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Efficacy evaluation of a novel oral silica-based vaccine in inducing mucosal immunity against *Mycoplasma hyopneumoniae*

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ABSTRACT

Mycoplasma hyopneumoniae, the main etiological agent of Porcine Enzootic Pneumonia, is widely spread in swine production worldwide. Its prevention is of great interest for the productive system, since its colonization in the lung tissue leads to intense production losses. This study aimed to compare the *M. hyopneumoniae* shedding and acute-phase response in 30 pigs submitted to different vaccination protocols: an experimental oral vaccine using a nanostructured mesoporous silica (SBA-15) as adjuvant (n = 10); an intramuscular commercially available vaccine at 24 days of age (n = 10); and a control group (n = 10) following experimental challenge with *M. hyopneumoniae*. Laryngeal and nasal swabs were collected weekly and oral fluids were collected at 7, 10, 14, 17, 23, 28, 35, 42, and 49 days post-infection to monitor pathogen excretion by qPCR. Nasal swabs were also used to detect anti-*M. hyopneumoniae* IgA by ELISA. Blood samples were collected for monitoring acute phase proteins. The antibody response was observed in both immunized groups seven days after vaccination, while the control group became positive for this immunoglobulin at 4 weeks after challenge. Lung lesion score was similar in the immunized groups, and lower than that observed in the control. SBA-15-adjuvanted oral vaccine provided immunological response, decreased shedding of *M. hyopneumoniae* and led to mucosal protection confirmed by the reduced pulmonary lesions. This study provides useful data for future development of vaccines against *M. hyopneumoniae*.

1. Introduction

Constantly, new technologies have been used for the development of vaccines in Human Medicine, aiming an effectively results in treatment of diverse diseases. Nanoparticles are popular tools utilized to selectively deliver drugs for identification and treatment of disease. Since the development of the first ordered mesoporous silica in 1992 (Kresge et al., 1992), the number of its possible applications in Medicine, such as an efficient drug delivery and vaccine carrier, has increased considerably (Huang et al., 2014), especially in oral vaccines due the capacity of drug delivery transmucosal (Sreeharsha et al., 2022). The Mesoporous Santa Barbara Amorphous-15 (SBA-15) has several applications in oral vaccines (Hoseini et al., 2021). In Veterinary Medicine, few studies have shown the capacity of use this nanotechnology as carrier of antigens, especially in animal production (Mechler-Dreibi et al., 2021; Oliveira et al., 2022).

In this context, *Mycoplasma hyopneumoniae* is the main pathogen of Porcine Enzootic Pneumonia (PEP) and can affect swine of all ages. The involvement of the respiratory system with clinical signs and injuries that can range from mild to severe, lethargy, occasional sneezing, and increased respiratory rates and coughing are commonly observed in infected pigs. Affected lungs also show pneumonia lesions, characterized

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by cranioventral consolidation with diffuse loss of cilia caused by the adhesion of the pathogen, which can lead to immunosuppression, opening doors for secondary infections (Pieters and Maes, 2019).

As it is a disease that causes great economic losses in swine production due to decreased animal performance and higher costs with treatment (Pieters and Maes, 2019), better control and prevention are of great interest to the production system. Studies have shown the effectiveness of antibiotics, such as tetracycline, lincosamides, aminoglycosides, florfenicol, fluoroquinolones, and pleuromutilins against *M. hyopneumoniae*, with a reduction in lung lesions and clinical signs, in addition to improving animal performance (Maes et al., 2018). However, with the need to reduce the use of antimicrobials in pig farming (McEwen and Collignon, 2018), other control strategies are needed to minimize the damage caused by this pathogen.

M. hyopneumoniae induces innate and adaptive immune responses, which can prevent significant systemic spread of the organisms (Pieters and Maes, 2019). However, the immune system is unable to rapidly clear pulmonary airways infection, resulting in a prolonged localized inflammatory and cellular immune response, also responsible for the majority of gross and microscopic lesions (Pieters and Maes, 2019). As the immune response of pigs is not always successful in eliminating *M. hyopneumoniae* infection, it can persist for up to 254 days after infection, and carrier pigs can infect susceptible animals for up to 214 days (Pieters et al., 2009). Current vaccines have limitations, as they do not prevent adhesion and colonization of respiratory tract epithelia by the pathogen (Maes, 2014) and are develop using several technologies (Maes et al., 2021). Despite this, they are used worldwide, as they reduce clinical signs and lung lesions, improve performance, and decrease the microbial load in the respiratory tract (Meyns et al., 2006; Vranckx et al., 2012).

Mucosal immunity has the potential to control pathogens at their point of entry. Thus, it would be advantageous to develop vaccines that trigger a mucosal and systemic immune response rather than simply stimulating the systemic immune system (Wilson and Obradovic, 2015) which still a challenge in the role of *M. hyopneumoniae* vaccines (Maes et al., 2021). Several studies have been carried out to develop oral vaccine adjuvants that are resistant to biological solutions of different pH existing in the body and efficient in presenting antigens to the intestinal mucosa, as the ordered mesoporous silica (SBA-15), which can act as an adjuvant for this purpose (Scaramuzzi et al., 2016).

New technologies have been used for the development of vaccines against *M. hyopneumoniae* to improve the host's immune response and minimize economic losses for pig producers vaccines (Maes et al., 2021). An oral vaccine developed in partnership with USP (University of São Paulo), UNIFESP, and the Butantan Institute using a mesoporous silica, named SBA-15, as adjuvant, proved to be effective in reducing pulmonary consolidation lesions in slaughter pigs and might be a promising alternative for the control of this pathogen (Mechler-Dreibi et al., 2021). Therefore, the present study aimed to evaluate the efficacy of the newly developed oral vaccine in the SBA-15 adjuvant in reducing *M. hyopneumoniae* impacts in challenged piglets, having as a comparator an injectable commercial vaccine with good results in the field, which is widely used worldwide in pig farming.

2. Materials and methods

2.1. Animal selection and experimental design

Thirty weaned piglets (21 days old) with an average weight between 6 and 7 kg were purchased from a free-*M. hyopneumoniae* certified farm (Agroceres PIC, Minas Gerais, Brazil), where no vaccination against *Mycoplasma hyopneumoniae* is performed, and with a good sanitary status regarding respiratory diseases. The animals were housed until 65 days of age in nursery pens and then transferred to fattening pens, where they remained until 130 days of age. The piglets received feed according to the production stage, free of antibiotics and water *ad libitum*. All piglets were tested for anti-*M. hyopneumoniae* antibodies and bacterial shedding at arrival.

Both stables were located in the facilities of the Swine Medicine Laboratory at FCAV/Unesp (Jaboticabal/São Paulo), with appropriated isolation and biosafety standards. At the moment of the arrival, all piglets underwent blood collection to measure antibodies, and laryngeal swabs were collected to certify the absence of *M. hyopneumoniae*. The individual piglets were identified with numbered ear tags and were randomly distributed into three groups of 10 animals each.

The Commercial Vaccine (CV) group included piglets that were immunized at 24 days of age with an injectable commercial vaccine (M + PAC®, MSD Animal Health). The OV (Oral Vaccine) adjuvanted with SBA used in this work is described in the 2.2 topic, and the group was composed of piglets that received a dose of the oral vaccine at 24 days of age; and CONT (Control) included the non-immunized animals. After 49 days, all groups were challenged with a pathogenic strain of *M. hyopneumoniae* (strain 232). During 56 days after the challenge, oral fluid collections were performed every three days after challenge, while laryngeal and nasal swabs were weekly collected to assess the detection/shedding of *M. hyopneumoniae*.

All procedures performed in this experiment were approved by the Ethics Committee on the Use of Animals (CEUA) of FCAV/Unesp Jaboticabal, under the protocol number 005174/18.

2.2. Oral vaccine preparation

2.2.1. Cultivation and preparation of M. hyopneumoniae for immunization

A small fraction of a pathogenic strain of *M. hyopneumoniae* (232) imported from Iowa State University (Minion et al., 2004), certified free of any other respiratory pathogens was taken for cultivation in Friis medium. The process of culturing the pathogen and preparing the oral vaccine was carried out according to Mechler-Dreibi et al. (2021), and resulted in a culture concentration of 10^7 CCU/mL, which was observed upon serial dilutions. Sterility test was performed for both the Friis medium and the *M. hyopneumoniae* strain on blood agar and McConkey media, which remained for three days in an oven at 37 °C.

The contents of the culture flask were centrifuged in appropriate tubes, previously autoclaved, in an ultracentrifuge at 13,700*g* for 45 min. Bacterial cells were deposited at the bottom of the tubes, forming pellets, which were resuspended in 15 mL of PBS for washing. The tubes were centrifuged again at 21,000*g*, and this procedure was repeated twice until obtaining a clean pellet. Each pellet was finally resuspended in 10 mL of PBS and stored in sterile falcon tubes. Subsequently, this content was sonicated three consecutive times in a sonicator (Soni-tech Ultrasonic Cleaning) at a frequency of 20 Hz during one minute, with intervals of one minute between processes. An aliquot of the content was removed to confirm the presence of *M. hyopneumoniae* by qPCR, using specific primers and probes for the agent (described in 2.5 topic), and the analysis indicated positivity for the bacterium. To determine the protein concentration in the cell lysate, the Bradford method (ThermoFisher Scientific®, Wilming, USA) was used, followed by measuring on NanoDrop™ One Spectrophotometer (ThermoFisher Scientific®, Wilming, USA), within the Bradford method.

2.2.2. Oral vaccine production and immunization procedures

The oral vaccine was prepared according to Mechler-Dreibi et al. (2021), and was recently developed by our research group in partnership with important research centers in Brazil, such as USP, UNIFESP, and Butantan Institute,. For that, ordered mesoporous silica (OMS) with antigen encapsulation capacity was used. Aiming at the ability of the vaccine to cross the digestive tract for its absorption by the intestinal mucosa, the commercial polymer Eudragit® (Evonik Industries, Germany) was used to coat the molecule. This polymer resists the acidic environment of the stomach and when in contact with the basic medium, provides a slow release of the desired molecule (Mariano-Neto et al., 2014).

Briefly, 200 μg of antigen proteins adsorved in silica, coated with Eudragit®, was resuspended in 5 mL of acidified water (Selko pH, Throw Nutrition). The doses were individually supplied to the piglets through gavage to ensure that all animals received the same dose. The water was acidified to a pH of 4.5 to avoid early dissociation of the polymer and consequent unwanted release of the antigen in the stomach environment. The commercial vaccine was administered in the neck region, by intramuscular route.

2.3. Infectious challenge with M. hyopneumoniae and collection of biological samples

At 73 days of age, the piglets of all groups were individually challenged with that the same strain used for the oral vaccine. All animals were intratracheally inoculated with 5 mL of 10^6 CCU/mL of *M. hyopneumoniae* cells (strain 232). Challenge was performed with the aid of a sterile probe and a laryngoscope. Venous blood samples were collected three times: at 24 days of age (D0), one day after challenge with *M. hyopneumoniae*, and one week after challenge. Blood serum was obtained using sterile tubes with a clot activator, followed by centrifugation at 1500 ×*g* for 10 min. Blood serum was aliquoted and stored at −20 °C until further processing.

The oral fluid samples were collected weekly since the arrival of the piglets, and in ten moments post-infection, namely 0, 7, 10, 14, 17, 23, 28, 35, 42, and 49 dpi. To facilitate the collection of the oral fluid, each pen was equipped with cotton ropes (Ø = 10 mm). Each time, two ropes were left in the room for 20–30 min. Then, the lower parts of the ropes were individually inserted into an autoclaved plastic bag, in which the oral fluids were extracted by mechanical compression. The samples were transferred to 5 mL graduated tubes and stored at −20 °C, until testing by ELISA and qPCR. After challenge, samples of laryngeal and nasal swabs were collected weekly from all animals to obtain quantitative data on the *M. hyopneumoniae* dynamics of shedding by qPCR,). Upon animal containment, the swab samples were collected from soft friction of the cotton swab on the laryngeal and nasal mucosa. The samples were collected in duplicates. Then, the swabs were stored in graduated 2 mL plastic microtubes free of DNases and RNases containing 300 μL of Phosphate Buffered Saline (PBS), pH = 7.4, and kept at −80 °C until further testing.

2.4. DNA extraction and conventional PCR for the mammals-gapdh gene

Nasal and laryngeal swabs and oral fluid samples were centrifuged) at 13000 *g* at 4 °C for 20 min, followed by DNA extraction protocol by Tris-HCl (Kuramae-Izioka, 1997). DNA concentration was assessed through NanoDrop™ One Spectrophotometer (ThermoFisher Scientific®, Wilming, USA), and, to acceptance, all samples had to reach the purity of 1.8 to 2.0 in the 260/280 ratio. All samples were submitted to a conventional PCR targeting the endogenous Glyceraldehyde-3-phosphate dehydrogenase gene (gapdh) (Birkenheuer et al., 2003) to rule out the presence of inhibitors in the extracted DNA samples and the occurrence of false negatives in qPCR for *M. hyopneumoniae*.

2.5. Detection and quantification of M. hyopneumoniae by qPCR based on the p102 gene in nasal and laryngeal swabs and oral fluid samples

Nasal swab samples were tested by qualitative real-time PCR, while for laryngeal swabs and oral fluid samples, quantification was performed by qPCR. The qPCR reaction was optimized from a previously published protocol, as described by Almeida et al. (2020). The primer pairs used in the reaction were based on *M. hyopneumoniae* p102 adhesion protein gene sequence (forward 5'-AAGGGTCAAAGTCAAAGTC-3′; reverse 5′- AAATTAAAAGCTGTTCAAATGC-3′; and hydrolysis probe 5'-FAM-AACCAGTTTCCACTTCATCGCC-BHQ2–3′).

The amplification reaction was performed in a CFX 96 Thermocycler (BioRad Laboratories, California, USA). Ten-fold dilutions were used to determine the different concentrations of synthetic DNA in the Gblock (IDT, USA) containing the target sequence (10^7 copies/μL to 10^1 copies/μL), which were also used as positive controls. The amplification efficiency was calculated from the slope of the standard curve in each run, following the established guidelines proposed by the Minimum Information for Publication of Quantitative real-time PCR Experiments (MIQE) (Bustin et al., 2009). As negative control, sterile ultrapure water (Nuclease-Free Water, Promega®, Madison, Wisconsin, USA) was used.

2.6. Detection and quantification of IgA antibodies by enzime-linked immunosorbent assay

For the detection of IgA antibodies in nasal swabs, a standardization was performed using the sensitized plates and components of *M.hyo Ab test* (Idexx, USA) with modifications, as described by Mechler-Dreibi et al. (2021). Briefly, ELISA plates were blocked with 1.5% ovalbumin in PBS. To detect IgA antibodies in nasal swabs, the sample liquid fraction (100 μL) was 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 HRP-conjugated goat anti-pig IgA (Bethyl Laboratories Inc., USA), at a dilution of 1:500 using the diluent provided by the kit, followed by incubation for 60 min at room temperature. The washing processes and all the following steps were performed according to the protocol of the kit *M.hyo Ab test* (Idexx, USA). The mean optical densities (OD) for each of the test samples (ODs) were related with the OD found for the negative and positive controls ($\overline{NC}$; $\overline{PC}$) in order to calculate the S/*P* values (sample/positive ratio) according to the formula: $S/P = (OD_{sample} - \overline{NC})/(\overline{PC} - \overline{NC})$. The threshold between positive and negative samples was calculated from the value of S/P $\overline{NC} + 3\times$ standard deviation. Nasal swab samples were considered positive if $S/P > 0.4$.

2.7. Total protein concentration and Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

The serum concentration of total proteins was determined by the biuret method in a semiautomatic spectrophotometer (Labquest, Labtest Diagnóstica, Lagoa Santa, Minas Gerais, Brazil), using a set of commercial reagents (Labtest Diagnóstica, Lagoa Santa, Minas Gerais, Brazil).

Protein fractionation was performed using the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), according to the protocol described by Laemmli (1970). After fractionation, the gel was stained for 10 min in a 0.25% coomassie blue solution and detained in a 7% acetic acid solution to remove excess dye until the protein fractions were clear. The concentrations of these proteins were determined in a computerized densitometer (Shimadzu CS-9301 PC, Tokyo, Japan). As a reference, we used a marker solution (Sigma, St Louis, MO, USA) with different molecular weights. Besides, the purified proteins transferrin, haptoglobin, ceruloplasmin, α1-antitrypsin, and porcine IgG, were also used as markers.

2.8. Lung lesion scoring at slaughter

At the end of the sampling period, all piglets were euthanized with an intramuscular administration of a combination of ketamine and xylazine (6 mg/kg and 4 mg/kg, respectively), followed by intravenous administration of saturated potassium chloride solution (approved by Federal Council of Veterinary Medicine, Brazil). The lungs were evaluated by a blinded investigator, and the extent of the consolidation lung lesions were classified according to the European Pharmacopeia method, in which the percentage of each lobe affected area is multiplied by the lobe relative weight and summed to provide the total weight percentage of affected lung (Ph. Eur, 2013).

2.9. Statical analysis

For the data obtained in the analysis of oral fluids and laryngeal swabs, basic descriptive averages were obtained, such as mean, standard deviation and standard error of the mean, considering all observations of each group throughout the study. The assumptions for carrying out the parametric tests of difference between the means were then verified. The data were transformed to meet the assumptions of normality and homoscedasticity, calculated by the Shapiro Wilk and Bartlett tests, respectively. Parametric tests were performed to evaluate differences between means, through multiple comparisons using Tukey's test at the level of $p < 0.05$.

The analyzes between the log10 values of the quantification averages of laryngeal swabs over time were performed by the linear mixed effects model (linear mixed effect model) in the R program using the packages: "lmerTest", "arm". The difference between the means (*post hoc*) was calculated by the last mean square ("last square mean") adjusted by the Tukey method.

The results of acute phase protein were submitted to descriptive statistical analysis; the values obtained, expressed as mean and standard deviation, were analyzed by the analysis of variance (ANOVA), using the Tukey test for comparison between the means, after checking the homogeneity of the samples. The significance level was checked at 5% probability.

3. Results

3.1. Laryngeal and nasal swab samples

At 7 dpi, it was possible to detect *M. hyopneumoniae* DNA in at least 70% of the laryngeal swab samples in all groups (Table 1). At 14 dpi, samples from all animals in the CONT group were positive for the pathogen, while for the immunized groups, 90% of the animals were positive. At 21 dpi, 100% of the animals in the OV and CONT groups were positive, which only occurred with the CV group at 28 dpi. All animals from all groups had positive samples until the end of the experimental period, with the exception of the OV group at 49 dpi, which had 50% of positive piglets (Table 1).

The highest estimate of statistically significant bacterial load in the CV group was at 21 dpi (1.17×10^4 copies/μL), while the lowest load was observed at 7 dpi. In the OV group, the highest load occurred at 28 dpi (2.47×10^4 copies/μL), and lower loads were observed at 7 and 49 dpi. In the CONT group, the highest bacterial load occurred later, at 42dpi (7.81×10^4 copies/μL), although it was not statistically different (Fig. 1). The lowest bacterial load detected in this group occurred at 7 dpi and was statistically different from all the other time points. At 49 dpi, the bacterial load in the CONT was statistically higher than the CV and OV groups (Tukey's test, $p < 0.05$), the latter being similar to each other (Fig. 1). It was also noted that at 14 dpi, 21 dpi, 35 dpi, and 42 dpi, the mean bacterial load detected in the laryngeal swab samples in the CV and OV groups remained lower than that observed in the CONT, but no statistically significant difference was observed.

Quantification of *M. hyopneumoniae* in nasal swab samples could not be evaluated due to the Monte Carlo effect (Bustin et al., 2009), which is related to the low concentration of the target DNA in the samples. This results in large variation in Cq values between duplicates, leading to inaccurate quantification values (Bustin et al., 2009). Qualitative assessment of nasal swab samples showed that at 7 dpi piglets were shedding the pathogen through the nasal route in all groups (Table 1), which was also observed in the laryngeal swab samples. However, the number of positive animals at this time was lower than the number of positive animals in the laryngeal swab samples. Excretion was fluctuating in all groups, and only at 56 dpi, 100% of shedding was observed. There were no significant differences between groups (Tukey test $p < 0.05$).

3.2. Oral fluid samples

The quantification of *M. hyopneumoniae* DNA by qPCR in oral fluid samples showed that in the CV group, there were positive animals at all collection dates, except at 17 dpi, while the OV group had negative samples on 7, 10, and 14 dpi. The CONT group was negative only at the 10 dpi (Table 2; Fig. 2). The highest estimated concentration of *M. hyopneumoniae* in the CONT group was at 23 dpi (3.22×10^5 copies/μL), when the CV and OV groups showed a mean estimate value of 2.89×10^1 copies/μL and 3.17×10^3 copies/μL, respectively. Despite the numerical difference, there was no statistical difference between the groups.

3.3. Detection of IgA and-M. hyopneumoniae antibodies in nasal swabs

The IgA antibody response in nasal swabs of vaccinated piglets were observed initially at 14 days post immunization, while the control group only became positive for IgA antibodies 28 days post infection. Considering that this was a relative analysis, vaccinated groups were not compared to each other, but to the CONT group individually. The IgA responses of CV and OI at D42 and D49 were statistically different from CONT (*). At 7dpi, 21dpi and 35dpi, CV and OV were different from from CONT(**). At 28dpi, CV was different CONT, but OV did not differ from CONT (***). From 42dpi onwards, no differences were found between experimental groups (Fig. 3).

3.4. Acute-phase proteins

The SDS-PAGE technique enabled the detection of 23 proteins, whose molecular weights ranged from 22.000 Da to 306.000 Da. Of

Table 1
Percentage of *Mycoplasma hyopneumoniae* positive animals in nasal and laryngeal swab samples by qPCR from 7 to 56-day post experimental infection (dpi) by this pathogen.

	7 dpi		14 dpi		21 dpi		28 dpi	
	NS	LS	NS	LS	NS	LS	NS	LS
CV	20%	80%	70%	80%	70%	90%	80%	100%
OV	40%	70%	90%	80%	40%	100%	40%	100%
CONT	50%	70%	60%	100%	50%	100%	40%	100%

	35 dpi		42 dpi		49 dpi		56 dpi	
	NS	LS	NS	LS	NS	LS	NS	LS
CV	100%	100%	40%	100%	80%	100%	100%	100%
OV	60%	100%	60%	100%	80%	50%	100%	100%
CONT	100%	100%	100%	100%	100%	100%	100%	100%

*NS: Nasal swab samples; LS: Laryngeal swab samples.

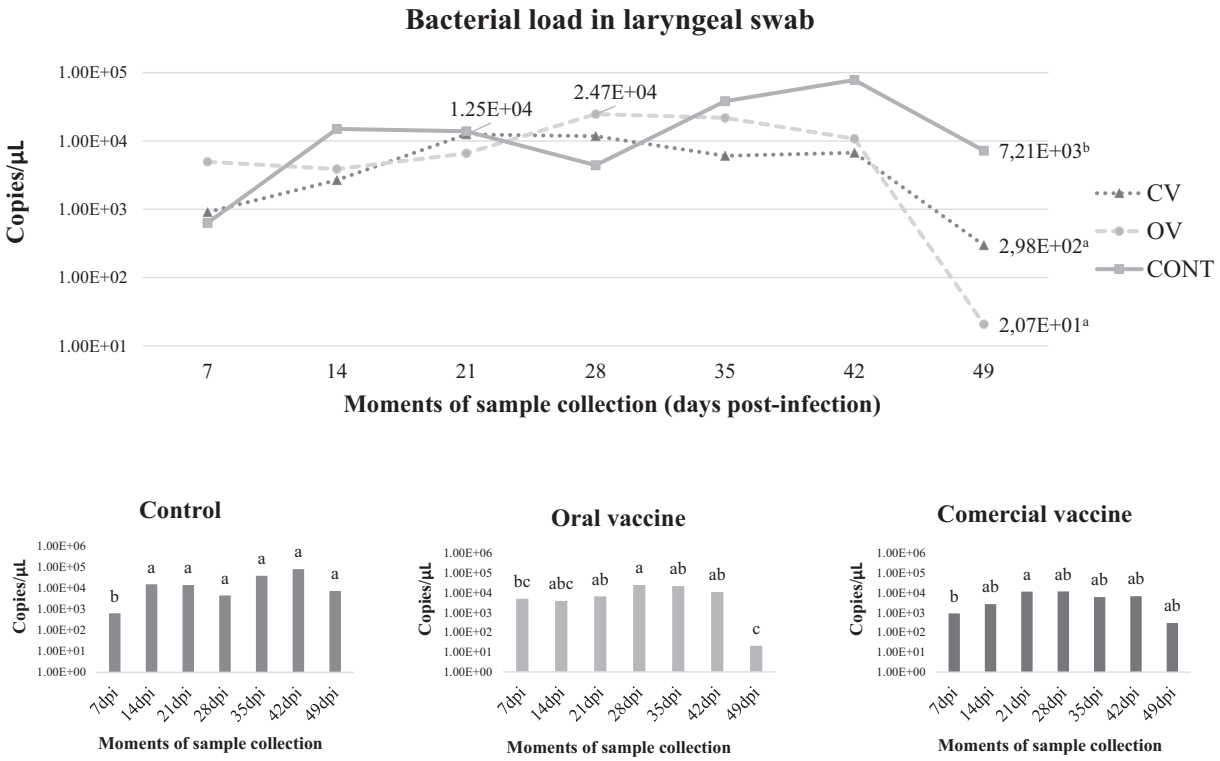

Fig. 1. Bacterial load detected in laryngeal swabs of piglets immunized against *Mycoplasma hyopneumoniae* with a comercial vaccine (CV), an experimental oral vaccine (OV) and a non vaccinated group in moments post infectious challenge with *Mycoplasma hyopneumoniae* strain 232.

Table 2
Detection of *Mycoplasma hyopneumoniae* in oral fluid samples, from each group, collected from the day of infection to 49-day post-infection (dpi).

	0 dpi	7 dpi	10 dpi	14 dpi	17 dpi
CV	–	+	+	+	–
OV	–	–	–	–	+
CONT	–	+	–	+	+

	23 dpi	28 dpi	35 dpi	42 dpi	49 dpi
OV	+	+	+	+	+
OV	+	+	+	+	+
CONT	+	+	+	+	+

CV: Comercial vaccine; OV: Oral vaccine; CONT: Control group.

these, ceruloplasmin (113.000 Da), transferrin (77.000 Da), albumin (66.000 Da), α1-antitrypsin (55.000 Da), haptoglobin (43.000 Da), α1-acid glycoprotein (38.000 Da), nominally unidentified protein of Molecular Weight 23.000 Da (MW 23.000 Da) were evaluated for their diagnostic importance. The results of total proteins concentration and the seven protein fractions analyzed are shown in Table 3.

In all groups, except in G1, there was a significant difference between the post-challenge period (D509 and D56) and D0 ($p < 0.05$; Table 3). No significant differences were observed in the serum concentration of total proteins between the experimental groups at different times. The serum concentration of ceruloplasmin, transferrin, albumin, α1-antitrypsin, haptoglobin, α1-acid glycoprotein and MW 23,000 proteins were evaluated according to the sampling times and experimental groups.

An increase in the concentration of transferrin in all groups was observed. Significant differences were noted in the serum concentration of this protein between the groups at D56 (Table 3). CV groups had a concentration of 353(±34.9) mg/dL; OV piglets showed 432(±23.2) mg/dL, while in CONT the concentration was 363(±34.0) mg/dL. All groups showed significant differences at D50 and D56 compared to D0. Additionally, OV was statistically different from CV. Serum α1-antitrypsin concentration was significantly higher in CONT at D509 (264 mg/dL) than D0 and was numerically lower in CV and OV at D56 than the other moments of sampling (156 mg/dL, 169 mg/dL, respectively), but with no statistical difference. Regarding α1-acid glycoprotein, there was an increase in concentration in OV at D509 (8.58 mg/dL), and CONT at D50 (8.85 mg/dL) and D56 (10.2 mg/dL) (Table 3). The concentratiosn of ceruloplasmin, albumin, and haptoglobin were not statistically different between the groups and time points. Numerically, ceruloplasmin occurred in lower concentration in CV at D56 (64.3 mg/dL) (Table 3). There was a higher concentration of albumin in group OV at D56 (3668 mg/dL). At D50, there was a higher concentration of haptoglobin in OV (53.1 mg/dL). At D56, there was a higher concentration of haptoglobin in OV (52 0.8 mg/dL). MW 23.000 presented similar response in all groups and all moments of sampling.

3.5. Lung score score

Macroscopic lung consolidation lesions were observed in the apical and cardiac lobes, cranio-ventral portions of diaphragmatic lobe, and in some cases in portions of the intermediate lobe. Both immunized groups had statistically lower lung lesion score when comparing with the control (Dunn test, $p < 0.05$). CV group had a 5.0% of average percentual of lung lesion consolidation score, OV had 4.8%, and the control had 25.6% (Fig. 4). No significant differences were observed between the immunized groups.

4. Discussion

Control and prevention of Porcine Enzootic Pneumonia are related to

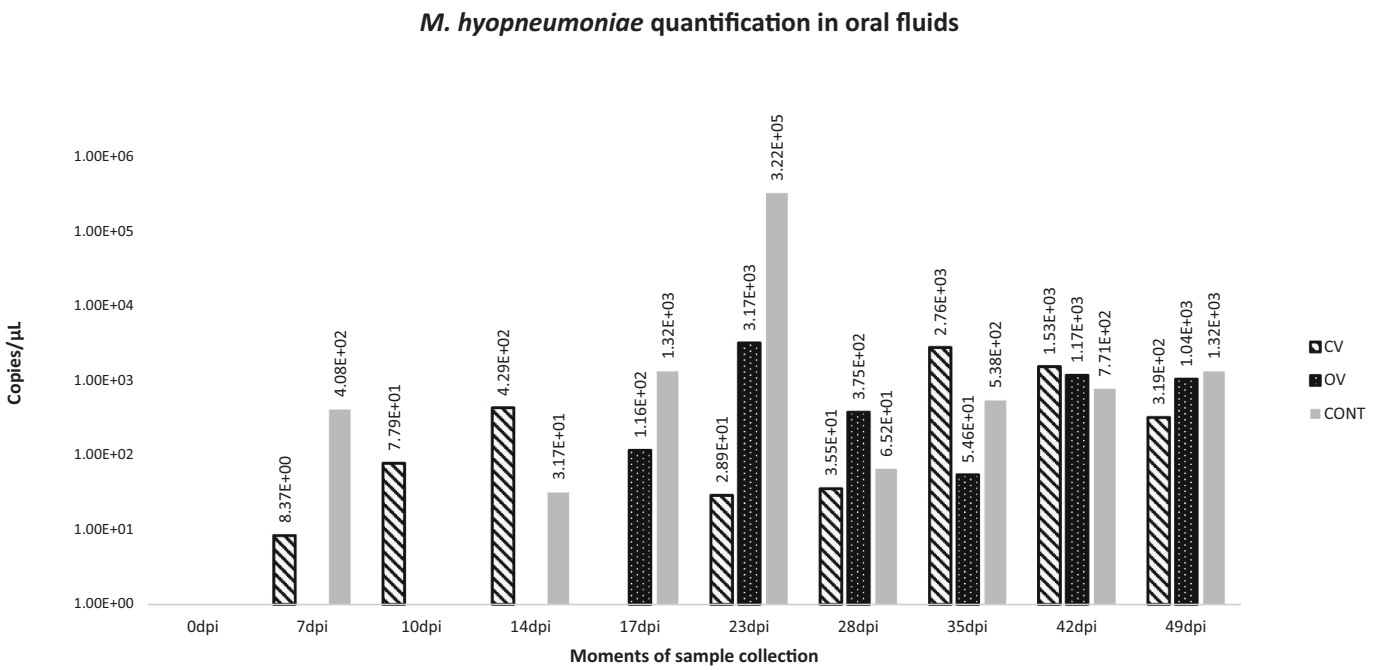

Fig. 2. Fig. 1. Bacterial load detected in oral fluids of piglets immunized against *Mycoplasma hyopneumoniae* with a comercial vaccine (CV), an experimental oral vaccine (OV) and a non vaccinated group in moments post infectious challenge with *Mycoplasma hyopneumoniae* strain 232.

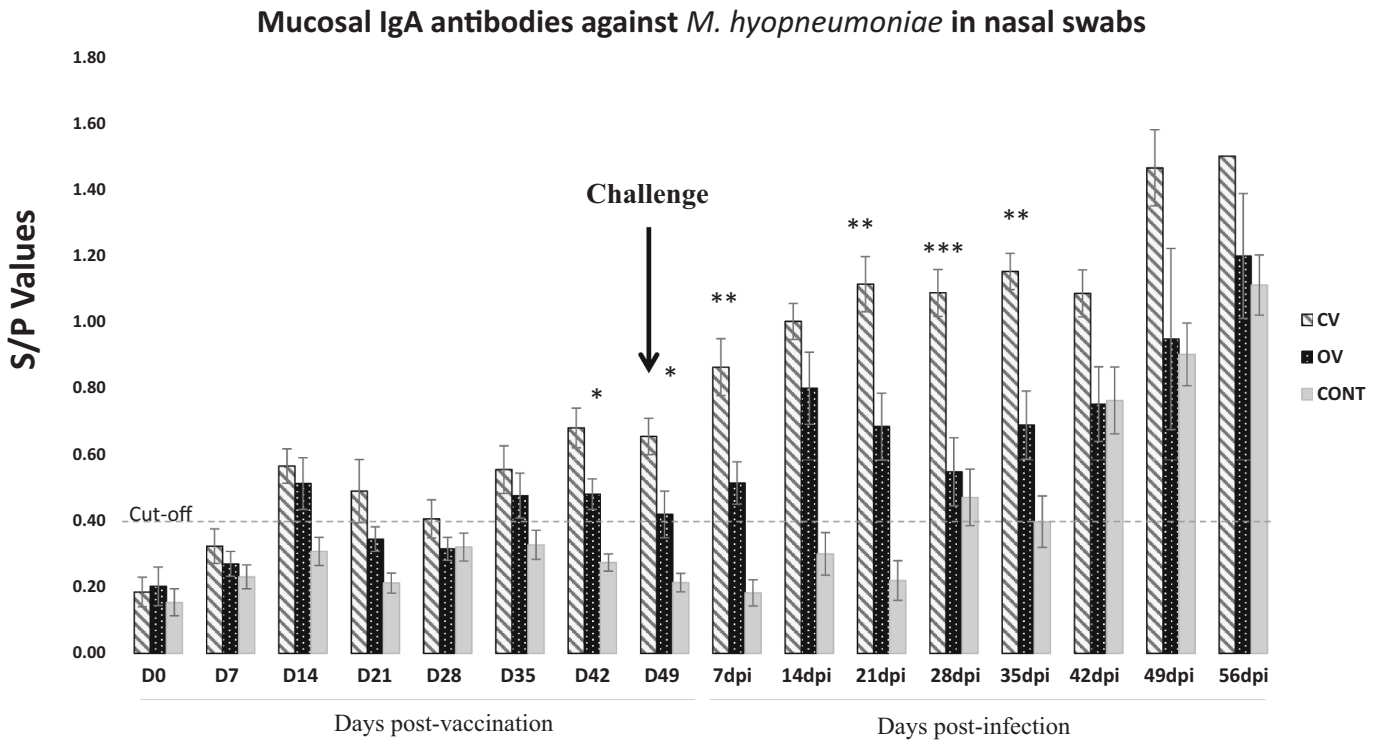

Fig. 3. Mucosal IgA antibodies detected in nasal swabs of piglets immunized against *Mycoplasma hyopneumoniae* with a comercial vaccine (CV), an experimental oral vaccine (OV) and a non vaccinated group in moments post infectious challenge with *Mycoplasma hyopneumoniae* strain 232.
The IgA responses of CV and OI at D42 and D49 were statistically different from the control group (*). At 7dpi, 21dpi and 35dpi, CV and OV were different from CONT(**).At 28dpi, CV was different CONT, but OV did not differ from CONT (***). From 42dpi onwards, no differences were found between experimental groups.

the ability to immunize pig herds and minimize the transmission of the pathogen and the occurrence of clinical signs. In the present study, an oral vaccine recently developed by our research group in partnership with important research centers in Brazil, such as USP, UNIFESP, and Butantan Institute, was used to evaluate the shedding of piglets experimentally infected with *M. hyopneumoniae* by analyzing samples of laryngeal and nasal swabs, and oral fluids. A commercial intramuscular vaccine was used as main comparison method since it is the main control method used in swine herds. A group of animals that did not receive immunization was used as a positive control of infection. Although we

Table 3
Mean ± standard deviation of serum concentrations of total proteins and protein fractions from piglets immunized with the different vaccine protocols. CV: piglets immunized with commercial; OV: piglets immunized with the oral vaccine; CONT: piglets not immunized. D0: Vaccination Day; D50: one day after the *M. hyopneumoniae* challenge; and D56: one week after the challenge.

	Groups				Groups		
	CV	OV	Cont		CV	OV	Cont
Total proteins (g/dL)				α1-antitrypsin (mg/dL)			
D0	5.02 ± 0.38Aa	4.86 ± 0.32Aa	4.71 ± 0.14Aa	D0	166 ± 22.8Aa	122 ± 19.9Aa	157 ± 18.6Aa
D50	5.49 ± 0.25Aa	5.89 ± 0.49Ab	5.75 ± 0.70Ab	D49	225 ± 41.7Aa	209 ± 56.2Aa	264 ± 82.3Ab
D56	5.39 ± 0.39Aa	6.06 ± 0.31Ab	5.96 ± 0.40Ab	D56	156 ± 38.5Aa	169 ± 37.2Aa	223 ± 83.2Aab
Ceruloplasmin (mg/dL)				Haptoglobin (mg/dL)			
D0	65.6 ± 12.0Aa	74.6 ± 16.9Aa	82.5 ± 24.2Aa	D0	32.0 ± 23.4Aa	22.8 ± 10.4Aa	27.1 ± 25.2Aa
D50	77.3 ± 5.25Aa	94.4 ± 41.3Aa	93.3 ± 43.1Aa	D50	24.7 ± 12.7Aa	53.1 ± 49.9Aa	24.3 ± 15.1Aa
D56	64.3 ± 12.4Aa	105 ± 50.7Aa	87.3 ± 30.6Aa	D56	29.0 ± 8.25Aa	52.8 ± 66.8Aa	39.7 ± 28.1Aa
Transferrin (mg/dL)				α1-acid glycoprotein (mg/dL)			
D0	331 ± 25.9Aa	308 ± 38.6Aa	316 ± 42.3Aa	D0	4.90 ± 0.88Aa	4.26 ± 1.39Aa	4.30 ± 0.99Aa
D50	417 ± 24.7Ab	426 ± 37.2Ab	391 ± 47.6Ab	D50	6.73 ± 1.28Aa	8.58 ± 2.39Ab	8.85 ± 0.94Ab
D56	353 ± 34.9Aa	432 ± 23.2Bb	363 ± 34.0Aab	D56	7.82 ± 1.62Aa	6.87 ± 1.85Aab	10.2 ± 1.855Ab
Albumin (mg/dL)				MW 23,000 (mg/dL)			
D0	3.531 ± 395Aa	3460 ± 378Aa	3104 ± 164Aa	D0	155 ± 29.5Aa	134 ± 13.6Aa	151 ± 22.6Aa
D50	3344 ± 177Aa	3481 ± 614Aa	3067 ± 343Aa	D50	189 ± 40.4Aa	167 ± 34.8Aa	175 ± 29.9Aa
D56	3362 ± 383Aa	3668 ± 535Aa	3.261 ± 290Aa	D56	214 ± 44.6Aa	195 ± 28.8Aa	205 ± 79.9Aa

*Means followed by equal uppercase letters in the same row and equal lowercase letters in the same column did not differ by Tukey's test ($p > 0.05$).
CV: piglets immunized with a commercial vaccine; OV: piglets immunized with the oral vaccine based on the 232 strain.

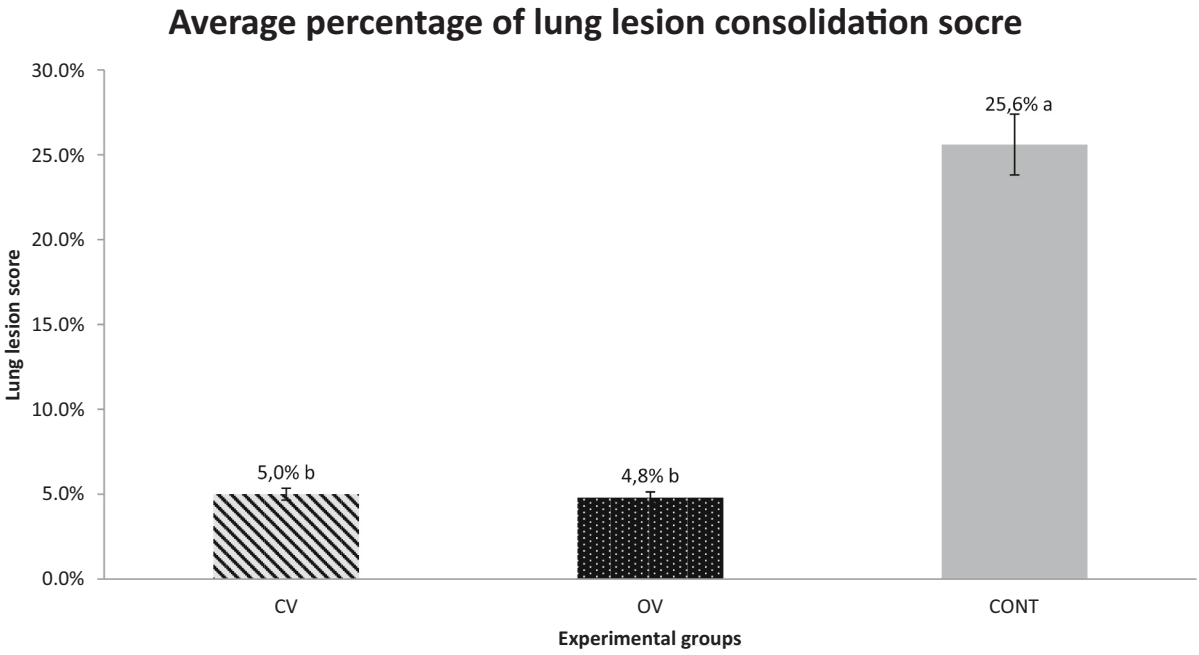

Fig. 4. Average percentage of lung lesion consolidation score from piglets immunized against *Mycoplasma hyopneumoniae* with a comercial vaccine (CV), an experimental oral vaccine (OV) and a non vaccinated group 56 days post infectious challenge with *Mycoplasma hyopneumoniae* strain 232.

have used vaccines from different administration routes, we chose an intramuscular administered vaccine as a parameter due to its wide use and the fact that oral and nasal vaccines against this pathogen remain under development and are not commercially available. Furthermore, most studies using oral vaccines did not perform an infection challenge with *M. hyopneumoniae*, which limits the comparison of our results.

De Conti et al. (2022) showed that animals from a farm that promoted mass vaccination against *M. hyopneumoniae* showed more antibodies against the pathogen than animals from a farm that did not adopt this vaccination strategy. The effect was also evident in the litters, indicating that vaccination plays an important role in the disease control. In the present study, the piglets were acquired from a free-*M. hyopneumoniae* farm, and there was no influence of the maternally-derived immunity in the results of this study.

With regard to the sensitivity of each type of sample collected, it was noted that, as previously observed (Pieters et al., 2017), laryngeal swab samples had the highest sensitivity for detecting the pathogen, while the oral fluids and nasal samples had the lowest sensitivity in the qPCR based on the p102 gene, asthese findings also shown that *M. hyopneumoniae* is more accurately detected in samples collected from the lower respiratory tract; consequently, lower Cq values were obtained from deep tracheal swabs than from nasal and tonsil swab samples (Fablet et al., 2010). Unfortunately, the Monte Carlo effect (Bustin et al., 2009) is an inherent limitation of the qPCR technique commonly seen in samples with a low target DNA copy concentration. In such samples, quantification cannot be accurately performed. Thus, it is likely that this effect prevented us from properly quantify *M. hyopneumoniae* shedding in nasal swab samples.

Under natural conditions, shedding duration and dynamics are a challenge to be understood, as the pathogen may be present at low concentrations in the upper respiratory tract of pigs (Sibila et al., 2009), and its shedding can be irregular and inconsistent (Fano et al., 2005). This fluctuant shedding may be the reason why piglets experimentally infected and proven positive for *M. hyopneumoniae* in the laryngeal swab were negative in the nasal swab at some points in the present study. Under experimentally conditions (Almeida et al., 2020), *M. hypneumoniae* strain 232 had higher loads at 28 dpi (4.6×10^3 copies/μL) for cranial trachea, and 2.0×10^5 copies/μL at 56 dpi for medium and lower trachea samples. In our study, the group orally immunized showed, at all sampling points (except for the 14dpi), a smaller number of piglets shedding the bacteria by the nasal route when compared to the CONT. Besides, the estimated *M. hyopneumoniae* load in laryngeal swab samples was also numerically lower in the groups immunized with intramuscular commercial vaccine.

It is believed that specific locally secreted IgA play a protective role, preventing or decreasing the pathogen's adhesion to the ciliated epithelium (Martelli et al., 2014; Gomes Neto et al., 2014). In the present work, IgA antibodies against *M. hyopneumoniae* in nasal swabs were induced by both oral and commercial vaccines, while in the control group this immunoglobulin was only detected at 4 weeks post-infection. Considering that the nasal swab samples may vary in quantity between animals and no absolute comparison is possible, the values observed were used to compare the vaccinated groups with the non-vaccinated control group, but not between vaccinated groups. The S/*P* values of anti-*M. hyopneumoniae* IgA showed that both vaccination protocols were able to induce anti-*M. hyopneumoniae* IgA when compared to the non-vaccinated group. The oral vaccine used in the present study was able to induce a specific mucosal immune response, most likely by participating in the protection and prevention against invasion and adherence of *M. hyopneumoniae* (Mechler-Dreibi et al., 2021).

Commercial vaccines are known to reduce the number of organisms in the respiratory tract (Meyns et al., 2006; Vranckx et al., 2012) and decrease the level of infection in the herd (Sibila et al., 2007). The exact mechanisms of protection provided by vaccines are not fully understood, since there is a great variety of mechanisms of action and responses of the organism in the face of immunization (Maes et al., 2021). On the other hand, studies under experimental (Meyns et al., 2006) and field conditions (Pieters et al., 2009; Villarreal et al., 2011) have shown that vaccination provided only a limited reduction in the transmission rate of *M. hyopneumoniae*.

The absence of statistical difference between the estimated bacterial load in the upper respiratory tract and oral fluid samples of the vaccinated and non-vaccinated infected groups may indicate that vaccination alone does not significantly reduce the bacterial load and would not eliminate PEP in swine herds (Meyns et al., 2006; Ogawa et al., 2009). These data would also justify nasal shedding not differing between groups over time (Villarreal et al., 2009). In addition, several factors such as challenge dose, post-infection time, strain virulence, and individual pig immune responses can influence the number of *M. hyopneumoniae* organisms and their nasal clearance (Almeida et al., 2020; Sibila et al., 2007). The number of animals used in this experiment may also have been a limiting factor for the statistical evaluation. Therefore, data collection under field conditions with a bigger sample size could provide more information about these assessments.

It's well known that IgG antibodies perform a minor role in protection against *M. hyopneumoniae* (Maes et al., 2021). On the other hand, specific locally secreted IgA is supposed to act against the pathogen adhesion to the ciliate epithelium (Martelli et al., 2014). In this study, both oral and IM vaccines were capable of inducing IgA antibodies production, which was found in the respiratory tract, as all immunized piglets were positive for this Ab at 14 days post-vaccination. Since both groups showed significantly reduced lung consolidation lesions compared to the control, mucosal IgA probably participates in the protection and prevention against *M. hyopneumoniae* invasion and adherence, corroborating previous studies (Martelli et al., 2014; Li et al., 2019; Mechler-Dreibi et al., 2021; Martelli et al., 2021).

Regarding APP, our results showed an increase in the serum concentration of most of the evaluated APPs, which may be related to the intensity of the immune response caused by the infection and/or vaccine response (Rubio and Moreira, 2014). Moreover, an increase in the serum concentration of proteins was noted in all groups from D0 to D49. The production and response of APPs occur between 24 and 48 h after the inflammatory stimulus (Cerón et al., 2005; Jain, 1993; Kaneko et al., 2008). Thus, an increase in the concentration of APPs in challenged piglets was expected, representing response to the inflammatory stimulus generated by the pathogen.

Our findings showed a higher concentration of haptoglobin in OV group at D49 (53.1 mg/dL) and D56 (52 0.8 mg/dL). These results are in agreement with published data that indicate a relationship between haptoglobin serum levels and ADWG in pigs younger than 13 weeks old (Eurell et al., 1992; Grellner et al., 2002) and also during the critical phase of post-weaning piglets and fattening pigs (Clapperton et al., 2005; Piñeiro et al., 2007). Moreover, higher concentrations of serum haptoglobin was highly correlated with *M hyopneumoniae* titers (Grellner et al., 2002). Transferrin and albumin are known as "negative" acute-phase proteins because their concentration drops during a process of acute inflammation. Transferrin is a glycoprotein responsible for the transport of iron ions in the circulation, whose serum content tends to decrease in the presence of an inflammatory condition and its present in mucosal receptors (Grange and Mouricout, 1996). By analyzing the transferrin protein profile between the day after the challenge (D49) and one week after the challenge (D56), it was possible to observe that the animals vaccinated with the oral vaccine had higher transferrin values. Consequently, this may indicate a lower intensity of the inflammatory process caused by the challenge. Differently, the serum concentration in the group of piglets immunized only with the commercial vaccine (CV) and in the control group (CONT) was lower than in OV, which may indicate a more intense inflammatory reaction in these groups.

Although no statistically significant difference was observed between the mean values of serum albumin, it was noted that the control group (non-immunized and challenged) had lower values than the others after challenge. These findings agree with the presentation of the clinical disease, which becomes more severe in the challenged control group than in the vaccinated groups, as previously observed (Rudoler et al., 2015). We can infer that vaccination can lead to an acute-phase protein reaction, which is part of the innate immune system, generating an inflammatory response that results in homeostatic changes. Differences in acute-phase reaction in response to an inflammatory stimulus may vary between individuals due to genetic, nutritional, age, or other factors. Nonetheless, the development of new vaccines requires intense studies to understand their mechanisms of action upon challenge. Therefore, this study is a valuable tool for the continuous improvement of this oral vaccine, recently developed by our research group. Both immunization protocols showed reduced *Mycoplasma*-like lung lesion, and all groups presented a significant difference in lung lesion score when compared to the control group, as previously observed by Mechler-Dreibi et al. (2021).

5. Conclusions

Detection and shedding of *M. hyopneumoniae* in laryngeal and nasal swabs and oral fluid samples may have been influenced by the use of vaccine using nanoporous silica SBA-15, since lower bacterial loads were observed in the orally immunized group of piglets. In addition, immunization of piglets against *Mycoplasma hyopneumoniae* with a newly developed oral vaccine and a commercial intramuscular vaccine, followed by infectious challenge with a homologous strain, resulted in significant different, positive and negative, acute-phase protein responses. Thus, this may reflect a milder innate inflammatory response conferred by vaccine protection, minimizing tissue damage. Both

vaccines used in this study were able to minimize the effects of *M. hyopneumoniae* infection in piglets. Last but not least, the present study provided useful data for the future development of vaccines using nanoporous silica SBA-15, that could be useful in veterinary medicine, but still requires studies under field to elucidate its potential for the effective control and prevention of *M. hyopneumoniae* in swine production.

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Declaration of Competing Interest

The authors declare to have no conflicts of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.rvsc.2023.03.018.

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