

Article

Comparative Safety and Efficacy of Commercial Porcine Vaccines Against *Mycoplasma hyopneumoniae* and Porcine Circovirus Type 2 (PCV2)

Meritxell Simon-Grifé *, Alexandra Moros , Cecilia Pedernera, Ester Puigvert, Lucía Acal, Elena Plantalech, Mercè Roca, Jordi Montané, Ricard March  and Marta Sitjà

HIPRA S.A., Avda. La Selva 135, Amer, 17170 Girona, Spain; sandra.moros@hipra.com (A.M.); cecilia.pedernera@hipra.com (C.P.); ester.puigvert@hipra.com (E.P.); lucia.acal@hipra.com (L.A.); elena.plantalech@hipra.com (E.P.); merce.roca@hipra.com (M.R.); jordi.montane@hipra.com (J.M.); ricard.march@hipra.com (R.M.); marta.sitja@hipra.com (M.S.)

* Correspondence: meritxell.simon@hipra.com; Tel.: +34-662-193-747

Simple Summary

Respiratory diseases caused by *Mycoplasma hyopneumoniae* (Mhyo) and porcine circovirus type 2 (PCV2) are common in pig farms and can seriously affect animal health, wellbeing, and productivity. Vaccination is essential to prevent these problems, but available vaccines differ in both administration route and efficacy. In this study, several commercial vaccines were compared under controlled conditions, including products administered either intramuscularly or intradermally using a needle-free device. All vaccines proved to be safe and did not negatively affect the animals' growth. When pigs were exposed to both infectious agents, all vaccines controlled the virus responsible for circovirus disease. However, only the intradermal recombinant Mhyo-PCV2 vaccine and the intramuscular inactivated Mhyo + intramuscular inactivated PCV2 vaccine markedly reduced the lung damage associated with *Mycoplasma* infection. These results can provide practical information for farmers and veterinarians, showing how certain vaccination strategies may offer better disease protection while also supporting animal welfare and efficient farm management.

Abstract

Mycoplasma hyopneumoniae (Mhyo) and porcine circovirus type 2 (PCV2) infections cause enormous economic losses in the swine production sector. Vaccination remains the most effective strategy against the complex clinical entities caused by these pathogens. While effective vaccines against PCV2 are available, immunisation against Mhyo continues to be challenging. In parallel, intradermal (ID) vaccination can optimise time and costs while providing faster and user-safe administration. This study compares the safety and efficacy of commercial porcine vaccines against Mhyo and PCV2, including intramuscular (IM) and ID ones: (1) ID recombinant Mhyo-PCV2, (2) ID recombinant PCV2 + ID inactivated Mhyo, (3) IM bivalent inactivated Mhyo and recombinant PCV2, (4) IM bivalent inactivated Mhyo and inactivated chimeric PCV1-PCV2 and (5) IM inactivated Mhyo + IM inactivated PCV2. Safety, evaluated through clinical signs, adverse effects, local reactions, and average daily weight gain, has been confirmed for all vaccines. Following challenge with both pathogens, all products reduced PCV2 viremia and faecal shedding, proving efficacy against PCV2 infection. In contrast, only the ID recombinant Mhyo-PCV2 and the IM inactivated Mhyo + IM inactivated PCV2 vaccines, significantly reduced lung lesions related to Mhyo infection, indicating a better protection compared to the other vaccines.



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1. Introduction

Mycoplasma hyopneumoniae (Mhyo) and porcine circovirus type 2 (PCV2) infections pose significant challenges to the global swine industry, leading to substantial economic losses and compromising porcine health and welfare. Mhyo, the etiological agent of porcine enzootic pneumonia [1], is highly contagious and primarily affects the respiratory tract, leading to chronic respiratory lesions and decreased pulmonary function. This results in reduced growth rates, increased medication costs, and compromised animal welfare [2]. This bacterium is also the main causal agent of pneumonia lung lesions observed at the slaughterhouse in pigs, with frequencies varying from 19% to 79% [3,4]. Mhyo infection in pigs typically causes purple to grey areas of lung tissue consolidation in the apical, cardiac, and intermediate lobes and in the cranial part of the diaphragmic lobes [5]. This cranioventral consolidation of lung lobes results in respiratory distress, dehydration, heavy breathing, non-productive cough, fever, high morbidity (the prevalence can reach 100% in a naïve population), and increased mortality as a result of secondary infections [1,5,6].

Mhyo and a variety of bacterial and viral pathogens such as PCV2 are associated with porcine respiratory disease complex (PRDC). It manifests with coughing, sneezing, nasal discharge, fever, and poor growth, or with more severe signs such as pneumonia, lung lesions, decreased feed efficiency, and increased mortality [7]. PCV2 is also the primary agent of porcine circovirus-associated diseases (PCVAD), a multifactorial entity characterised by immunosuppression, lymphoid depletion, and various clinical manifestations, such as wasting, respiratory distress, reproductive failure, and increased mortality [8,9]. It has been shown that Mhyo is a frequent co-infectious agent in PCVAD [10,11], aggravating PCV2-associated disease when inoculated two weeks before PCV2 infection [12]. The inflammatory response, which is a characteristic feature of lung infection induced by Mhyo, plays a crucial role in facilitating the invasion and survival of the PCV2 organism within alveolar macrophages. This process enables subsequent dissemination to vital organs such as the liver, spleen, kidneys, and lymph nodes [13,14]. The economic losses caused by PRDC and PCVAD in the swine industry worldwide are enormous due to the decrease in growth performance and the increase in mortality and treatment-derived costs [2,15].

Regarding the current scenario of increasing antimicrobial resistance, proper prevention and control of such infectious entities can only be achieved through the implementation of effective and efficient vaccination plans [14,16,17]. Since Mhyo and PCV2 vaccines are administered at a similar age in piglets, double vaccination has gained attention, as it aims to avoid such a complex disease picture while reducing both the labour involved and animal stress during vaccination [18,19]. Common vaccines include ready-to-mix (RTM) [20] and ready-to-use (RTU) vaccines [16,21]. While efficacy of the existing vaccines against PCV2 has been reported so far in different conditions [22,23], immunisation with vaccines against Mhyo has not always been effective [13,24]. Recent field data have shown that the performance of bivalent PCV2–*Mycoplasma hyopneumoniae* vaccines may vary considerably between farms, particularly regarding the reduction in Mhyo-associated lung lesions, highlighting challenges in achieving consistent protection [25].

Intradermal (ID) vaccination in pigs is being explored for Mhyo and PCV2 with interesting results, and some products have reached the market [26]. The concept of ID vaccines is not new, but it is currently under the spotlight of some veterinary firms for its potential to reduce iatrogenic infections through needles [27], which increases animal welfare [28,29], and as a time-effective, user-safe vaccination route in pig production

systems. Moreover, since the dermis and epidermis are rich in antigen-presenting cells, ID vaccination can induce a higher immunisation than intramuscular (IM) or subcutaneous routes with a lesser number of doses [30,31]. One of these ID vaccines, MHYOSPHERE® PCV ID, goes one step beyond with a novel biological entity: an inactivated recombinant Mhyo strain expressing an embedded PCV2 capsid protein, achieving the simultaneous immunisation against both pathogens in one single injection.

The purpose of this study is to evaluate the safety and efficacy of four commercial porcine vaccines, as well as the ID recombinant Mhyo-PCV2 vaccine, in piglets challenged with a co-infection model involving Mhyo and PCV2. Safety is evaluated by clinical signs, adverse effects, local reactions at the administration site, and average daily weight gain (ADWG). Efficacy is assessed after a challenge infection through lung lesion evaluation for Mhyo, viremia and virus shedding for PCV2, and serological analysis for both pathogens.

2. Materials and Methods

2.1. Study Design and Vaccination

All procedures involving animals were conducted in accordance with the European Union Guidelines for Animal Welfare (Directive 2010/63/UE) and approved by the Ethical Committee of HIPRA Scientific SLU and the Department of Territori i Sostenibilitat of the Catalan Government (file: 9650; approval date: 16 December 2020).

A total of 90 commercial hybrid healthy piglets (3 weeks of age) of both genders were used in this study. The animals in the study were free of antibodies against Mhyo and with low levels of maternally derived antibodies against PCV2. The criteria used to exclude pigs from the study were (1) seropositivity against Mhyo, (2) positive Mhyo PCR for nasal swab samples, and (3) PCV2 viraemia (positive serum qPCR). Piglets were introduced to the test facilities and allowed to acclimatise for 5 days before the start of the trial. Housing conditions followed the Directive 86/609/EEC [32]. The animals were randomly distributed into 6 groups and were individually identified by double ear tags (one tag in each ear). Random allocation to treatment groups was performed using a computer-generated randomization list created with Microsoft Excel (Microsoft 365; Microsoft Corporation, Redmond, WA, USA). As shown in Table 1, each group received a different vaccine at 3 weeks of age on the same day by blinded investigators (study day 0, D0). The volume and method of administration followed the manufacturer's instructions. All treatments were administered to the right side of the neck except for group 5, who received two intramuscular vaccines against Mhyo and PCV2 on the right and left sides of the neck, respectively. Pigs in the control group were inoculated with 0.2 mL of phosphate-buffered saline (PBS) intradermally using Hipradermic 3.0 (HIPRA, Amer (Girona), Spain). The devices used in this study were commercially available needle-free ID delivery systems that could administer vaccines into the dermis using jet-injection technology. Hipradermic 3.0 is a manually operated device intended for single-dose administration, whereas IDAL 3G Twin (Merck, Rahway, NJ, USA) is an electronically controlled system that allows sequential administration of two formulations.

Table 1. Groups, commercial vaccines, and study design.

Group	Vaccines	Via ¹	Device	Volume (mL)	N
1	MHYOSPHERE® PCV ID (ID recombinant Mhyo-PCV2)	ID	Hipradermic 3.0 (HIPRA, Amer (Girona), Spain)	0.2	15

Table 1. Cont.

Group	Vaccines	Via ¹	Device	Volume (mL)	N
2	Porcilis [®] PCV ID + Porcilis [®] M Hyo ID ONCE (ID recombinant PCV2 + ID inactivated Mhyo)	ID	IDAL 3G Twin (Merck, Rahway, NJ, USA)	0.2 + 0.2	15
3	Porcilis [®] PCV M Hyo (IM bivalent inactivated Mhyo and recombinant PCV2)	IM	Needle & Syringe	2	15
4	SUVAXYN [®] CIRCO + MH RTU (IM bivalent inactivated Mhyo and inactivated chimeric PCV1-PCV2)	IM	Needle & Syringe	2	15
5	HYOGEN [®] + CIRCOVAC [®] (IM inactivated Mhyo + IM inactivated PCV2)	IM	Needle & Syringe	2 + 0.5	15
Control	PBS	ID	Hipradermic 3.0	0.2	15

¹ ID, intradermic; IM, intramuscular.

2.2. Clinical Observations, Sampling and Weighing

All pigs were observed daily to evaluate and record clinical abnormalities for the first 3 days of the study, and then weekly until the challenge test, when they were observed daily and clinical signs related to the experimental infection were recorded. Observations of the local reaction at the administration site were performed at the same time points before experimental infection. Local reactions were evaluated by inspection and palpation and scored as 0 (absence), 1 (mild; 0–3 cm), 2 (moderate; >3–5 cm), and 3 (severe; >5 cm) according to the inflammation diameter. Body temperatures were measured on the day of vaccination (D0) and then on days 1 (D1), 2 (D2), and 3 (D3) after vaccination. Blood samples from each pig were collected from the marginal vein at D-4, D14, D21, D28, D35, D41, D49, D56, and D64, and were transferred to the laboratory site under refrigeration conditions (2–8 °C). Samples were centrifuged to obtain the serum, which was stored at ≤−15 °C until use. Finally, pigs were weighed at the beginning of the study (D0) and at D14, D41, and D64 (end of the study) to calculate the ADWG at the corresponding periods as a safety variable. During the study, pigs were euthanized for ethical reasons with an intravenous overdose of sodium pentobarbital (Dolethal[®]; Vetoquinol, Madrid, Spain) before death when showing severe depression or severe respiratory clinical signs. Before euthanasia, pigs were anaesthetized intramuscularly with 0.2 mL/kg of a mixture of Xilagesic[®] (Calier, Barcelona, Spain) and Zoletil 100[®] (Virbac, Barcelona, Spain).

2.3. Experimental Infection

The appropriate measures were taken to avoid Mhyo and PCV2 natural infection. All groups were challenged with a very virulent Mhyo strain (isolated in Denmark and kindly provided by Prof. Dr. N. Friis) inoculum grown in Friis broth intranasally 6 weeks after vaccination (D42). Each animal received 5 mL of inoculum using a syringe (2.5 mL/nostril, 2.8×10^{10} CCU/animal) on 3 consecutive days (D42, D43, and D44). Challenge infection with PCV2 consisted of a single intranasal administration of 5 mL of a PCV2b inoculum (2.5 mL/nostril, 1.7×10^5 CCID₅₀/animal) at D42. The challenge strain corresponded to a PCV2b isolate obtained from porcine serum (GenBank accession number GU049342).

2.4. Mhyo Lung Lesion Scoring

As Mhyo causes decreased pulmonary function accompanied with chronic respiratory lesions, 3 weeks after the challenge infection with Mhyo (12 weeks of age), the pigs were euthanized and the lung lesions related to Mhyo were assessed. Lung scoring was

performed by trained evaluators blinded to the vaccination status of the animals following the method recommended by the European Pharmacopoeia (Ph. Eur. monograph no. 2448). Each lung lobe was scored from 0 to 5 according to the surface of tissue affected by Mhyo lesions (i.e., lung consolidation). In order to calculate the total percentage of affected lung, the contribution of each lung lobe was taken into account to obtain a weighed score as previously described [33]. The main variables to determine the vaccination programme efficacy were (1) the affected lung surface and (2) the lung lesions' severity.

2.5. PCV2 Quantification by qPCR

Tissue samples (lung, tonsils, inguinal and mesenteric lymph nodes) were weighed (approximately 1–5 g per tissue) and homogenised on PBS (one-half weight/volume dilution) with stainless steel beads using a Bullet Blender (Next Advance Inc., Troy, NY, USA) following the manufacturer's protocol, and then centrifuged at 1000 g to collect the supernatant. Faecal swabs were suspended in 1 mL of PBS. DNA was extracted from 200 µL of serum and from processed faecal and tissue samples using the MagMAX™ CORE Nucleic Acid Purification kit (Applied Biosystems by Thermo Fischer Scientific, Austin, TX, USA) following the manufacturer's instructions. The DNA obtained was suspended in 90 µL of elution buffer. The quantity of isolated DNA from tissues was quantified by spectrophotometry (NanoDrop One, Thermo Fisher Scientific). The samples were kept at −80 °C until processing. The commercial qPCR kit VetMAX™ Porcine PCV2 Quant Kit (Applied Biosystems by Thermo Fisher Scientific, Lissieu, France) was used to quantify PCV2 DNA in serum, faecal, and tissue samples. PCV2 qPCR results were expressed as PCV2 copies per millilitre for serum and faecal samples and as PCV2 copies per microgram of total extracted DNA in the case of tissue samples, normalising viral copy numbers to the total DNA extracted for each sample. The total number of days of viremia or faecal shedding were calculated considering the last time point of the study when the animal was positive for viraemia or presented viral shedding.

2.6. Antibody Measurement

The levels of antibodies in serum samples against Mhyo were analysed with the commercial ELISA kit CIVTEST® SUI5 MHYO (HIPRA, Amer (Girona), Spain). The results were expressed as the relative percent index (IRPC). The levels of antibodies against PCV2 in serum samples were tested with the commercial PCV2 antibody ELISA kit (BioChek, Ascot, UK), and the results were expressed as the sample-to-positive (S/P) ratio according to the manufacturer's instructions.

2.7. Statistical Analysis

The minimum sample size was estimated based on simulations of the expected results for the primary efficacy variable (percentage of lung surface affected), using Ene 3.0 software (Universitat Autònoma de Barcelona, Barcelona, Spain) and data from previous studies. Mean expected values of 12.5% for the control group and 8% for the vaccinated group were assumed. To achieve a statistical power of 80% to detect differences under the null hypothesis ($H_0: \mu_1 = \mu_2$) for two independent samples, with a significance level of 5%, and allowing for an expected drop-out rate of 10%, it was determined that at least 11 pigs per group were required.

Individual pigs were the experimental unit. Data were tested for normality with Shapiro–Wilks tests, and the most appropriate statistical test was used with the aim of evaluating the efficacy and safety of each vaccine. The severity of lung lesions was evaluated using two-tailed Z-tests for independent proportions with Bonferroni adjustment of the *p* value. Serological longitudinal data were analysed using linear mixed effects models. Differences between factor levels were determined using estimated marginal means with

multivariate *t*-distribution correction for multiple testing. The other parameters were analysed by Student's *t*-test or Mann–Whitney U test, performing comparisons between each vaccinated group and the control group. Fisher's exact test was used to compare the proportion of animals presenting clinical signs by performing comparisons between each vaccinated group and the control group. IBM® SPSS Statistics V22.0 (IBM Corp., Armonk, NY, USA) and GraphPad Prism software (v6.0 GraphPad Software, San Diego, CA, USA) were used for data analysis. A significance level of *p* value < 0.05 was used for all the variables evaluated in this study.

3. Results

3.1. General Clinical Signs

None of the pigs in all groups presented noticeable general clinical signs or adverse reactions after vaccine administration. Coughing, depression, and/or respiratory symptoms (dyspnoea) attributable to Mhyo or PCV2 infection were recorded from D42 to D64 of the study. Coughing was observed in 10 out of 15 animals (66.7%) in groups 1 and 5; 11 out of 15 (73.3%) in group 2; 13 out of 15 (86.7%) in group 3; and 14 out of 15 (93.3%) in both group 4 and the control group. Depression was observed in 2 out of 15 animals (13.3%) in groups 1 and 3; 4 out of 15 (26.7%) in group 2; 7 out of 15 (46.7%) in group 4; 1 out of 15 (6.7%) in group 5; and 5 out of 15 (33.3%) in the control group. Respiratory symptoms at rest were recorded in 1 animal (6.7%) in groups 1 and 5; 2 animals (13.3%) in group 2; 6 animals (40.0%) in group 3; and 4 animals (26.7%) in both group 4 and the control group. Respiratory symptoms after encouraging movement were observed in 0 animals (0.0%) in group 1; 2 animals (13.3%) in groups 2 and 3, as well as the control group; and 1 animal (6.7%) in groups 4 and 5. No statistically significant differences (*p* value > 0.05) were observed in the proportion of animals showing clinical signs between any vaccinated group and the control group. Additionally, 8 animals that died before the end of the study were euthanised prior to the challenge in accordance with the endpoint criteria (severe depression or severe respiratory signs). These cases were unrelated to vaccination procedures. Necropsy findings identified complications associated with blood collection (haematoma formation), intestinal intussusception, and septicaemia as the causes of death.

As expected, in all groups the average body temperature increased significantly on D1, but it returned to normal levels on D2. The differences in temperature compared to the control group were not statistically significant after vaccination (Table 2). As required by the European Medicines Agency (EMA) guidelines for safety assessment, the mean body temperature increase did not exceed 1.5 °C in any individual, and the average increase in any group did not exceed 2 °C after vaccination.

Table 2. Body temperature (mean ± SEM) at vaccination day (D0) and three days post-vaccination.

Group	D0	D1	D2	D3
1	39.2 ± 0.2	39.6 ± 0.1	39.6 ± 0.1	39.6 ± 0.1
2	39.2 ± 0.1	39.3 ± 0.1	39.4 ± 0.2	39.5 ± 0.1
3	39.2 ± 0.1	39.3 ± 0.1	39.3 ± 0.1	39.5 ± 0.1
4	39.3 ± 0.1	39.8 ± 0.1	39.3 ± 0.1	39.4 ± 0.1
5	39.3 ± 0.2	39.8 ± 0.1	39.5 ± 0.1	39.6 ± 0.1
Control	39.0 ± 0.1	39.3 ± 0.1	39.2 ± 0.1	39.4 ± 0.1

SEM, standard error of mean. No significant differences were observed between vaccinated and control groups (*t*-test comparisons between each vaccinated group and control group; *p* value > 0.05).

3.2. Evaluation of Local Reactions at the Administration Site

Local reactions at the administration site of the different vaccines (including intramuscular and intradermic vaccines) were evaluated by palpation and by inspection. All the

reactions noted were mild (none of the pigs in any of the groups presented a severe or moderate local reaction), so this variable was evaluated based on the frequency at different time points. As expected, local reactions at the administration site detectable by palpation appeared at higher frequencies in pigs that received ID vaccination (groups 1 and 2) compared to IM-administered groups, where the percentage of local reactions remained below 10% (Figure 1). The group with the highest percentage of palpable local reactions along time was group 2, in which 73.3% to 84.6% of pigs presented local reactions from D1 to D14. At D28, mild local reactions persisted in 46.2% of the animals. In addition, there were two cases of scabs on the administration site in group 2 (from D3 to D21 and from D7 to D14, respectively). In group 1, 100% of the animals showed mild local reactions at D1, but the frequency rapidly decreased to 46.7% the next day and the decreasing trend continued until D28, when none of the pigs showed local reactions. Regarding evaluation by visual inspection, as all reactions were mild, they were only detectable in around 50% of the animals in group 2 until D28, and around 10% in group 1 until D14.

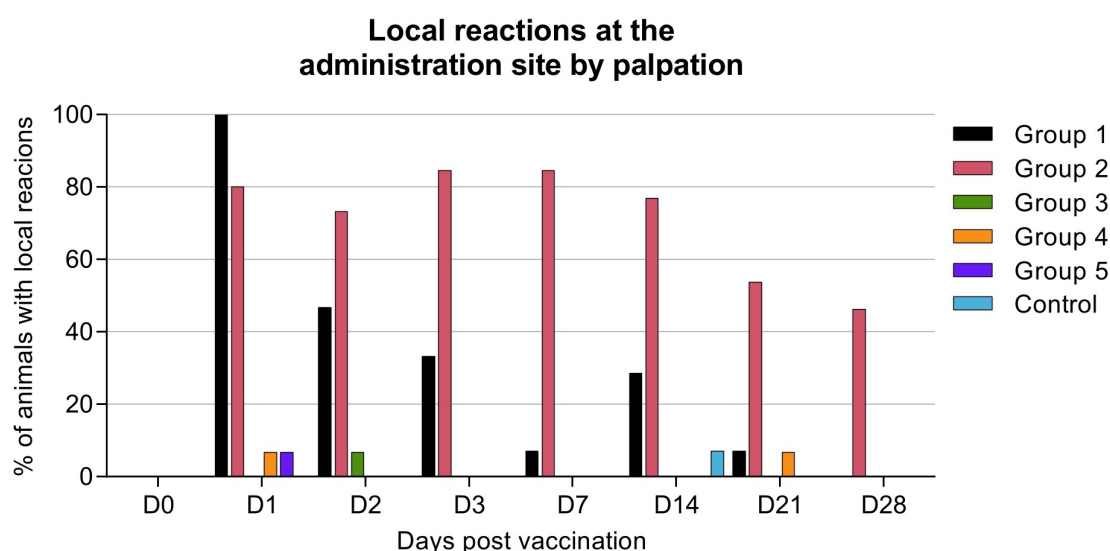


Figure 1. Percentages of pigs showing local reactions at the administration site over time. All the detected local reactions were mild (score 1 on a 0–3 scale).

3.3. Average Daily Weight Gain Post-Vaccination and Post-Challenge

Control animals presented an ADWG from D0 to D14 post-vaccination (3 to 5 weeks old) of 293.3 g, and the differences with vaccinated groups were not statistically significant (p value > 0.05). There were also no significant differences with the control group in the ADWG from D41 to D63 post-challenge (9 to 12 weeks old) (p value > 0.05; Table 3).

Table 3. Mean body weight (BW $\pm$ SEM) and mean average daily weight gain (ADWG $\pm$ SEM) 2 weeks post-vaccination (D0 to D14) and 3 weeks post-challenge (D41 to D63).

Group	BW D0 (kg)	BW D14 (kg)	ADWG D0 to D14 (g)	BW D41 (kg)	BW D63 (kg)	ADWG D41 to D63 (g)
1	6.7 $\pm$ 0.4	10.9 $\pm$ 0.6	312.9 $\pm$ 20.7	22.7 $\pm$ 1.4	34.4 $\pm$ 1.9	585.2 $\pm$ 34.3
2	7.0 $\pm$ 0.4	10.6 $\pm$ 0.5	271.0 $\pm$ 16.3	21.7 $\pm$ 1.1	34.8 $\pm$ 1.4	592.8 $\pm$ 28.5
3	6.4 $\pm$ 0.4	10.0 $\pm$ 0.5	253.5 $\pm$ 38.1	21.4 $\pm$ 1.1	33.0 $\pm$ 1.4	527.3 $\pm$ 47.2
4	7.0 $\pm$ 0.3	11.2 $\pm$ 0.5	297.4 $\pm$ 20.5	22.8 $\pm$ 1.3	35.9 $\pm$ 2.0	592.5 $\pm$ 43.0
5	7.2 $\pm$ 0.4	11.3 $\pm$ 0.7	285.9 $\pm$ 50.5	23.8 $\pm$ 1.4	36.7 $\pm$ 2.3	585.7 $\pm$ 46.3
Control	6.9 $\pm$ 0.3	10.9 $\pm$ 0.5	293.3 $\pm$ 15.5	22.4 $\pm$ 0.8	35.1 $\pm$ 1.5	578.6 $\pm$ 34.3

SEM, standard error of mean. No significant differences were observed between vaccinated and control groups (t -test comparisons between each vaccinated group and control group; p value > 0.05).

3.4. Mhyo-Compatible Lung Lesions Evaluation

Pigs in group 1 presented the lowest median lung lesion surface of 8.50% (range: 0–21.90%), which was significantly lower than the median of the control group (14.22%, range: 10.94–28.26%) (p value < 0.05 ; Table 4), representing a 40.2% relative reduction. Group 5 showed a median of 9.18% (range: 2.56–18.26%), also significantly reduced compared to the control (p value < 0.05), with a relative reduction of 35.4%. Animals in group 3 presented a median percentage of affected lung surface of 11.82% (range: 1.90–25.24%), which did not differ significantly from the controls (p value > 0.05). In contrast, groups 2 and 4 showed median percentages of affected lung surface of 14.24% (range: 4.76–29.24%) and 19.47% (range: 0–55.42%), respectively, with no significant differences from the control group (p value > 0.05)—the latter group 4 representing a 36.9% numerical relative increase.

Table 4. Lung lesions evaluation at necropsy on D22 after challenge infection with Mhyo. Median of the percentage of affected lung surface in each group.

Group	% of Affected Lung Surface (Range)
1	8.50 (0–21.9) ^a
2	14.24 (4.76–29.24) ^{ab}
3	11.82 (1.9–25.24) ^{ab}
4	19.47 (0–55.42) ^{ab}
5	9.18 (2.56–18.26) ^a
Control	14.22 (10.94–28.26) ^b

^{a,b} Different superscripts indicate statistically significant differences with the control group (Mann–Whitney U test comparisons between each vaccinated group and the control group; p value < 0.05).

Additionally, the proportion of animals with severe lung lesions was also significantly lower in groups 1 and 5 compared to the control group (p value < 0.05 ; Figure 2), corresponding to a relative reduction of 67% in both cases.



Figure 2. Lung lesion severity at necropsy at D22 after challenge infection with Mhyo. * p value < 0.05 , two-tailed Z-test for independent proportions with Bonferroni adjustment of the p value.

3.5. Evaluation of Antibodies Against Mhyo

Figure 3 shows the mean IRPC of antibodies against Mhyo in each group weekly until the end of the study by an enzyme-linked immunosorbent assay (ELISA; positive threshold considered as $\text{IRPC} \geq 35$). At D14 after vaccine administration, pigs from groups 1 and 5 already exhibited a significantly higher mean IRPC than the control group (p value < 0.05), although they did not reach seroconversion. At D21, groups 1, 3, and 5 were above the seroconversion threshold and, together with group 2, were significantly higher than the control group (p value < 0.0001). The mean IRPC in group 4 did not increase until D49 (one-week post-challenge infection) and was not considered as seroconversion until D56 (two-weeks post-challenge). In all groups, once animals seroconverted, the mean IRPC remained positive and significantly higher than the control group until the end of the study (p value < 0.0001 ; D63).

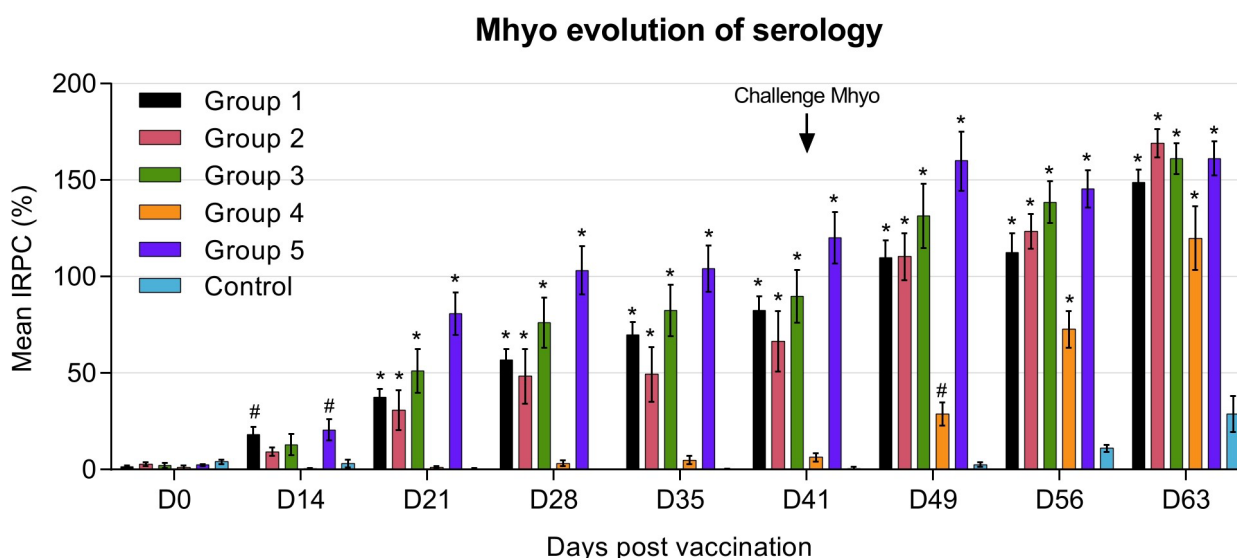


Figure 3. Mean relative percent index (IRPC, %) for Mhyo antibodies in serum over time after vaccination. Bars show mean $\pm$ SEM. #, p value < 0.05 ; *, p value < 0.0001 compared to the control group by linear mixed effects model with multivariate t -distribution correction.

3.6. PCV2 Viraemia and Shedding

PCV2 viraemia was negative in all animals at the challenge day (D41). At D49 (one-week post-challenge), viraemia was detected at low levels in some vaccinated individuals from each group except for group 3. In all groups except group 4, values were significantly lower than in the control group (p value < 0.05). Viraemia clearly increased in the control group but remained significantly low in all vaccinated groups after 2 and 3 weeks from PCV2 challenge inoculation (Figure 4A). Moreover, at the end of the study, all vaccinated groups showed a significantly lower number of viraemia-positive days than the control group (p value < 0.05 ; Table A1).

Faecal shedding in all groups showed a high level of PCV2 DNA copies after the first week post-challenge, without significant differences with the control group (p value > 0.05). However, on D56 and D63, all vaccinated groups experienced a reduction in viral faecal shedding compared to the control group (p value < 0.05 ; Figure 4B). Finally, the total number of days of faecal shedding in all vaccinated groups was significantly lower than the control group (p value < 0.0001 ; Table A2).

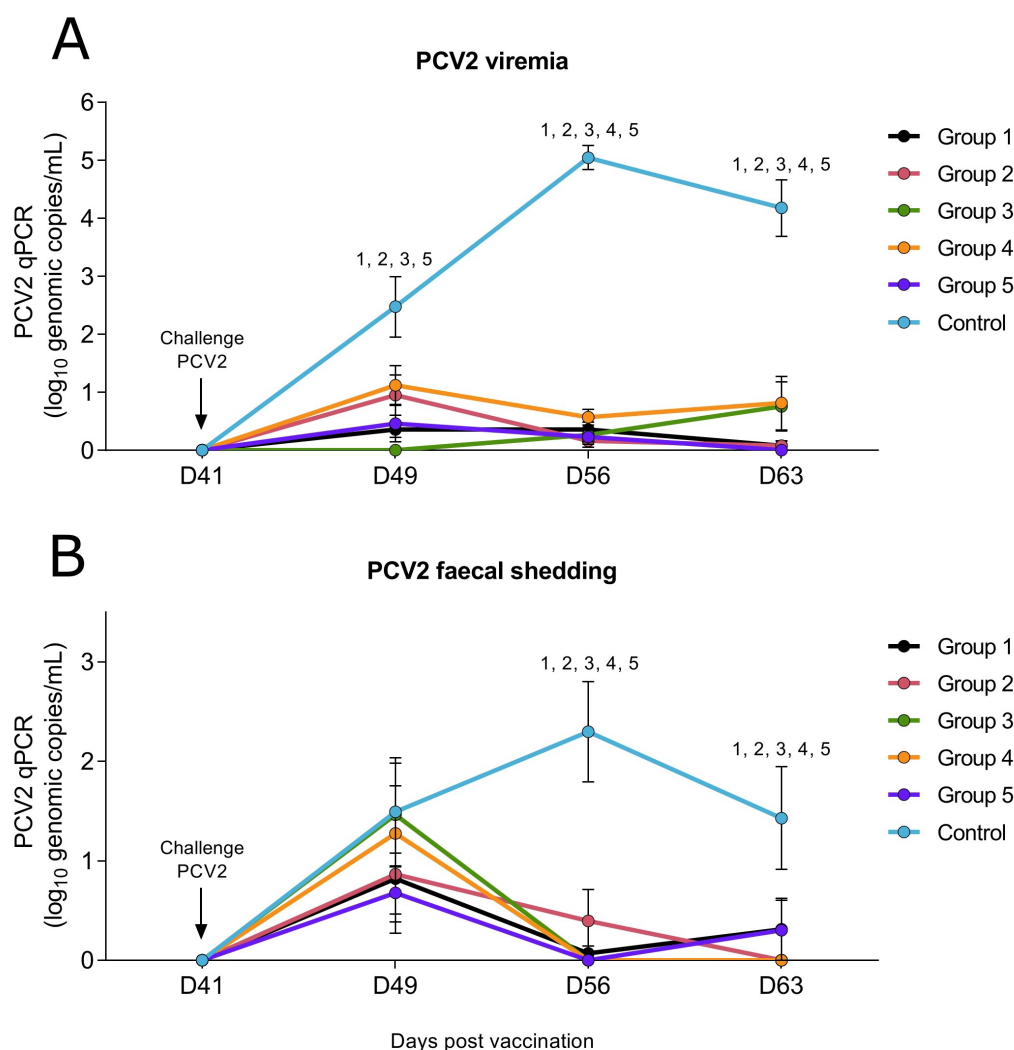


Figure 4. Weekly evolution post-challenge infection of PCV2 measured by qPCR. **(A)** Viraemia and **(B)** faecal shedding. ^{1, 2, 3, 4, 5} Numbers on top of time points indicate significant differences in the respective groups with the control group (Mann–Whitney U test comparisons between each vaccinated group and the control group; *p* value < 0.05).

3.7. PCV2 Tissue Colonisation

The PCV2 load was measured in the lungs, tonsils, inguinal lymph nodes, and mesenteric lymph nodes at the end of the study (D63). All samples of the different tissues showed the presence of PCV2 (only one lung sample in group 1 and one in group 5 showed a value of zero and were included in the calculations). As shown in Table 5, the PCV2 load was significantly lower in the five vaccinated groups than in the control group in all the tissues analysed (*p* value < 0.05). Group 4 exhibited a numerically higher viral load in tissues compared to the other vaccinated groups.

Table 5. PCV2 load in tissues (log₁₀ PCV2 genomic copies/μg DNA). Values are shown as logarithm10 (mean ± SEM).

Group	Lung	Tonsil	Inguinal Lymph Node	Mesenteric Lymph Node
1	3.46 ± 0.44 ^a	3.04 ± 0.33 ^a	2.43 ± 0.31 ^a	2.43 ± 0.22 ^a
2	3.69 ± 0.27 ^a	3.03 ± 0.38 ^a	2.76 ± 0.40 ^a	2.36 ± 0.31 ^a
3	3.69 ± 0.26 ^a	2.37 ± 0.29 ^a	2.72 ± 0.34 ^a	2.50 ± 0.24 ^a
4	4.70 ± 0.25 ^a	3.42 ± 0.30 ^a	3.33 ± 0.32 ^a	3.17 ± 0.23 ^a

Table 5. Cont.

Group	Lung	Tonsil	Inguinal Lymph Node	Mesenteric Lymph Node
5	3.41 ± 0.35 ^a	2.32 ± 0.33 ^a	2.25 ± 0.33 ^a	1.78 ± 0.26 ^a
Control	6.27 ± 0.22 ^b	6.58 ± 0.30 ^b	6.37 ± 0.29 ^b	6.08 ± 0.31 ^b

^{a,b} Different superscripts indicate statistically significant differences with the control group (Mann–Whitney U test comparisons between each vaccinated group and the control group; *p* value < 0.05).

3.8. Evaluation of Antibodies Against PCV2

Figure 5 shows the S/P ratio ELISA values in each group during the study. Two weeks after vaccination (D14), groups 3 and 5 showed a statistically significant higher S/P ratio than the control group (*p* value < 0.05). From D21 until the finalisation of the study, the S/P ratio for all vaccinated groups was significantly higher than the control group (*p* value < 0.01 at D21; *p* value < 0.0001 from D28 to D63).

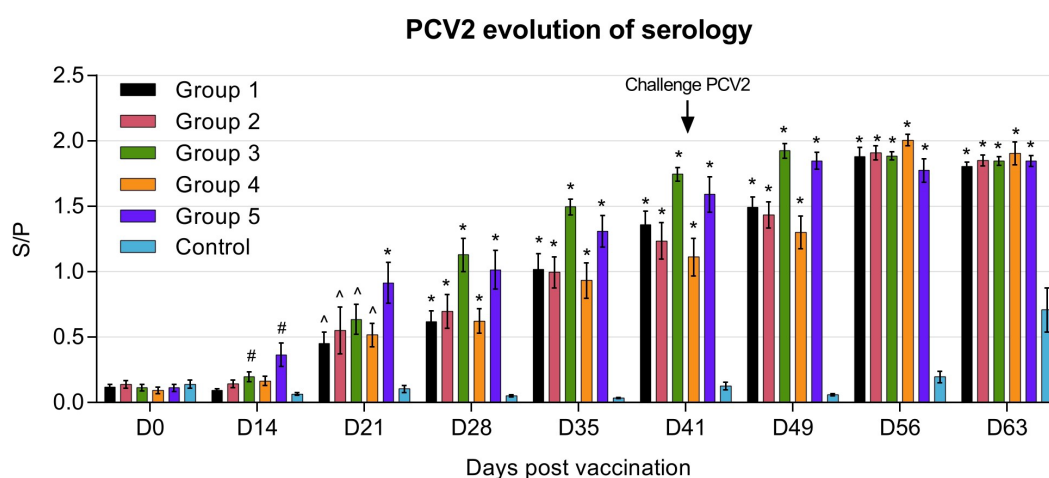


Figure 5. S/P (Sample to Positive) ratio for PCV2 antibodies in serum over time after vaccination until the end of the study. Bars show mean ± SEM. #, *p* value < 0.05; ^, *p* value < 0.01; *, *p* value < 0.0001 compared to the control group by the linear mixed effects model with multivariate *t*-distribution correction.

4. Discussion

Mhyo and PCV2 are two of the most prevalent pathogens in the swine industry that entail large profit losses and declines in health. Different vaccination strategies have been used in the field that include separate or RTU vaccines by an IM or an ID route, with varying levels of protection [34]. This article has provided a comparison of commonly used commercial porcine vaccines against both pathogens. All vaccines in the study proved to be safe in terms of observation of adverse reactions and body temperature. These were foreseeable results, as these vaccines have already been approved by the EMA. Almost all animals administered with the ID vaccines presented a mild local reaction at palpation, as expected for this administration route [35], which gradually disappeared in the ID recombinant Mhyo-PCV2 vaccinated group until none of the animals showed local reactions at day 28. On the other hand, local reactions in the ID recombinant PCV2 + ID inactivated Mhyo group were persistent in almost half of the animals at 4 weeks after vaccination. However, in another study with ID recombinant PCV2 + ID inactivated Mhyo, they observed that at 4 weeks post-vaccination local reactions had disappeared in >76% of the animals [26].

ADWG post-challenge was another measured variable, although no differences were observed between groups. Similar controlled challenge studies have shown that infections

with Mhyo and PCV2b often resulted in subclinical effects, with no differences in the growth between vaccinated and control animals and no manifestation of clinical signs [36]. In a similar experimental study, pigs vaccinated with the IM inactivated Mhyo + IM inactivated PCV2 also showed no differences in growth compared to control animals [19]. Likewise, another experimental study using a combined vaccine against PCV2a/PCV2b and Mhyo (CircoMax Myco) reported no impact of vaccination on the body weight of the animals [37]. Nonetheless, in a dual challenge study, Suh et al. [38] reported improved growth performance in pigs vaccinated with the ID recombinant Mhyo-PCV2 vaccine compared to an unvaccinated control group.

In general, experimental infection models may not fully reproduce field disease dynamics and can often result in subclinical outcomes, as reported in PCV2 experimental challenge models [39]. Accordingly, the primary objective of this model was not to replicate the full spectrum of clinical disease observed under field conditions, but rather to allow a standardised and controlled evaluation of virological parameters and Mhyo-associated lung lesions across different vaccination strategies. While Mhyo and PCV2b infections have often led to a limited impact on body weight under experimental conditions, field studies have demonstrated a more evident positive impact of vaccination on growth performance. For instance, in a clinical study comparing the ID recombinant Mhyo-PCV2 vaccine with three IM commercial porcine vaccines against the same pathogens (including the IM inactivated Mhyo-recombinant PCV2 and the IM inactivated Mhyo + IM inactivated PCV2 vaccines), the overall ADWG for the ID recombinant Mhyo-PCV2 vaccine was higher than for the other vaccines during the nursery period [40]. Similar results for ADWG were obtained in another field study that compared the ID recombinant Mhyo-PCV2 and the IM inactivated Mhyo + IM inactivated PCV2 vaccines, showing that piglets vaccinated with the IM vaccine stopped feeding for about one day and exhibited depressed behaviour, resulting in higher animal weights for the ID vaccine group on the first weeks of growth [41].

Efficacy was evaluated separately for each of the pathogens. The main parameter for efficacy against Mhyo was the evaluation of lung lesions after the challenge infection [42]. A plethora of Mhyo strains have been in circulation across pig farms with different levels of virulence which determines the clinical course of the infection [43,44]. Noticeably, an infection with a low virulence strain does not confer protection against a high virulence strain [45]. In the present study, where animals were challenged with a highly virulent strain, the average affected lung surface in control animals was around 14%, and approximately half of them presented severe macroscopic lesions. The ID recombinant Mhyo-PCV2 and the IM inactivated Mhyo + IM inactivated PCV2 were the vaccines with the highest capacity for reducing lung lesion surfaces, as well as for decreasing their severity. It has also been described in other studies that the ID recombinant Mhyo-PCV2 vaccine can decrease pulmonary and lymphoid lesions [38] and that the IM inactivated Mhyo vaccine can reduce lung lesion severity [46], alone or in combination with the IM inactivated PCV2 vaccine [19,41]. In contrast, the reduction in lung lesions observed in the ID recombinant PCV2 + ID inactivated Mhyo and IM bivalent inactivated Mhyo and recombinant PCV2 groups was limited and did not reach statistical significance compared to controls. Although one of the products was also administered via the intradermal route, its performance differed from that of the ID recombinant Mhyo-PCV2 vaccine. This suggested that vaccine formulation and antigen design, rather than the route of administration alone, may play a determinant role in protection against Mhyo-associated lung lesions. Moreover, the ID devices used are manufactured by different companies, and although both are licenced needle-free delivery systems, minor technical differences between devices cannot be completely excluded as a potential source of variability between ID vaccination groups.

Additionally, animals vaccinated with the IM bivalent inactivated Mhyo and inactivated chimeric PCV1-PCV2 showed a numerical increase in the affected lung surface compared to controls. This trend, although not statistically significant, suggested limited protection against Mhyo-induced lung lesions under the present experimental conditions. Similar results were reported by Hsueh et al. [25], where the IM bivalent inactivated Mhyo and recombinant PCV2 vaccine did not achieve statistically significant differences in lung lesion indices under field conditions despite showing improvements in other parameters of disease control. Lung lesions have been well-recognised indicators of respiratory health and associated with long-term impacts on animal performance and increased susceptibility to secondary infections under field conditions [3,11]. Consequently, the differences observed at the pathological and virological level may translate into productive benefits in commercial farms, even if such effects cannot be fully captured in short-term experimental models.

There was also a difference between groups in the serology pattern, where antibodies began to increase two weeks after vaccination in the groups vaccinated with the ID recombinant Mhyo-PCV2 and with the IM inactivated Mhyo + IM inactivated PCV2 vaccines. While all groups had seroconverted by day 27 after vaccination, the IM bivalent inactivated Mhyo and inactivated chimeric PCV1-PCV2 group did not show an increase in antibody levels until two weeks after the challenge infection, suggesting that seroconversion was triggered by the experimental infection and not by the administered vaccine—although this could potentially impact field protection. The numerically higher viral loads and delayed seroconversion observed in this group may reflect differences in vaccine formulation or immune kinetics between products, potentially influencing the timing and magnitude of protection. Nevertheless, protection against PCV2 is multifactorial and not exclusively dependent on early antibody detection.

Pigs in the current study were challenged with PCV2 genotype b. Although most commercial vaccines are based on the PCV2a genotype, cross-protection has been demonstrated for PCV2b and PCV2d, which are currently the most widespread genotypes in the field [47,48]. The efficacy against PCV2 of the five vaccines was demonstrated by the reduction in viraemia and virus faecal shedding combined with the decreased viral load in tissues. Results for PCV2 vaccines in experimental conditions can be extrapolated to the field as numerous studies have also proven, even in the case of intradermal vaccination against both Mhyo and PCV2 [26,40].

The present comparative study, along with the others discussed above, has demonstrated that while all vaccines were safe and efficacious against PCV2, the ID recombinant Mhyo-PCV2 and the IM inactivated Mhyo + IM inactivated PCV2 vaccines promoted better protection against Mhyo pulmonary lesions. Notably, the ID recombinant Mhyo-PCV2 vaccine is an intradermal vaccine, whereas the IM inactivated Mhyo + IM inactivated PCV2 vaccine requires two intramuscular injections. The ID route has demonstrated the potential of needle-free delivery systems which can reduce the risk of needlestick injuries, iatrogenic infections, and cross-contamination. Although reduced animal stress and shorter vaccination times have been reported in previous studies [29,49], these parameters were not directly measured in the present study and should therefore be taken with caution. Nonetheless, the present findings should be interpreted within the context of the experimental model used and considered primarily as a comparative assessment of vaccine performance under standardised conditions.

5. Conclusions

In conclusion, the present study has shown that all vaccines proved to be safe, no appearance of clinical signs or decrease in ADWG were observed, and all significantly reduced PCV2 viremia and tissue viral loads compared to controls. Nevertheless, only the

ID recombinant Mhyo-PCV2 and the IM inactivated Mhyo + IM inactivated PCV2 vaccines showed a significantly lower percentage of Mhyo pulmonary lesion surfaces and severity compared to the control group.

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Institutional Review Board Statement: The study was conducted in accordance with the European Union Guidelines for Animal Welfare (Directive 2010/63/UE) and approved by the Ethical Committee of HIPRA Scientific SLU and the Department of Territori i Sostenibilitat of the Catalan Government (file: 9650; approval date: 16 December 2020).

Informed Consent Statement: Not applicable. All animals used were owned by HIPRA, as they were purchased specifically for the experiment. Therefore, there were no external owners from whom consent had to be obtained.

Data Availability Statement: Data from this study are available upon reasonable request from the corresponding author.

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Conflicts of Interest: All the authors are currently employees of HIPRA. MHYOSPHERE® is a commercial vaccine from HIPRA.

Appendix A

Table A1. Total number of days of PCV2 viraemia per individual in each group.

Individual	Group 1	Group 2	Group 3	Group 4	Group 5	Control
1	14	7	0	0	14	21
2	7	0	0	21	0	21
3	0	7	21	21	0	21
4	7	21	21	7	7	21
5	14	14	0	21	14	21
6	0	7	0	14	-	21
7	14	0	0	-	14	-
8	-	0	21	7	0	21
9	14	-	14	0	0	21
10	0	-	0	14	0	21
11	0	7	21	14	0	21
12	0	-	14	0	0	14
13	0	7	0	14	0	14
14	21	14	0	21	0	21
15	0	7	0	14	-	21
<i>p</i> value compared to control ^a	<0.0001	<0.0001	<0.05	<0.05	<0.0001	

^a Mann–Whitney U test comparisons between each vaccinated group and the control group. -: animal died before the end of the study.

Table A2. Total number of days of PCV2 faecal shedding per individual in each group.

Individual	Group 1	Group 2	Group 3	Group 4	Group 5	Control
1	21	7	0	0	0	14
2	7	0	7	0	7	21
3	0	0	0	7	0	21
4	0	0	0	7	0	14
5	0	14	0	7	0	21
6	0	0	7	7	-	21
7	14	0	0	-	0	-
8	-	7	0	0	21	14
9	7	-	0	0	0	21
10	0	-	7	0	0	21
11	0	0	7	7	0	21
12	0	-	0	7	0	14
13	0	0	7	0	7	14
14	0	14	7	0	0	14
15	0	7	0	0	-	21
<i>p</i> value compared to control ^a	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	

^a Mann–Whitney U test comparisons between each vaccinated group and the control group. -: animal died before the end of the study.

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