the oral vaccine has potential immunological benefits as SBA-15, when administered orally, has been shown to diffuse to several systems, including the respiratory tract, in considerable concentrations.

2. Literature review

2.1. Epidemiology of *Mycoplasma hyopneumoniae* infection

Mycoplasma hyopneumoniae is a pathogen that infects wild boars (*Sus scrofa scrofa*) (Maes et al., 2008) and domestic pigs (*Sus scrofa domesticus*) being present in over 70% of swine herds globally. Control and eradication strategies rely on vaccination, biosafety measures, and treatment with highly effective antimicrobial molecules (Maes et al., 2018). *M. hyopneumoniae,* the causative of Swine Enzootic Pneumonia (PES) is considered the most prevalent pulmonary bacterial infection in pigs worldwide (Dutra et al., 2021) generally associated with other bacteria or viruses, such as Swine Reproductive and Respiratory Syndrome (PRRS), influenza virus, and circovirus, among others, leading to the respiratory disease syndrome known as Porcine Respiratory Diseases Complex (PRDC) (Pieters & Maes, 2019).

M. hyopneumoniae infections are commonly diagnosed based on clinical signs in growing and finishing pigs, although positive piglets can be detected during nursery and farrowing phases (Vranckx et al., 2012). Lesions associated with *M. hyopneumoniae* infection are typically observed in the lungs of slaughtered pigs. Fibrotic lesions may indicate a previous acute infection that was resolved over time, leading to loss of viable lung area. Consolidated lung lesions are commonly seen in animals that experienced reduced weight gain during the growing and finishing phases,

chronic cough, and decreased animal welfare, which are the main impacts of *M. hyopneumoniae* infection on commercial pig production (Ferraz et al., 2020).

Experimental infection models based on serological and microbiological tests are commonly used for epidemiological and experimental studies aimed at assessing the prevalence of *M. hyopneumoniae* infection in swine herds. Vaccination is widely implemented as a control measure in commercial farms and aligns with the industry's efforts to reduce antimicrobial usage. Although vaccination can help in controlling, it is not entirely effective in preventing *M. hyopneumoniae* infection (Maes et al., 2008).

Regarding the prevalence of *M. hyopneumoniae* in herds, studies demonstrate that the infection can occur in the early stages of life through close contact between the infected breeding female and their litter (Calsamiglia & Pijoan, 2000; Fano et al, 2007). The prevalence tends to increase over the growing phases, as observed by Vranckx et al. (2012), who detected 35% of positive individuals at six weeks of age and 96% positive with 26 weeks. Similar results were shown in herds in the Netherlands and Belgium, where 27% and 29% of piglets aged 3 to 11 months were positive nasal swab samples were detected, respectively (Vangroenweghe et al., 2015). High prevalences were also observed in finishing pigs in China (76.99%), Spain (76.7%), and Italy (94.7%) (Yue et al., 2021; Garza-Moreno et al., 2019; Merialdi et al., 2012).

Like other pathogens, *M. hyopneumoniae* can alter its antigenic expression, particularly during DNA replication (Vergnaud and Pourcel, 2009). Several approaches have been developed to molecularly characterize this microorganism, such as nucleic acid amplification, genomic sequencing, and identifying differences in surface-adhesin loci using Variable Number Tandem Repeat (VNTR) analysis (Mayor et al., 2007; Bogema et al., 2012; Minion et al., 2004; Vasconcelos et al., 2005; de Castro et al., 2006; Vranckx et al., 2011 and dos Santos et al. (2015)). The genetic makeup of *Mycoplasma hyopneumoniae* varies between herds and even within individual herds. Nearly half of the field isolates have less than 55% homology with the vaccine strains and reference samples. Although there is a heterogeneity, the sources of these molecular variabilities are still poorly explored and the variability of *M. hyopneumoniae* can result in different clinical manifestations and even alter the dynamics of infection, leading to more severe lung lesions (Michiels et al., 2017).

As previously noted, *M. hyopneumoniae* infections have a significant impact on production and are challenging to control, despite the application of immunization, treatment, and biosafety measures. High prevalence of pathogens in swine herds, combined with genetic variability, can complicate infection dynamics, making successful implementation of control and eradication protocols difficult. This is due to the pathogen's entrenchment in the production process, which poses a challenge for controlling and eradicating the disease (Yeske et al., 2020). Therefore, studies on the epidemiology of *M. hyopneumoniae*, infection dynamics (Betlach et al., 2020), acclimation strategies (Poeta Silva et al., 2021), diagnostic techniques (Moiso et al., 2020; Poeta Silva et al., 2021), and eradication programs (Yeske et al., 2020) are crucial in the search for alternative measures to control and eradicate the disease, which are aligned with the reality of large-scale commercial production.

Although countries such as Switzerland, Norway, and Finland have demonstrated successful models for eradicating *M. hyopneumoniae*, achieving this goal is more complex in countries with vast territories like Brazil or the United States (Holst et al., 2015). Eradication or drastic reduction of *M. hyopneumoniae* prevalence in commercial pig farming can improve productivity and reduce costs associated with infection (Clavijo et al., 2021). The measures adopted and the results of eradication in countries with *M. hyopneumoniae*-free swine production provide valuable information for producers and veterinarians showing the importance of making efforts to eradicate the pathogen in other countries, not only aiming for free farms but also for an entire national production free from *M. hyopneumoniae*.

2.2. Epidemiology of *Mycoplasma hyopneumoniae* infection in pigs in Brazil

High prevalence of lung lesions and positive individuals for *M. hyopneumoniae* have been reported in different states of Brazil. In the state of Rio Grande do Sul, *M. hyopneumoniae* was detected in 83.3% of samples analyzed for different pneumonia-causing agents (De Conti et al., 2021). In the same state, even higher prevalence rates were observed in a slaughterhouse, with 95.8% of the 120 samples collected testing positive for *M. hyopneumoniae* (Pacce et al., 2019). In Minas Gerais, 55.6% of the sampled lungs from a slaughterhouse were positive for *M. hyopneumoniae* (Assao et al., 2019).

In addition to prevalence studies, molecular techniques have provided valuable information about *M. hyopneumoniae* infection. The use of qPCR on samples of laryngeal, nasal, tracheal, and tonsil swabs can provide information about the onset and excretion dynamics (Almeida et al., 2021; Sponheim et al., 2020). Moreover, multiple locus variable-number tandem repeats analysis (MLVA) can indicate the genetic diversity, sources, and transmission routes of different strains in farms. Takeuti et al. (2017) demonstrated the epidemiological significance of acclimated females in the herd and the genetic diversity of *M. hyopneumoniae* introduced into the herd using these techniques. Similarly, dos Santos et al. (2015) detected multiple variants of *M. hyopneumoniae* in swine herds in the USA, Brazil, Mexico, and Spain, indicating a wide distribution of the pathogen and its potential impact on the global swine industry.

In Brazil, 16 genes related to virulence and/or immune response were identified using molecular techniques demonstrating the genetic diversity of Brazilian *M. hyopneumoniae* field strains (Assao et al., 2019). Interestingly, the study also revealed a high level of clonality of *M. hyopneumoniae*, with PES outbreaks having low genetic diversity, and five new and unique strains being identified on Brazilian farms (Balestrin et al., 2019). These findings suggest that differences between strains may affect the pathogenicity of *M. hyopneumoniae*. Molecular epidemiology studies offer further insight into genetic, antigenic, pathogenic, proteomic, and transcriptomic variations of *M. hyopneumoniae*, and can be valuable tools for the development of control techniques, including the selection of vaccine candidates (Leal Zimmer et al., 2020).

As previously mentioned, *M. hyopneumoniae* infections are often linked to other pathogens, including both bacteria and viruses (Balestrin et al., 2022). Co-infections of *Pasteurella multocida* and *M. hyopneumoniae* are the most prevalent in Brazil, accounting for 43.9% of the samples analyzed by Balestrin et al. in 2022 and found in 97.2% of the lung lesions analyzed by Takeuti et al. (2013). These results highlight the significant role of co-infections in the context of PES in the country. In contrast, *Actinobacillus pleuropneumoniae* was identified in 5.6% of the samples positive for *M. hyopneumoniae*, which is similar to the findings reported by Hillen et al. in 2014 in Germany. In commercial pig production, infections caused by *Actinobacillus pleuropneumoniae* are often associated with risk factors such as batch mixing, variations in climate, and overcrowding, which can increase the incidence of the

disease. In addition, co-infections with *M. hyopneumoniae* have been shown to exacerbate lung lesions at slaughter. Along with bacterial agents, viruses such as Swine Influenza A Virus (swIAV) and Porcine Circovirus type 2 (PCV-2) have also been linked to *M. hyopneumoniae* infections in Brazil, with prevalence rates of 7.2% and 15.6% respectively among samples positive for *M. hyopneumoniae* (Balestrin et al., 2022).

In addition to co-infections, *M. hyopneumoniae* may interact with other microorganisms such as mycoplasmas (Ferreira et al., 2021; Petri et al., 2020), as well as *Streptococcus suis* and *Glaesserella parasuis* (Balestrin et al., 2022). Studies have demonstrated that exposure to mycotoxins can predispose animals to *M. hyopneumoniae* infection, leading to more severe disease and increased mortality rates. Additionally, mycotoxins can exacerbate the pathogenicity of other respiratory pathogens such as the influenza virus, further compromising respiratory health (Posa et al., 2013, 2016; Michiels et al., 2018). Although limited research has been conducted on the direct impact of these factors on respiratory health, they are important considerations in the epidemiology of respiratory diseases. It's important to take into account risk factors associated with respiratory diseases, as they significantly impact disease prevalence, as highlighted by Merialdi et al. (2012) and Sobestiansky & Barcellos (2012).

2.3. *Mycoplasma hyopnemoniae* **infection: pathogenicity and virulence factors**

Mycoplasma hyopneumoniae is recognized for its ability to adhere to and colonize the ciliated epithelium of the trachea, bronchi, and bronchioles, promoting ciliostasis, loss of cilia, and eventual death of epithelial cells (Debey et al., 1994). The infection by *M. hyopneumoniae* begins with adherence to mucosal epithelial cells, initiating replication and inflammatory response. As the bacterium multiplies on the ciliary epithelial layer, a process called cilioestasis occurs, impairing respiratory defense, increasing mucus production, and predisposing co-infection with other pathogens, such as viruses and bacteria (Haesebrouck, 2004; Pieters & Maes, 2019).

Among the constituents of *M. hyopneumoniae* that interact with the host organism, 35 proteolytic proteins have been described, including adhesins, lipoproteins, and multifunctional cytosolic proteins that cover the bacterium's surface

(Tacchi et al., 2016). *M. hyopneumoniae* has the ability to reduce populations of immune cells through bacterial proteins displayed on its surface, which induce apoptosis and weaken the immune system (Bai et al., 2013). Lipid-associated membrane proteins (LAMPs) and surface lipoproteins play crucial roles in inducing apoptosis in porcine immune cells by increasing levels of nitric oxide and reactive oxygen species, and by activating caspase-3 (Thanawongnuwech et al., 2003). This cytopathogenic mechanism is observed in other mycoplasma species, including *M. hyorhinis*, *Mycoplasma synoviae*, and *M. pneumoniae*, and is associated with the production and release of nitric oxide and reactive oxygen species (ROS) (Leal Zimmer et al., 2020).

The adhesins P97 and P102, including P102, are involved in adhesion to cilia and have multifunctional binding abilities, respectively, and are associated with virulence (Adams et al., 2005). Both P97 and P102 can recruit plasminogen and fibronectin to the surface of *M. hyopneumoniae*, suggesting that they play important roles in virulence (Seymour et al., 2012). Interestingly, some cytosolic and metabolic proteins, such as glutamyl aminopeptidase MHJ_0125 and leucine aminopeptidase MHJ_0461 act as adhesion molecules and facilitate the conversion of plasminogen to plasmin on the cell surface (Robinson et al., 2013). Recently, P68 protein was described as a lipoprotein that binds to cilia of immune cells during *M. hyopneumoniae* infection (Jarocki et al., 2015; Liu et al., 2019; Robinson et al., 2013). Furthermore, it contributes to the induction of pro-inflammatory cytokines TNF-α and IL-1ß (Liu et al., 2019).

In animals infected with *M. hyopneumoniae*, the bronchus-associated lymphoid tissue (BALT) undergoes significant changes, including the release of various cytokines. Studies have shown that the levels of pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), and interleukin-6 (IL-6) are increased in the BALT of infected animals (Lorenzo et al., 2006). Additionally, the levels of interferon-gamma (IFN-γ), a cytokine associated with cell-mediated immunity, are also elevated and correlated with macroscopic lung lesion score (Almeida et al., 2020). In the pulmonary epithelium, *M. hyopneumoniae*-triggered release of IL-5 and IL-13 cytokines has also been identified as a potential cause of tissue damage (Rodríguez et al., 2016). Upon adherence and multiplication of *M. hyopneumoniae*, an

inflammatory process is initiated, activating cellular immunity and recruiting defense cells while increasing the expression of pro- and anti-inflammatory cytokines that contribute to an exacerbated inflammatory response, resulting in pulmonary lesions. Factors such as the release of nitric oxide and reactive oxygen species by macrophages, as well as the stimulation of apoptosis of defense mononuclear cells, trigger tissue damage with loss of lung function (Bai et al., 2015). As a result of the pathogenic effects of the infection, dry and chronic coughs are observed, as well as a reduction in production and weight gain during the growth and finishing stages (Rodríguez et al., 2016).

Coughing is one of the most relevant clinical signs and an essential mecanism in transmission since particles containing *M. hyopneumoniae* are expelled into the environment and enter the respiratory tract of other animals that can become infected directly through nose-to-nose contact (Marois et al., 2010). In infected individuals, the pathogen can be detected by molecular techniques using nasal swab, oropharyngeal swab, or even oral fluid samples (Pieters et al., 2017). It is described that the first signs of coughing occurring around 11 days, and intensify around 28 days post infection, when lung lesions reach their maximum degree (Castro et al., 2006; Villarreal et al., 2011).

Defining the minimum infectious dose of *M. hyopneumoniae* is a challenging task, as experimental infection dynamics may differ from the complex transmission mechanism in the field and may be influenced by genetic differences, virulence variability, and other risk factors in commercial production (Pieters & Maes, 2019). However, Marois et al. (2010) reported a minimum infectious dose of 10^8 viable cells/mL, which corresponds to 10^5 color-changing units (CCU) measured by the coloration of the Friis culture medium (Maes et al., 2018).

Studies suggest an association between lung injury scores and the quantification of *M. hyopneumoniae* in bronchoalveolar lavage fluid (BALF) samples from naturally infected finishing phase pigs (Casalmiglia et al., 2000; Vranckx et al., 2012). This indicates that the pathogenesis and virulence of *M. hyopneumoniae* may be related to the rate of multiplication and the number of microorganisms colonizing the respiratory tract. Additionally, Meyns et al. (2007) found that virulent strains had a higher transmission rate, possibly due to their higher multiplication rate and increased

excretion into the environment. Nevertheless, the presence and extent of lung injury in animals cannot be reliably correlated with the load of *M. hyopneumoniae* colonizing the animals, as high disease prevalence and incidence in a herd do not necessarily imply the presence or extent of lung lesions (Vicca et al., 2003; Woolley et al., 2012).

2.4. *Mycoplasma hyopneumoniae*: **evasion and modulation of the immune response**

Variations in pathogenicity and virulence of *M. hyopneumoniae* have been observed due to various factors, such as the variability of field isolate involved in the infection, protein expression, and individual host characteristics (Vicca et al., 2003). In general, the chronic nature of *M. hyopneumoniae* infections and the difficulty in controlling them indicate that the bacterium has the ability to modulate the host's immune system and persist among the species that make up the microbiota of infected animals. As previously described, the mechanical defense apparatus of the mucosal surface is impaired by the adhesion and multiplication of *M. hyopneumoniae*, and the pathogen also promotes modulation of the immune response at the cellular level (Shen, et al, 2017). Studies have shown that *M. hyopneumoniae* membrane proteins induce a higher release of the anti-inflammatory cytokine IL-10 in rats when compared to orthologous proteins of *Mycoplasma flocculare*, another pathogen of pigs (Leal et al., 2016). This finding may be related to the agent's ability to modulate the host's pro-inflammatory response and persist in the tissues, causing damage.

The interaction of *M. hyopneumoniae* with actin on the surface of mucosal epithelial cells promotes rearrangements on its surface, allowing it to be phagocytosed, being one of the hypotheses for its perpetuation within the organism the resistance to phagocytosis when it lodges inside the cytoplasm (Tacchi et al., 2016). In this way, *M. hyopneumoniae* not only evades the immune system but also disseminates to other organs protected from the interaction with defense cells and systemic antibodies (Raymond et al., 2018).

Also, it is described that Mycoplasmas, including *M. hyopneumoniae*, have potent membrane nucleases such as MnuA which can degrade DNA-based extracellular traps formed by immune cells (Mitiku, et al., 2018). This allows the bacteria to escape the host immune defense and use the trapped nucleotides and proteins for their own

proliferation. Also, the extracellular DNA allows the formation of a biofilm protecting the pathogen against the host immune system (Raymond, et al., 2018).

Another hypothesis described in the literature is that *M. hyopneumoniae* can penetrate the epithelial barrier by disrupting tight junctions, increasing permeability and tissue damage, possibly through the activation of plasminogen to plasmin (Leal Zimmer et al., 2020). Through this mechanism or via its phagocyte invasion mechanism, *M. hyopneumoniae* can persist in the infected individual's organism.

Another mechanism described in the literature for *Mycoplasma genitalium*, *Mycoplasma mycoides*, and *M. hyopneumoniae* is the *Mycoplasma* Ig binding protein (MIB) and *Mycoplasma* Ig protease (MIP) MIB-MIP, which would bind to IgGs promoting their degradation, but little information is available on this mechanism (Arfi et al., 2016). Although further studies are necessary to investigate the mechanisms of *M. hyopneumoniae*'s evasion from the immune system (Pieters & Maes, 2019), it is possible to realize the variety of possibilities that *M. hyopneumoniae* can use to evade the immune response, making systemic antibodies (IgG) less effective and causing chronic infection for long periods of time.

2.5. Mechanisms of protection

M. hyopenumoniae is induces the production of inflammatory cytokines through the activation of TLR2 and TLR6 receptors in immune cells of the host, such as macrophages and dendritic cells (Muneta et al., 2003). Activation of these receptors triggers an intracellular signaling cascade that culminates in the production of pro-inflammatory cytokines, such as tumor necrosis factor alpha (TNF-α) (Okusawa et al., 2004) and interleukin-6 (IL-6), which contribute to the host's immune response against *M. hyopenumoniae* infection. In addition, the expression of TLR2 and TLR6 receptors is elevated in lung cells during *M. hyopenumoniae* infection, suggesting an important role of these receptors in the pathogenesis of the disease (Muneta et al., 2003).

Cell-mediated responses are crucial for defending against *M. hyopneumoniae* infection (Maes et al., 2021). T lymphocytes are activated upon recognition of specific *M. hyopneumoniae* antigens presented by antigen-presenting cells, including dendritic cells and macrophages. Upon activation, T cells proliferate and differentiate into effector cells that migrate to the site of infection and exert their functions, such as

cytokine production, cytotoxicity, and activation of other immune cells (Zuckerman and Husmann, 1996). T cells are categorized into two major groups based on the expression of cell surface receptors and effector functions. T helper cells (Th) express the co-receptor CD4, while cytotoxic T lymphocytes (CTLs) express CD8 on their surface (Alberts et al., 2002).

Studies have demonstrated the presence of *M. hyopneumoniae*-specific T cells, particularly CD4+ T cells, in the lungs of infected pigs, and their crucial role in clearing the infection, possibly by activating macrophages through IFN-γ, given that *M. hyopneumoniae* is primarily an extracellular pathogen (Dobbs et al., 2009). The pathogenesis of *M. hyopneumoniae* is complex and involves the activation of the host immune system, including the differentiation of Th17 cells. Th17 cells are activated and recruited to the lungs, where they secrete pro-inflammatory cytokines that promote neutrophil recruitment and activation, epithelial regeneration, and an increase in mucosal IgA levels (Biebaut et al., 2021). CD8+ T cells are responsible for recognizing and killing infected cells through the release of cytotoxic molecules, including perforin and granzymes, and the production of cytokines, such as IFN-γ, which activate other immune cells and enhance the killing of *M. hyopneumoniae* (Mach et al., 2013, Biebaut et al., 2021).

In general, cell-mediated responses, including the activation of both CD4+ and CD8+ T cells, are critical for controlling *M. hyopneumoniae* infection. Further research is needed to understand the mechanisms underlying these responses and to develop more effective strategies for preventing and treating this important respiratory disease in pigs and to better understand the mechanisms involved and to develop more effective vaccination strategies (Summerfield, 2021).

Cell-mediated immune responses are involved in the recognition and elimination of *M. hyopneumoniae*-infected cells, while the activation of humoral immunity leads to the production of antibodies that can directly neutralize the pathogen or mediate its clearance through complement activation and phagocytosis. These two arms of the immune system work in concert to provide protection against *M. hyopneumoniae* infections. Following infection, IgG levels are detected between 21 to 28 days, peaking at 11-12 weeks (Kobisch et al., 1993), while IgA can be detected around 6 days post-infection but declines more rapidly than IgG (Chae et al., 2021).

Commercial vaccines generate antibodies against *M. hyopneumoniae*. However, the percentage of animals that seroconvert after vaccination can range from 30 to 100% (Sibila et al., 2004), and vaccines may produce different serological reactions (Thacker et al., 1998). The immunity produced, with increased IgG levels, does not correlate with the severity of pulmonary lesions, demonstrating the secondary role of systemic immunity in controlling the infection (Djordjevic et al., 1997).

Variations in adjuvants, administration methods, antigen doses, levels of antigen expression in bacterins, vaccination strategy (single or double vaccination), and individual animal responses can all result in variable serological responses following vaccination against *M. hyopneumoniae* (Fisch et al., 2016; Matthijs et al., 2019). After a two-dose vaccination, serum antibodies are typically detected two to four weeks later and remain detectable for several weeks and months. The serological response after a single vaccine is usually less pronounced than after a double vaccination, unless there is concurrent infection that boosts the immune system. Antibody titers decrease below detectable levels one to three months after vaccination, unless the immune system is stimulated by spontaneous illness (Maes et al., 1999). However, systemic antibodies are not reliable indicators of protective immunity against *M. hyopneumoniae*, as there is no direct relationship between antibody concentration and protection (Djordjevic et al., 1997; Thacker et al., 1998).

2.6. *M. hyopneumoniae* **control**

The current approaches employed for porcine enzootic pneumonia (PES) control involve pathogen surveillance, biosecurity measures, piglet and sow vaccination, and antimicrobial administration. Despite these efforts, controlling *M. hyopneumoniae* infections remains challenging and often ineffective, given the continued high prevalence of mycoplasma in swine production (Maes et al., 2018).

In general, risk factors should be controlled to prevent the entry of different strains into a production system, as well as to mitigate other predisposing factors for respiratory diseases such as mixing of batches, high stocking density, air quality, and thermal variation (Nathues et al., 2013). One potential point to be explored in a protocol for controlling *M. hyopneumoniae* in production is the acclimation of females, since

breeding animals are considered a source of early infection for the piglets (Sibila et al., 2008).

Maintaining females for an acclimatization period, in which they are exposed early to infected animals, has shown positive effects, as the bacterial loads excreted tend to be lower when these females approach the farrowing date and with advancing age in animals exposed early to *M. hyopneumoniae* within the production system, reducing pathogen amplification and consequently early infection of piglets during the farrowing (Takeuti et al., 2023; Garza-Moreno et al., 2018). A thorough acclimation process allows the gilts to build up natural immunity to the pathogen, reducing the risk of disease transmission and improving overall herd health. This process may include testing and treatment for *M. hyopneumoniae*, as well as implementing strict biosecurity measures to prevent introduction of new infections (Pieters et al., 2018).

Proper management practices and judicious use of antimicrobials are crucial in controlling infections caused by *M. hyopneumoniae*. Considering therapeutic protocols, several classes of antimicrobials can be used, such as tetracyclines, 15- and 16-membered ring macrolides, lincosamides, pleuromutilins, fluoroquinolones, florfenicol, aminoglycosides, and aminocyclitols (Felde et al., 2018). Pleuromutilins and macrolides are antimicrobial molecules allowed as a last resort for veterinary use (EMEA, 1998) and according to a study by Stingelin et al., 2022, positive results in productivity, with lower mortality and lung bacterial load, were observed in animals medicated with therapeutic protocols involving molecules from both classes, especially protocols that used valnemulin, which is described as being 30 times more effective than tiamulin against Mycoplasma sp. in vitro (Burch et al., 2013).

Although the use of antimicrobial drugs is a viable alternative showing positive results, it alone is not capable of eradicating the infection, and its usage should be implemented rationally to avoid the development of antimicrobial resistance (Gautier-Bouchardon, 2018). Therefore, studies indicating highly effective molecules, presenting sensitivity profiles for respiratory pathogens, as well as the molecule's distribution and concentration pattern in different systems, are essential to define the best approach considering the application of antimicrobials in the control or eradication of *M. hyopneumoniae*.

Vaccination against *M. hyopneumoniae*, in combination with biosecurity measures and judicious use of antimicrobials, can significantly reduce the incidence and severity of infections in swine herds (Maes et al., 2008). From an immunological standpoint, intramuscular vaccination can decrease the clinical symptoms of the disease, but it fails to elicit complete immunity since the stimulation of mucosal immunity can insufficient and does not prevent bacterial colonization (Murtaugh, 2014). The precise mechanism of protection provided by the intramuscular vaccines commercially available is not yet fully comprehended (Simionatto et al., 2013).

Most commercial vaccines for PES use inactivated whole-cell preparations adjuvanted with strains J, which was first identified in a field outbreak of moderate enzootic pneumonia in sows in 1963 (Goodwin and Whittlestone, 1963). However, the exact mechanism of protection provided by these vaccines is not yet fully understood (Maes et al., 2021). These commercial bacterin vaccines are available for single or double vaccination and can also be combined with Glaesserella (Haemophilus) parasuis or porcine circovirus type 2 (PCV-2).

2.7. Oral vaccine against *M. hyopenumoniae*

Oral vaccination against *M. hyopneumoniae* has emerged as a promising alternative to traditional injectable vaccines, offering potential advantages such as ease of administration and needle-free delivery (Maes et al, 2018). The induction of mucosal immunity, particularly secretory IgA, is critical for protecting against respiratory pathogens such as *M. hyopneumoniae*. Oral vaccines have been shown to stimulate mucosal IgA responses, making them a potentially effective strategy for controlling respiratory infections in swine herds (Mechler – Dreibi et al., 2021).

IgA is one of the defense mechanisms responsible for mitigating the adhesion of microorganisms to the mucous epithelium, acting as the main immunoglobulin involved in membrane surface protection. The role of mucosal immunization is to induce local production of IgA on the mucosal surface and on distant surfaces, made possible using a carrier-adjuvant (Lin et al., 2003). Secretory IgA (SIgA) can also be used to monitor the diagnosis of the disease showing the stimulation of the immune system challenges by the bacterium (Parida et al., 2006).

Studies to develop oral vaccines aiming to promote respiratory immunity stimulation response have been carried out in rabbits (Stinson et al., 2016), rats (Joyce et al., 2018), dogs (Ellis et al., 1983), and humans (Teo et al., 2017) showing positive results. In pigs, the ability to stimulate the mucous membranes of different systems through oral immunization with other pathogens has been reported. However, few studies have been conducted for development of oral vaccines focused on the respiratory effect in the species (Hyland et al., 2004)

It is estimated that 70% of the industrial swine herd around the world are vaccinated against *M. hyopneumoniae*, a number that has increased due to the technification of the production process (Martelli et al., 2014). Recently, taking into account the dynamics of bacterial infection, new studies have been conducted to develop vaccines that act directly by stimulating an immune response that offers primary protection against the agent, focused, therefore, on inducing the local SIgA response. Efforts have been focused on obtaining better results concerning mucosal immunity, as this is the entry for pathogens, instead of stimulating a systemic immune response that is not efficient in the sense of controlling the local infection (Wilson & Obradovic, 2015).

Commercial vaccines are composed of whole and inactivated bacterial cells combined with an adjuvant for intramuscular application. It is reported that after vaccination, individuals who have not come into contact with the agent have anti -*Mhyo* antibodies below the detection threshold, anti -*Mhyo* serum antibodies are not suitable for assessing protective immunity, since there is no direct relationship between anti -*Mhyo* serum antibody levels and protection against bacterial adhesion to the epithelium and multiplication (Maes et al., 2008). Regarding the adjuvant, there are limiting factors for the choice, such as toxicity, stimulation of the specific immune response against the agent and not against the adjuvant, in addition to various side effects (Petrovsky & Aguilar, 2004). In addition, the compound used must have the ideal characteristics considering the route of administration. Among the 13 commercial vaccine formulations available on the market, 12 are for intramuscular administration, and one is for intradermal administration. Concerning adjuvants, the most used are aluminum hydroxide, associations with mineral oil, in addition to other compounds, such as paraffin oil, carbapol, and squalene (Maes et al., 2018)

Work carried out by the Department of Applied Physics at USP (São Paulo) in collaboration with the Butantan Institute demonstrated that ordered mesoporous silica (SBA-15) has the ideal characteristics for oral use and vaccine development (Scaramuzzi et al., 2011). The physicochemical characteristics of silica, such as chemical, mechanical and thermal stability, high surface area (above 800 m 2 g -1), and mesopores that can vary from 8 to 50 nm, with a narrow distribution of mesopores, allow molecules to be incorporated and released, unlike other adjuvants such as aluminum hydroxide, which, despite meeting the assumptions of non-toxicity and efficiently stimulating the immune system, has an amphoteric characteristic and cannot incorporate the antigen and promote its release at the site of action (Mercuri et al., 2006). In the case of oral administration, in particular, they must also be resistant to biological solutions of different pH existing in the body and efficient in presenting antigens to the intestinal mucosa.

In addition, the ability of non-toxic SBA-15 nanoparticles to increase the immunogenicity and repair the responsiveness of constitutively poor responders was demonstrated, inducing the production of IgG2a and IgG1 isotypes regardless of the compromised immune cell, and conditioning the low phenotype (Carvalho et al., 2010). However, it is well known that antibodies Serum IgG anti - *M. hyopneumoniae* play a secondary role in protecting against SEP, the local mucosal response with IgA production being more significant (Djordjevic et al., 1997; Thacker et al., 1998). Ordered mesoporous silica (SBA-15) used as an adjuvant in vaccine formulations has been described as a potential substitute for aluminum hydroxide in injectable formulations, due to the stimulation of mixed responses of the Th1/Th2 type, with an additional anti-inflammatory effect mediated by IL-10, inducing satisfactory and desirable cellular and humoral responses (Virginio et al., 2017).

The use of a commercial polymer called Eudragit ®, produced and marketed by Evonik Industries, has been used to coat the complex formed by silica combined with immunogenic particles, conferring resistance to stomach pH. Among the main characteristics of the compound is its ability to resist acidic pH and dissociate slowly and gradually in basic pH, ideal for protecting the compound during gastrointestinal transit and enabling the immunostimulating action of the oral vaccine on the intestinal mucosa. Studies demonstrate that the use of the polymer enables the potentiation of

the immune response and the local inflammatory response, evidenced by the recruitment of pro-inflammatory cells and the increase of phagocytosis by antigen-presenting cells (Carvalho et al., 2010; Garcia et al., 2016; Scaramuzzi et al., 2011).

A specific oral vaccine against *M. hyopneumoniae*, using the adjuvant carrier SBA-15 has been developed, demonstrating its capacity to stimulate the immune system associated with the intestinal mucosa, showing promising results with the decrease in the extent of lung lesions at slaughter in animals experimentally challenged with a homologous strain (232) used in the vaccine composition (Mechler-Dreibi et al., 2021).

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Use of Nanostructured Silica SBA-15 as an Oral Vaccine Adjuvant to Control *Mycoplasma hyopneumoniae* in Swine Production

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Abstract: *Mycoplasma hyopneumoniae* is a difficult-to-control bacterium since commercial vaccines do not prevent colonization and excretion. The present study aimed to evaluate the performance of an orally administered vaccine composed of antigens extracted from *Mycoplasma hyopneumoniae* and incorporated into mesoporous silica (SBA-15), which has an adjuvant-carrier function, aiming to potentiate the action of the commercial intramuscular vaccine. A total of 60 piglets were divided into four groups (n = 15) submitted to different vaccination protocols as follows, Group 1: oral SBA15 + commercial vaccine at 24 days after weaning, G2: oral vaccine on the third day of life + vaccine commercial vaccine at 24 days, G3: commercial vaccine at 24 days, and G4: commercial vaccine + oral vaccine at 24 days. On the first day, the piglets were weighed and, from the third day onwards, submitted to blood collections for the detection and quantification of anti-*Mycoplasma hyopneumoniae* IgG. Nasal swabs were collected to monitor IgA by ELISA, and oropharyngeal swabs were used to assess the bacterial load by qPCR. Biological samples were collected periodically from the third day of life until the 73rd day. At 41 days of life, 15 individuals of the same age, experimentally challenged with an inoculum containing *M. hyopneumoniae*, were co-housed with the animals from groups (1 to 4) in a single pen to increase the infection pressure during the nursery period. At 73 days, all piglets were euthanized, and lungs were evaluated by collecting samples for estimation of bacterial load by qPCR. Quantitative data obtained from physical parameters and laboratory investigation were analyzed by performing parametric or non-parametric statistical tests. Results indicate that animals from G2 showed smaller affected lung areas compared to G3. Animals from G2 and G4 had a low prevalence of animals shedding *M. hyopneumoniae* at 61 days of age. Additionally, no correlation was observed between lung lesions and *M. hyopneumoniae* load in lung and BALF samples in animals that received the oral vaccine, while a strong correlation was observed in other groups. In the present study, evidence points to the effectiveness of the oral vaccine developed for controlling *M. hyopneumoniae* in pig production under field conditions.

Keywords: animal health; immunology; infectious diseases; respiratory diseases; vaccinology

1. Introduction

Mycoplasma hyopneumoniae (*M. hyopneumoniae*) is the primary agent of porcine enzootic pneumonia (PES), which is one of the diseases of the respiratory complex of pigs [1]. PES mainly affects animals during the growing to finishing period that presents a chronic condition of non-productive cough, reduced weight gain, and feed conversion [2], as well as increased mortality and costs associated with the control and treatment of animals [3]. The bacterium acts on the mucous membranes of the respiratory epithelium by adhering to the cilia and changing the frequency of beats, promoting ciliostasis, epithelial cell death, modulating cellular and humoral immunity, and resulting in an exacerbated inflammatory reaction in the lungs [4].

Piglets are considered free of *M. hyopneumoniae* at birth, as in utero transmission has not been documented, and the first exposure events occur during the lactation period when piglets are in contact with sows that are shedding the microorganism, so this moment is considered of highest risk for the colonization of piglets by *M. hyopneumoniae*, related to the stress of parturition [5].

Currently, attempts to control PES involve monitoring the pathogen, attention to biosecurity, vaccination, and antimicrobial usage. However, control is difficult, considering that even applying all the measures, the prevalence of *M. hyopneumoniae* in swine production is high [4]. From an immunological point of view, the intramuscular vaccine helps to reduce the clinical signs; however, vaccines do not prevent the infection, only reduce the impact of the infection on the performance of the pigs [6]. The mechanism of action of commercial vaccines has been studied to understand the dynamics of the cellular and humoral immune response in these animals [7].

IgA is one of the defense mechanisms responsible for mitigating the adhesion of microorganisms to the epithelium and possibly one of the main components responsible for protecting the mucous membranes, whose production by commercial vaccines has been evaluated in recent studies. The role of mucosal immunization is to induce local production of IgA on the mucosal surface and on distant sites, made possible using the carrier-adjuvant [8].

Studies have been conducted to develop vaccines that act directly by stimulating an immune response that offers primary protection against the pathogen, involving the evaluation of the local IgA response [9]. Oral vaccines that promote induction of the respiratory and immune response have been developed and evaluated in several species [10–12], showing positive results. In pigs, the ability to stimulate the mucous membranes of different systems through oral immunization with other pathogens is reported. However, until now, there have been few studies focused on the development of oral vaccines against respiratory pathogens [13].

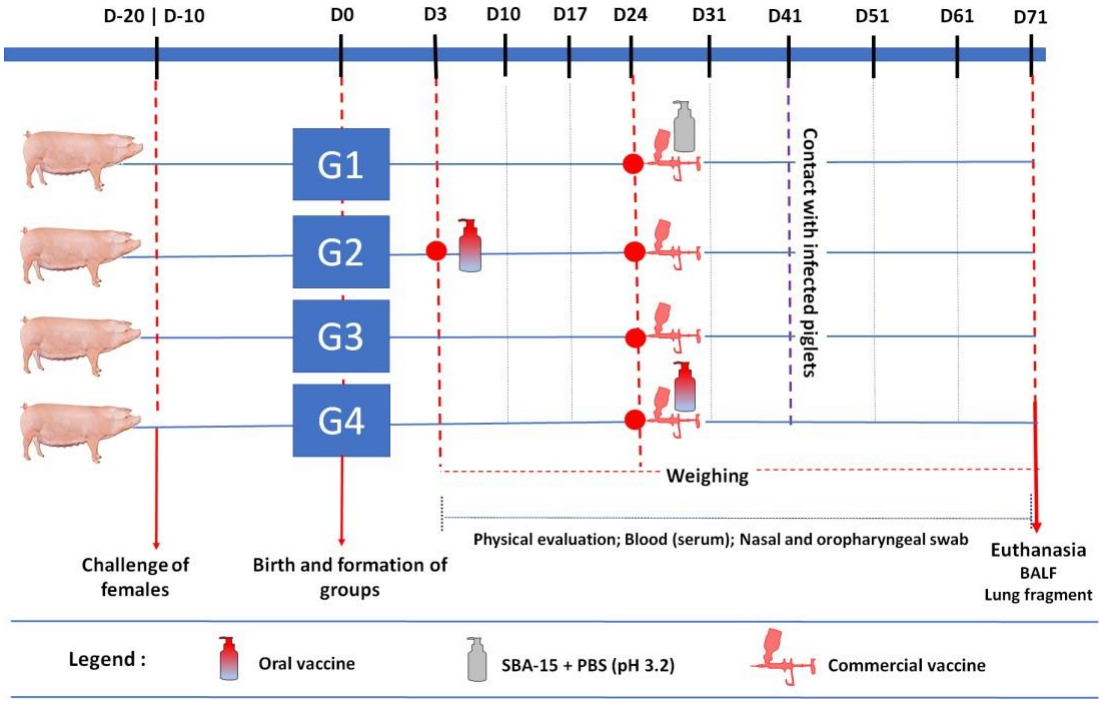
The Department of Applied Physics at the University of Sao Paulo—USP, in collaboration with the Butantan Institute, found that ordered mesoporous silica (SBA-15) is suitable for oral use and vaccine development. The chemical, mechanical, and thermal stability of silica, along with its high surface area and mesopores in the range of 8–50 nm with a narrow distribution, allows for the incorporation and release of molecules. This sets it apart from other adjuvants, such as aluminum hydroxide [14,15].

A specific oral vaccine against *M. hyopneumoniae*, using the adjuvant carrier SBA-15 has been developed, demonstrating its capacity to stimulate the immune system associated with the intestinal mucosa, showing promising results with the decrease in the extent of lung lesions at slaughter in animals experimentally challenged with a homologous strain (232) used in the vaccine composition [16].

This study aims to evaluate the use of the previously developed oral vaccine, experimentally under field conditions, against an uncharacterized field strain of *Mhyo*, considering the dynamics of the infection and the main challenges of commercial pig farming involving *M. hyopneumoniae.*

2. Result

Four pregnant females were challenged with a field strain of *M. hyopneumoniae* at 94 and 104 days of gestation. The litters born to the four *M. hyopneumoniae*-positive females were randomly assigned to groups as follows: G1 (gilt number 275), G2 (gilt number 350), G3 (gilt number 933), and G4 (gilt number 794). Throughout the study, the piglets were regularly weighed, and their physical parameters were evaluated. Periodic biological samples were collected from the third day of life until the 73rd day. All study groups received the intramuscular vaccine against *M. hyopneumoniae*. In addition to the commercial vaccine, G2 and G4 received the oral vaccine, while G1 received pure SBA-15 in an acidified PBS solution (pH 3.2). Group 3 was the only group that received only the intramuscular vaccine, as a standard vaccination protocol applied in commercial swine production. To increase infection pressure during the nursery period, 15 piglets of the same age as the others were challenged with *M. hyopneumoniae* on the 41st day of life and added to the pen with the animals from G1 to G4. Finally, at the conclusion of the study, on the 73rd day, all piglets were euthanized and underwent necropsy (Scheme 1).



Scheme 1. Assessments and sample collections in piglets.

From the date the breeding females were transferred to the farrowing, the first signs of coughing were observed. Throughout the period, the four females whose litters composed the experimental groups presented sporadic episodes of dry cough. Female number 275, whose litter made up G1, presented recurrent episodes of anorexia from the 14th day after parturition.

The oral vaccine and SBA-15 + PBS were easily administrated, and there was no reflux or extravasation of any volume. Diarrhea did not occur at the time of vaccination or in the three days following the application of the compounds orally. On D24, when all animals received intramuscular vaccination, no temperature higher than 39.0 °C was recorded, and there were no noteworthy changes at the injection site up to 48 h after administration.

Information on the physical assessment of the piglets, changes in health condition, stool appearance, mucous membranes color, nasal discharge, and behavior are described in Table 1. Changes in health conditions were observed throughout the entire study, except for D51, in all groups. Diarrhea was observed from birth until the fifth day in G2

and G3, and from D27 to D41 in all groups, except in G3. Mucosal color change was observed only once on D51 in one of the G4 piglets. Nasal discharge was observed on D51 and D61 in all groups. Changes in behavior were observed on D25 and D27 in G1 and G4 and on D61 in G3 (Table 1).

Table 1. Physical parameters evaluated in animals from groups 1 to 4 throughout the study period with individual identification, group identification, and degree of alteration.

Physical Exam					
Day	**Health Status**	**Stool Consistency**	**Mucous Membrane**	**Nasal Discharge**	**Behavior**
D0		G2 = 22, 26 (+) [1] G3 = 39, 42, 43 (+)			
D4		G2 = 21 (+)			
D5	G1 = 9 (+)	G2 = 21 (+)			
D10					
D17	G1 = 9 (+)				
D25	G1 = 9 (++) G2 = 20 (+) G4 = 40 (++)				G1 = 9 (++) G4 = 36, 40 (++)
D27	G1 = 9 (++) G4 = 40 (+)	G4 = 50 (+)			G1 = 9 (++)
D31	G1 = 9 (+)	G1 = 14 (+) G2 = 17, 22, 29 (+); 21, 24, 25, 26 (++) G4 = 59 (++)			
D41	G2 = 22 (+)	G1 = 3 (+) G4 = 50 (+)			
D51				G1 = 13 (+) G2 = 26 (+) G3 = 38, 40, 42 (+) G4 = 55, 56 (+)	
D61	G3 = 38 (+)			G1 = 5, 11 (+) G2 = 23 (+) G3 = 35, 38 (+) G4 = 58 (+)	G3 = 38 (+)
D72	G2 = 26 (+) G3 = 38 (++) G4 = 45 (++)		G4 = 50 (+)		
Classification	(+) regular (++) bad	(+) mild diarrhea (++) severe diarrhea	(+) pale	(+) present	(+) apathy (++) uncoordinated

[1] The information is presented as follows: Group = ID (classification).

Regarding body weight, no difference was observed between litters on the third day of life with mean in kilograms ± standard error of G1 (2.41 ± 0.1), G2 (2.32 ± 0.09), G3 (2.55 ± 0.07), G4 (2.54 ± 0.14) nor on D72 days among the groups G1 (11.27 ± 0.7), G2 (11.15 ± 0.71), G3 (11.94 ± 0.72), and G4 (11.57 ± 0.7) ($p = 0.34$); however, at 24 days, a difference in weight was observed between the vaccinated groups ($p = 0.008$) when G2 (6.06 ± 0.21) (mean ± standard error) did not differ from G1 (5.25 ± 0.29), G3 (6.32 ± 0.21), and G4 (6.48 ± 0.28), while the mean of G1 was smaller than G3 and G4. Regarding the daily weight gain (DWG) recorded during farrowing, from the 3rd to the 24th day, there was a lower weight gain in G1 (0.14 ± 0.01) when compared to G2 (0.19 ± 0.01), G3 (0.19 ± 0.01), and G4 (0.2 ± 0.01) which did not differ from each other by Tukey's test ($p < 0.001$). The DWG

throughout the nursery period indicated that there was no difference between groups according to Tukey's test ($p = 0.68$).

Regarding rectal temperature, it was observed that on days 0, 5, 10, 41, 51, 61, and 71, no differences were recorded between the means of the groups by the Tukey test at the level of ($p < 0.05$) (Table S1). On D4, the mean temperature of G3 was higher than that of G4. On D17, the mean temperature of G1 was lower when compared to the temperature of G4. Regarding D24, the temperature of G1 was lower than the temperature of the other groups. From D26 to D31, G1 averages were lower than G2 and G4, while G3 did not differ from the others.

Multiple comparisons between different rectal temperature measurement points within each group were performed to observe whether there was a variation in rectal temperature associated with the vaccination protocol. A decrease in rectal temperature over time was observed when comparing D0, D3, and D5 with the values recorded from D17 to D41 in G1, followed by an increase in temperature on D51 to D71 that differed from D41 to D61, which were superior to D24 and D31. In G2, the values recorded from D0 to D10 were higher than D17 to D71. In G3, the temperature of D0, D3, and D10 was higher than those from D17 to D41 and D71, and D5 was higher than D17 to D51 and D71. Additionally, in G3, an increase in rectal temperature was observed, with higher values on D51 compared to D41 and D61 when compared to D31 and D41. In G4, the values recorded from D0 to D10 were higher than D24 and from D26 to D51. Additionally, an increase in temperature was observed between D41 and D61 at the level of $p < 0.05$ by the generalized linear mixed model (Table S2 and Figure 1).

Figure 1. Parallel coordinate boxplots of rectal temperature measurements (°C) recorded over time considering animals from G1 to G4. The lines in a parallel coordinates plot represent the trajectories of observations across different time points. The bold dots indicate the outliers.

2.1. Anti-M. hyopneumoniae IgA and IgG in Nasal Swabs and Serum

Serum samples and nasal swabs collected from D3 to D72 were submitted to detection and relative quantification of immunoglobulin concentration (IgA and IgG), whose results were expressed in S/P value and compared between groups in each collection point and within each group over time.

Regarding the IgG quantifications in the piglets' serum, differences were observed when comparing the medians between groups since the first collection. On D3, lower IgG concentrations were observed in G2, and the highest in G3, followed by G1 and G4, with all groups significantly differing from each other by the Kruskal–Wallis test at the level of $p < 0.05$. On D10, D17, and D24, both G1 and G3 were superior to G2 and G4. On D31, IgG titers were higher in G3 compared to the others, and G1 was higher than G2 and G4. On D41, all groups differed significantly from each other, with the highest concentration of IgG recorded for G3, followed by G1 and G4, with the lowest median observed for G2. On D51, it was observed that G3 had the highest measurement when compared to the other groups, followed by G1, which differed from G2. On D61, the S/P value in G3 was higher than in G2 and G4. Finally, on D71, no differences were found between the groups at the level of $p < 0.05$ by the non-parametric Kruskal–Wallis test (Table S3).

Multiple comparisons over time demonstrated that for G1, the IgG levels on D71, D61, and D51 were significantly lower than the moments from D3 to D31. On D24 and D41, significant reductions in the titles were observed, compared to D3 and D17. In G2, the IgG levels on D71 were higher than those observed on D24 and D41. On D61, it was higher than on D41. Therefore, over time, in G2, there was a significant decrease in titles on D24 and D41 compared to moments from D3 to D17. In G3, a pattern similar to G1 was observed, with D71 and D61 significantly differing from D3 to D31 and D51 from D3, D17, and D31. On D41, a significant titer decrease was observed when compared to D3, D17, and D31 on G3. In G4, the levels of D71 differed only from D3, while D61 and D51 and D41 differed from D10 to D17 by Friedman's non-parametric test for comparisons of repeated measures over time $p < 0.05$ (Figure 2 and Table S4).

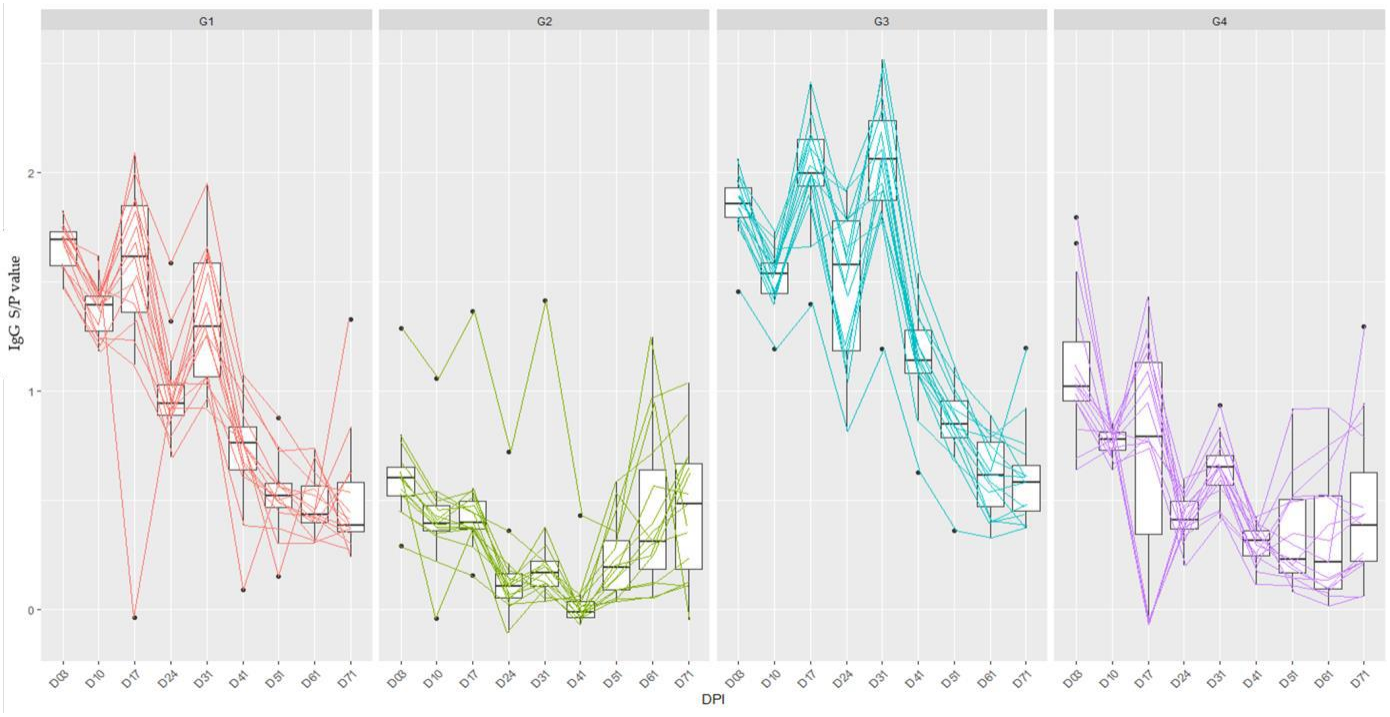


Figure 2. Parallel coordinates boxplots of S/P values for IgG anti-*M. hyopneumoniae* recorded over time considering animals from G1 to G4. The lines in a parallel coordinates plot represent the trajectories of observations across different time points. The bold dots indicate the outliers.

Regarding IgA, on D3, D41, and D51, no differences were recorded between the medians of the groups by the non-parametric Kruskal–Wallis test ($p = 0.31$). At D10, the S/P value was lower in G2 and G4 when compared to G1 and G3. On D17, the median of G4 was higher when compared to G1 and G2. On D31, G3 was higher than G4. On D61, the smallest measure was G3 when compared to the others. On D71, the median S/P was

higher in G4 than in G3, while G1 and G2 did not differ from the others by the Kruskal–Wallis test at $p < 0.05$ (Table S5).

Regarding multiple comparisons for IgA concentrations in the nasal swab, an increase in S/P values was observed in all groups, with the highest values presented on D71, D61, and D41. In G1, D71 and D61 differed from the values recorded from D3 to D41. In G2, the values on D71, D61, and D51 differed from D3 to D24. From D31 onwards, there was an increase in the S/P values, and, from that moment on, no significant differences were observed. As for G3, differences were observed when comparing D71 with moments from D3 to D24 and D61 and comparing D51 with D10 to D24. In G3, differences were also observed between D31 and moments from D10 to D24, demonstrating an increase in IgA levels from D31. In G4, differences were observed when comparing D71 with the moments from D10 to D31 and D61. On D51 (G4), significant differences were registered when compared to the moments from D10 to D24. Still, in G4, a significant increase was observed from D41 onwards, which differed from D10 and D24 by Friedman's non-parametric test for comparisons of repeated measures over time at $p < 0.05$ (Table S6 and Figure 3).

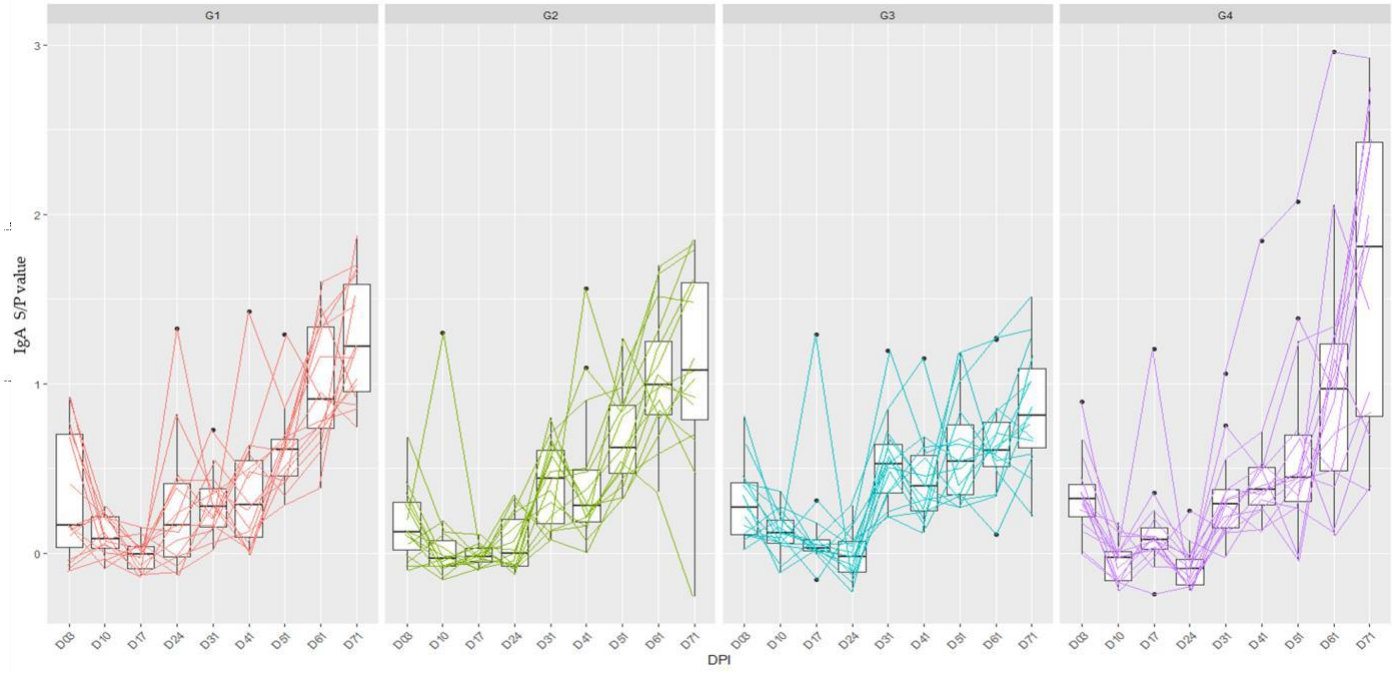


Figure 3. Parallel coordinates boxplots of S/P values for IgA anti-*M. hyopneumoniae* recorded over time considering animals from G1 to G4. The lines in a parallel coordinates plot represent the trajectories of observations across different time points. The bold dots indicate the outliers.

2.2. Assessment of Lung Lesions

Determination of the Pneumonia Index

The lungs of the piglets in each group were evaluated individually, recording the degree of consolidation lesions in each lobe so that the total affected area could be calculated (Figure 4). Regarding the extension of macroscopic lung lesions (%), a difference was observed between G2 and G3. Significant differences were observed between G2 and G3 ($p = 0.005$), and a trend towards significance was noted between G4 and G3 ($p = 0.07$) (Table 2).

Table 2. Percentage of pulmonary consolidation lesions determined by macroscopic evaluation and Pneumonia Index for G1 to G4 and a group of challenged animals.

Groups	Affected Lung Area (%) [1]	Animals with Lung Injury	Pneumonia Index
G1	4.40 ± 2.61	12/15	1.07
G2	0.00 ± 0.68 [b]	6/15	0.80
G3	6.00 ± 2.75 [a]	12/14	1.33
G4	1.40 ± 3.13	9/15	0.93
p-value	0.006		–

[1] Medians followed by different letters on the same column differ significantly by Kruskal–Wallis test ($p < 0.05$).

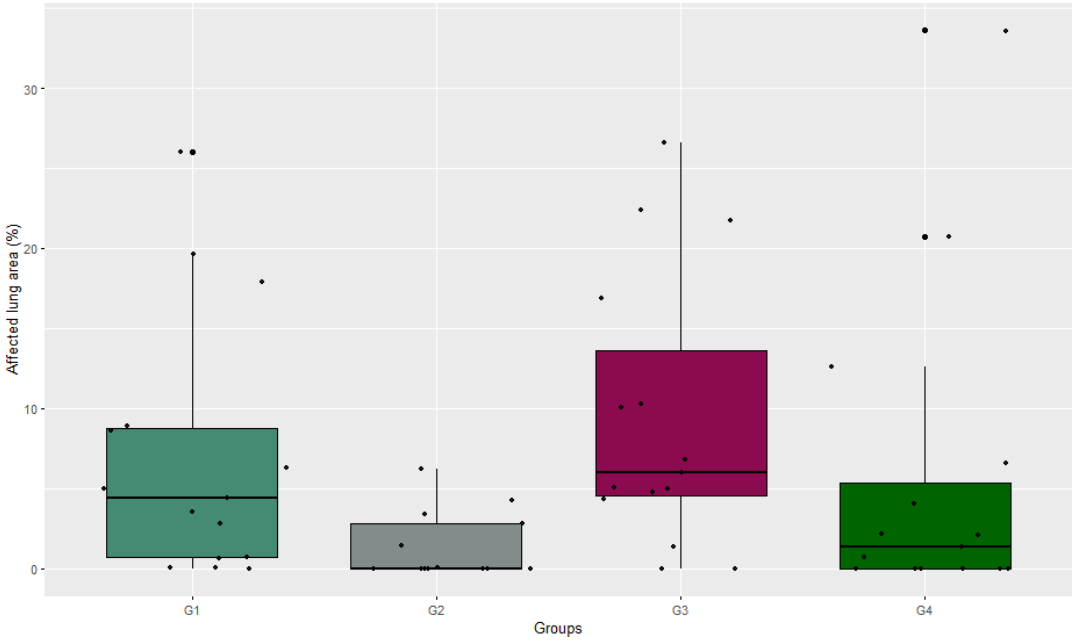


Figure 4. Boxplot demonstrating degrees of injury between groups in the macroscopic evaluation. Light dots represent each observation. The bold dots, centered on the boxplot, indicate the outliers.

2.3. Estimation of Bacterial Load of M. hyopneumoniae in Oropharyngeal Swab, BALF, and Lung by qPCR

Samples of oropharyngeal swabs collected from piglets in groups 1 to 4 over the period from D3 to D72, as well as tracheobronchial lavage samples and lung fragments collected at euthanasia, were subjected to DNA extraction and detection of *M. hyopneumoniae* present in the samples (Figure 5).

Swab samples collected from D3 to D51 were negative for the presence of *Mhyo*, demonstrating that positive animals were not detected during this period. On D61, 20 days after the start of the challenge with animals experimentally exposed to lung homogenate, nine vaccinated piglets were detected as positive in qPCR. Altogether, two animals from G1, individuals number 5 and 10, totaling 13.33% (3.74 ± 37.88 % CI), one individual from G2, number 17, totaling 6.67% (1.19 ± 29.82 % CI), five positive individuals from G3, animals number 32, 33, 36, 40, and 41, totaling 35.71% of prevalence, and one individual from G4, number 54, resulting in a total of 6.67% (1.19 ± 29.82 % CI) (Table 3). The results of quantification of *M. hyopneumoniae* in the BALF, as well as in the lung, indicated that there was no difference between the vaccinated groups by Kruskal–Wallis test (Table 4).

Table 3. Identification of animals positive for p102 gene detection in the oropharyngeal swab by qPCR followed by the prevalence and confidence interval of each group (G1 to G4) at D61.

Groups	Positives on D61 Oropharyngeal Swab (ID)	Prevalence % (IC)
G1	# 5 # 10	2/15 = 13.33 % (3.74 ± 37.88)
G2	# 17	1/15 = 6.67 % (1.19 ± 29.82)
G3	# 32 # 33 # 36 # 40 # 41	5/14 = 35.71 % (16.34 ± 61.24)
G4	# 54	1/15 = 6.67 % (1.19 ± 29.82)
p-value	>0.05	

Table 4. Median ± median standard error of p102 gene detection and quantification values expressed in copies/μL and proportion of positive animals in analyzes of tracheobronchial and lung lavage samples by qPCR for groups from G1 to G4.

Groups	BALF	BALF Positive/ Analyzed	Lung	Lung Positive/ Analyzed	BALF vs. Lung p [1]-Value
G1	$2.02 \times 10^{+2} \pm 8.62 \times 10^{+3}$	15/15	$40.00 \pm 3.56 \times 10^{+4}$	8/15	0.10
G2	$6.73 \pm 1.25 \times 10^{+4}$	13/15	$11.90 \pm 1.62 \times 10^{+3}$	12/15	0.49
G3	$24.90 \pm 2.28 \times 10^{+3}$	13/14	$6.29 \pm 1.14 \times 10^{+3}$	11/14	4×10^{-2}
G4	$2.83 \times 10^{+2} \pm 1.61 \times 10^{+4}$	14/15	$1.94 \pm 1.21 \times 10^{+3}$	14/15	6×10^{-3}
p-value	0.282		0.973		

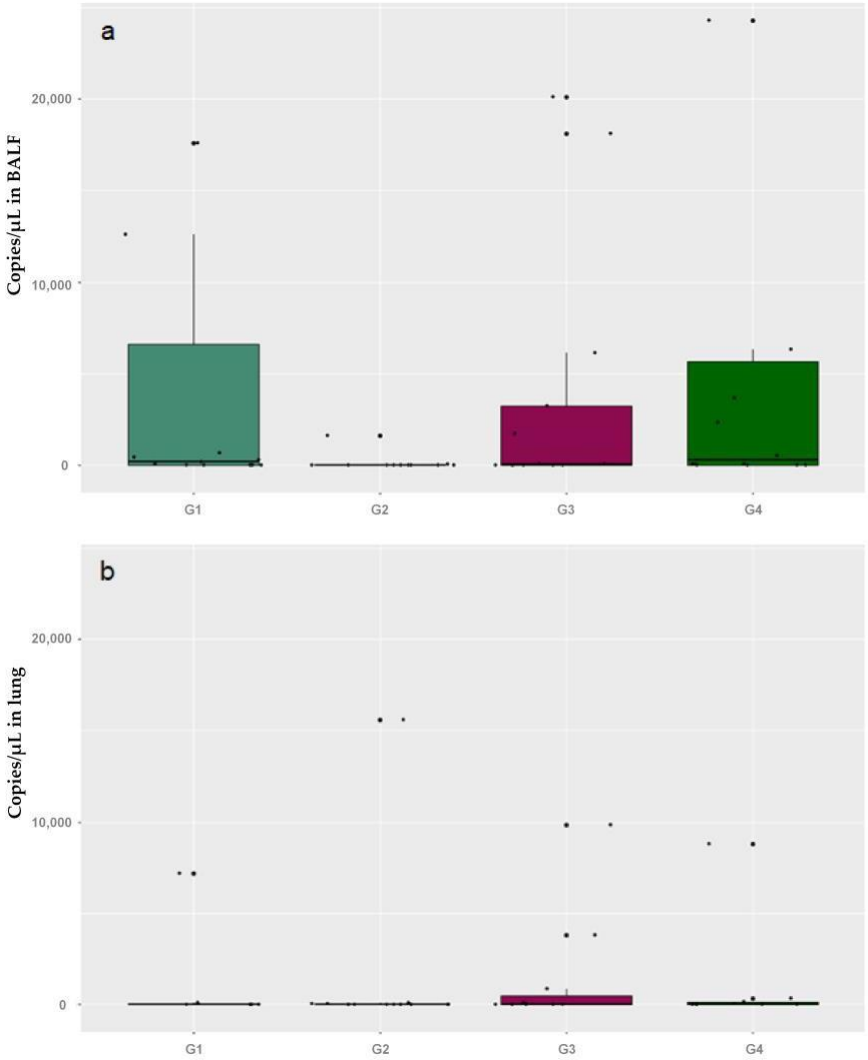


Figure 5. Boxplot with *Mhyo* quantification values in tracheobronchial and lung lavage samples for groups from G1 to G4 (**a**,**b**). The light dots represent each observation. The bold dots, centered on the boxplot, indicate the outliers.

2.4. Correlation Analysis

Correlation analyses were performed considering measurements observed over time (IgA, IgG, rectal temperature, and body weight) and also measurements obtained from post-mortem sample measurements and analyses. The correlation between body weight and the S/P value of IgA indicated that for the vaccinated groups (G1 to G4), the correlations were significant ($p < 0.05$) by the Spearman test. The value of rho varied from 0.30 for G3 to 0.58 for G1, indicating that the samples exhibit different levels of correlation between IgA and weight values. G1 has the strongest correlation, while G3 has the weakest correlation. For G2, rho was 0.43, and for G4, it was 0.41.

The correlation between IgA and IgG was analyzed in four groups (G1 to G4). Significant negative correlations were found in G1 ($p < 0.001$) and G3 ($p < 0.01$), with G1 having the strongest correlation (tau = −0.42) and G3 having the weakest (tau = −0.31). In G4, a significant negative correlation was observed (rho = −0.524, $p = 0.045$) for IgG and BALF quantification indicating that higher values of IgG would be correlated with lower concentrations of *M. hyopneumoniae* in BALF samples.

No correlations were found between bacterial loads in BALF and lung samples in any of the groups using Kendall's test ($p > 0.05$). Although directly proportional correlations were observed between lung lesions at euthanasia and the quantification of

M. hyopneumoniae, only in G1 and G3 (G1, *p*-value = 0.009 and R = 0.64; G3, *p*-value = 0.001 and R = 0.80), indicating a strong correlation between lung lesions and *M. hyopneumoniae* load. In G2 and G4, the recorded *p*-values were 0.43 and 0.51, respectively, by Pearson's test. On the other hand, when analyzing the correlation between *M. hyopneumoniae* load in lung samples and lung lesions, no significant correlation was found in any of the groups.

3. Discussion

There is still little data in the literature regarding the use of SBA-15 associated with antigens in the composition of oral vaccines, especially for animals such as the one produced in the present study. A previous study of characterization and use of the same oral vaccine under laboratory conditions of controlled infection showed promising results in stimulating immunity and reducing lung lesions at slaughter [16].

Especially in pig production, zootechnical performance is a fundamental aspect that can suffer positive or negative impacts due to the action of drugs and vaccines [17]. Regarding the administration of the oral vaccine produced in the present study, no significant alterations in rectal temperature, no occurrence of diarrhea, and no alteration of the clinical picture were observed either in animals that received the oral vaccine at three days (G2) or in piglets vaccinated in the weaning group (G4). Changes in stool consistency were observed only on D31. However, they are likely associated with dietary changes after weaning [18]. Considering the post-challenge period in the nursery phase, in which experimentally challenged piglets were mixed with vaccinated piglets (G1-G4), the emergence of animals with nasal discharge at D51 and D61 in the four groups, animals that were positive for *M. hyopneumoniae* in samples collected after euthanasia, was observed, indicating a possible clinical picture of respiratory disease.

Regarding body weight and weight gain, differences were only observed in G1, which in turn may be associated with anorexia of female number 275, which may have resulted in lower quality and quantity of milk produced in some moments during the suckling period that reflected in the performance of the animals of the litter. Although there was no evidence of a difference between the weights of the piglets at slaughter, it is necessary to emphasize that the study only covered the period of farrowing and weaning, so, possibly, if the study was prolonged throughout growth and finishing, differences in weight gain and performance became more evident, which is a limitation of the present study.

As for body temperature, a reduction was observed over time in the groups, which is physiologically consistent with the variation in body temperature over the lifetime of pigs [19]. Variation in body temperature was observed between moments D41 and D51, being significant only in G1 and G3. Such a variation may be associated with an inflammatory process [20] since it is consistent with the initial challenge period caused by the mixing of infected animals. It is possible to observe, analyzing the plots and the recorded values, that such differences are present in the four groups, although there are indications that in G2 and G4, such temperature variation may have occurred more mildly when compared to the others. It was previously observed that the application of the oral vaccine acted in the modulation of the cellular immune response with an increase in the concentrations of anti-inflammatory cytokines, which was indicated as an effect possibly related to a lower incidence and extent of lung lesions in vaccinated animals [16].

In the swine species, passive immunity is of great relevance in the acquisition of antibodies that will help protect piglets during the farrowing period and the beginning of the nursery phase, with IgG being one of the main components involved in the process of transferring colostral immunity, therefore absorbed, shortly after birth [21]. In gilts, variability in passive immunity may result in differences between litters considering colostrum uptake [22]. In the present study, it was observed that more expressive amounts of anti-*M. hyopneumoniae* IgG were detected in G3 and G1 compared to G4, and especially in G2. This difference in the immunity of piglets from the different groups remained

during practically the entire period, with a decrease in the concentration of immunoglobulins, which persisted even after vaccination of the groups, both with the oral vaccine and with the commercial intramuscular vaccine, with perhaps an only exception on the part of G2, in which an increase in the concentration of IgG was registered from D51, a fact that meant that on D71 no significant differences were registered between the groups regarding the concentration of such immunoglobulin.

Based on parallel coordinates plots and the analysis of the repeated measurements, it was observed that G2 was the only group that presented an increase in anti-*M. hyopneumoniae* IgG concentrations. In the other groups, there were no signs of an increase in antibody titers. However, it was not possible to determine that there was no stimulation of IgG production since humoral immunity may have raised antibody titers as the concentration of anti-*M. hyopneumoniae* IgG from colostral immunity decreased in G1, G3, and G4. Although IgG anti-*M. hyopneumoniae* does not have a direct role as a protective factor against *M. hyopneumoniae* infection, it is an indicator of cellular and humoral immune response stimulation. Considering passive immunity, a higher IgG titer may indicate a more significant contribution of colostrum-derived defense cells, which would affect piglet protection against infections [23].

Considering that IgG has no direct action in guaranteeing protection against *M. hyopneumoniae* and, therefore, mild effectiveness in controlling PES, much has been discussed about the effect of mucosal immunity in protection against colonization of the respiratory tract of pigs by controlling *M. hyopneumoniae* infection. In a previous study, an oral vaccine with SBA-15 produced following the same methodology as in the present study showed positive results regarding the stimulation of mucosal immunity in animals vaccinated at weaning against a homologous strain of *M. hyopneumoniae* (232) [16]. In the present study, the development of mucosal immunity in animals submitted, in addition to vaccination at weaning, to early vaccination on the third day of life, as well as the nonspecific effect of stimulation of IgA production by the carrier adjuvant (SBA- 15) were effects that have not been considered in the previously proposed model. Regarding the differences in the concentration of IgA between the groups, it was observed that, despite the differences up to D31, there was no increase in the concentration in any of the groups. From D31, increases in immunoglobulin concentration were observed, also in all groups, and significantly higher concentrations were recorded in groups G2 and G3 with more noticeable peaks, as observed in the parallel coordinate plots. From D41 onwards, there was an increase in IgA levels in the groups. Significant differences were observed from D61 with lower IgA concentration in G3, which had been vaccinated only with the commercial intramuscular vaccine. On D61 and D71, there were no differences between G1, G2, and G4, although, numerically, the values observed in G4 are higher than the others. Therefore, there are indications of a nonspecific stimulation by SBA-15 and that the oral vaccine produced and administered had a positive effect in stimulating the production of IgA when combined with the commercial intramuscular vaccine. Another point to be highlighted is that, numerically, the S/P values recorded in G4 are superior to the others. However, no significant difference was evidenced, probably due to the greater variability of the data in G4 and the number of animals per group, which are limitations for statistical models in animal studies.

A lower degree of lung injury, associated with the increase in IgA levels in animals that received the oral vaccine, was observed in animals that received the new vaccine in the previously conducted study [16]. In the present study, vaccinated piglets also showed a lower degree of lung injury, regardless of the vaccination protocol. In addition, both G2 and G4 animals had a smaller area of lung injury, indicating that oral vaccination, administered both early and at weaning, was effective in reducing lung lesions.

Correlation analyses were performed between S/P values for IgG and IgA, resulting in significant correlation only for groups G1 and G3. This suggests that in these groups, as maternal IgG levels decreased, an increase in IgA levels was observed. It is possible

that in the other groups, where no significant IgG titers were derived from passive immunity, such a correlation was not observed.

The correlation between the *M. hyopneumoniae* load in the BALF samples and lung lesions in G1 and G3 indicates that infection by the bacterium is strongly associated with the occurrence of lesions in these individuals. In the groups that received the oral vaccine, G2 and G4, the correlation between *M. hyopneumoniae* and lung lesions, considering *Mhyo* quantification in lung and BALF samples, suggests that the observed lesions may not be directly related to *M. hyopneumoniae*, indicating the effectiveness of the oral vaccine in reducing lung lesions caused by *M. hyopneumoniae*.

By analyzing the detection of *M. hyopneumoniae* between the groups over time, no positive animals were observed until D41. Although field situations were simulated in the present study, complex events such as the spread of *M. hyopneumoniae* during farrowing may occur differently in a commercial production environment. The gilts in the present study were initially free from *M. hyopneumoniae* and were exposed to the pathogen only 20 days before parturition. Although positive during the farrowing period, they possibly shed less *M. hyopneumoniae* when compared to nulliparous females in the environment of production, in which the infection occurs endemically, resulting in early exposure to *M. hyopneumoniae*. Considering that the multiplication of *M. hyopneumoniae* has the characteristic of being fastidious [7] when infected early, the bacterial growth would occur for a more prolonged period, resulting in a higher bacterial load in the lung and, consequently, increasing the odds ratio that the bacteria is shed steadily and in greater concentration during the farrowing period.

On the other hand, in the present study, there are indications that the spread of *M. hyopneumoniae* occurred from the challenge during the nursery period with the mixture of infected animals. After mixing on D41, clinical respiratory signs were observed, and the first positive piglets were identified on D61 in oropharyngeal swab samples. Numerically, a difference can be observed when comparing the proportions of positive animals between groups G3, G2, and G4, although based on the confidence interval, it is not possible to show a significant difference. A limiting factor for comparisons between proportions is the number of samples analyzed, and, in the case of a study with animals, there are few options for analyzes and models that are actually appropriate to determine differences between groups with a reduced number of experimental units.

Based on the present study, measures based on vaccination were essential in protecting the animals, although they were not fully effective in inhibiting the dissemination or infection by *M. hyopneumoniae*, demonstrating that the control of the bacteria depends, in addition to vaccination, on other joint actions such as the rational use of efficient antimicrobial molecules [24] and especially effective biosecurity measures in production [6]. Given the results of the present study, there is evidence that oral vaccination with a vaccine produced using the carrier-adjuvant has been effective in reducing lung lesions and the dissemination of *M. hyopneumoniae* in production. Future studies may explore formulations based on SBA-15 in other ways and associated with *M. hyopneumoniae* antigens produced from national strains facing homologous and heterologous challenges.

4. Material and Methods

4.1. Animal Selection

For the study, six pregnant primiparous females with 80 days of gestation were acquired from a producer located in the municipality of Itu, SP, and sent to the Swine Medicine Laboratory, where they remained in gestation pens (0.3 female/m^2) for up to 10 days before the expected parturition date. After this period, the gilts were transferred into individual farrowing pens. Upon arrival, they were subjected to blood samples collection to obtain serum for antibody quantification and laryngeal swab collection to verify the females' status regarding *M. hyopneumoniae* infection. After farrowing, the groups were

defined, and the litters were kept with the gilts during the farrowing phase (24 days). After weaning, the females returned to the collective pens, and the four experimental groups (G1 to G4) were kept together in a single nursery pen until they were 72 days old (2 piglets/m^2). At 41 days, 12 animals, positive for *M. hyopneumoniae*, randomly chosen from the litters of the other two breeding gilts, were mixed with the other piglets from G1 to G4. Strict biosecurity measures were followed to avoid cross-infection and minimize external interference, such as the use of specific, clean clothes and not having contact with any other pigs during the experimental period. The animals received feed according to the production phase, free of antibiotics, and water ad libitum. All procedures of this study were approved by the Ethics Committee for the Use of Animals (CEUA), Faculty of Agrarian and Veterinary Sciences, Unesp Campus of Jaboticabal–SP, under protocol number 00915/20.

4.2. Experimental Design

Pregnant females were, at 94 and 100 days of gestation, exposed to *M. hyopneumoniae* via the intratracheal and nasal route using a positive inoculum (10^5 copies⁄µL) for *M. hyopneumoniae*, produced from lungs with lesions suggestive of PES, obtained from a slaughterhouse located in the city of Guariba, SP. A total of 60 piglets born to four females positive for *M. hyopneumoniae* were divided into four groups (n = 15) and submitted to different vaccination protocols as follows, G1: SBA-15 in acidified PBS (pH 3.2) without *M. hyopneumoniae* antigens + commercial vaccine at 24 days, G2: oral vaccine at the third day + commercial vaccine at 24 days, G3: commercial vaccine at 24 days, and G4: commercial vaccine + oral vaccine at 24 days. On the first day, the piglets were weighed, and from the third day on, they were subjected to blood collections for the detection and quantification of anti-*Mycoplasma hyopneumoniae* IgG, as well as nasal swabs for monitoring IgA by ELISA and oropharyngeal swabs to estimate the bacterial load of *M. hyopneumoniae* by qPCR. Biological samples were collected periodically until the 72nd day. At 41 days of life, 15 individuals of the same age from two other pregnant females previously exposed to *M. hyopneumoniae* were mixed with the animals from groups (1 to 4) to increase the infection pressure during the nursery period. At the end of the study, at 72 days, all piglets were euthanized and necropsied for evaluation of the lungs and collection of lung samples for estimation of bacterial load by qPCR. Samples of bronchoalveolar fluid (BALF) were also collected and submitted to the qPCR test.

4.3. Inoculum

For the inoculum production, five lungs of animals at approximately 150 days of age, which presented consolidation lesions in varying degrees in the portions of the right and left cardiac lobes, were collected in a slaughterhouse placed in the municipality of Guariba, SP. The lungs were sent refrigerated to the Swine Medicine Laboratory. Fragments of the apical and cardiac lobes of the lungs were selected, with the aid of a sterilized scalpel blade, sectioned into smaller portions of approximately 1 mm^3, which in turn were transferred to a clean and sterilized plastic package, to which 500 mL of PBS (pH 7.2) heated to 37 ºC were added, homogenizing the content, pressing the fragments and stirring the volume. The homogenate was then filtered using a clean and disinfected sieve, and part of the filtered volume was aliquoted in fractions of 1 mL in plastic tubes free of DNAse and RNAse intended for analysis to estimate the concentration of *M. hyopneumoniae* in the inoculum. DNA from the inoculum samples was immediately extracted [25] and subjected to qPCR, indicating a quantification of 10^5 copies/µL.

4.4. Challenge of Pregnant Females

Before the challenge, the pregnant females were subjected to blood sample collection to obtain blood serum and oropharyngeal swabs. Blood samples were collected using a vacuum tube with clot-activating gel through a jugular vein puncture using an attached

25 × 0.8 mm needle. The oropharyngeal swab samples were collected using a mouth opener and a laryngoscope by rubbing the cotton swab in the region posterior to the epiglottis of the females.

The pregnant females were challenged on two occasions because they had been vaccinated on the farm of origin at 180 and 200 days of life with the vaccine RespiSure (Pfizer, New York), which was also verified by testing positive in the ELISA with S/P values for anti-*M. hyopneumoniae* IgG in: 1.76 (female 275), 0.78 (female 350), 1.53 (female 933), and 1.06 (female 794). Although vaccination does not prevent infection, it was expected that not all females would become infected. The litters born to the four *M. hyopneumoniae*-positive females were randomly assigned to groups as follows: G1 (gilt number 275), G2 (gilt number 350), G3 (gilt number 933), and G4 (gilt number 794). Additionally, the litter of two negative females for *M. hyopneumoniae* composed a group of animals experimentally challenged against *M. hyopneumoniae.*

The first exposure of the females was performed about 20 days before the expected parturition (94 days of gestation) and the second 10 days before farrowing (104 days). At both times, the gilts were exposed to the lung homogenate through the nasal and tracheal routes. For that, the females were restrained using a rope and with the oral cavity opened using a mouth opener and a laryngoscope. In each exposure, 10 mL of inoculum were then administered directly into the trachea of the females using an adapted insemination pipette. After exposure through the tracheal route, nasal administration was performed with 5 mL in each nostril, instilling the volume gradually and keeping the female with her head elevated so that there was no extravasation of the administered volume.

About 24 h before farrowing, the females were submitted to oropharyngeal swab collection to determine their status for *M. hyopneumoniae* infection and define the groups. Swabs were collected and transferred to 2 mL plastic tubes containing PBS (pH 7.2) and then immediately processed for DNA extraction and subjected to qPCR for *M. hyopneumoniae* detection and quantification in the sample.

qPCR results indicated that of the six females challenged, four of them were positive 24 h before parturition. The four females gave birth on the same date. A total of 15 piglets were born in each of the litters from sows: 275, 350, and 933, while sow 794's litter consisted of 16 piglets. Litter standardization was not carried out, nor were transfers of piglets between sows.

Before the collection of samples and the definition of the formulations that each group would receive, all animals, from G1 to G4, were weighed, their temperature was measured, nasal swabs and blood were collected, and they were identified with earrings, defining a total of 15 piglets per group. The drawing of formulations among the different litters was carried out in the R Project software version 4.0.3 using the "sample" function. The vaccination protocols were randomly defined to each group as follows: G1: oral SBA15 + commercial vaccine at 24 days, G2: oral vaccine on the third day + commercial vaccine at 24 days, G3: commercial vaccine at 24 days, and G4: commercial vaccine + oral vaccine at 24 days.

4.5. Production of the Oral Vaccine

The oral vaccine production process followed the same protocols described by Mechler-Dreibi [16]. A pathogenic strain of *Mycoplasma hyopneumoniae* (232) was purchased from Iowa State University and certified free of other pathogens. Initially, a pre-culture was performed using 5 mL of Friis medium. Then, four flasks containing 100 mL were inoculated, with 1 mL of the previous culture kept at 37 °C in an oven. The procedure was performed twice to obtain all the necessary culture volume. The color was observed daily, which showed changes about seven days after inoculation. The flasks were removed from the oven when the culture medium turned orange. A volume of 700 mL of Friis medium with *M. hyopneumoniae* was obtained. The final volume was transferred to 50 mL falcon tubes and kept at −80 °C until processing. A sterility test was conducted on blood agar and McConkey media kept in the incubator at 37°C for three

days to evaluate the microbiological growth indicative of contamination. *M. hyopneumoniae* was also cultivated in a modified Friis Agar solid medium, and in seven days, it was possible to observe the presence of bacterial colonies.

The concentration of *M. hyopneumoniae* was determined through successive dilutions of the samples in Friis medium, varying from 10^{-1} to 10^{-7}. In addition to estimating the quantification of *M. hyopneumoniae* through successive dilutions, samples were submitted to qPCR. The results obtained from qPCR indicated concentrations between 1.8 x 10^6 and 1.3 × 10^7 copies/µL. The culture was centrifuged to form a bacterial cell pellet at 13,700× *g* for 45 min at 4 °C. Three washing steps with PBS (pH 7.4) were performed, and the *M. hyopneumoniae* was suspended in PBS. The suspension was subjected to a sonication process (Soni -tech Ultrasonic Cleaning) at a frequency of 20 Hz for 1 min, with one-minute intervals between processes. To determine the protein concentration in the bacterial lysate, the Bradford method was applied (Thermo Fisher Scientific, Rockford, Illinois, USA) followed by spectrophotometer reading (NanoDrop One, Thermofisher Scientific, USA), which provided a concentration of 1125 µg/ mL.

About seven days before the oral vaccination administration, the dry fraction of the vaccine was prepared, measuring the number of proteins suspended in PBS (pH 7.2). The dose used for the production of the oral vaccine was 200 µg per piglet, mixed with silica in the proportion 1:35 defined in previous studies [14,26]. *Mycoplasma hyopneumoniae* protein lysate was added to SBA-15, previously macerated and activated, and a drying step at 37 °C for 72 h was performed to obtain the dry fraction [16].

About two days prior to administration, the complete oral vaccine and SBA-15 compound in acidified PBS without *M. hyopneumoniae* antigens were prepared. For the production of the complete oral vaccine, the Eudragit® L30 D-55 polymer (Evonik) was added to the dry fraction consisting of SBA-15 + *M. hyopneumoniae* lysate, and the mixture was macerated until a paste was formed. After the addition of Eudragit®, a new drying step was carried out at 37 °C for 48 h until the content was completely dried. The dry antigen + silica fraction, coated with Eudragit®, was macerated again, and acidified PBS (pH 3.2) was added with 2M HCl. The addition of the acidifier was performed just moments before product administration to the animals. Acidified PBS (pH 3.2) was added, considering a final vaccine volume of 3 mL per individual. Before administration, the vaccine was vigorously shaken to ensure complete homogenization of the mixture in the bottle.

For the production of the compound without *M. hyopneumoniae* antigens, the silica was weighed on an analytical balance considering the same proportion of silica administered to animals that received the complete oral vaccine (7 mg per animal). Pure SBA-15 was macerated using a glass rod, and acidified PBS solution (pH 3.2) was added. In both the production of the complete oral vaccine and the pure SBA-15, the addition of the acidifier was performed just moments before administration to the animals. In both cases, acidified PBS (pH 3.2) was added, considering a final solution volume of 3 mL per individual. Before administration, the bottles containing the complete vaccine and the acidified SBA-15 without antigens were vigorously shaken for complete homogenization of the mixtures in the bottles.

4.6. Vaccination of Piglets

The administration of the oral vaccine and SBA-15 + acidified PBS was performed using a 5 mL syringe coupled to a urinary catheter 16 Fr cut with the aid of disinfected scissors. The probe size was reduced to three centimeters, enough to direct the content directly into the oral cavity of the animals, similar to the administration using a pump bottle dispenser.

The commercial vaccine used was Hyogen® (CEVA Santé Animale, Libourne Cedex, France), a mineral oil emulsion with non-toxic LPS from Escherichia coli J5 as an immunostimulant and inactivated *M. hyopneumoniae* BA 2940-99 field isolate as an antigen.

The commercial Hyogen® vaccine (batch: 005/21, manufacture: 4/21, and expiry date: 4/23) was applied following the manufacturer's guidelines, with 2 mL per individual being administered to all piglets in groups G1 to G4 by intramuscular route in the neck region using 25 × 0.8 mm disposable needles and 5 mL syringes. In G1 and G4, the application of the commercial vaccine was performed shortly after the application of the oral vaccine.

The vaccine injection site was evaluated beforehand and after 48 h. Regarding the volume administered intramuscularly, no extravasation of the vaccine was observed in any of the animals. After the oral vaccine and SBA-15 + acidified PBS application, the individuals remained under observation for about 1 h to observe any occurrence indicative of reflux or vomiting in these animals.

For three days, the piglets were evaluated for signs of diarrhea and submitted to body temperature measuring 48 h after the application of the commercial vaccine.

4.7. Assessments and Sample Collections in Piglets

Nasal swabs and blood samples were collected over time. Then, at D72, piglets were euthanized and necropsied. The animals were weighed at 3 days of life, 24 days, and 71 days. At three days old, the piglets received 2 mL of iron dextran solution (Ferdex, Fabiani Saúde Animal, São Paulo -SP) and Baycox ® 5% (Elanco, Brazil). Clinical assessments were carried out periodically and consisted of temperature measurement, evaluation of the general state of the animals (classified as good, regular, or bad), mucous membrane color, evaluation of the animals' behavior (normal, apathetic, or uncoordinated), evaluation of the occurrence of diarrhea (normal, mild diarrhea or severe diarrhea), and the presence of nasal or ocular discharge.

4.7.1. Blood Collection

At each time point, after the physical evaluation of the animals, they were submitted to the collection of blood samples by puncturing the jugular vein with sterile needles, size 25 × 0.8 mm, and deposited in vacuum tubes (red cap), free of anticoagulant and with clot activating factor and centrifuged at 3000 rpm for 10 min (Centrifuge 5804 R, Eppendorf ®, Germany). Blood serum was aliquoted in 1 mL fractions into 2 mL sterile microtubes in duplicate and stored in a −20 °C freezer and later analyzed for the detection and quantification of anti-*M. hyopneumoniae* IgG using the m.hyo Ab Test Kit (IDEXX Laboratories, Inc., Westbrook, Maine, USA, Lot: DT583; Expiration: 09/23/2022).

4.7.2. Nasal and Oropharyngeal Swab Collection

Nasal swab collections were performed using sterile cotton swabs. With the individuals properly restrained, the swab was lightly rubbed on the nasal mucosa of both nostrils. At the time of collection, contact with other external areas of the animal's nostril was avoided. Immediately after collection, the swabs were placed in sterile microtubes with a buffer solution (PBS—pH 7.2) and transferred to a freezer at −20 °C, where they were kept until the moment of analysis for IgA Ab detection using the indirect ELISA technique using the commercial M.hyo kit Ab. Test (IDEXX Laboratories, Inc., USA, Lot: DT583; Expiration: 09/23/2022) standardized [16].

Swabs were collected from the animals to confirm the presence or absence of *Mycoplasma hyopneumoniae* through the detection of the p102 gene. For collection, sterilized and individually wrapped plastic swabs with a cotton tip were used. With the animal physically restrained and with the aid of a laryngoscope with a light source, the oral cavity was opened. The lingual torus was pressed downwards to facilitate access to the oropharynx region, very close to the tonsils, and then the swabs were lightly rubbed in rotating movements on the swab stem so that the entire extremity had contact with the mucosa. Contact with other internal areas of the animal's mouth was avoided when introducing and removing the swab, and immediately after it was placed in a sterile

microtube, duly identified with the animal's number and time of collection, the swab stem was cut with the aid of clean scissors with 70° alcohol to each animal and stored at −20 °C. Oropharyngeal swab samples were subjected to DNA extraction by qPCR for the detection and quantification of *M. hyopneumoniae DNA.*

4.8. Nursery Phase and Sanitary Challenge

The piglets were weaned at 24 days of age, after which the sows were transferred, and the piglets from G1 to G4 were housed together in a pen. Two out of the six female litters that were not initially selected to join the study groups were separated at 24 days of age and placed in a separate nursery. About seven days after vaccinating groups 1 to 4 (at 31 days of age), 15 piglets from the two additional litters that did not belong to any study group were separated from the 60 vaccinated pigs and challenged with 10 mL of the same inoculum at a concentration of 10^5 copies/μL, administered intratracheally to pregnant sows. The challenged piglets were housed separately from those in G1 to G4.

Ten days after the challenge, at 41 dpi, the challenged animals (n = 15) were co-housed with the vaccinated animals, G1 to G4, in a single nursery pen, totaling 75 animals. The aim of placing infected animals together with those subjected to vaccination protocols was to simulate the natural transmission of the pathogen between piglets that were exposed experimentally to *Mhyo* (G1 to G4) and those that were not exposed through the air and nose-to-nose contact.

4.9. Euthanasia of Piglets, Assessment of Lungs, and Collection of Samples

At the end of the experimental period, at 71 days of age, all animals were euthanized. For euthanasia, the piglets received an intramuscular injection of acepromazine (5 mg/kg), intramuscular administration of xylazine and ketamine (5 mg/kg and 7 mg/kg, respectively), and intravenous administration of propofol (5 mg/kg). Finally, 2% lidocaine was injected intrathecally to promote cardiorespiratory arrest. The animals were then subjected to bleeding and necropsied to obtain the respiratory set (trachea + lung), which was subjected to bronchoalveolar fluid (BALF) collection. BALF collection was performed by introducing 20 mL of 1X PBS into the cranial portion of the trachea. After pouring all the liquid, the lung was lightly massaged, and the liquid was aspirated by pipette, recovering an approximate volume of 10 mL. The aspirate was transferred to 15 mL Falcon tubes, stored in a freezer at −80 °C, and aliquoted in duplicate in 2 mL graduated microtubes, free of dNases and rNases (Kasvi, Brazil), for qPCR analysis.

The lungs were evaluated for the presence and extent of lesions by a unique blinded investigator. The extent of lung lesions was quantified using the European Pharmacopoeia method, in which the percentage of each affected area of the lobe was multiplied by lobe relative weight and summed to provide the percentage of the total weight of the affected lung [27]. Lung tissue fragments for qPCR were obtained with individual scalpel blades and sterile tweezers. The tweezers were kept in boiling water between collections and previously disinfected using 70% alcohol. These fragments were collected in duplicate and transferred to dNase and RNAse-free plastic packages kept at −80 °C. Subsequently, the lung fragments were processed to standardize the weight and region from where the lung fragments were obtained and destined for extraction and qPCR.

4.10. Detection and Quantification of IgA and IgG Antibodies by ELISA

Detection and quantification analyses of antibodies (IgA and IgG) were performed in the nasal swab and blood serum, respectively. To detect serum IgG, the commercial kit M.hyo Ab test (IDEXX Laboratories, Inc., Westbrook, Maine, USA) was used. For the detection of IgA Ab in nasal swabs, an adaptation using the same commercial IgG detection kit was used as described previously [15]. For IgA detection, initially, the plates were blocked with 1.5% ovalbumin in PBS, followed by incubation at 37 °C for 30 min. Negative and positive samples of nasal swabs from a previous experimental infection

study were selected and homogenized in the form of a pool to compose a negative (CN) and a positive (CP) control that was aliquoted to be used in all ELISA plates. To detect IgA Ab in nasal swabs, 100 µL of the sample's liquid fraction were used, as the swabs were deposited in 500 µL of PBS, which were quickly homogenized in a vortex and placed, without further dilution, in each microplate well. The conjugate from the kit was replaced by an immunoenzymatic conjugate of goat anti-Pig IgA Antibody HRP Conjugated (Bethyl Laboratories Inc., USA) at a dilution of 1:500 using the diluent provided by the kit. At the end of the respective protocols, both the IgG and IgA analysis plates were read in a iMark microplate reader (Bio-Rad Laboratories Inc., Hercules, California, USA) at a wavelength of 650 nm.

The mean optical densities (OD) for each of the test samples were related to the OD found for the negative and positive controls (NC$\underline{x}$, PC$\underline{x}$) to calculate the S/P values (positive sample ratio) according to the formula: S/P = oDs−NC$\underline{x}$/PC$\underline{x}$−NC$\underline{x}$. The threshold between positive and negative samples was calculated from the value of S/P NC$\underline{x}$+ 2 × standard deviation. Serum samples and nasal swabs were considered positive if S/P > 0.3.

4.11. DNA Extraction from Nasal Swabs, Lung Samples, and BALF

DNA extraction was performed using the in-house Tris–- HCl protocol [25]. For nasal swabs and BALF samples, centrifugation (Centrifuge 5804 R, Eppendorf, Germany) at 13,000 × g at 4 °C for 20 min was performed before the DNA extraction protocol. For lung samples, 0.05 g of tissue were used. Extracted DNA was stored at −20 °C until qPCR analysis. The concentration of DNA present in the extraction product was evaluated utilizing spectrophotometry, with the aid of the Thermo Scientific NanoDrop 2000 (Thermo Fisher Scientific®, Waltham, Massachusetts, USA), with the exclusion factor being samples that did not reach a purity of 1.8 to 2.0 in the 260/280 ratio before performing the qPCR. To rule out the presence of inhibitors in the extracted DNA samples and the occurrence of false negatives in the qPCR for *M. hyopneumoniae,* all samples were subjected to a conventional PCR targeting the endogenous gene Glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) [28]. Amplified products of the 437 bp *gapdh* gene were detected after horizontal electrophoresis on a 1% agarose gel stained with ethidium bromide (0.5 µL/mL) in TEB running buffer pH 8.0 at a current of 90 V/ 50 mA for 90 min.

4.12. qPCR Analysis

Absolute real-time quantitative PCR analysis (qPCR) was used to detect *Mhyo* in oropharyngeal swab samples, lung fragments, and BALF. For *M. hyopneumoniae*, the primers used in the reaction were designed to the bacterium p102 adhesion protein gene sequence. All samples were tested in duplicate, and the qPCR analysis followed a previously optimized published protocol [29] adapted [30]. The nucleotide sequences used were forward primer 5″-AAGGGTCAAAGTCAAAGTC-3″, reverse primer 5″-AAATTAAAAAGTGTTCAAATGC-3″, and hydrolysis probe 5″-FAM-AACCAGTTTCCACTTCATCGCC-§BHQ2-3″ [29].

Only results in which the standard deviation between duplicates was less than or equal to 0.5 were accepted. Otherwise, samples were retested in triplicate. Sterile ultrapure water (Nuclease-Free Water, Promega®, Madison, Wisconsin, USA) q.s.p. was used as a negative control in the qPCR reactions. Serial dilutions were performed to determine the standard curve generated with different concentrations of synthetic DNA (gBlock®, IDT, Iowa City, IA, USA) containing the target sequence (10^7 copies/µL to 10^1 copies/µL), which were also used as positive controls. Synthetic DNA was diluted as per the manufacturer's recommendations and maintained at a stock concentration of 10^7 molecules/µL.

Quantification was performed using 10-fold serial dilutions (starting at 10^7 to 10^1 copies/µL) of synthetic DNA (gBlock ®, IDT, Iowa City, IA, USA) containing the 150 bp fragment amplified by the pair of primers used in qPCR. The quantification data based on

the standard curve were only validated if the reaction efficiency was between 90 and 105% [31].

5. Statistical Analysis

The variables in each group for each time point were assessed for normality and homoscedasticity by the Shapiro–Wilk and Bartlett tests, respectively. The difference among means was calculated by Tukey's test ($p < 0.05$). Variables that did not meet the assumptions were submitted to the non-parametric Kruskal–Wallis test ($p < 0.05$), and in cases where significance was observed, the Dunn test (Post hoc) was applied. The Friedman non-parametric test [32] and generalized linear mixed models (GLMMs) were used for multiple comparisons among the time points $p < 0.05$. For proportion analysis, differences between groups by date were calculated using Wilson's scoring interval method. Comparison between two paired measurements was performed using the Wilcoxon paired test. Values of measurements recorded over time and at euthanasia were subjected to a correlation analysis using the parametric Pearson and non-parametric Spearman tests, as well as Kendall analyses, to measure the association between the two variables. For this, the R software version 4.0.3 was used with the following packages: "agricolae" [33], "LmerTest" [34]; "arm" [35]; "Emmeans" [36]; "Car" [37]; " Nortest" [38]; "MASS" [39] with R software version 4.0.3.

6. Conclusions

The present study showed the positive effects of the administration of the oral vaccine, produced with SBA-15 carrier-adjuvant and strain 232 antigens associated with a standard intramuscular vaccination protocol, against a natural challenge with a field strain of *M. hyopneumoniae*. Higher levels of mucosal immunity and reduction of lung lesions at euthanasia were observed, being more evident results in animals that received the oral vaccine early. The application of the oral vaccine under field conditions can, even associated with the conventional vaccine protocol, provide better control of *M. hyopneumoniae* infection, substantially reducing the effects of infection in swine and possibly promoting better performance and quality of life results for pigs in commercial production environments.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1.

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Table S1. Means ± standard error of rectal temperature measurements recorded for groups 1 to 4 over the study period.

	Rectal temperature (ºC)[1]				
Day	G1	G2	G3	G4	P value
D3	38.96 ± 0.1	39.17 ± 0.08	38.99 ± 0.08	38.84 ± 0.07	6.02×10^{-2}
D4	39.01 ± 0.07	39.03 ± 0.05	39.06 ± 0.07[a]	38.8 ± 0.06[b]	1.53×10^{-2}
D5	38.90 ± 0.09	39.03 ± 0.06	39.21 ± 0.11	38.95 ± 0.09	9.55×10^{-2}
D10	39.28 ± 0.19	39.14 ± 0.1	38.99 ± 0.12	38.88 ± 0.15	2.44×10^{-1}
D17	37.95 ± 0.11[b]	38.17 ± 0.1	38.34 ± 0.11	38.39 ± 0.12[a]	3.05×10^{-2}
D24	37.39 ± 0.16[b]	38.31 ± 0.12[a]	38.11 ± 0.16[a]	37.93 ± 0.09[a]	1.27×10^{-4}
D26	37.85 ± 0.07[b]	38.29 ± 0.12[a]	38.12 ± 0.1	38.33 ± 0.09[a]	7.30×10^{-3}
D31	37.85 ± 0.13[b]	38.29 ± 0.21[a]	38.12 ± 0.16	38.33 ± 0.23[a]	9.40×10^{-1}
D41	37.10 ± 0.15	37.32 ± 0.19	37.01 ± 0.11	37.41 ± 0.19	4.55×10^{-1}
D51	38.32 ± 0.15	38.33 ± 0.14	38.35 ± 0.14	37.84 ± 0.23	1.23×10^{-1}
D61	38.76 ± 0.13	38.37 ± 0.08	38.54 ± 0.15	38.64 ± 0.15	2.56×10^{-1}
D71	38.29 ± 0.19	38.19 ± 0.16	37.98 ± 0.23	38.37 ± 0.16	4.56×10^{-1}

[1]Means followed by different letters in the same row differ significantly by Tukey's parametric test ($p < 0.05$).

Table S2. P-values from multiple comparisons between rectal temperature measurements recorded over time

Comparation	G1	G2	G3	G4
D3-D0	1.00[1]	1.00	1.00	1.00
D5-D0	1.00	1.00	1.00	1.00
D10-D0	1.00	1.00	1.00	1.00
D17-D0	9.88×10^{-3}	4.19×10^{-4}	1.50×10^{-1}	4.27×10^{-1}
D24-D0	9.16×10^{-6}	1.53×10^{-2}	4.61×10^{-3}	1.60×10^{-3}
D26-D0	6.35×10^{-3}	1.01×10^{-2}	6.46×10^{-3}	1.68×10^{-1}
D31-D0	3.85×10^{-4}	1.75×10^{-7}	4.40×10^{-6}	5.16×10^{-4}
D41-D0	1.72×10^{-7}	6.10×10^{-8}	5.82×10^{-10}	4.88×10^{-7}
D51-D0	6.14×10^{-1}	1.08×10^{-2}	8.14×10^{-2}	5.80×10^{-3}
D61-D0	1.00	1.53×10^{-2}	6.86×10^{-1}	9.53×10^{-1}
D71-D0	4.47×10^{-1}	1.99×10^{-3}	6.46×10^{-3}	2.67×10^{-1}
D5-D3	1.00	1.00	1.00	1.00
D10-D3	1.00	1.00	1.00	1.00
D17-D3	2.52×10^{-3}	1.45×10^{-3}	1.23×10^{-1}	5.95×10^{-1}
D24-D3	1.08×10^{-6}	3.68×10^{-2}	3.29×10^{-3}	3.95×10^{-3}
D26-D3	1.38×10^{-3}	2.42×10^{-2}	4.61×10^{-3}	2.80×10^{-1}
D31-D3	6.02×10^{-5}	1.61×10^{-6}	2.32×10^{-6}	1.50×10^{-3}
D41-D3	5.75×10^{-8}	2.54×10^{-7}	3.01×10^{-10}	1.07×10^{-5}
D51-D3	3.59×10^{-1}	2.58×10^{-2}	6.48×10^{-2}	1.41×10^{-2}
D61-D3	9.99×10^{-1}	3.68×10^{-2}	6.31×10^{-1}	9.87×10^{-1}
D71-D3	2.27×10^{-1}	6.07×10^{-3}	4.61×10^{-3}	4.09×10^{-1}
D10-D5	9.98×10^{-1}	1.00	9.99×10^{-1}	1.00
D17-D5	2.31×10^{-2}	9.52×10^{-4}	2.23×10^{-2}	3.27×10^{-1}

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D24-D5	3.55×10^{-5}	2.92×10^{-2}	2.57×10^{-4}	8.04×10^{-4}
D26-D5	1.46×10^{-2}	1.79×10^{-2}	4.03×10^{-4}	1.15×10^{-1}
D31-D5	1.19×10^{-3}	1.28×10^{-6}	1.76×10^{-7}	2.74×10^{-4}
D41-D5	1.08×10^{-6}	3.05×10^{-8}	3.17×10^{-12}	1.61×10^{-7}
D51-D5	7.69×10^{-1}	2.01×10^{-2}	9.96×10^{-3}	3.11×10^{-3}
D61-D5	1.00	2.92×10^{-2}	2.54×10^{-1}	9.08×10^{-1}
D71-D5	6.14×10^{-1}	4.39×10^{-3}	4.03×10^{-4}	1.90×10^{-1}
D17-D10	4.23×10^{-4}	5.58×10^{-4}	2.67×10^{-1}	6.49×10^{-1}
D24-D10	7.06×10^{-8}	1.79×10^{-2}	1.24×10^{-2}	5.40×10^{-3}
D26-D10	3.20×10^{-4}	1.19×10^{-2}	1.58×10^{-2}	3.27×10^{-1}
D31-D10	7.99×10^{-6}	2.57×10^{-6}	3.47×10^{-5}	2.02×10^{-3}
D41-D10	1.04×10^{-9}	4.13×10^{-8}	7.92×10^{-9}	4.92×10^{-6}
D51-D10	1.59×10^{-1}	1.29×10^{-2}	1.60×10^{-1}	1.88×10^{-2}
D61-D10	9.85×10^{-1}	1.79×10^{-2}	8.42×10^{-1}	9.93×10^{-1}
D71-D10	8.75×10^{-2}	2.91×10^{-3}	1.58×10^{-2}	4.64×10^{-1}
D24-D17	9.53×10^{-1}	9.99×10^{-1}	9.96×10^{-1}	8.00×10^{-1}
D26-D17	1.00	1.00	9.98×10^{-1}	1.00
D31-D17	1.00	9.85×10^{-1}	3.77×10^{-1}	6.32×10^{-1}
D41-D17	6.69×10^{-1}	8.40×10^{-1}	9.96×10^{-3}	5.14×10^{-2}
D51-D17	8.99×10^{-1}	1.00	1.00	9.40×10^{-1}
D61-D17	5.51×10^{-2}	9.99×10^{-1}	9.99×10^{-1}	9.99×10^{-1}
D71-D17	9.64×10^{-1}	1.00	9.98×10^{-1}	1.00
D26-D24	9.76×10^{-1}	1.00	1.00	9.68×10^{-1}
D31-D24	1.00	6.11×10^{-1}	9.69×10^{-1}	1.00
D41-D24	1.00	2.64×10^{-1}	2.40×10^{-1}	9.59×10^{-1}
D51-D24	8.75×10^{-2}	1.00	1.00	1.00
D61-D24	2.35×10^{-4}	1.00	7.38×10^{-1}	2.01×10^{-1}
D71-D24	1.59×10^{-1}	1.00	1.00	9.17×10^{-1}
D31-D26	1.00	7.00×10^{-1}	9.53×10^{-1}	8.98×10^{-1}
D41-D26	7.54×10^{-1}	3.40×10^{-1}	2.03×10^{-1}	1.79×10^{-1}
D51-D26	8.42×10^{-1}	1.00	1.00	9.97×10^{-1}
D61-D26	3.75×10^{-2}	1.00	7.86×10^{-1}	9.68×10^{-1}
D71-D26	9.33×10^{-1}	1.00	1.00	1.00
D41-D31	9.83×10^{-1}	1.00	9.80×10^{-1}	9.91×10^{-1}
D51-D31	4.10×10^{-1}	6.83×10^{-1}	5.39×10^{-1}	1.00
D61-D31	3.63×10^{-3}	6.11×10^{-1}	4.43×10^{-2}	1.07×10^{-1}
D71-D31	5.76×10^{-1}	9.07×10^{-1}	9.53×10^{-1}	8.00×10^{-1}
D51-D41	1.32×10^{-2}	3.24×10^{-1}	2.23×10^{-2}	8.40×10^{-1}
D61-D41	5.84×10^{-6}	2.64×10^{-1}	2.61×10^{-4}	1.58×10^{-3}
D71-D41	2.93×10^{-2}	6.11×10^{-1}	2.03×10^{-1}	1.07×10^{-1}
D61-D51	9.08×10^{-1}	1.00	9.95×10^{-1}	3.92×10^{-1}
D71-D51	1.00	1.00	1.00	9.85×10^{-1}
D71-D61	8.00×10^{-1}	1.00	7.86×10^{-1}	9.91×10^{-1}

[1] p-values for comparison between time-points by linear mixed effect model ($p < 0.05$).

Table S3. Medians ± standard error of median of S/P of IgG recorded for groups 1 to 4 over the study period.

S/P values for IgG anti - *M. hyopneumoniae*[1]					
Day	**G1**	**G2**	**G3**	**G4**	**P value**
D3	1.69 ± 0.03^{b}	0.6 ± 0.06^{d}	1.85 ± 0.04^{a}	1.02 ± 0.09^{c}	1.52×10^{-10}
D10	1.39 ± 0.03^{a}	0.39 ± 0.06^{b}	1.54 ± 0.03^{a}	0.78 ± 0.02^{b}	5.44×10^{-11}
D17	1.61 ± 0.13^{a}	0.4 ± 0.07^{b}	1.99 ± 0.06^{a}	0.79 ± 0.14^{b}	3.77×10^{-9}
D24	0.94 ± 0.06^{a}	0.1 ± 0.05^{b}	1.58 ± 0.09^{a}	0.41 ± 0.03^{b}	7.45×10^{-11}
D31	1.29 ± 0.08^{b}	0.17 ± 0.09^{c}	2.06 ± 0.09^{a}	0.65 ± 0.04^{c}	7.30×10^{-11}
D41	0.76 ± 0.06^{b}	-0.01 ± 0.03^{d}	1.14 ± 0.06^{a}	0.31 ± 0.02^{c}	1.31×10^{-10}
D51	0.52 ± 0.05^{b}	0.19 ± 0.05^{c}	0.86 ± 0.05^{a}	0.23 ± 0.06^{bc}	2.98×10^{-7}
D61	0.43 ± 0.03	0.31 ± 0.09^{b}	0.61 ± 0.05^{a}	0.22 ± 0.07^{b}	1.12×10^{-2}
D71	0.39 ± 0.07	0.48 ± 0.08	0.58 ± 0.06	0.38 ± 0.09	2.61×10^{-1}

[1] Medians followed by different letters on the same row differ significantly by Kruskal-Wallis test ($p < 0.05$).

Table S4. P-values from multiple comparisons between anti - *M. hyopneumoniae* IgG S/P measurements recorded over time

Comparisons	G1	G2	G3	G4
D71-D3	2.70×10^{-9}	8.98×10^{-1}	1.55×10^{-6}	1.66×10^{-3}
D71-D10	1.36×10^{-4}	9.99×10^{-1}	1.50×10^{-3}	1.16×10^{-1}
D71-D17	2.24×10^{-7}	1.00	3.00×10^{-10}	4.96×10^{-1}
D71-D24	4.53×10^{-2}	2.77×10^{-3}	5.85×10^{-3}	1.00
D71-D31	2.71×10^{-4}	1.60×10^{-1}	3.00×10^{-10}	8.71×10^{-1}
D71-D41	7.26×10^{-1}	2.25×10^{-6}	3.22×10^{-1}	8.98×10^{-1}
D71-D51	1.00	5.56×10^{-2}	9.69×10^{-1}	9.79×10^{-1}
D71-D61	1.00	9.69×10^{-1}	1.00	9.69×10^{-1}
D61-D3	2.70×10^{-9}	2.16×10^{-1}	3.99×10^{-7}	1.05×10^{-5}
D61-D10	1.36×10^{-4}	1.00	$^{x}10^{-3}$	2.79×10^{-3}
D61-D17	2.24×10^{-7}	9.91×10^{-1}	4.49×10^{-10}	3.72×10^{-2}
D61-D24	4.53×10^{-2}	1.15×10^{-1}	4.37×10^{-3}	9.91×10^{-1}
D61-D31	2.71×10^{-4}	8.40×10^{-1}	4.49×10^{-10}	1.86×10^{-1}
D61-D41	7.26×10^{-1}	8.59×10^{-4}	2.84×10^{-1}	1.00
D61-D51	1.00	5.90×10^{-1}	9.57×10^{-1}	1.00
D51-D3	1.51×10^{-7}	2.46×10^{-4}	2.12×10^{-4}	1.05×10^{-5}
D51-D10	9.04×10^{-4}	2.83×10^{-1}	8.11×10^{-2}	3.60×10^{-3}
D51-D17	4.72×10^{-6}	9.71×10^{-2}	1.05×10^{-6}	4.55×10^{-2}
D51-D24	1.36×10^{-1}	9.95×10^{-1}	1.86×10^{-1}	9.95×10^{-1}
D51-D31	1.51×10^{-3}	1.00	1.05×10^{-6}	2.16×10^{-1}
D51-D41	9.21×10^{-1}	3.62×10^{-1}	9.57×10^{-1}	1.00
D41-D3	8.11×10^{-5}	1.45×10^{-10}	3.04×10^{-2}	1.00×10^{-6}
D41-D10	9.64×10^{-2}	9.60×10^{-5}	7.26×10^{-1}	8.76×10^{-4}
D41-D17	2.54×10^{-3}	8.25×10^{-6}	7.58×10^{-4}	1.52×10^{-2}
D41-D24	8.98×10^{-1}	8.98×10^{-1}	8.98×10^{-1}	9.57×10^{-1}
D41-D31	1.36×10^{-1}	1.60×10^{-1}	7.58×10^{-4}	9.73×10^{-2}

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D31-D3	5.91×10^{-1}	1.57×10^{-3}	9.86×10^{-1}	1.86×10^{-1}
D31-D10	1.00	5.43×10^{-1}	2.16×10^{-1}	9.21×10^{-1}
D31-D17	9.57×10^{-1}	2.49×10^{-1}	1.00	1.00 -
D31-D24	9.21×10^{-1}	9.41×10^{-1}	9.66×10^{-2}	7.67×10^{-1}
D24-D3	3.04×10^{-2}	2.25×10^{-6}	6.37×10^{-1}	7.78×10^{-4}
D24-D10	8.71×10^{-1}	3.00×10^{-2}	1.00	6.75×10^{-2}
D24-D17	2.17×10^{-1}	5.78×10^{-3}	9.66×10^{-2}	3.63×10^{-1}
D17-D3	9.98×10^{-1}	8.05×10^{-1}	9.86×10^{-1}	5.43×10^{-1}
D17-D10	9.79×10^{-1}	1.00	2.16×10^{-1}	9.98×10^{-1}
D10-D3	6.82×10^{-1}	4.96×10^{-1}	8.40×10^{-1}	9.41×10^{-1}

[1] p-values for comparison between time-points by Friedman test ($p < 0.05$).

Table S5. Medians ± standard error of median of S/P for IgA recorded for groups 1 to 4 over the study period.

S/P values for IgA[1]					
Day	**G1**	**G2**	**G3**	**G4**	**P value**
D3	0.32 ± 0.1	0.17 ± 0.05	0.3 ± 0.06	0.34 ± 0.06	3.09×10^{-1}
D10	0.08 ± 0.03[a]	-0.03 ± 0.09[b]	0.12 ± 0.03[a]	-0.02 ± 0.03[b]	2.19×10^{-3}
D17	0 ± 0.02[b]	-0.02 ± 0.02[b]	0.03 ± 0.09	0.08 ± 0.08[a]	1.51×10^{-2}
D24	0.17 ± 0.13[a]	0 ± 0.05	-0.02 ± 0.05	-0.09 ± 0.04[b]	3.50×10^{-2}
D31	0.28 ± 0.05[b]	0.44 ± 0.06	0.53 ± 0.07[a]	0.29 ± 0.07[b]	3.31×10^{-3}
D41	0.28 ± 0.12	0.28 ± 0.14	0.4 ± 0.09	0.37 ± 0.13	6.38×10^{-1}
D51	0.61 ± 0.06	0.62 ± 0.07	0.54 ± 0.08	0.44 ± 0.15	5.11×10^{-1}
D61	0.91 ± 0.12[a]	0.99 ± 0.12[a]	0.59 ± 0.12[b]	0.97 ± 0.24	2.12×10^{-2}
D71	1.22 ± 0.09	1.08 ± 0.15	0.81 ± 0.1[b]	1.81 ± 0.23[a]	4.39×10^{-2}

[1] Medians followed by different letters on the same row differ significantly by Kruskal-Wallis test ($p < 0.05$).

Table S6. P-values from multiple comparisons between anti - *M. hyopneumoniae* IgA S/P measurements recorded over time

Comparisons	G1	G2	G3	G4
D71-D3	1.55×10^{-3}	4.44×10^{-4}	4.42×10^{-3}	8.14×10^{-2}
D71-D10	6.69×10^{-7}	1.83×10^{-6}	2.60×10^{-5}	1.48×10^{-9}
D71-D17	9.37×10^{-11}	2.12×10^{-6}	6.53×10^{-6}	6.32×10^{-6}
D71-D24	5.97×10^{-5}	1.27×10^{-5}	9.10×10^{-8}	1.08×10^{-10}
D71-D31	2.67×10^{-3}	1.86×10^{-1}	8.98×10^{-1}	4.60×10^{-3}
D71-D41	3.65×10^{-3}	1.86×10^{-1}	3.62×10^{-1}	2.16×10^{-1}
D71-D51	3.63×10^{-1}	9.79×10^{-1}	9.21×10^{-1}	5.90×10^{-1}
D71-D61	1.00	1.00	9.69×10^{-1}	9.79×10^{-1}
D61-D3	1.22×10^{-2}	2.13×10^{-4}	1.60×10^{-1}	6.37×10^{-1}
D61-D10	1.27×10^{-5}	2.12×10^{-6}	3.46×10^{-3}	2.83×10^{-6}
D61-D17	4.00×10^{-8}	1.64×10^{-7}	2.16×10^{-3}	1.61×10^{-3}
D61-D24	6.72×10^{-4}	1.60×10^{-5}	4.63×10^{-6}	2.28×10^{-6}
D61-D31	1.97×10^{-2}	1.16×10^{-1}	1.00	1.36×10^{-1}

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D61-D41	2.39×10^{-2}	1.16×10^{-1}	9.69×10^{-1}	8.71×10^{-1}
D61-D51	7.26×10^{-1}	9.41×10^{-1}	1.00	9.95×10^{-1}
D51-D3	6.82×10^{-1}	3.02×10^{-2}	2.48×10^{-1}	9.86×10^{-1}
D51-D10	2.95×10^{-2}	4.44×10^{-4}	7.41×10^{-3}	1.67×10^{-4}
D51-D17	1.82×10^{-4}	2.13×10^{-4}	4.42×10^{-3}	3.72×10^{-2}
D51-D24	2.16×10^{-1}	2.47×10^{-3}	2.60×10^{-5}	4.43×10^{-5}
D51-D31	7.67×10^{-1}	8.40×10^{-1}	1.00	6.37×10^{-1}
D51-D41	8.05×10^{-1}	8.40×10^{-1}	9.91×10^{-1}	1.00
D41-D3	1.00	7.26×10^{-1}	8.40×10^{-1}	1.00
D41-D10	7.67×10^{-1}	1.16×10^{-1}	1.36×10^{-1}	2.65×10^{-3}
D41-D17	8.06×10^{-2}	6.75×10^{-2}	9.66×10^{-2}	1.86×10^{-1}
D41-D24	9.91×10^{-1}	2.84×10^{-1}	1.47×10^{-3}	8.83×10^{-4}
D41-D31	1.00	1.00	9.95×10^{-1}	9.41×10^{-1}
D31-D3	1.00	7.26×10^{-1}	2.84×10^{-1}	9.95×10^{-1}
D31-D10	8.05×10^{-1}	1.16×10^{-1}	9.50×10^{-3}	1.60×10^{-1}
D31-D17	9.74×10^{-2}	6.75×10^{-2}	5.55×10^{-3}	9.22×10^{-1}
D31-D24	9.95×10^{-1}	2.84×10^{-1}	3.74×10^{-5}	8.14×10^{-2}
D24-D3	9.98×10^{-1}	9.99×10^{-1}	2.16×10^{-1}	4.60×10^{-3}
D24-D10	9.98×10^{-1}	1.00	9.21×10^{-1}	1.00
D24-D17	5.43×10^{-1}	1.00	9.57×10^{-1}	8.05×10^{-1}
D17-D3	1.36×10^{-1}	9.41×10^{-1}	9.21×10^{-1}	4.05×10^{-1}
D17-D10	9.41×10^{-1}	1.00	1.00	9.22×10^{-1}
D10-D3	8.71×10^{-1}	9.79×10^{-1}	9.57×10^{-1}	1.23×10^{-2}

[1] p-values for comparison between time-points by Friedman test ($p < 0.05$).