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Understanding and predicting porcine circovirus 2 (PCV2) epidemiology and vaccination outcomes

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PhD Thesis

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Ceva

IRTA CReSA Centre de Recerca en Sanitat Animal

Understanding and predicting porcine circovirus 2 (PCV2) epidemiology and vaccination outcomes

Doctoral thesis submitted by **Mònica Sagrera** i **Cozar** to obtain the degree of Doctor within the Doctoral Program in *Animal Medicine and Health* of the *Faculty of Veterinary Medicine* at the *Universitat Autònoma de Barcelona*, under the supervision of **Dr. Joaquim Segalés Coma**, **Dr. Marina Sibila Vidal** and **Dr. Laura Garza Moreno**.

Bellaterra, 2026

Dr. Marina Sibila Vidal, researcher at the *Institute of Agrifood Research and Technology – Centre for Research in Animal Health* (IRTA-CReSA); Dr. Laura Garza Moreno, Veterinary Services Specialist at Ceva; and Dr. Joaquim Segalés Coma, Full Professor of the *Department of Animal Health and Anatomy* of the *Faculty of Veterinary Medicine* at the *Universitat Autònoma de Barcelona* (UAB) and adscribed researcher at IRTA-CReSA,

Certify,

that the research work developed in the doctoral thesis *Understanding and predicting porcine circovirus 2 (PCV2) epidemiology and vaccination outcomes*, submitted by Ms. Mònica Sagrera i Cozar to obtain the degree of *Doctor in Animal Medicine and Health*, has been carried out under our supervision and guidance, and we authorize its submission to be evaluated by the corresponding committee.

In witness thereof, and for all legal purposes, we sign this declaration.

Supervisor	Supervisor	Supervisor and advisor	PhD Candidate
Dr. Marina Sibila Vidal	Dr. Laura Garza Moreno	Dr. Joaquim Segalés Coma	Ms. Mònica Sagrera i Cozar

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Als meus pares i el meu germà,

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Imagino el dia d'escriure aquestes pàgines des del mateix moment en què vaig començar la tesi. És arran d'un text de l'escriptora Ester Vallès que em va impactar especialment, on fa una reflexió que em va quedar gravada: com n'és d'important aprendre a dir-nos les coses en vida. Com ens és molt més senzill llançar un retret que verbalitzar allò bonic. Donant-hi voltes, em vaig adonar que aquest pensament connecta amb un aprenentatge essencial que he rebut des de casa; l'ús i el valor de tres expressions que ho canvien tot: si us plau, ho sento i gràcies.

Amb els anys, però, hi ha un altre text de la mateixa autora que també m'ha acompanyat, gairebé com un recordatori silenciós en els moments més complexos:

> *La tovallola no es tira ni s'abandona*
>
> *El que t'ensenya el temps és que la tovallola no es tira ni s'abandona. Es recull tants cops com faci falta. El primer pas per entendre el camí és que va més enllà de números, resultats i pressió. Hi ha tantes coses que tenim davant i no aconseguim veure. Olors, tactes, sentiments. Això és el que importa. Ferir-se i supurar la ferida. Per reobrir-ne una altra de nou. I seguir.*
>
> *Seguir sentint. El bo d'això és que la tovallola no es tira ni s'abandona.*
>
> *Es torna a recollir.*
>
> *Tants cops com faci falta.*

Durant aquesta tesi, així com al llarg dels quasi deu anys de trajectòria universitària que porto, he entès moltes vegades què vol dir això de recollir la tovallola, de tornar a començar i de seguir endavant, tants cops com ha calgut. I si alguna cosa he après, és que hi ha tovalloles que no es recullen soles: hi ha mans que t'hi ajuden, mirades que t'hi sostenen i presències que t'empenyen a continuar. Hi ha dies en què ets tu qui s'ajup per tornar-la a agafar, i n'hi ha d'altres en què és algú altre qui la recull, fins i tot, abans que toqui a terra. Perquè el camí no s'avança només amb constància, sinó també amb companyia; amb aquells que celebren cada passa, que et recorden la direcció quan el trajecte es fa llarg i que caminen al teu costat mentre tot agafa sentit.

I és precisament quan mires enrere i veus totes aquestes mans, que entens que res d'això no hauria estat igual sense elles. Potser per això aquest moment és tan especial: perquè arriba l'hora d'agrair. D'agrair a totes aquelles persones que, d'una manera o altra, heu fet possible que aquest somni immens que és la meva tesi doctoral hagi arribat fins aquí.

Els primers responsables que hagi pogut fer realitat aquest somni sou vosaltres tres: en Quim, la Marina i la Laura. Sou extraordinaris, no només pel vostre rigor i saviesa professional, sinó (i sobretot) per la vostra qualitat humana.

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Dani, la incorporació (ja no tan) recent, però que estàs donant-ho tot. Gràcies per les ganes, l'alegria i aquesta actitud fresca que ho fa tot tan fàcil. Perquè entre rialles, moments inesperats i algun concert improvisat de Raphael, has fet que el camí fos molt més lleuger. I per un PhD que segur que arribarà ben aviat.

Dani, la incorporación (más) reciente. Por entrar en esta tesis cuando ya tiene historia, pero también mucho futuro. Tienes ante ti una oportunidad de oro (de esas que no pasan muchas veces en la vida) y que, sin duda, puede convertirse en una de las mejores experiencias de tu trayectoria. Exprímela y disfrútala al máximo.

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I... un agraïment, que hagués sigut inesperat a l'inici de la tesi, va pel crossfit. Qui m'havia de dir que, començar a l'equador de la tesi, en un moment de molta feina i estrès, m'ajudaria tant no només a nivell físic, sinó també a optimitzar el meu rendiment. Però, més enllà de l'esport en sí, una persona ha esdevingut una part fonamental d'aquest procés: la **Gisela**. Y, como no, también su marido, **Aday**, que nos ha seguido allí a donde hemos ido. Juntes vam formar el nostre equip, *Les Matasanos*, i ha estat una font incondicional de suport i energia positiva. Per això, també vull agrair a en **Joan**, a l'**Anna**, la **Janet** i a tota la gent del box per la seva confiança, el seu suport constant i per animar-nos sempre a seguir endavant, sense perdre mai el somriure. Hem arribat al final de la tesi amb un petit parón… però només per tornar-hi amb més empenta encara.

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LIST OF ABBREVIATIONS

A

ADWG	Average daily weight gain
AKT	Protein kinase B
A. pleuropneumoniae	*Actinobacillus pleuropneumoniae*
AUC	Area under the curve

B

BW	Body weight

C

Cap	Capsid protein
cGAS	Cyclic GMP-AMP synthase
CI	Confidence interval
C-ISH	Conventional *in situ* hybridisation
CMI	Cell-mediated immunity
cRPMI	Complete RPMI 1640 medium
CTL	Cytotoxic T lymphocyte
CV	Coefficient of variation

D

DNA	Deoxyribonucleic acid
dpi	Days post-infection

E

E	Exposed
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
ERK	Extracellular signal-related kinase

F

FBS	Foetal bovine serum
FCR	Feed conversion rate

G

gC1qR	Receptor for the globular head domains of complement component 1q

H

H	High
HE	Haematoxylin-eosin

I

IFN-α	Interferon alpha
IFN-β	Interferon beta
IFN-γ	Interferon gamma
IFN-I	Type I interferon
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IHC	Immunohistochemistry
IL-6	Interleukin 6
IL-10	Interleukin 10
IMM	Immunised
IPC	Internal positive control
IPMA	Immunoperoxidase monolayer assay
ISG	Interferon-stimulated gene
ISH	*In situ* hybridisation
ISGF3	Interferon-stimulated gene factor 3 complex

L

L	Low
LD	Lymphocyte depletion
LDF	Lactation diet feed

LOD	Limit of detection
LOQ	Limit of quantification
LW	Live weight

M

M	Medium
M. hyopneumoniae	*Mycoplasma hyopneumoniae*
MAPK	Mitogen-activated protein kinase
MDA	Maternally derived antibodies
MDI	Maternally derived immunity
MHC-II	Major histocompatibility complex class II

N

NA	Neutralising antibodies
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
Non_IMM	Non-immunised
NPM1	Nucleophosmin 1
nt	Nucleotides
NV	Not vaccinated

O

OD	Optical density
OOI	Onset of immunity
ORF	Open reading frame

P

PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PCV	Porcine circovirus
PCV1	Porcine circovirus 1
PCV2	Porcine circovirus 2
PCV3	Porcine circovirus 3

PCV4	Porcine circovirus 4
PCV5	Porcine circovirus 5
PCV2a – PCV2i	PCV2 genotypes a to i
PCV2-AD	Porcine circovirus 2 associated diseases
PCV2-RD	Porcine circovirus 2 reproductive disease
PCV2-SD	Porcine circovirus 2 systemic disease
PCV2-SI	Porcine circovirus 2 subclinical infection
PCV3-RD	Porcine circovirus 3 reproductive disease
PCV3-SD	Porcine circovirus 3 systemic disease
PCVD	Porcine circovirus diseases
pDC	Plasmacytoid dendritic cell
PDNS	Porcine dermatitis and nephropathy syndrome
PK-15	Porcine kidney cell line 15
PMWS	Post-weaning multisystemic wasting syndrome
PPV	Porcine parvovirus
PRDC	Porcine respiratory disease complex
PRRSV	Porcine reproductive and respiratory syndrome virus

Q

qPCR	Quantitative polymerase chain reaction

R

R	Recovered
R-ISH	RNA *in situ* hybridisation
Rep	Replication-associated protein
RT-qPCR	Real time quantitative polymerase chain reaction

S

S	Susceptible
SEIR	Susceptible-exposed-infected-recovered
SF	Supplementary figure
SIV	Swine influenza virus
SOCS3	Suppressor of cytokine signalling 3

SP1	Specificity protein 1
ssDNA	Single-stranded DNA
ST	Supplementary table
STAT1	Signal transducer and activator of transcription 1
STAT2	Signal transducer and activator of transcription 2
S phase	Synthesis phase (of the cell cycle)

T

Th1	T helper type 1
TLR9	Toll-like receptor 9
TNF-α	Tumor necrosis factor alpha
TTSuV1a	Torque teno sus virus 1a

U

UPR	Unfolded protein response
USA	United States of America

V

V	Vaccinated
VH	Very high
VLP	Virus-like particle

W

woa	Weeks of age
wpc	Weeks post-challenge
wpv	Weeks post-vaccination

ABSTRACT

Porcine circovirus 2 (PCV2) remains a ubiquitous pathogen in global pig production despite nearly two decades of extensive vaccination. While current vaccines have markedly reduced clinical disease, they are not sterilising and cannot fully prevent viral circulation. Under these conditions, maternally derived immunity (MDI) has become a central determinant of PCV2 epidemiology and vaccine performance, particularly in the context of co-circulating immunomodulatory pathogens such as porcine reproductive and respiratory syndrome virus (PRRSV). This doctoral thesis investigated how MDI, vaccination, and viral coinfections interact to shape PCV2 infection dynamics, integrating field epidemiology, vaccine efficacy, digital pathology, and predictive modelling.

The first Chapter analysed serological and virological data from Spanish farrow-to-finish herds collected in 2020 and 2022. The study revealed that lower PCV2 maternally derived antibodies (MDA) levels in piglets coincided with a higher frequency of early-life PCV2 viremia, particularly in farms with concurrent PRRSV circulation. These results demonstrate how herd-level humoral immunity landscapes, modulated by both PCV2 vaccination pressure and PRRSV status, among others, determine the timing of PCV2 infection and the window of susceptibility before piglet vaccination.

The second Chapter evaluated experimentally the efficacy of a combined PCV2d and *Mycoplasma hyopneumoniae* vaccine in piglets with high and low MDA values. Vaccination significantly reduced PCV2 viremia, viral load in lymphoid tissues, and lymphoid histopathological lesions following a heterologous PCV2b challenge regardless the MDA level. Additionally,

cytokine profiling indicated a vaccine-induced modulation of both pro-inflammatory and regulatory responses, reflecting an effective immune activation even in the presence of high MDA. Given the subclinical nature of the challenge, conventional histopathology did not reveal visible lesions in most animals. However, RNAscope™ *in situ* hybridisation (ISH) successfully identified variable amounts of viral genome, demonstrating its higher sensitivity and confirming a markedly lower viral load in vaccinated compared to not vaccinated pigs.

The third Chapter expanded that diagnostic approach by developing an automated, pixel-based RNAscope ISH quantification method for objective detection of PCV2 genome in formalin-fixed, paraffin-embedded tissues. This digital pathology tool, validated with an extended set of lymphoid samples, provided a reproducible and high-sensitivity platform to visualise and quantify PCV2 distribution.

Finally, building upon the conceptual framework established in the previous epidemiological and experimental studies, a stochastic individual-based model was developed to simulate PCV2 prevalence, MDA decay, and vaccination outcomes in farrow-to-finish herds. The model was parameterised using data from the current scientific literature and predicts how different combinations of MDA levels, infection pressure, and vaccination schedules influence viral dynamics, shedding, and production performance, providing a decision-support framework for optimising PCV2 control at the herd level.

Overall, this PhD thesis advances in the understanding of PCV2 epidemiology and immunity by connecting passive protection, vaccination efficacy, digital diagnosis and predictive modelling.

RESUMEN

El circovirus porcino 2 (PCV2) sigue siendo un patógeno ubicuo en la producción porcina mundial a pesar de casi dos décadas de vacunación intensiva. Aunque las vacunas disponibles han reducido de forma notable la enfermedad clínica, no son esterilizantes y no pueden prevenir completamente la circulación viral. En estas condiciones, la inmunidad maternal (MDI) se ha convertido en un determinante central de la epidemiología del PCV2 y de la eficacia vacunal, especialmente en el contexto de patógenos inmunomoduladores co-circulantes como el virus del síndrome respiratorio y reproductivo porcino (PRRSV). Esta tesis doctoral investigó cómo la MDI, la vacunación y las coinfecciones virales interactúan para modelar la dinámica de infección por PCV2, integrando epidemiología de campo, eficacia vacunal, patología digital y modelización predictiva.

En el primer capítulo se analizaron datos serológicos y virológicos de granjas españolas de ciclo cerrado recogidos en 2020 y 2022. El estudio reveló que niveles más bajos de anticuerpos de origen maternal (MDA) frente a PCV2 en lechones coincidían con una mayor frecuencia de viremia temprana, especialmente en granjas con circulación concurrente de PRRSV. Estos resultados muestran cómo el contexto inmunitario de la granja, modulado por la presión vacunal frente a PCV2 y el estatus sanitario frente a PRRSV, entre otros factores, determinan el momento de la infección de PCV2 y la ventana de susceptibilidad previa a la vacunación de los lechones.

En el segundo capítulo se evaluó experimentalmente la eficacia de una vacuna combinada frente a PCV2d y *Mycoplasma hyopneumoniae* en lechones con valores altos y bajos de MDA. La vacunación redujo significativamente la

viremia por PCV2, la carga viral en tejidos linfoides y las lesiones histopatológicas tras un desafío heterólogo con PCV2b, independientemente del nivel de MDA en el momento de la vacunación. Además, el perfil de citoquinas indicó una modulación vacunal tanto de respuestas proinflamatorias como regulatorias, reflejando una activación inmunitaria eficaz incluso en presencia de MDA elevados. Dado el carácter subclínico del desafío, la histopatología convencional no reveló lesiones visibles en la mayoría de los animales. Sin embargo, la hibridación *in situ* RNAscope™ (ISH) identificó cantidades variables de genoma viral, demostrando su mayor sensibilidad y confirmando una carga viral marcadamente menor en los cerdos vacunados en comparación con los no vacunados.

En el tercer capítulo se profundizó en este enfoque diagnóstico mediante el desarrollo de un método automatizado de cuantificación de ISH por RNAscope basado en píxeles, diseñado para la detección objetiva del genoma de PCV2 en tejidos fijados en formalina e incluidos en parafina. Esta herramienta de patología digital, validada con un conjunto ampliado de tejidos linfoides, ofreció un sistema reproducible y de alta sensibilidad para visualizar y cuantificar la distribución de PCV2.

Finalmente, aprovechando el marco generado por los estudios epidemiológico y experimental anteriores, se desarrolló un modelo estocástico individual que simula la prevalencia de PCV2, el descenso de los MDA y los efectos de distintos programas vacunales en granjas de ciclo cerrado. Parametrizado con datos recientes de la literatura, el modelo permite evaluar cómo la combinación de niveles de MDA, presión de infección y pautas de vacunación modula la dinámica viral, la prevalencia y los resultados

productivos, ofreciendo una herramienta útil para optimizar el control del PCV2 en las granjas.

En conjunto, esta tesis doctoral profundiza en la comprensión de la epidemiología y la inmunidad frente a PCV2, integrando conocimientos sobre protección pasiva, eficacia vacunal, diagnóstico digital y modelización predictiva.

RESUM

El circovirus porcí 2 (PCV2) continua sent un patogen ubic en la producció porcina mundial malgrat gairebé dues dècades de vacunació intensiva. Tot i que les vacunes actuals han reduït de manera notable la malaltia clínica, no són esterilitzants i no impedeixen completament la circulació viral. En aquest context, la immunitat maternal (MDI) s'ha convertit en un factor clau per entendre l'epidemiologia del PCV2 i l'eficàcia vacunal, especialment en presència de patògens immunomoduladors co-circulants, com el virus de la síndrome respiratòria i reproductiva porcina (PRRSV). Aquesta tesi doctoral ha investigat com la MDI, la vacunació i les coinfeccions víriques interactuen per modelar la dinàmica d'infecció per PCV2, integrant epidemiologia de camp, immunologia experimental, patologia digital i modelització predictiva.

Al primer capítol es van analitzar dades serològiques i virològiques de granges espanyoles de cicle tancat recollides el 2020 i el 2022. L'estudi va mostrar que la presència de nivells baixos d'anticossos d'origen maternal (MDA) en els garrins en el moment de la vacunació s'associaven a una major freqüència de virèmia precoç, especialment en granges amb circulació concomitant de PRRSV. Aquests resultats il·lustren com el context immunitari de la granja, modulat per la pressió vacunal contra el PCV2 i l'estatus de PRRSV, entre altres factors, condiciona el moment de la infecció i la finestra de susceptibilitat prèvia a la vacunació dels garrins.

Al segon capítol es va avaluar experimentalment l'eficàcia d'una vacuna combinada front a PCV2d i *Mycoplasma hyopneumoniae* en garrins amb nivells alts i baixos de MDA en el moment de la vacunació. Aquesta vacunació va reduir significativament la virèmia per PCV2, la càrrega viral en teixits

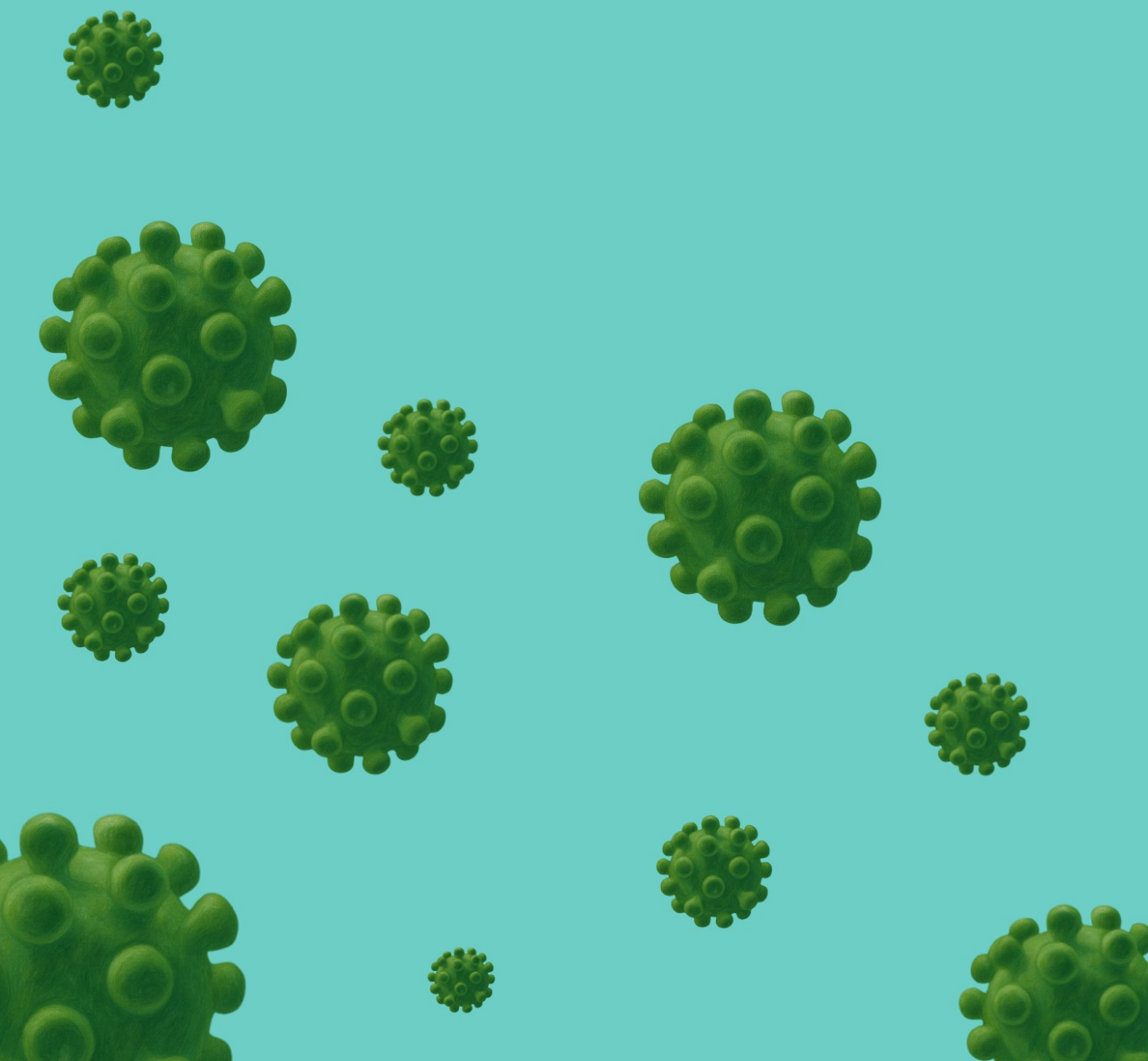
limfoides i les lesions histopatològiques després d'un desafiament heteròleg amb PCV2b, independentment del nivell d'MDA. A més, el perfil de citoquines va mostrar una modulació de respostes proinflamatòries i reguladores induïda per la vacunació, reflectint una activació immunitària eficaç fins i tot en presència de MDA elevats. Atès el caràcter subclínic del desafiament, l'anàlisi histopatològic convencional no va evidenciar lesions en la majoria dels animals. No obstant això, la hibridació *in situ* (ISH) per RNAscope™ va detectar quantitats variables de genoma viral, demostrant una major sensibilitat i confirmant una càrrega viral molt inferior en els animals vacunats en comparació amb els no vacunats.

Al tercer capítol es va aprofundir en aquest enfoc diagnòstic mitjançant el desenvolupament d'un mètode automatitzat de quantificació d'ISH per RNAscope basat en píxels, destinat a la detecció objectiva del genoma de PCV2 en teixits fixats en formalina i inclosos en parafina. Aquesta eina de patologia digital, validada amb un conjunt més ampli de limfonodes, va proporcionar un sistema reproduïble i d'alta sensibilitat per visualitzar i quantificar la distribució del PCV2.

Finalment, a partir del marc establert pels estudis epidemiològic i experimental previs, es va desenvolupar un model individual que simula la transmissió del PCV2, el descens dels MDA i els efectes de diferents estratègies de vacunació en granges de cicle tancat. Parametritzat amb dades recents de la literatura científica, el model permet analitzar com la combinació de nivells d'MDA, pressió d'infecció i pautes vacunals influeix en la dinàmica viral, l'excreció i els resultats productius, constituint una eina útil per optimitzar el control del PCV2 a nivell d'explotació.

En conjunt, aquesta tesi doctoral aprofundeix en la comprensió de l'epidemiologia i la immunitat davant del PCV2, integrant coneixements sobre protecció passiva, resposta vacunal, diagnòstic digital i modelització predictiva.

General introduction



1. OVERVIEW

1.1. Porcine circoviruses

Porcine circoviruses (PCVs) belong to the genus *Circovirus*, within the family *Circoviridae* (phylum *Cressdnaviricota*, class *Arfiviricetes*, and order *Cirlivirales*), which also includes the genus *Cyclovirus* (Varsani et al., 2024). Members of this viral family are small, non-enveloped viruses with covalently closed circular single-stranded DNA (ssDNA) genomes (Varsani et al., 2024) and exhibit icosahedral symmetry, measuring approximately 15 to 25 nm in diameter (Todd et al., 1991; Crowther et al., 2003; Rosario et al., 2017). This family has been expanded in the recent years with the recognition of 54 new species, classified according to a species demarcation threshold of less than 80% genome-wide pairwise identity (Varsani et al., 2024). Among *Circovirus* species, four can infect swine: PCV1, PCV2, PCV3, and PCV4 (Varsani et al., 2024). Recently, a novel circular single-stranded DNA virus, tentatively named PCV5, has been identified in China, although phylogenetic analyses may suggest clustering outside the *Circoviridae* family (Liu et al., 2025).

The first PCV was identified in 1974 as a non-cytopathic viral contaminant of the porcine kidney cell line 15 (PK-15) (Tischer et al., 1974). Due to its small size and morphology, this virus was initially considered similar to picornaviruses. Later, considering its genomic characterization and the confirmation of being a DNA virus, it was classified as the first circovirus and named PCV (Tischer et al., 1982). Despite its widespread presence and ubiquitous nature, this circovirus was regarded as non-pathogenic (Tischer et al., 1986; Allan et al., 1995; Tischer et al., 1995). Due to the subsequent discovery of a second PCV (Clark, 1997; Harding and Clark, 1997), it was proposed to name Porcine circovirus 1 (PCV1) to the cell culture contaminating agent.

A second porcine circovirus was identified in the late 1990s in association with postweaning multisystemic wasting syndrome (PMWS), a new emerging disease in pigs characterised by jaundice, progressive weight loss, respiratory distress, increased mortality, enlarged lymph nodes, and histopathological lesions mainly affecting lymphoid organs (Clark, 1997; Harding and Clark, 1997). Comparative genomic analysis revealed a 68% nucleotide similarity between the existing PCV (current PCV1) and the novel virus, leading to its classification as a distinct species and being termed as porcine circovirus 2 (PCV2) (Allan et al., 1998; Hamel et al., 1998; Meehan et al., 1998). The capsid measures 12 to 23 nm in diameter (Hamel et al., 1998; Crowther et al., 2003; Rodríguez-Cariño and Segalés, 2009), and its genome spans 1,766 to 1,768 nucleotides (nt) with an ambisense organization that allows bidirectional transcription from both DNA strands (Meehan et al., 1998; Hamel et al., 1998; Mankertz et al., 1998, 2004). Retrospective analyses of archived tissues revealed that PCV2 had been circulating in swine populations well before the clinical recognition of PMWS. Specifically, polymerase chain reaction (PCR) studies detected PCV2 DNA in pig samples dating back to 1962 in Germany, although no disease association was established at that time (Jacobsen et al., 2009). Since its emergence, PCV2 has been associated to different conditions that were collectively known as porcine circovirus diseases (PCVDs) in Europe (Segalés et al., 2005a) or porcine circovirus-associated diseases (PCV-AD) in North America (Opriessnig et al., 2007). Very recently, an effort to homogenize nomenclature has suggested the use of PCV2-associated diseases (PCV2-AD) to group the conditions linked to this virus (Segalés et al., 2025). PMWS, currently referred to as PCV2-systemic disease (PCV2-SD) (Segalés, 2012), is one of the clinical manifestations of PCV2-AD, which also comprises PCV2-subclinical infection (PCV2-SI), PCV2-reproductive disease (PCV2-RD), and porcine dermatitis and nephropathy syndrome (PDNS) (Segalés, 2012).

Porcine circovirus 3 (PCV3) was first identified in 2015 in a farm of USA using metagenomic sequencing in sows suffering from reproductive failure and PDNS (Palinski et al., 2016). Similarly to PCV2, retrospective analyses have revealed that PCV3 had been circulating in pig populations for decades prior to its formal discovery (Klaumann et al., 2018b; Sun et al., 2018; Rodrigues et al., 2020). Since its initial identification, PCV3 has been detected in swine populations from multiple countries across different continents, highlighting its broad geographical distribution (Silva et al., 2024). Clinically, PCV3 has been associated with a wide spectrum of clinical signs, including reproductive failure, respiratory distress, enteric disorders, PDNS and multisystemic inflammation (Phan et al., 2016; Faccini et al., 2017; Zhai et al., 2017b; Kedkovid et al., 2018; Klaumann et al., 2018a; Shen et al., 2018; Arruda et al., 2019; Deim et al., 2019; Qi et al., 2019a). Based on its detection within histological lesions (lymphohistiocytic, systemic arteritis/periarteritis) and its correlation with clinical signs, two clinical presentations, collectively known as PCV3-associated disease, have been proposed for PCV3: reproductive disease (PCV3-RD), primarily linked to stillbirths, foetal mummification and weak-born piglets; and systemic disease (PCV3-SD), characterised by wasting, anorexia and generalized inflammation (Saporiti et al., 2021a,b). The outcome from a PCV-3 experimental infection study in pregnant gilts suggested that PCV3-SD can be the evolution of those animals surviving from PCV3-RD originated by viral intrauterine infection (Cobos et al., 2023).

Porcine circovirus 4 (PCV4) was identified for the first time in 2019 in pigs from China exhibiting respiratory signs and a condition resembling PDNS (Zhang et al., 2020a). Although few studies are available to date, current data suggest a wider geographical distribution, as it has been detected in Mongolia (Ha et al., 2021), South Korea (Nguyen et al., 2022), Thailand (Sirisereewan et al., 2023b), Malaysia

(Tan et al., 2023), Spain (Holgado-Martín et al., 2023), and USA (Kroeger et al., 2024). The frequency of PCV4 detection seems to be much lower than that of PCV2 or PCV3 (Vargas-Bermudez et al., 2022). Nevertheless, retrospective analyses have detected PCV4 DNA in tissues from 2012 (Hou et al., 2022) and antibodies as early as 2008 (Ge et al., 2021), indicating that the virus may have been circulating undetected for years. Clinically, the role of PCV4 remains unclear, as most of the available evidence comes from field cases with coinfections involving PCV2 or PCV3. This scenario makes difficult to attribute the observed clinical signs, such as respiratory distress, PDNS-like lesions, and reproductive failure, to PCV4 (Zhang et al., 2020a; Hou et al., 2022; Tian et al., 2021; Holgado-Martín et al., 2023). However, a recent experimental infection in 3-week-old piglets indicated that PCV4 is infectious and pathogenic, inducing growth retardation, diarrhoea, respiratory signs, and multisystemic lesions, with viral DNA detected in multiple organs, including the brain (Zheng et al., 2025). However, this preliminary information is yet to be contrasted.

What has been referred to as PCV5 has been detected in pigs exhibiting respiratory signs, diarrhoea and reproductive failure, with viral DNA identified across a wide range of organs in affected animals (Liu et al., 2025). In addition, the study reported that viral copy numbers strongly mirrored the severity of clinical signs, with intestinal and lymphoid tissues consistently showing the highest levels of PCV5 DNA (Liu et al., 2025). So far, the putative PCV5 has generated more questions than answers to date, and its eventual pathogenicity has to be disentangled in the future.

1.2. Economic and productive relevance of PCVs in swine health

Porcine circovirus 2 is among the most economically impactful pathogens in modern pig production systems (Alarcon et al., 2013a; Boeters et al., 2023). This virus has

been associated with increased mortality, reduced average daily weight gain (ADWG), poor feed conversion, reproductive failure, and higher frequency of concomitant infections in PCV2-SD affected pigs (Segalés, 2012), translating into substantial economic losses (Alarcon et al., 2013a; López-Soria et al., 2014). Moreover, the intensification of swine production in recent decades has facilitated complex clinical presentations driven by coinfections with pathogens such as porcine reproductive and respiratory virus (PRRSV), porcine parvovirus (PPV) 1, and *Mycoplasma (M.) hyopneumoniae*, which further exacerbate disease severity and production losses (Holtkamp et al., 2013; Boeters et al., 2023). In the UK, the specific economic burden of PCV2 before vaccination was estimated at £52.6 million annually, peaking at £88 million during epidemic phases (Alarcon et al., 2013a). Similar trends have been reported globally, especially in intensive systems, where PCV2-SI may go unnoticed while still impairing productivity (Segalés et al., 2005a; Alarcon et al., 2013b; Boeters et al., 2023).

Porcine circovirus 3 has been associated with reproductive disorders and systemic inflammation, with growing evidence linking it to reduced litter size, abortions, mummified and stillborn foetuses, as well as weak-born piglets exhibiting poor growth (Arruda et al., 2019; Molossi et al., 2022; Cobos et al., 2023) representing potential sources of productivity loss. Since the prevalence of such clinical cases in swine production is largely unknown, the economic impact of PCV3 remains unquantified. However, its global spread and presence in clinically affected pigs may suggest a relevant role compromising growth performance and overall productivity (Pranoto et al., 2023; Cobos et al., 2024).

Porcine circovirus 4 has been associated with respiratory distress, PDNS-like lesions and reproductive failure (Zhang et al., 2020a; Hou et al., 2022; Holgado-Martín et al., 2023). These clinical signs suggest that PCV4 may impair growth performance

and survival in affected pigs, raising concerns abouts its potential relevance, although its economic impact has not yet been quantified.

Although the economic impact of the recently described PCV5 has not yet been quantified, the virus has been detected in farms experiencing respiratory, enteric and reproductive disorders, with high detection rates in clinically affected piglets (Liu et al., 2025). These findings suggest that PCV5 circulation in production systems may contribute to performance losses, particularly in outbreaks where viral loads are elevated across multiple tissues (Liu et al., 2025). Anyway, more research is needed to ascertain its impact in swine production.

Globally, while PCV2 remains the most extensively characterised and impactful species among PCVs in swine health, the concurrent circulation of other circoviruses warrants continued monitoring, diagnostic refinement, and the development of integrated control strategies, including vaccination approaches if needed. The present PhD Thesis will address these aspects in relation to PCV2.

2. PORCINE CIRCOVIRUS 2 GENETIC CHARACTERISTICS AND DIVERSITY

2.1. Genomic organization

The PCV2 genome contains up to eleven predicted open reading frames (ORFs) (Hamel et al., 1998; Shang et al., 2009). Among these, four ORFs (ORF1 to ORF4) have been extensively characterised and are well-established as encoding proteins with defined biological functions (*Figure 1*):

- The ORF1 protein (945 nt), oriented in the clockwise direction, encodes the replicase proteins Rep and Rep'. These two proteins are essential for viral DNA replication through the rolling-circle replication mechanism (Cheung, 2003; Mankertz et al., 2004).
- The ORF2 protein (702 nt), transcribed in the counterclockwise direction, encodes the major capsid protein (Cap). This protein plays a central role in immune recognition, acting as the main target of neutralising antibodies (NA) (Nawagitgul et al., 2000; Blanchard et al., 2003), and constitutes the most variable region of the PCV2 genome, supporting its use as a phylogenetic marker (Olvera et al., 2007).
- The ORF3 protein (315 nt) overlaps ORF1 in the opposite direction and encodes a non-structural protein that induces apoptosis in infected cells, including porcine peripheral blood mononuclear cells (PBMCs) (Lin et al., 2011) and PK-15 cells (Liu et al., 2005). This pro-apoptotic activity has also been demonstrated *in vivo*, where it contributes to exacerbated tissue damage (Liu et al., 2006).
- In contrast, the ORF4 protein (180 nt) located within ORF3 protein and expressed in the same orientation, appears to counterbalance the pro-apoptotic effect of ORF3 protein by inhibiting virus-induced apoptosis, potentially promoting cell survival during early infection stages (He et al., 2013; Gao et al., 2014a).

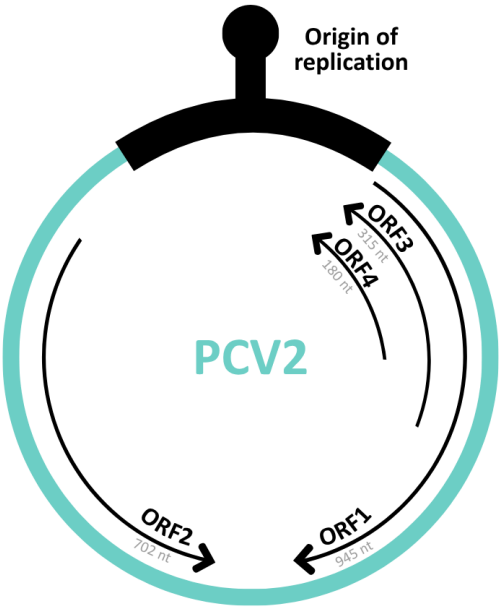


Figure 1. Schematic representation of the PCV2 genome organization. The circular single-stranded DNA genome includes the four main ORFs: ORF1 (Rep and Rep'), ORF2 (Cap), ORF3 and ORF4 (non-structural proteins). The origin of replication is in the intergenic region between ORF1 and ORF2. nt: nucleotides; ORF: open reading frame.

In addition, ORF5 protein, which overlaps with ORF1 protein and is transcribed in the same direction, encodes a protein that promotes viral replication by inducing autophagy pathways in infected cells (Lv et al., 2020). Similarly, ORF6, located within ORF2 protein and transcribed in the same direction, regulates caspase activity and modulates the expression of various cytokines *in vitro*, suggesting a potential role in shaping host immune responses (Li et al., 2018b). Nevertheless, further *in vivo* studies are required to confirm and fully elucidate the functional relevance of these two ORFs. Finally, the remaining predicted ORFs (ORF7–ORF11) have only been identified through bioinformatic analyses, and their transcriptional activity and biological significance remain unconfirmed (Gao et al., 2024).

2.2. Genotypes and evolutionary trends

Nowadays, PCV2 is classified based on sequence divergence in eight genotypes (from PCV2a to PCV2h) (Franzo and Segalés, 2018). In addition, the designation of a further genotype, PCV2i, has been suggested (Wang et al., 2020a). Given its single-stranded DNA nature and a nucleotide substitution rate similar to that of RNA viruses, PCV2 exhibits a high potential for genomic mutation, making the emergence of new genotypes in the future a probable scenario (Firth et al., 2009; Franzo et al., 2024).

Whereas PCV2a, PCV2b, and PCV2d are commonly detected worldwide (Wang et al., 2020a), the remaining genotypes have only been reported occasionally and/or in geographically restricted locations (Franzo and Segalés, 2018). The predominant genotype worldwide, from its discovery until the early 2000s, was PCV2a (Cortey et al., 2011a; Franzo et al., 2024). Later, coinciding temporally with severe PCV2-SD outbreaks (Cortey et al., 2011a), a genotype shift occurred with PCV2b becoming the most prevalent one in many regions, such as in North America and Europe (Dupont et al., 2008; Carman et al., 2008; Timmusk et al., 2008; Cortey et al., 2011a; Franzo et al., 2024). Subsequently, from approximately 2010-12 onwards, PCV2d gradually displaced PCV2b as the most frequently found genotype in various parts of the world (Xiao et al., 2015; Franzo et al., 2016; Tsai et al., 2019; Sibila et al., 2021).

Recent findings suggest that PCV2 evolution is driven more by fluctuations in the prevalence of existing genotypes than by the emergence of entirely new ones (Franzo et al., 2024). Considering that immunity, natural or vaccine-induced, is built against dominant genotypes, selective pressures may favour variants with even subtle immunological advantages (Franzo et al., 2024). This process, potentially consistent with negative frequency-dependent selection, may have contributed to

the sequential replacement of PCV2a by PCV2b and subsequently by PCV2d (Franzo et al., 2024). Vaccination efforts, while effective, may have accelerated the latest shift, reinforcing the importance of continued genomic surveillance to assess vaccine efficacy, detect emerging strains, and anticipate potential genotype shifts that could compromise long-term control strategies (Sibila et al., 2021; Franzo et al., 2024).

3. EPIDEMIOLOGY OF PCV2

3.1. Prevalence and genotype distribution

Porcine circovirus 2 is one of the most ubiquitous swine pathogens worldwide, with evidence of infection reported across all continents (Franzo et al., 2024). This global spread probably reflects its remarkable environmental persistence and high transmission efficiency (Firth et al., 2009; Meng, 2012; Franzo et al., 2025).

In Europe, the virus is highly prevalent, with PCV2a, PCV2b, and PCV2d being the most frequently identified genotypes (Segalés et al., 2013; Saporiti et al., 2020; Franzo et al., 2024). A comparable scenario is observed in Asia, where large-scale surveys (particularly in China) have revealed high prevalence rates, often exceeding 40%, and a similar predominance of PCV2a, PCV2b, and PCV2d (Liu et al., 2020). However, their relative frequencies may vary between countries (Kim et al., 2018; Dinh et al., 2021; Flay et al., 2022; Sivamurthy et al., 2022; Sirisereewan et al., 2023a).

In the Americas, PCV2 is also widespread and considered endemic in both North and South America, being detected in clinically affected and subclinically infected pigs across diverse production systems (Vargas-Bermudez et al., 2022; Cezar et al., 2024; Franzo et al., 2024; Miotto et al., 2024; Koszegi et al., 2025; Rodrigues et al., 2025). Like the situation in Asia and Europe, PCV2a, PCV2b, and PCV2d remain the predominant genotypes reported in this geographical area (Wang et al., 2020a; Franzo et al., 2024).

The few available studies on the prevalence of PCV2 in Africa confirm its presence in multiple countries (Laisse et al., 2018; Wilfred et al., 2018; Afolabi et al., 2019; Adebowale et al., 2022). In this continent, several genotypes, including PCV2g, often co-circulate, and phylogeographic analyses indicate multiple introductions from

other continents alongside the emergence of unique African clusters (Franzo et al., 2022; Afolabi et al., 2023).

In Oceania, PCV2 has also been documented in both Australia and New Zealand, where PCV2b predominates in the few studies available (Raye et al., 2005; Garkavenko et al., 2005; Mone et al., 2020). However, updated large-scale epidemiological data of this swine production area are lacking.

3.2. Transmission routes

In general terms, PCV2 can be transmitted through horizontal and vertical routes (Patterson and Opriessnig, 2010).

3.2.1. Horizontal transmission

Among the various horizontal transmission routes, close contact (including nose-to-nose interactions and commingling in shared pens) and faecal-oral exposure are considered the most relevant ones (Albina et al., 2001; Bolin et al., 2001; Dupont et al., 2009; Kristensen et al., 2009; Rose et al., 2012). Nasal secretions, saliva and faeces are recognised as the main shedding routes (Sibila et al., 2004; Segalés et al., 2005b; Patterson et al., 2011b,c). However, viral excretion has been also documented in urine, colostrum, milk, semen and respiratory and ocular secretions, highlighting the broad range of possible excretion pathways (Krakowka et al., 2000; Larochelle et al., 2000; Shibata et al., 2003,2006; Ha et al., 2009).

In contrast, indirect transmission via contaminated fomites, aerosolized particles, or environmental surfaces has also been reported (Madson and Opriessnig, 2011; Patterson et al., 2011a). Nevertheless, its efficiency is generally lower than that of direct contact (Andraud et al., 2008; Kristensen et al., 2009).

3.2.2. Vertical transmission

Vertical transmission denotes the passage of PCV2 from the sow to its offspring, either transplacentally during gestation, at parturition, or in the immediate postnatal period. Experimental studies have confirmed that PCV2 is able to cross the placental barrier and replicate in embryos and foetuses, especially when sows are infected during the late stages of gestation (Sánchez et al., 2001; Johnson et al., 2002; Park et al., 2005; Ha et al., 2008; Dong et al., 2016). This transplacental transmission has been associated with various reproductive disorders, including abortions, increased rates of stillbirths, mummified or weak-born piglets, and eventually, early embryonic death (West et al., 1999; O'Connor et al., 2001; Kim et al., 2004; Mateusen et al., 2007; Brunborg et al., 2007; Madson and Opriessnig, 2011). Additionally, infected foetuses may show myocarditis, highlighting the pathogenic potential of PCV2 during gestation (Brunborg et al., 2007). However, field data suggest that reproductive failure directly attributable to PCV2 is relatively uncommon in many regions, and prevalence estimates vary widely between countries and production systems (Ladekjaer-Mikkelsen et al., 2001; Kim et al., 2004; Pensaert et al., 2004; Maldonado et al., 2005; Shen et al., 2010a).

In addition to transplacental transmission, PCV2 has also been detected in semen (Larochelle et al., 2000). Experimental inseminations with PCV2-spiked semen demonstrated that the virus can be shed through semen and transmitted to foetuses, but the viral load was not sufficient to consistently infect pregnant sows by artificial insemination (Madson et al., 2009a,b; Sarli et al., 2012). Reproductive failure was only reproduced under specific experimental conditions (Madson et al., 2009c). Nevertheless, field evidence indicates that transmission of PCV2 via semen is of limited epidemiological relevance due to the generally low prevalence and viral loads detected in boars (Rose et al., 2012; Fan et al., 2023).

During the lactation period, both sows and piglets can exhibit viremia. This enables PCV2 transmission through suckling or contact with contaminated maternal secretions (Eddicks et al., 2022; Vargas-Bermudez et al., 2025).

3.3. Infection dynamics and modelling approaches

Previous studies indicate that PCV2 viremia can be detected as early as 3-7 days post-inoculation (dpi), with viral excretion peaking between 14 and 21 dpi (Krakowka et al., 2000; Ladekjaer-Mikkelsen et al., 2002; Rovira et al., 2002; Resendes et al., 2004; Chung et al., 2005; Caprioli et al., 2006; Andraud et al., 2008). While these studies describe the temporal patterns of infection at the individual level, modelling approaches have been developed to better quantify transmission dynamics and to extrapolate these findings to the population scale (Andraud et al., 2008; Andraud et al., 2009a,b).

In a first study, PCV2 within- and between-pen transmission was quantified by estimating the daily transmission rate (β) and the basic reproduction number (R_0) using a stochastic susceptible-exposed-infected-recovered (SEIR) model fitted to serial transmission experiments (Andraud et al., 2008). This approach revealed a relatively high transmission potential within pens (R_0 ranging from 5.5 to 8.9, depending on assumptions regarding the end of infectiousness), while transmission between pens was markedly lower (R_0≈0.6) (Andraud et al., 2008). A subsequent deterministic model incorporated a time-dependent transmission rate, providing a more accurate description of infectiousness dynamics and estimating a latency period of approximately 8 days, a basic R_0 of 5.9, and a mean generation time of 18.4 days (Andraud et al., 2009a). Finally, a stochastic individual-based modelling framework was applied to farrow-to-finish systems to evaluate the influence of husbandry practices and vaccination (Andraud et al., 2009b). This study highlighted

three major results: 1) reducing piglet mixing significantly decreased the risk of early infection, 2) sow vaccination delayed transmission until the waning of maternal immunity, and 3) piglet vaccination markedly reduced infection pressure and the overall number of cases (Andraud et al., 2009b).

Despite these advances, existing modelling approaches remain limited in scope. At the broader epidemiological level, most previous models are deterministic compartmental frameworks, which are valuable for describing overall infection trends but often fail to capture the stochasticity of transmission events and the heterogeneity of individual immune responses (Ezanno et al., 2020). In the specific context of PCV2, these models do not account for the variability in maternally derived antibody (MDA) levels or heterogeneous vaccination responses, known to influence infection outcomes and growth performance. These limitations emphasise the need for more flexible and mechanistic modelling frameworks able to integrate biological, immunological, and management-related factors (Picault et al., 2019; Ezanno et al., 2020). Such new models would help gaining a deeper understanding of PCV2 epidemiology and to support the development of more effective control strategies.

3.4. Environmental stability and persistence

The virus is remarkably stable in the environment. This feature contributes to its long-term persistence in swine production systems and may indirectly facilitate transmission between animals (Rose et al., 2012; Dvorak et al., 2013; López-Lorenzo et al., 2019, 2022). Moreover, previous *in vitro* studies have shown that PCV2 is highly resistant to thermal inactivation, especially under dry conditions, requiring exposure to temperatures above 75°C for at least one hour to achieve a significant

reduction in viral infectivity (Welch et al., 2006; O'Dea et al., 2008; Kim et al., 2009a).

Additionally, PCV2 also exhibits high resistance to chemical disinfectants containing iodine, alcohol, phenol, or formaldehyde (Royer et al., 2001; Yilmaz and Kaleta, 2004; Martin et al., 2008). In contrast, products containing oxidizing agents, halogens, or sodium hydroxide are among the few compounds capable of effectively reducing viral titres with relatively short exposure times (Kim et al., 2009). A field trial corroborated these *in vitro* findings, demonstrating that effective disinfection protocols can significantly reduce PCV2 contamination and prevent infection in exposed animals (Patterson et al., 2011c).

3.5. Host range beyond domestic swine

Although *Sus scrofa domesticus* is the primary host of PCV2, evidence suggests that other animal species may also harbour the virus, either as incidental hosts or mechanical vectors, thereby contributing to its maintenance in the environment (*Table 1*). Nevertheless, their overall contribution to the epidemiology of PCV2 appears to be limited, and further research is needed to clarify the significance of these cross-species detections.

Table 1. Reported hosts of PCV2 beyond domestic pigs.

Host	Evidence type	Epidemiological role	References
Wild boar	• PCR detection in natural hosts • Clinical cases (PCV2-SD reported) • Genetic similarity with strains detected in domestic pigs	• Reservoir • Possible vector to domestic pigs	Ellis et al., 2003 Schulze et al., 2004 Vicente et al., 2004 Lipej et al., 2007 Hohloch et al., 2015 Dei Giudici et al., 2019 Franzo et al., 2020b Rudova et al., 2022
Peccary	• PCR detection in natural hosts	• Possible incidental host	De Castro et al., 2014
Rodents (mice, rat)	• PCR detection in natural hosts • Experimental infection	• Replication confirmed in mice • Potential local reservoirs and mechanical vectors	Kiupel et al., 2001,2005 Cságola et al., 2008 Opriessnig et al., 2009a Lorincz et al., 2010 Pinheiro et al., 2013 Zhai et al., 2016
Rabbit	• Experimental infection	• Incidental or experimental hosts. • No evidence of clinical disease or significant epidemiological role confirmed.	Quintana et al., 2002
Sheep			Allan et al., 2000a
Cattle			Ellis et al., 2001 Kappe et al., 2010
Buffalo	• PCR detection in natural hosts		Zhai et al., 2017a
Fox			Song et al., 2019b
Mink			Wang et al., 2016
Racoon dog			Song et al., 2019a
Housefly	• PCR detection in natural hosts	• Potential mechanical vectors	Blunt et al., 2011
Stable fly			Schwarz et al., 2020
Mosquito			Yang et al., 2012

4. PORCINE CIRCOVIRUS 2 ASSOCIATED DISEASES: CLINICAL AND PATHOLOGICAL OUTCOMES

Porcine circovirus 2 associated diseases (PCV2-AD) encompass four primary clinical presentations: PCV2-SD, PCV2-SI, PCV2-RD, and PDNS (Segalés et al., 2025). These conditions are defined not only by their clinical manifestations, but also by characteristic macroscopic and histopathological lesions, which are summarised in *Table 2*.

The PCV2-SD is considered the most significant clinical form of PCV2-ADs (Segalés, 2012). Its clinical relevance has been extensively documented, particularly in non-immunised pigs, where the morbidity rate could range from 4% to 30% (occasionally exceeding 50%) and mortality may reach up to 20% (Segalés and Domingo, 2002). Both PCV2-SD and PCV2-SI occur mainly during the nursery-to-finishing period; the latter is currently the most widespread form and exerts its economic impact through impaired growth performance and reduced productivity (Kristensen et al., 2011).

In contrast, PCV2-RD affects breeding herds and is among the least commonly diagnosed manifestations of PCV-ADs, largely due to the widespread immunity among adult sows, which reduces the likelihood of clinical outcomes following infection (Pensaert et al., 2004; Segalés, 2012). However, in populations with a high proportion of naïve gilts or seronegative sows, transplacental infection may lead to reproductive failures (West et al., 1999; Opriessnig et al., 2007; Togashi et al., 2011). The outcome of foetal infection varies with gestational stage, ranging from embryonic death and reduced litter size in early gestation, to mummification and abortion in mid gestation, and stillbirths or weak-born piglets in late gestation (Madson and Opriessnig, 2011). Sows rarely develop overt clinical signs, although transient hyperthermia or anorexia during viraemia has been described (Cariolet et al., 2002; Park et al., 2005). Although infrequent, atypical reproductive

presentations including delayed farrowing (>118 days of gestation) and pseudo-gestation have also been reported (Josephson and Charbonneau, 2001; Madson and Opriessnig, 2011). Given its variable presentation, PCV2-RD should be included in the differential diagnosis of reproductive disorders, especially in herds with suboptimal immunity and/or irregular vaccination protocols.

Another infrequent manifestation within the PCV2-AD spectrum is PDNS, a condition of presumed immune-mediated origin that most commonly affects pigs during the late weaning and growing–finishing phases, although sporadic cases have also been reported in sows (Segalés et al., 1998; Drolet et al., 1999). In non-vaccinated herds, its prevalence is generally low (<1%), although case fatality rates can range from 50% to 100% (Wellenberg et al., 2004). The aetiology of PDNS has remained controversial for decades: while epidemiological associations with PCV2 have been repeatedly reported, experimental reproduction of the syndrome using PCV2 alone has been unsuccessful (reviewed by Segalés and Sibila, 2022). This finding supports the hypothesis of an immune-complex-mediated pathogenesis (Wellenberg et al., 2004). Indeed, experimental coinfections with other pathogens as *Torque teno sus* virus 1a (TTSuV1a), PRRSV or PCV3, have also failed to fully reproduce the clinical and pathological spectrum of PDNS (Krakowka et al., 2008; Jiang et al., 2019), casting doubt on their etiological role. Recently, a pivotal shift in understanding has emerged with a study by Cobos et al. (2024), in which all retrospectively examined PDNS cases were real-time quantitative PCR (qPCR) positive for PCV2, and a selected subset also tested positive by ISH RNAscope™ in lymphoid and/or renal tissues. These findings strongly reinforce the hypothesis of a causal link between PCV2 and PDNS (and not with other circoviruses), suggesting that viral presence may act as a trigger for the immune-mediated pathology rather than being directly a cytopathic effect (Cobos et al., 2024).

Table 2. Diagnostic overview of PCV2-AD clinical-pathological presentation (adapted from Segalés, 2012, and Segalés and Sibila, 2022).

	Clinical presentation	Macroscopic findings	Histopathological findings
PCV2-SD	Progressive emaciation, poor body condition, rough hair coat, respiratory distress and diarrhoeic episodes.	• Generalized lymph node enlargement • Thymic atrophy • Non-collapsing lungs with a tan-mottled appearance • Pale and atrophic liver with granular surface • White areas in the renal cortex • Catarrhal enteritis	A hallmark lesion is lymphocyte depletion associated with granulomatous infiltration in lymphoid organs. Inflammatory lesions may also include interstitial pneumonia, interstitial nephritis, and lymphohistiocytic hepatitis, along with granulomatous enteritis. Inflammation involving lymphohistiocytic cell populations can be present in various tissues.
PCV2-SI	Reduced growth performance (10-40 g/day) without overt clinical signs.	• No remarkable gross pathological changes observed at necropsy.	Lymphoid tissues may present absent or minimal depletion of lymphocytes, occasionally accompanied by mild granulomatous inflammation.
PCV2-RD	Reproductive disorders such as abortions, stillbirths, return to oestrus, embryonic mortality, and foetal mummification.	• Enlarged and congested liver in foetus, along with cardiac hypertrophy showing pale multifocal regions in the myocardium • Foetal oedema, ascites, and hydrothorax	Non-suppurative fibrotic or necrotic myocarditis in foetuses. Hepatic congestion and mild pneumonia may also be noted in foetal tissues.
PDNS	Irregular red to purple macules and papules, on hind limbs and perineal area.	• Subcutaneous haemorrhages and oedema in skin • Kidneys with generalized cortical petechiae and pelvic oedema • Enlarged lymph nodes may also be present	Systemic necrotizing vasculitis and fibrino-necrotizing glomerulonephritis, often with non-suppurative interstitial nephritis. Mild to moderate lymphoid depletion and granulomatous infiltration can occur.

PCV2-SD: porcine circovirus 2 systemic disease; PCV2-SI: PCV2 subclinical infection; PCV2-RD: PCV2 reproductive disease; PDNS: porcine dermatitis and nephropathy syndrome.

5. DIAGNOSTIC AND MONITORING TECHNIQUES

5.1. Diagnostic criteria for PCV2-AD

Given the ubiquitous nature of PCV2 infection in pig populations, the mere detection of the virus or antibodies is not sufficient to establish a definitive diagnosis of PCV2-ADs (Segalés et al., 2005a; Segalés and Sibila, 2022). Instead, the diagnosis of these conditions relies on a combination of three essential criteria: (1) presence of compatible clinical signs, (2) confirmation of microscopic lesions characteristic of PCV2-ADs, and (3) detection of PCV2 within those histological lesions (Segalés et al., 2005a). Based on these principles, specific diagnostic criteria have been proposed for each clinical presentation (Segalés and Sibila, 2022), which are summarised in *Table 3*.

5.2. Available techniques for diagnosis and monitoring

Microscopic evaluation of lymphoid tissues remains essential, since lymphocyte depletion (LD) and histiocytic replacement (HR) are the primary histopathological features and the diagnostic hallmarks of PCV2-SD (Sorden, 2000). However, PCV2 infection is not restricted to the immune system, as viral replication has also been observed in the lungs, liver, kidneys, heart, gastrointestinal tract, and reproductive tissues (Rosell et al., 2000; Segalés et al., 2004; Mikami et al., 2005; Brunborg et al., 2007; Yu et al., 2007; Hansen et al., 2010; Szeredi et al., 2012; Baró et al., 2015; Oropeza-Moe et al., 2017; Lippke et al., 2024).

Table 3. Diagnostic criteria for PCV2-ADs (adapted from Segalés, 2012, and Segalés and Sibila, 2022).

	Diagnostic criteria
PCV2-SD	1. Marked weight loss and paleness of the skin, occasionally accompanied by respiratory and/or gastrointestinal signs. 2. Moderate to severe lymphoid depletion, typically with granulomatous inflammation (which can also affect other tissues). 3. Intermediate to high concentrations of PCV2 load in lymphoid tissues, with variable levels found in other tissues.
PCV2-SI	1. Absence of noticeable clinical manifestations. 2. Lack of significant histopathological alterations, with possible mild lesions restricted to lymphoid structures. 3. Limited detection of PCV2, typically restricted to small amounts in follicular regions of lymphoid organs. Criteria 2 and 3 can alternatively be addressed by applying PCV2 detection methods, as for example conventional PCR or qPCR.
PCV2-RD	In case of abortions or mummified foetuses: 1. Reproductive losses occurring in late gestation may resemble SMEDI-like condition (stillbirth, mummification, embryonic death and infertility, applying this las condition to return-to-oestrus scenario). 2. Myocarditis in affected foetuses often shows fibrotic and/or necrotic patterns. 3. Moderate to high levels of PCV2 DNA can be detected in foetal myocardial tissue. In sows with return to oestrus: 1. Irregular return to oestrus may indicate infertility issues linked to infection. 2. PCV2 exposure can be confirmed by seroconversion and/or PCR or qPCR detection around the time of return to oestrus.
PDNS	1. Haemorrhagic and necrotizing skin lesions and/or swollen and pale kidneys with generalized cortical petechiae. 2. Systemic necrotizing vasculitis, and necrotizing and fibrinous glomerulonephritis.

PCV2-SD: porcine circovirus 2-systemic disease; PCV2-SI: porcine circovirus 2-subclinical infection; PCV2-RD: porcine circovirus 2-reproductive disease; PDNS: porcine dermatitis and nephropathy syndrome; SMEDI: stillbirth, mummification, embryonic death and infertility; PCR: polymerase chain reaction; qPCR: quantitative polymerase chain reaction.

Beyond histopathology, multiple laboratory techniques have been developed for the detection of PCV2, primarily targeting either the viral genome or antigen, or its antibodies (Segalés and Sibila, 2022). The most used approaches include PCR, ISH, and immunohistochemistry (IHC) (Rosell et al., 1999; Segalés et al., 2004; Yu et al., 2007; Segalés and Sibila, 2022) for viral detection and enzyme-linked immunosorbent assay (ELISA) tests for antibodies (Sibila et al., 2004; Grau-Roma et al., 2009).

In situ hybridisation detects PCV2 genome, while IHC reveals the presence of viral antigen within tissues. Both methods are typically applied to formalin-fixed, paraffin-embedded samples and allow associating viral presence with microscopic lesions (Rosell et al., 1999; Segalés et al., 2004; Segalés and Sibila, 2022). The intensity of staining is visually evaluated and semi-quantified into categories depending on the amount of viral genome or antigen present (Rosell et al., 1999; Chianini et al., 2003; Szczotka et al., 2011). More recently, digital image analysis tools have been introduced to enhance the objectivity and reproducibility of IHC quantification, as demonstrated in PCV2-infected pig tissues (Horváth et al., 2025). In parallel, advanced RNA-based ISH methods such as RNAscope have been applied to detect PCV2, offering higher sensitivity and resolution compared to conventional ISH (Cobos et al., 2024).

In addition, PCR (and particularly qPCR) is widely used for detecting and quantifying PCV2 DNA in a broad variety of clinical samples: blood, tissues, saliva, nasal secretions, colostrum, semen, faeces, and urine (Larochelle et al., 2000, 2003; Segalés et al., 2005b; Caprioli et al., 2006; Shibata et al., 2006; Ramirez et al., 2012). The qPCR generally provides faster turnaround and a lower risk of cross-contamination than conventional PCR, while offering comparable or sometimes higher sensitivity depending on assay design (Caprioli et al., 2006; Zhao et al., 2010).

Various assay formats (such as SYBR Green I-based and TaqMan-based assays) with different analytical sensitivities have been described (Yang et al., 2007; Grau-Roma et al., 2009; Zhao et al. 2010) and some TaqMan-based qPCRs can discriminate between PCV2 genotypes (Yang et al., 2019; Wang et al., 2020b; Chen et al., 2021). The qPCR has also proven useful for detecting viral DNA in environmental matrices such as air, water, manure, sow and piglet skin surfaces, and various farm equipment surfaces (Verreault et al., 2010; Viancelli et al., 2012; Dvorak et al., 2013; López-Lorenzo et al., 2022).

The viral load determined by qPCR serves as a proxy indicator for distinguishing between clinical and subclinical infections. A strong correlation has been established between high tissue viral loads and the severity of lymphoid lesions (Olvera et al., 2004). These results would support the use of qPCR as a diagnostic proxy in samples with microscopic lesions consistent with PCV2-AD from animals with a compatible clinical picture. In this scenario, qPCR may potentially replace ISH or IHC (Grau-Roma et al., 2009; Segalés and Sibila, 2022) and the detection of high viral load in serum or tissue samples would help confirming a PCV2-AD diagnosis. Animals affected by PCV2-SD tend to show viral loads equal to or greater than 10^7 copies/mL in serum, while in lymphoid tissues are generally higher (Brunborg et al., 2004; Olvera et al., 2004; Fort et al., 2007). However, this proposed threshold to discriminate diseased versus non-diseased pigs was established by means of a particular qPCR method, and very likely the current commercial techniques should define a different cut-off. In addition to its diagnostic role, PCV2 qPCR is widely applied in epidemiological surveillance and herd monitoring, where it is typically performed on serum to evaluate infection dynamics and prevalence (Segalés and Sibila, 2022).

Different serological assays have been developed to detect PCV2-specific antibodies in serum, including immunoperoxidase monolayer assay (IPMA), immunofluorescence assay (IFA), and ELISA (Allan and Ellis, 2000; Rodríguez-Arrioja et al., 2000; Ge et al., 2012). However, antibody detection is not suitable for diagnosing PCV2-AD, as PCV2 is a ubiquitous virus and seropositivity may result from colostrum immunity, vaccination, or infection (Segalés and Sibila, 2022). Nevertheless, serological techniques remain important tools for surveillance purposes, assessing antibody kinetics in experimental trials, monitoring vaccination programs, and evaluating the potential interference of maternally derived immunity (MDI) with vaccine efficacy (Sibila et al., 2004; McKeown et al., 2005; Fachinger et al., 2008; Fort et al., 2009b; Grau-Roma et al., 2009; Turlewicz-Podbielska et al., 2020).

6. PATHOGENESIS AND IMMUNE RESPONSES IN PCV2 INFECTION

6.1. Mechanisms of viral replication and persistence

Porcine circovirus 2 lacks an own DNA polymerase and, therefore, its replication will depend on host cells under division cycle (Tang et al., 2013). Firstly, it adheres to membrane receptors of actively dividing cells, especially those in lymphoid tissues such as macrophages, dendritic cells (DCs) and epithelial cells (Darwich et al., 2004; Meng et al., 2013). After entering these cells through endocytosis, the virus DNA reaches the nucleus, where the viral replication takes place during the S phase of the host cell cycle (Tang et al., 2013).

The virus replicates via a rolling-circle mechanism in which the viral Rep and Cap proteins cooperate with host factors to amplify viral genomes (Lv et al., 2014; Rosario et al., 2017). Non-structural viral proteins contribute to viral persistence and pathogenicity (Fehér et al., 2023). Among them, ORF4 protein prevents early apoptosis to maintain the pool of infected cells, while ORF3 and ORF5 proteins promote apoptosis and endoplasmic reticulum (ER) stress at later stages, facilitating viral release and dissemination (Liu et al., 2005; Wei et al., 2009; He et al., 2013; Gao et al., 2014a; Lv et al., 2015; Zhou et al., 2016; Lv et al., 2020).

Beyond genome replication, PCV2 has evolved strategies to optimise its intracellular environment (Féher et al., 2023). Specifically, the Cap protein interacts with the host receptor gC1qR activating the PI3K/AKT pathway and prolonging cell survival during early infection (Féher et al., 2023). Later, activation of p38-MAPK and ERK signalling supports viral exit and replication (Choi et al., 2015; Wang et al., 2019). Cap also assists nuclear transport by interacting with dynein and promoting α-tubulin acetylation, ensuring efficient intracellular transport (Cao et al., 2015).

Another mechanism that contributes to PCV2 persistence is the virus capacity to manipulate host immune signalling, enabling persistent infection (Fehér et al., 2023). Its genome, rich in unmethylated CpG motifs, can modulate IFN-α production through both TLR9-dependent and independent mechanisms (Hasslung et al., 2003; Vincent et al., 2007; Wilkstrom et al., 2007; Hansoongnern et al., 2022). In addition, PCV2 interferes with DC functions, impairing endocytosis, antigen presentation, and cytokine secretion (Balmelli et al., 2011).

Finally, PCV2 persistence contributes to LD, a hallmark of PCV2-SD as a result from a combination of high viral replication, ER stress, and apoptosis induced by Rep and Rep', Cap, and ORF3 and ORF5 proteins (Tang et al., 2013; Lv et al., 2015; Zhou et al., 2016; Lv et al., 2020; Sun et al., 2021). These changes establish a favourable environment for the virus while altering the host immune landscape. *Table 4* summarises the main mechanisms underlying PCV2 replication and persistence and provides additional molecular details.

Table 4. Schematic overview of the mechanisms involved in PCV2 replication and persistence within host cells.

Virus entry and intracellular transport

- PCV2 binds **gC1qR** at the cell surface.
- Internalization and transport to the nucleus is via **dynein** and **acetylated α-tubulin** (Cap-dependent).

Nuclear replication (S phase)

- Rep and Cap initiate **rolling-circle replication** (RCR) inside the nucleus.
- Replication only in dividing cells (**S phase dependency**).
- Cap and Rep contribute to local **chromatin remodeling**.

Non-structural ORFs and functional outcomes

- **ORF4 protein:** inhibits apoptosis (early phase) → favors replication.
- **ORF3 protein:** induces apoptosis (late phase) → may assist in viral egress.
- **ORF5 protein:** triggers ER stress → UPR → autophagy + apoptosis → viral release.

Host signaling pathways

- Early: **PI3K/Akt** pathway activated → anti-apoptosis.
- Late: **p38-MAPK, ERK** → supports replication and exit.
- **Cap–gC1qR** binding triggers signaling cascade.

Immune evasion and modulation

- Unmethylated **CpG motifs** in viral DNA:
  - Modulate **IFN-α** via **TLR9-dependent and -independent pathways**.
  - **Synthetic CpG-ODNs** used as adjuvants.
- Circular DNA structure interferes with **dendritic cell function**:
  - ↓ Endocytosis
  - ↓ Antigen presentation
  - ↓ Cytokine secretion

Persistence strategies

- Cap and Rep induce:
  - **Oxidative stress**
  - **ER-associated degradation (ERAD)**
- p53 phosphorylation → S phase arrest → continued replication (especially in alveolar macrophages) (Pan et al., 2018; Deng et al., 2022).

cGAS: cyclic GMP-AMP synthase; CpG: cytosine-phosphate-guanine motifs; DC: dendritic cell; ERAD: endoplasmic reticulum-associated degradation; gC1qR: globular head C1q receptor; MAPK: mitogen-activated protein kinase; ORF3: open reading frame 3; ORF4: open reading frame 4;ORF5: open reading frame 5; p53: tumor protein p53; PI3K/AKT: phosphoinositide 3-kinase / protein kinase B pathway; PK: protein kinase; S phase: synthesis phase (of the cell cycle); TLR9: toll-like receptor 9; UPR: unfolded protein response.

6.2. Molecular mechanisms of PCV2 infection pathogenesis and immunosuppression

Porcine circovirus 2 infection outcomes range from PCV2-SI to severe PCV2-SD (Segalés and Sibila, 2022). The virus triggers molecular mechanisms that impair antiviral defences, disrupt immune homeostasis, and lead to LD and immunosuppression (*Figure 2*).

The clinical outcome depends on the balance between viral immune evasion and host antiviral responses (Féher et al., 2023). The innate immune system, through pattern recognition receptors (PRRs) such as TLR9, recognises pathogen-associated molecular patterns and induces type I interferons (IFN-α, IFN-β) and pro-inflammatory cytokines to initiate early antiviral responses (Ablasser and Hur, 2020; Rojas et al., 2021).

Beyond TLR9, PCV2 blocks cytosolic DNA sensing via cGAS–STING, reducing IFN-β and other type I interferons (Li et al., 2018a; Zhang et al., 2020b; Wang et al., 2021; Wu et al., 2021). As a result, interferon-stimulated gene expression declines, limiting viral control (Féher et al., 2023). The virus also upregulates SOCS3, a negative regulator of NF-κB, suppressing TNF-α and IL-6 (Zhu et al., 2016). Together, these early alterations weaken innate and adaptive immune activation (Ablasser and Hur, 2020; Rojas et al., 2021).

The virus also interferes with type I interferon signalling. Cap protein blocks STAT1/2 phosphorylation, preventing the ISGF3 complex formation and interferon-stimulated gene induction (Wang et al., 2022). The Rep protein promotes IL-10 overexpression via p38-MAPK, enhancing NF-κB p50 and SP1 recruitment to its promoter (Wu et al., 2019). Together, impaired type I interferon signalling and elevated IL-10 create a tolerogenic environment that weakens early innate defences during the initial stages of the infection (Féher et al., 2023). Viral-induced

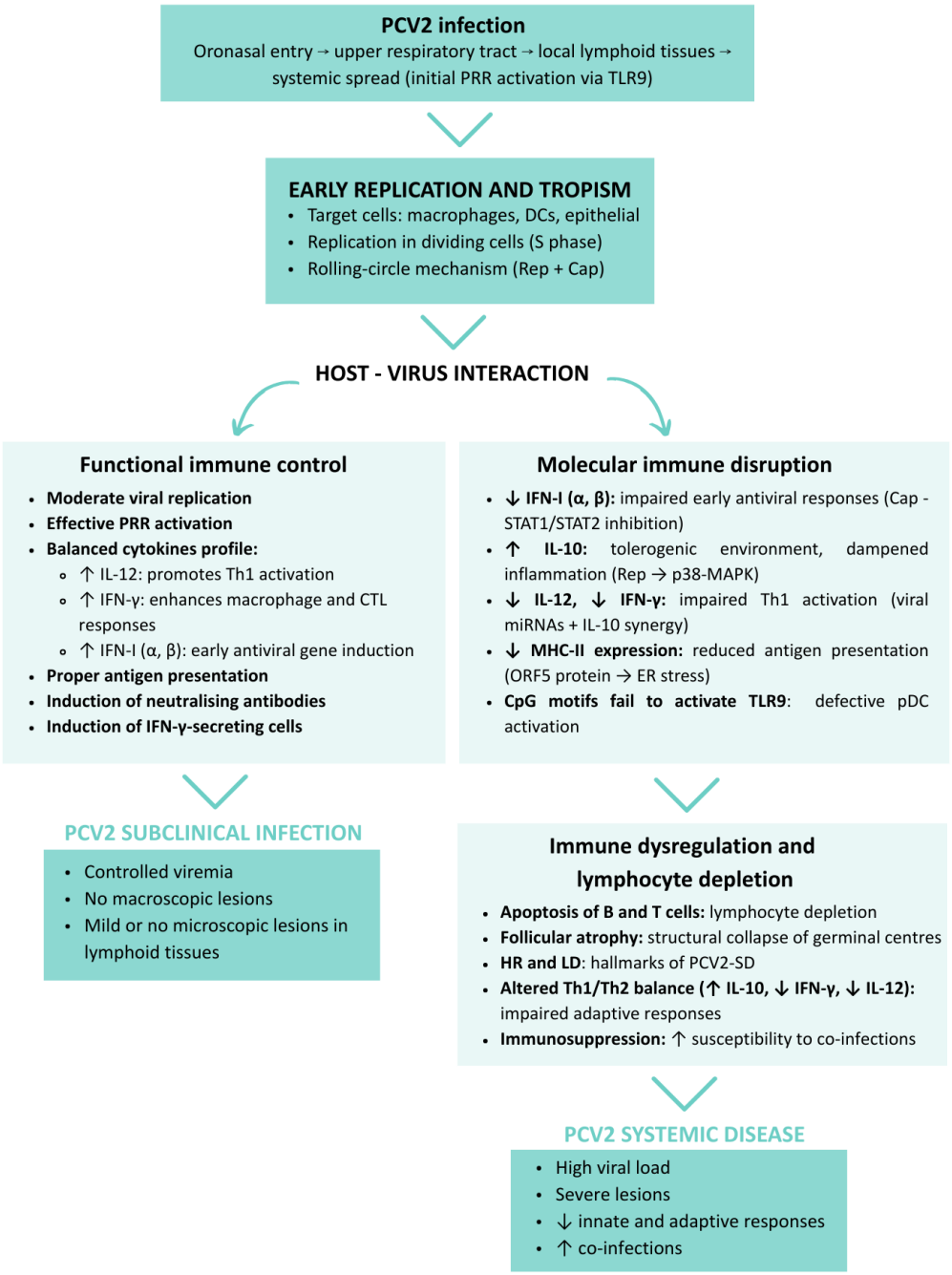
microRNAs further suppress IL-12 and IFN-γ, reinforcing this regulatory state (Núñez-Hernández et al., 2015; Du et al., 2016, 2018).

The ORF5 protein induces ER stress, downregulating MHC and suppressing cytokine secretion, which compromises adaptive immune activation (Lv et al., 2020). The viral genome, rich in unmethylated CpGs, also interacts with innate sensors in a sequence-dependent manner (Vincent et al., 2007; Wikström et al., 2007). Rather than activating TLR9-mediated IFN-α in plasmacytoid dendritic cells (pDCs), these motifs may fail to trigger or even inhibit the pathway, dampening early antiviral signalling (Vincent et al., 2005; Balmelli et al., 2011).

Collectively, these molecular triggers shift the infection from a state of controlled replication to immune dysregulation. When early mechanisms fail to contain viral replication, this imbalance drives the progression towards lymphocyte depletion, immunosuppression, and the development of PCV2-SD (Darwich et al., 2004; Féher et al., 2023).

A hallmark of PCV2-SD is the marked LD in secondary lymphoid organs, including lymph nodes, tonsils, spleen, and Peyer's patches (Darwich et al., 2004; Segalés, 2012). It results mainly from B and T cell apoptosis mediated by viral proteins such as ORF3, ORF5, and Cap (Liu et al., 2005; Lv et al., 2020). These proteins act directly and indirectly, through ER stress and altered cytokine signalling, to drive immune cell loss. As follicles are depleted, their architecture collapses and becomes infiltrated by macrophages and histiocytes, the HR, another diagnostic hallmark of PCV2-SD (Sorden, 2000; Darwich et al., 2004; Segalés, 2012).

Figure 2. Schematic overview of PCV2 pathogenesis and immune modulation.



B cells: B lymphocytes; Cap: capsid protein of PCV2; CTL: cytotoxic T lymphocytes; CpG motifs: cytosine-phosphate-guanine motifs; DCs: dendritic cells; ER: endoplasmic reticulum; HR: histiocytic replacement; IFN-I (α, β): type I interferons (alpha, beta); IFN-γ: interferon gamma; IL-10: interleukin 10; IL-12: interleukin 12; MHC-II: major histocompatibility complex class II; miRNAs: microRNAs; ORF5: open reading frame 5; p38-MAPK: p38 mitogen-activated protein kinase; pDCs: plasmacytoid dendritic cells; PCV2: porcine circovirus type 2; PCV2-SD: PCV2-systemic disease; PRRs: pattern recognition receptors; Rep: replicase protein of PCV2; STAT1/STAT2: signal transducer and activator of transcription 1 and 2; Th1/Th2: T-helper cell type 1 / T-helper cell type 2; TLR9: toll-like receptor 9; TNF-α: tumour necrosis factor alpha; T cells: T lymphocytes.

In addition to these structural changes, PCV2-SD affected pigs often show lymphopenia and depletion of B and T cell subsets, especially memory and activated T cells (Nielsen et al., 2003; Kekarainen et al., 2010). These alterations observed in diseased pigs are absent in PCV2-SI, highlighting impaired adaptive immunity in affected animals.

In parallel, activation of PI3K/AKT and p38-MAPK pathways leads to IL-10 overexpression in porcine macrophages (Féher et al., 2023). Elevated IL-10 creates a regulatory and anti-inflammatory environment, suppressing IL-12 and IFN-γ production, which are critical drivers of Th1 responses (Darwich et al., 2003a, 2003b; Sipos et al., 2004; Stevenson et al., 2006; Darwich et al., 2008; Gao et al., 2014b; Richmond et al., 2015). At the same time, virus-cell induced microRNAs reinforce this effect by directly targeting IL-12 and IFN-γ mRNAs (Du et al., 2016, 2018). Increased IL-10 levels have been positively correlated with the severity of LD, further linking cytokine dysregulation with PCV2-SD progression (Darwich et al., 2003b).

The combined effects of LD, HR, and cytokine dysregulation lead to systemic immunosuppression. Naïve B and T cell priming is impaired, antibody responses delayed or insufficient, and viral replication poorly controlled, making pigs highly susceptible to secondary infections.

6.3. Coinfections as disease enhancers

The virulence of PCV2 is often potentiated by concomitant infections with other immunosuppressive or immunomodulatory agents (Opriessnig and Halbur, 2012; Ouyang et al., 2019; Fehér et al., 2023). The most relevant pathogens associated with enhanced PCV2 replication and disease expression include PRRSV, PPV, and *M. hyopneumoniae* (Opriessnig and Halbur, 2012; Rose et al., 2012).

Several experimental studies have demonstrated that coinfection with PRRSV or PPV significantly increases the severity of microscopic lesions and clinical signs associated with PCV2 infection (Allan et al., 1999; Rovira et al., 2002; Shi et al., 2008; Butler et al., 2014; Park et al., 2014; Ouyang et al., 2019; Zhao et al., 2021). In particular, PRRSV enhances PCV2 replication in lymphoid tissues and exacerbates LD (Allan et al., 2000b). Similarly, PPV coinfection led to more extensive lesions and worsened clinical outcomes compared to single PCV2 infection (Allan et al., 1999; Kennedy et al., 2000; Saade et al., 2020).

At the immunological level, coinfections involving PCV2 result in cytokine dysregulation (Saade et al., 2020). While PCV2 alone induces negligible interferon responses in porcine PBMCs, it can suppress IFN-α production by pDCs and reduce IFN-γ secretion during coinfection, thereby blunting antiviral defences (Kekarainen et al., 2008; Gao et al., 2014a; Saade et al., 2020). In addition, PRRSV-induced IFN-γ and IL-2 responses are further inhibited by PCV2 (Shi et al., 2010), while PCV2-stimulated IL-10 production promotes an anti-inflammatory environment that suppresses IL-12 and other proinflammatory cytokines (Du et al., 2019). This cytokine imbalance compromises pathogen clearance and facilitates persistence of both viruses. Additionally, PRRSV-PCV2 coinfection leads to decreased expression of TNF-α and IL-1β in PBMCs, which compromises pathogen clearance and facilitates viral persistence (Tu et al., 2015; Li et al., 2022).

Another relevant pathogen within the porcine respiratory disease complex (PRDC) is *M. hyopneumoniae*. Although *M. hyopneumoniae* infection typically cause high morbidity but low mortality (Maes et al., 2018), its capacity to damage the mucociliary apparatus and modulate immune responses predisposes pigs to secondary viral and bacterial infections (Maes et al., 2018; Pieters and Maes, 2019; Saade et al., 2020). This scenario also contributes to the development of respiratory

signs such as coughing, dyspnoea, and lethargy, as well as reduced growth performance (Sibila et al., 2012; García-Morante et al., 2022).

Experimental studies demonstrated that coinfection with *M. hyopneumoniae* can exacerbate PCV2 replication and increase the incidence of PCV2-SD, when the bacterial infection is well established before viral challenge (Opriessnig et al., 2004). Mechanistically, chronic *M. hyopneumoniae* infection recruits immune cells into the lung, creating a pool of susceptible targets for PCV2 and other pathogens, and reduces macrophage phagocytic capacity against bacteria such as *Actinobacillus (A.) pleuropneumoniae* (Maes et al., 2018; Saade et al., 2020).

Beyond PRRSV, PPV and *M. hyopneumoniae,* additional pathogens have been reported to enhance PCV2 replication or exacerbate clinical outcomes (Saade et al., 2020). One example is swine influenza virus (SIV), which causes transient immunosuppression through epithelial damage and cytokine imbalance, thereby increasing susceptibility to PCV2 replication in the respiratory tract (Montoya et al., 2017; Bakre et al., 2021). Although experimental evidence is limited, field observations suggest that concurrent influenza outbreaks can intensify the clinical expression of PCV2-ADs (Saade et al., 2020).

Similarly, co-presence of PCV2 with *A. pleuropneumoniae* has been reported in pleuropneumonia and pneumonia-like lesions, which aggravates inflammatory responses (Saade et al., 2020). Experimental work further showed that PCV2 facilitates *A. pleuropneumoniae* survival in porcine alveolar macrophages by impairing oxidative responses and weakening bacterial clearance, thereby providing a mechanistic explanation for the enhanced pathogenicity of this coinfection (Qi et al., 2019b).

7. ADAPTIVE IMMUNE RESPONSE TO PCV2 INFECTION

The adaptive immune response to PCV2 is multifaceted and includes both passively acquired and actively developed immunity. However, this response may be delayed or functionally impaired, particularly in the presence of viral-induced immunomodulation (Ablasser and Hur, 2020). In this section, the mechanisms related to infection-induced immunity are discussed, while vaccine-induced immunity is addressed separately in *Section 8.2.5.*

7.1. Passive immunity: MDI

In swine, the placenta is epitheliochorial, which prevents prenatal immunoglobulin transfer (Macdonald and Bosma, 1985), despite a previous study suggested that small amounts of maternal antibodies may cross the placenta when it is damaged during gestation (Saha et al., 2014). Consequently, piglets are born immunologically naïve and rely exclusively on the ingestion of colostrum and milk to acquire MDI (Bandrick et al., 2014; Martínez-Boixaderas et al., 2022). This passive protection primarily involves MDA, alongside immune cells, cytokines, and bioactive molecules that are transferred during the first six hours of life (Poonsuk et al., 2018).

In the context of PCV2 infection, MDA are crucial during the early weeks of life, particularly before the development of the piglets own adaptive immunity and can persist for 4 to 12 weeks (Fachinger et al., 2008; Opriessnig et al., 2008a; Martelli et al., 2011, Feng et al., 2016; Martelli et al., 2016; Kiss et al., 2021). However, this duration depends on the initial antibody titre and the serological test used, as the MDA half-life reflects the time required for circulating antibody concentrations to decrease by 50% (Opriessnig et al., 2008a). Reported estimates of PCV2 antibody half-life show substantial variability, largely influenced by the type of serological assay and the calculation method applied, with values ranging from 16 to 45 days

(Opriessnig et al., 2008a; Fort et al., 2009b; Polo et al., 2012; Kiss et al., 2021; Sibila et al., 2022).

Presence of MDA has been shown to delay viremia onset, reduce viral replication, and mitigate PCV2 infection severity (Fort et al., 2009b; Oh et al., 2012; Poulsen Nautrup et al., 2021). Additionally, it has been associated with reduced mortality and improved clinical performance in the field (Figueras-Gourgues et al., 2019).

7.2. Active humoral response

Upon PCV2 infection, the host mounts a humoral immune response predominantly targeting the Cap protein, the major structural and immunogenic component of the virus (Blanchard et al., 2003; Fenaux et al., 2003; Kamstrup et al., 2004; Wang et al., 2006; Song et al., 2007; Fan et al., 2008). Although the immunogenic potential of the Rep protein has also been investigated, it has been characterized as a weak antigen in terms of humoral recognition (Blanchard et al., 2003).

Under field conditions, the seroconversion typically occurs around 7–15 weeks of age (Rodríguez-Arrioja et al., 2002). Nonetheless, the presence of high antibody titres in PCV2-infected pigs has been reported, suggesting that the humoral response alone may not be sufficient to entirely block or neutralise viral replication (Okuda et al., 2003; Meerts et al., 2006; Trible and Rowland, 2012).

The humoral immune response includes the generation of NA, which primarily target conformational epitopes on the Cap protein (Zhang et al., 2015, 2019; Kang et al., 2020). These NA have demonstrated cross-reactivity against various PCV2 genotypes, as for example between PCV2a and PCV2b (Opriessnig et al., 2008b; Kurtz et al., 2014). Conversely, in animals that develop PCV2-SD, high viral loads and

severe lymphoid lesions are typically associated with low or undetectable NA titres (Meerts et al., 2006; Fort et al., 2007).

7.3. Cell-mediated immune response

Humoral responses contribute to protection, although PCV2 antibodies alone are not sufficient to prevent viral replication and associated clinical outcomes (Rodríguez-Arrioja et al., 2002; Meerts et al., 2006; Trible and Rowland, 2012). This has reinforced the relevance of cell-mediated immunity (CMI) as a key protective mechanism.

Upon PCV2 infection, virus-specific T cell responses are mounted, primarily characterized by the induction of IFN-γ-secreting cells (IFN-γ-SC), which are typically associated with Th1 cells (Fort et al., 2009a; Kekarainen and Segalés, 2015). These cells produce IFN-γ upon recognition of PCV2 antigens and contribute to the antiviral state by enhancing macrophage activation and promoting cytotoxic T lymphocyte (CTL) responses (Fort et al., 2009a; Kekarainen and Segalés, 2015). The presence of PCV2-specific IFN-γ-SC has been inversely correlated with viral loads in serum, supporting their role in viral clearance (Seo et al., 2012). Moreover, IFN-γ-SC responses have been identified against both structural (Cap) and non-structural (Rep) viral proteins, indicating a broad antigenic recognition by the cellular compartment (Fort et al., 2010).

8. PCV2 CONTROL AND PREVENTION STRATEGIES

8.1. Risk factor-oriented strategies for PCV2-AD control

The clinical expression and epidemiological dynamics of PCV2-ADs are not solely determined by the presence of the virus but also the result from a multifactorial interplay involving immunological status, environmental stressors, and management practices (Rose et al., 2012). Consequently, identifying and addressing modifiable risk factors represents a fundamental pillar of disease control.

Non-vaccine-based strategies are particularly valuable in scenarios where vaccine efficacy may be compromised or where additional interventions are required to strengthen herd resilience (Rose et al., 2003, 2009; Czyżewska-Dors et al., 2018). One of the earliest and most influential frameworks in this field was “Madec’s 20-point plan” (Madec et al., 2000). Although it was developed prior to the introduction of commercial vaccines, this plan remains a valuable conceptual basis for biosecurity and management interventions. The main areas of intervention are summarized in *Table 5*, highlighting their associated risk factors and control strategies.

Economic modelling has also compared different risk factor-oriented strategies. Vaccination alone was the most cost-efficient option in moderately affected farms, whereas combining vaccination with improved biosecurity was most beneficial in highly affected herds (Alarcon et al., 2013). Other measures, such as dietary adjustments or reduced stocking density, provided only marginal benefits, reinforcing that while vaccination is pivotal, complementary management interventions can enhance long-term PCV2-AD control (Alarcon et al., 2013).

Table 5. Summary of non-vaccine-based, risk-factor-oriented strategies for PCV2-AD control.

Area of intervention	Main risk factors	Control strategies	References
Biosecurity	• Pathogen coinfection (PRRSV, *M. hyopneumoniae*, PPV) • Presence of wildlife, rodents and/or invertebrates	• Quarantine • Dedicated clothing and equipment • Controlled farm access • Strict disinfection protocols • Wildlife, rodent and insect control	Lorincz et al., 2010 Blunt et al., 2011 Opriessnig and Halbur, 2012 Rose et al., 2012 Choi and Park, 2015 Afghah et al., 2017 López-Lorenzo et al., 2022
Management and production practices	• Cross-fostering • Early weaning (<21 days) • Mixing of ages and weights • Inadequate gilt/sow management	• All-in/all-out strategies • Age segregation • Extended empty periods between batches • Reduced cross-fostering • Adequate weaning age and weight • Assurance of colostrum intake • Reduced peripartum stress	Madec et al., 2000 López-Soria et al., 2005 Woodbine et al., 2007 Rose et al., 2003, 2009, 2012
Housing and environmental conditions	• Poor air quality • High density • Inadequate ventilation, temperature and/or humidity	• Stocking density control avoiding overcrowding and excessive mixing • Ventilation adjustment • Air filtration	Rose et al., 2003, 2012 Patterson et al., 2015

Nutritional interventions	• Poor nutritional support • Oxidative stress	• Spray-dried plasma • Selenium • L-glutamine • L-proline • Conjugated linoleic acid (CLA) • Antioxidant additives (bioflavonoids, anthocyanins and essential oils)	Bassaganya-Riera et al., 2003 Donadeu et al., 2003 Dewey et al., 2006 Pan et al., 2008 Ren et al., 2013a, 2013b Liu et al., 2015
Host-related factors	• Genetic background susceptibility (Pietrain, Landrace) • Immune status (MDA)	• Genetic selection • Vaccination adapted to MDA	Rodríguez-Arrioja et al., 2002 López-Soria et al., 2004, 2005, 2011 Rose et al., 2003, 2005 Opriessnig et al., 2006, 2009b Grau-Roma et al., 2009
Microbiota	• Gut microbiota composition	• Increased microbiome diversity was associated with improved growth after PCV2/PRRSV coinfection • Faecal microbiota transplantation was linked to reduced PCV2-SD morbidity and mortality	Ober et al., 2017 Niederwerder et al., 2018

CLA: conjugated linoleic acid; MDA: maternally derived antibodies; PCV2: porcine circovirus 2; PCV2-AD: porcine circovirus 2 associated diseases; PPV: porcine parvovirus; PRRSV: porcine reproductive and respiratory syndrome virus.

8.2. Vaccination against PCV2

Vaccination has been the cornerstone of PCV2 control for nearly two decades, providing effective tools to mitigate clinical disease and reduce viral circulation in swine populations (Guo et al., 2022).

8.2.1. Types of PCV2 vaccines

Vaccination platforms against PCV2 converge on the Cap protein as the principal protective antigen because it contains conformational neutralising epitopes (Nawagitgul et al., 2002; Blanchard et al., 2003; Trible et al., 2011). The different vaccine platforms currently available or under development are all designed to present this antigen to the immune system in a form capable of inducing both humoral and cell-mediated responses (Guo et al., 2022).

The conformational state of Cap protein is critical for immunogenicity (Guarneri et al., 2021). When Cap protein is expressed and assembled into virus-like particles (VLPs), it retains its native three-dimensional structure and efficiently induces NA and PCV2-specific T-cell activation (Guo et al., 2022). In contrast, non-assembled Cap protein has been shown to elicit only weak humoral and cellular responses and does not confer protection upon viral challenge, highlighting that antigen structure, and not only antigen identity, determines protective efficacy (Guarneri et al., 2021).

Building on this principle, inactivated vaccines present the whole virus in a non-replicative form, while subunit vaccines rely on recombinant VLPs (Burrell et al., 2017). Other platforms, including chimeric live-attenuated, DNA-based and viral-vectored vaccines, explore alternative ways of presenting Cap to stimulate complementary immune mechanisms (Guo et al., 2022). Regardless of the technological approach, effective vaccination outcomes rely on achieving a

balanced humoral and cellular immune responses directed against Cap (Shi et al., 2021).

8.2.2. Active humoral response linked to vaccination

The humoral immune response is a key component of protection elicited by PCV2 vaccines (Segalés, 2015; Guo et al., 2022). The NA represent a major correlate of protection induced by PCV2 vaccines, as they primarily target conformational epitopes of the Cap protein (Nawagitgul et al., 2002; Blanchard et al., 2003). Both subunit vaccines based on VLPs and inactivated vaccines have been shown to induce NA against PCV2, which contribute to the reduction of viremia, nasal and faecal shedding and lymphoid lesions scores after challenge with different PCV2 isolates (Fort et al., 2008, 2009b; Seo et al., 2014a). However, comparisons between vaccine platforms should be interpreted with caution, since studies often differ in design, challenge conditions and immunological readouts (Shen et al., 2010b; Guo et al., 2022). However, it is important to note that none of the available vaccines are sterilising, and complete prevention of vertical or horizontal transmission has not been consistently achieved (Feng et al., 2014; Hemann et al., 2014; Segalés, 2015). Overall, both vaccine types have proven highly effective under experimental and field conditions, contributing substantially to the global control of PCV2 infections (Guo et al., 2022).

Experimental studies have demonstrated that vaccine-induced NA display broad cross-reactivity among PCV2 genotypes, particularly between PCV2a and PCV2b (Opriessnig et al., 2008b). Nevertheless, some antigenic variability has been observed in epitopic regions of the different genotypes, occasionally resulting in lower cross-neutralising titres compared with homologous strains (Saha et al., 2012; Kang et al., 2020; Yu et al., 2023). In pigs vaccinated with genotype-specific vaccines,

the highest neutralising titres were consistently detected against homologous strains, whereas titres against heterologous genotypes were significantly lower (Kang et al., 2020; Yu et al., 2023). While these differences rarely result in overt clinical vaccine failures, they may facilitate viral persistence and contribute to the gradual predominance of fitter strains such as PCV2d, thereby shaping the long-term epidemiological landscape (Franzo et al., 2024). Nevertheless, protective efficacy of vaccination should not be assessed solely on humoral responses, as CMI also plays a key role in protection against PCV2 (Martelli et al., 2011; Li et al., 2025). Indeed, from a field perspective, protection against disease can still be achieved even when antibody titres appear low or absent, underscoring that serological outcomes should not be interpreted alone when evaluating vaccine efficacy (Pérez-Martín et al., 2010).

8.2.3. Cell-mediated immune response linked to vaccination

The induction of CMI represents a central mechanism of protection following PCV2 vaccination (Féher et al., 2023). Both experimental and field studies demonstrated that immunisation triggers PCV2-specific IFN-γ-secreting cells, which inversely correlate with viral loads and contribute to infection, viral replication, promoting viral clearance, and complementing the protective effect of NA (Fort et al., 2009b; Martelli et al., 2011; Fort et al., 2012; Seo et al., 2012; Borghetti et al., 2013; Ferrari et al., 2014; Koinig et al., 2015). Interestingly, cytokine modulation related to the dam's vaccination status has also been observed, suggesting that maternal immunity may shape early immune activation patterns in piglets (Oliver-Ferrando et al., 2018b).

More recently, in an experimental study where the cellular immune responses elicited by different commercial vaccines was tested, a rapid T cell activation (7 days

post-vaccination) was detected. Such response was predominantly mediated by multifunctional $CD4^+$ T cells capable of producing IFN-γ, TNF-α, and IL-2 simultaneously (Li et al., 2025). In contrast, $CD8^+$ T cells exhibited weaker responses, with limited IFN-γ/TNF-α production and negligible cytotoxic activity, suggesting a less prominent role (Li et al., 2025). The study demonstrated that vaccine-induced T cells expanded upon PCV2d challenge, suggesting that vaccination establishes long-term cellular immunity capable of rapid recall responses (Li et al., 2025). Importantly, stronger multifunctional responses were observed in lymphoid tissues such as spleen and lymph nodes compared to peripheral blood, underscoring the significance of tissue-resident immunity (Li et al., 2025).

8.2.4. Commercial vaccines available in Europe

Building upon the different technological platforms described above, the first commercial PCV2 vaccines became available in Europe and North America in the mid-2000s, followed shortly after by their introduction in Asia (Zhai et al., 2014; Franzo and Segalés, 2020). A summary of the main commercial PCV2 vaccines currently authorised in Europe is detailed in *Table 6.*

Their rapid implementation was accompanied by a reduction in the incidence of PCV2-SD worldwide, confirming their effectiveness both under experimental and field conditions (Dvorak et al., 2016; Eddicks et al., 2016). Large-scale vaccination led to consistent improvements in health and performance indicators, including reduction of lymphoid lesions, lower mortality rates, higher ADWG, better feed conversion rate (FCR), and decreased medication costs (Horlen et al., 2008; Kixmöller et al., 2008; Chae, 2012; Eddicks et al., 2016).

Table 6. Characteristics of the principal commercially available PCV2 vaccines in Europe (European Medicines Agency, 2025a).

Company	Product	Target pathogens	Vaccine type	PCV2 genotype
Boehringer Ingelheim	**Ingelvac CIRCOFLEX®**	PCV2	Subunit (VLPs), baculovirus expressed Cap protein	PCV2a
ceva	**Circovac®**	PCV2	Inactivated (whole virus)	PCV2a
	CIRBLOC M Hyo®	PCV2 + *M. hyopneumoniae*	Subunit (VLPs), baculovirus expressed Cap protein + bacterin	PCV2d
HIPRA	**Myosphere® PCV ID**	PCV2 + *M. hyopneumoniae*	Inactivated *M. hyopneumoniae* expressing recombinant PCV2 capsid protein	PCV2b
MSD Animal Health	**Porcilis® PCV**	PCV2	Subunit (VLPs), baculovirus expressed Cap protein	PCV2a
	Porcilis® PCV ID	PCV2		
	Porcilis® PCV M Hyo	PCV2 + *M. hyopneumoniae*	Subunit (VLPs), baculovirus expressed Cap protein + bacterin	PCV2a
	Porcilis® PCV M Hyo ID	PCV2 + *M. hyopneumoniae*		
zoetis	**CircoMax®**	PCV2	Inactivated chimeric PCV1/PCV2 virus (+ bacterin in *M. hyopneumoniae* vaccines)	PCV2a/PCV2b
	CircoMax® Myco	PCV2 + *M. hyopneumoniae*		PCV2a/PCV2b
	Suvaxyn® Circo	PCV2		PCV2a
	Suvaxyn® Circo + MH RTU	PCV2 + *M. hyopneumoniae*		PCV2a

ID: intradermal; IM: intramuscular; *M. hyopneumoniae*: *Mycoplasma hyopneumoniae*; PCV1: porcine circovirus 1;…

Adjuvant	Target animals and schedule	Route	Onset of immunity (weeks post-vaccination)	Duration of immunity
Carbomer	Piglets: 1 × 1 mL ≥2 weeks old	IM	2 weeks	17 weeks
Light liquid paraffin	Sows/gilts: 2 × 2 mL pre-farrowing Piglets: 1 × 0.5 mL ≥3 weeks old	IM	2 weeks	23 weeks
Light liquid paraffin	Piglets: 1 × 2 mL ≥3 weeks old	IM	2 weeks (PCV2) 3 weeks (*M. hyopneumoniae*)	23 weeks
Light liquid paraffin	Piglets: 1 × 0.2 mL ≥3 weeks old	ID	2 weeks (PCV2) 3 weeks (*M. hyopneumoniae*)	22 weeks (PCV2) 23 weeks (*M. hyopneumoniae*)
Aluminium hydroxide + oil	Piglets: 1 × 2 mL ≥3 weeks old	IM	2 weeks	23 weeks
Dl-rac-α-tocopheryl acetate and light liquid paraffin	Piglets and sows: 1 × 0.2 mL ≥3 weeks old; revaccination every 26 weeks	ID	2 weeks	26 weeks
Aluminium hydroxide + oil	Piglets: single-dose schedule (1 × 2 mL ≥3 weeks old) or two-dose schedule (1 × 1 mL at 3 days and 3 weeks old)	IM	1 dose: 2 weeks (PCV2) and 4 weeks (*M. hyopneumoniae*) 2 doses: 18 days (PCV2) and 3 weeks (*M. hyopneumoniae*)	22 weeks (PCV2) 21 weeks (*M. hyopneumoniae*)
All-rac-α-tocopheryl acetate and squalane	Piglets and sows: 1 × 0.2 mL ≥3 weeks old	ID	2 weeks (PCV2) 4 weeks (*M. hyopneumoniae*)	26 weeks (PCV2) 18 weeks (*M. hyopneumoniae*)
Squalane emulsion (MetaStim®)	Piglets: single-dose schedule (1 × 2 mL ≥3 weeks old) or two-dose schedule (1 × 1 mL at 3 days and 3 weeks old)	IM	3 weeks (PCV2 and *M. hyopneumoniae*)	23 weeks
	Piglets: 1 x 2 mL ≥3 weeks old	IM	3 weeks	23 weeks
Squalane, poloxamer 401 and polysorbate 80	Piglets: 1 x 2 mL ≥3 weeks old	IM	3 weeks	23 weeks (PCV2) 16 weeks (*M. hyopneumoniae*)

… PCV2: porcine circovirus 2; RTU: ready-to-use; VLPs: virus-like particles.

8.2.5. Genotype shift and infection dynamics under widespread vaccination

Soon after the global implementation of mass vaccination, PCV2d progressively replaced PCV2a and PCV2b as the predominant genotype worldwide (Franzo et al., 2024). This genotype shift is thought to be largely driven by the selective pressure exerted by vaccination, favouring strains with partial antigenic divergence (Franzo et al., 2024).

Although PCV2d was initially proposed as a potential contributor to vaccine failures and suspected to be more virulent (Opriessnig and Langohr, 2013; Ssemadaali et al., 2015), no significant differences in virulence have been consistently observed among PCV2a, PCV2b, and PCV2d genotypes (Cho et al., 2020; Yu et al., 2023). Indeed, experimental data have demonstrated that current PCV2a-based vaccines remain effective against PCV2d experimental inoculations (Opriessnig and Langohr, 2013; Matzinger et al., 2016; Park et al., 2019; Opriessnig et al., 2020). Nevertheless, different studies have suggested reduced neutralizing activity of heterologous antibodies and increased viremia and transmission efficiency in animals infected with PCV2d compared to those infected with homologous strains (Liu et al., 2013; Opriessnig et al., 2013; Dvorak et al., 2016).

Under conditions of widespread vaccination, PCV2-SI has become the most common infection outcome (Young et al., 2011; Maity et al., 2023). At the same time, intensive use of vaccination has progressively led to the emergence of subpopulations of naïve gilts and sows with lower PCV2 immunity, especially in the absence of virus circulation (Segalés and Sibila, 2022). As a result, offspring from these animals may have reduced MDI, increasing the risk of early-life PCV2 infection (Martínez-Boixaderas et al., 2022). Thus, if the infection occurs before or at the time of piglet vaccination, the systemic disease may appear in a proportion of piglets even in vaccinated herds (Segalés and Sibila, 2022). These PCV2-SD cases typically

arise earlier than those observed in the pre-vaccine period, often between 6 and 8 weeks of age, and reflect the complex interplay between herd immunity, viral dynamics and management conditions (Segalés and Sibila, 2022).

8.2.6. Impact of maternally derived immunity on PCV2 vaccine efficacy

The interplay between MDI and piglet vaccination age represents one of the most critical factors for the design of effective PCV2 immunisation programmes (Martínez-Boixaderas et al., 2022). Sow vaccination efficiently induces PCV2-specific antibodies that are transferred to piglets via colostrum, ensuring protection during the first weeks of life (Madson et al., 2009d; Pejsak et al., 2010; Sibila et al., 2013; Lopez-Rodriguez et al., 2016). However, high MDA titres at the time of piglet vaccination can interfere with the development of active immunity, dampening seroconversion and reducing antibody titres post-vaccination (Fort et al., 2009a; Fraile et al., 2012b; Haake et al., 2014; Feng et al., 2016)

The specific half-life of PCV2 antibodies in piglets born from vaccinated sows has not been consistently defined and appears to vary according to sow vaccination protocols (Sibila et al., 2022). However, uncertainty regarding the kinetics of MDA does not necessarily translate into reduced protection, as vaccine-induced cellular immunity appears to remain unaffected (Martelli et al., 2013; Oh et al., 2014).

Moreover, under field conditions, interference with productive parameters has only been observed when piglets were vaccinated at very young ages or in the presence of exceptionally high antibody titres (Haake et al., 2014; Feng et al., 2016). In fact, high ELISA values equivalent to >17 $\log_2$ IPMA (Pileri et al., 2014), have been associated with reduced growth performance (Feng et al., 2016). This result suggest that only very high MDA levels are likely to compromise vaccine efficacy in terms of productive parameters and seroconversion.

8.2.7. Vaccination strategies

Within breeding herds, vaccination aims to establish a stable and homogeneous PCV2 immunity, ensuring adequate MDA in piglets during the early postnatal period (Segalés, 2015). This approach provides protection during the period of highest susceptibility and contributes to reducing viral circulation at the population scale (Guo et al., 2022).

Two approaches are commonly applied in gilts and sows:

- Vaccination during lactation, before mating or at early gestation, which contributes to immunological homogenisation along gestation (Gerber et al., 2011; Kurmann et al., 2011; O'Neill et al., 2012; Sibila et al., 2013).
- Vaccination at late gestation or before farrowing, which maximises colostral antibody transfer to piglets (Opriessnig et al., 2010; Fraile et al., 2012a; Pleguezuelos et al., 2021).

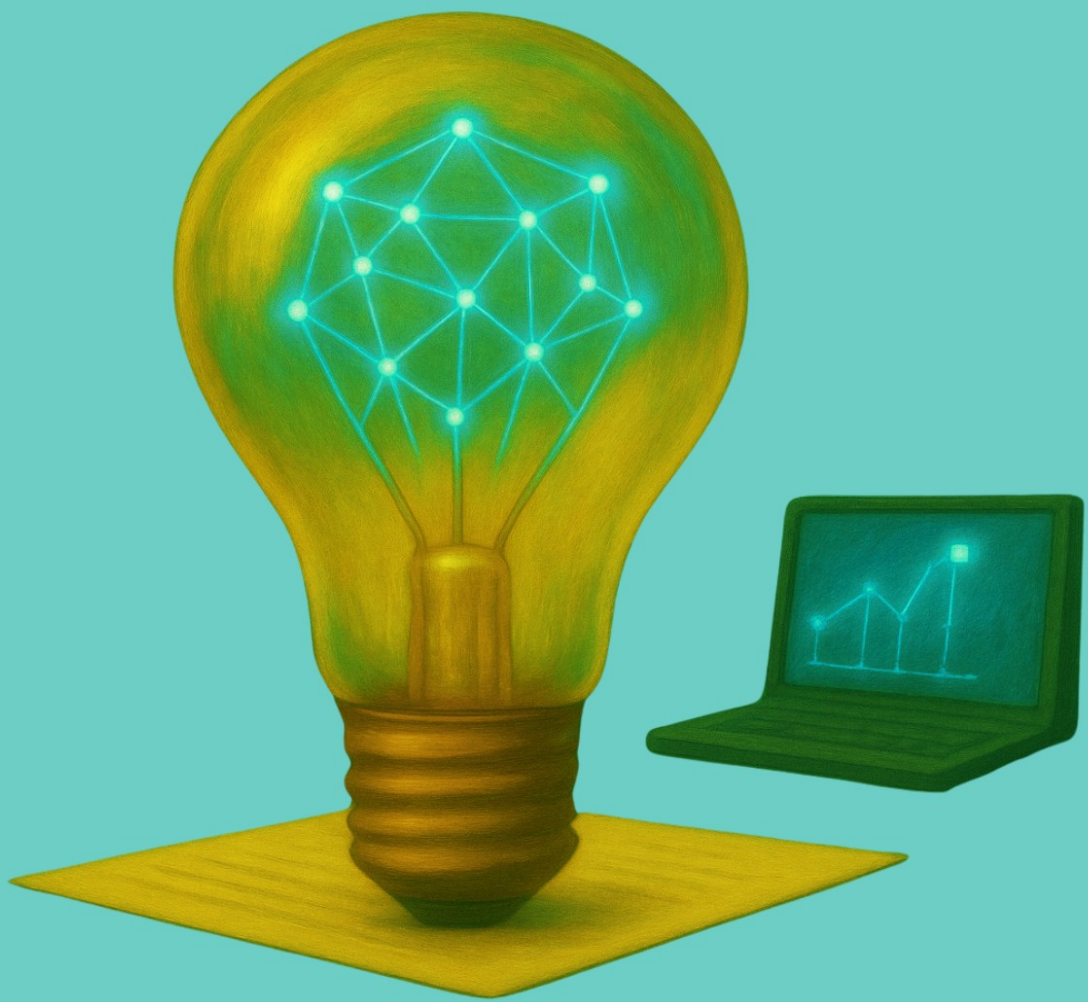
In parallel, whole-herd revaccination schemes, named blanket vaccination, are increasingly implemented to maintain immunity stability across reproductive cycles and reduce fluctuations in herd-level susceptibility (Pleguezuelos et al., 2021; Segalés and Sibila, 2025). Beyond the transfer of immunity to the offspring, long-term boosting in sows has been linked to improvements in reproductive outcomes, including increased litter size and enhanced piglet vitality (Pejsak et al., 2012; Oliver-Ferrando et al., 2018a; Pleguezuelos et al., 2021).

In piglets, vaccination at weaning (3–4 weeks of age) aims to induce active immunity before natural infection occurs (Segalés, 2015). Its efficacy has been repeatedly confirmed under both experimental and field conditions, with reductions in viraemia, shedding, viral loads in lymphoid tissues, microscopic lesions, and coinfections (Fachinger et al., 2008; Horlen et al., 2008; Kixmöller et al., 2008;

Segalés et al., 2009; Pejsak et al., 2010; Martelli et al., 2011; Heißenberger et al., 2013; Martelli et al., 2013; Oh et al., 2014; Opriessnig et al., 2017; Do et al., 2021; Krejci et al., 2025; Pálmai et al., 2025). Apart from clinical benefits, piglet vaccination has also been associated with consistent improvements in production parameters such as ADWG, reduction of PCV2 prevalence and PCV2 load in serum and faeces even under subclinical infection scenarios (Fraile et al., 2012b; Alarcon et al., 2013; Oliver-Ferrando et al., 2016).

A combined strategy including both sow and piglet vaccination has been evaluated in several studies, revealing additive benefits. These include reductions in the proportion of viraemic piglets, lower viral loads in blood and tissues, and improved morbidity, mortality, and growth rates (Opriessnig et al., 2010; Pejsak et al., 2010; Fraile et al., 2012a,b; Haake et al., 2014; Oh et al., 2014; Feng et al., 2016; Villa-Mancera et al., 2016; Martelli et al., 2016).

Hypotheses and objectives



Porcine circovirus 2 remains as one of the most relevant pathogens for the swine industry despite the widespread use of effective commercial vaccines (Franzo et al., 2024). Although immunisation strategies have markedly reduced the prevalence of overt PCV2-ADs, the virus still circulates widely under subclinical conditions (Young et al., 2011; Maity et al., 2023). This persistent circulation raises questions about the current epidemiological situation and the role of concurrent infections, particularly with immunomodulatory agents such as PRRSV, which has been shown to exacerbate PCV2-associated lesions and alter immune responses (Fehér et al., 2023). In addition, the recent emergence of the highly pathogenic PRRSV strains in Spain (Martín-Valls et al., 2023) have further increased concerns about the synergistic effects of coinfection in vaccinated populations (Lunney et al., 2016). These aspects highlight the need to update and better describe the epidemiology of PCV2–PRRSV coinfections under field conditions.

At the same time, MDA play a dual role in PCV2 control: while they confer early protection to piglets, excessively high titres may interfere with vaccine-induced immune responses (Bandrick et al., 2014; Feng et al., 2016; Martínez-Boixaderas et al., 2022). Indeed, very high levels of MDA have been associated with delayed seroconversion and reduced vaccine-induced antibody responses, potentially impairing the development of effective protection and negatively affecting growth performance (Feng et al., 2016; Poulsen Nautrup et al., 2021; Martínez-Boixaderas et al., 2022). Conversely, the widespread use of mass vaccination in piglets, together with different sow and gilt vaccination strategies, has progressively reduced virus circulation in many herds, leading to the emergence of naïve or partially immune sows that transfer lower MDA titres to their offspring (Segalés and Sibila, 2022). Such piglets may become susceptible to early PCV2 infection before vaccination, with an increased risk of PCV2-SD (Segalés and Sibila, 2022). These immunological

dynamics gain relevance in the context of the genotype shift, as PCV2d has become the predominant genotype worldwide, a change thought to be largely driven by the selective pressure of mass vaccination campaigns (Franzo et al., 2024). This scenario underscores the need to assess the cross-protection conferred by novel PCV2d-based vaccines against heterologous genotypes under varying MDA levels, as well as to better characterise the contribution of cellular immunity in vaccinated piglets with high MDA titres.

From a diagnostic perspective, PCV2 *in situ* detection techniques constitute one of the cornerstones in the PCV2-AD diagnostic criteria, as it enables direct visualisation of viral genome or antigen in affected tissues and cells (Segalés et al., 2005a). However, despite its diagnostic value, interpretation of ISH or IHC still relies on pathologist visual assessment, which limits its reproducibility and standardisation. Therefore, the development of automated, objective tools capable of reliably quantifying ISH or IHC signals, should help improving diagnostic precision and facilitating large-scale comparative field and experimental studies.

Despite advances in preventive and control measures, decision-making in PCV2 control is still largely empirical. Epidemiological studies provide valuable insights into infection dynamics under field conditions, yet they require extensive sampling, are costly to conduct, and may raise ethical concerns due to the repeated handling and invasive procedures applied to the animals. Also, experimental challenge studies are essential for evaluating vaccine efficacy and understanding PCV2 pathogenesis. Nevertheless, they are conducted under highly controlled conditions which, although necessary to ensure reproducibility, limit the direct extrapolation of results to field settings. In addition, such studies are associated with substantial costs and ethical considerations.

As a result, there is a growing need for integrative frameworks capable of synthesising empirical data while allowing the exploration of complex and heterogeneous scenarios. Mathematical modelling offers such potential, although current approaches remain limited. Previous models are useful for describing broad infection trends but remain insufficient to capture the stochasticity of transmission events and the heterogeneity of host immune responses (Ezanno et al., 2020). In the case of PCV2, such models have typically overlooked key drivers of variability, including MDA levels and individual heterogeneity in vaccine-induced protection, which strongly condition infection dynamics and growth performance, but are rarely explicitly represented. These limitations highlight the need for more flexible and mechanistic individually-based modelling tools capable of integrating biological, immunological, and management-related parameters to support decision-making and optimise control strategies (Picault et al., 2019; Ezanno et al., 2020).

Considering the abovementioned rationales, the present PhD thesis was structured into four complementary chapters, integrating field data, an experimental challenge study, diagnostic innovation insights, and mathematical modelling, to gain a broader understanding on PCV2 epidemiology and control. Thus, the general aim was to evaluate PCV2 infection and vaccine efficacy under diverse scenarios using a multidisciplinary approach combining epidemiology, pathology, immunology and modelling methodologies.

The specific objectives of this PhD thesis were:

1. To assess the prevalence of PCV2 and PRRSV coinfection in a Spanish swine integration system during two separate production cycles (2020 and 2022) (*Chapter 1*).

2. To evaluate the efficacy of a new PCV2d and *M. hyopneumoniae* based vaccine in piglets with high or low levels of MDA experimentally inoculated with PCV2b (*Chapter 2*).
3. To develop a pixel-based automated classifier for the quantification of PCV2 genome in PCV2 ISH RNAscope sections of different lymphoid tissues (*Chapter 3*).
4. To build and test an individual-based mechanistic model capable of simulating different PCV2 vaccination strategies across the pigs' production cycle, and to evaluate their epidemiological, productive, and economic impacts under varying MDA levels (*Chapter 4*).

Chapters

Chapter 1

Frequency of PCV2 viremia in nursery piglets from a Spanish swine integration system in 2020 and 2022 considering PRRSV infection status

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1.1. Introduction

Continuous monitoring of PCV2 infection patterns within pig farms remains essential, as viral circulation persists also in vaccinated populations (Segalés and Sibila, 2022). Although different experimental and field studies have demonstrated the performance of PCV2 vaccines reducing viremia, clinical signs and/or microscopic lesions in presence of MDA, a proportion of pigs may still develop PCV2-SI (Fort et al., 2008; Opriessnig et al., 2008a; Feng et al., 2016; Pleguezuelos et al., 2021). Importantly, the percentage of PCV2 early infections depends on the balance between the level of MDI and the infectious pressure existing on a particular farm and batch, as well as coinfections with different pathogens (Maity et al., 2023). Indeed, the intensification of the swine industry in the last three decades has favoured more complex clinical presentations due to coinfections, with PCV2 being found alongside PRRSV, PPV, and *M. hyopneumoniae*, among others (Kang et al., 2021).

Porcine reproductive and respiratory syndrome virus has been considered one of the most important viruses causing disease in pigs and great economic losses worldwide (Lunney et al., 2010; Nieuwenhuis et al., 2012; Holtkamp et al., 2013; Nathues et al., 2017). Importantly, both PRRSV and PCV2 target the host's immune cells by disrupting their function (Niederwerder et al., 2016; Ouyang et al., 2019) increasing their susceptibility to other pathogens. Such coinfections could affect their growth performance and the incidence and lethality of associated diseases (Niederwerder et al., 2016; Ouyang et al., 2019). Indeed, in some studies, PRRSV has been detected in coinfection in PCV2-SD cases up to 50%, suggesting that coinfection of these viruses is a significant factor contributing to overt disease expression (Drolet et al., 2003; Chen et al., 2019; Zhao et al., 2021). Moreover, it has been demonstrated that PRRSV infection at the time of PCV2 vaccination

jeopardizes the cellular immune response provided by the vaccine (Canelli et al., 2017). Thus, the surveillance of PRRSV and PCV2 in farms enables to establish the most effective management and vaccination programs.

Therefore, the objectives of the present study were (1) to determine and compare the frequency of early PCV2 viremia and antibody levels in piglets of different subclinically infected farms from a Spanish integration system in 2020 and 2022; and (2) to evaluate the frequency of PRRSV infection in those farms.

1.2. Materials and methods

1.2.1. Study design and sample collection

Forty-eight commercial farrow-to-weaning or farrow-to-nursery farms without PCV2-SD-like clinical signs were included in the study in 2020, which represented a total of 1860 tested piglets. From these 48 farms, 28 were tested again in 2022 (1140 piglets). The characteristics of each farm regarding herd size, production system and farrowing batches are presented in detail in *Supplementary Table (ST) 1.1*. Farms were located at different areas of Spain, being Aragón and Castilla-León the most represented areas in both years as these regions have the highest pig density in Spain (Ministerio de Agricultura, Pesca y Alimentación, 2022).

In 2020, between January and May, blood samples were collected from apparently healthy piglets at different ages in each selected farm. Sampling methodology included 10 (when farm size was < 1000 sows) or 30 (when farm size was ≥ 1000 sows) blood samples from piglets from different parity sows prior to vaccination (around 3–4 weeks of age), 10 samples at 6 weeks of age (woa), and 10 samples at 9 woa, respectively (*Figure 1.1*). The same sampling procedure was performed in

retested farms between January and August in 2022. The study followed a cross-sectional design, so, the piglets studied at different time-points were different.

Ten serum samples from the ones taken prior to vaccination per farm were subjected to PCV2 serology. Such sampling size allowed detecting a theoretical 25% seroprevalence of PCV2 with 95% confidence, as calculated using the WinEpi epidemiological tool (de Blas et al., 2006). Sera obtained from blood samples taken at all ages were tested by PCV2 and PRRSV qPCR and RT-qPCR, respectively. Specifically, for PCV2, 10 sera from all age-groups were tested in two pools of five samples each by qPCR. For PRRSV all available sera were processed through RT-qPCR in pools of five samples. This testing would allow detecting a theoretical frequency of infection of 10% at weaning and 25% at 6 and 9 woa for PRRSV with 95% confidence, as estimated using WinEpi (de Blas et al., 2006). These calculations considered the theoretical percentage of detection based on individual samples. Therefore, levels of sensitivity and specificity for detection of these viruses when using pools are expected to be slightly lower compared to individual sample testing (Rovira et al., 2007; Cortey et al., 2011b).

1.2.2. DNA and RNA extractions and detection of PCV2 and PRRSV by qPCR methods

Nucleic acids were extracted from 200 µL of each pool (of five samples) using the MagMAX™ Pathogen RNA/DNA Kit (Applied Biosystems, Thermo Fisher Scientific, Waltham, Massachusetts, USA) following the manufacturer's instructions. Negative controls were included to assess potential contamination during extraction.

To detect and quantify the PCV2 load, a commercial qPCR assay (LSI VetMAX™ Porcine Circovirus Type 2 Quantification, Applied Biosystems, Thermo Fisher Scientific, Waltham, Massachusetts, USA) was used. Each qPCR plate included a

negative control and an internal positive control (IPC) to monitor extraction and amplification procedures. Serum pools with <1.0 x 10^4 PCV2 genome copies/mL were considered positive but nonquantifiable. Pools with >1.0 x 10^4 PCV2 genome copies/mL were considered positive and quantifiable. Finally undetermined sample pools (Ct value ≥40) were considered negative. Viral load was expressed as the mean PCV2 genome copies/mL of pooled sera. To calculate the average genome copies per mL of pooled sera, those non-quantifiable positive values were given the cut-off value of 1.0 x 10^4 PCV2 genome copies/mL. A farm was considered positive when at least one of the tested pools was positive to PCV2 at any age.

To detect the PRRSV viremia, a commercial RT-qPCR assay (LSI VetMax™ PRRSV EU/NA 2.0, Applied Biosystems, Thermo Fisher Scientific, Waltham, Massachusetts, USA) was used. Each RT-qPCR plate included negative and IPC to monitor extraction and amplification procedures. Results were expressed as positive (Ct <40) or negative (Ct≥ than 40) for PRRSV. A farm was considered positive when at least one tested pool was positive to PRRSV at any tested age.

1.2.3. Indirect ELISA to detect anti-PCV2 IgG antibodies

Ten serum samples of piglets at 3–4 woa were individually tested by an indirect commercial ELISA assay (Ingezim Circo IgG 11.PCV.K1®, Gold Standard Diagnostics, Madrid, Spain) following manufacturer's instructions. All serum samples per farm were run on the same ELISA plate. The optical density (OD) was measured at 450 nm by the Sunrise™ reader (Tecan, Männendorf, Switzerland). Negative and positive cut-offs were calculated in each ELISA plate and results were expressed as mean S/P ratio (OD of sample/OD of positive control for each ELISA plate) per tested farm.

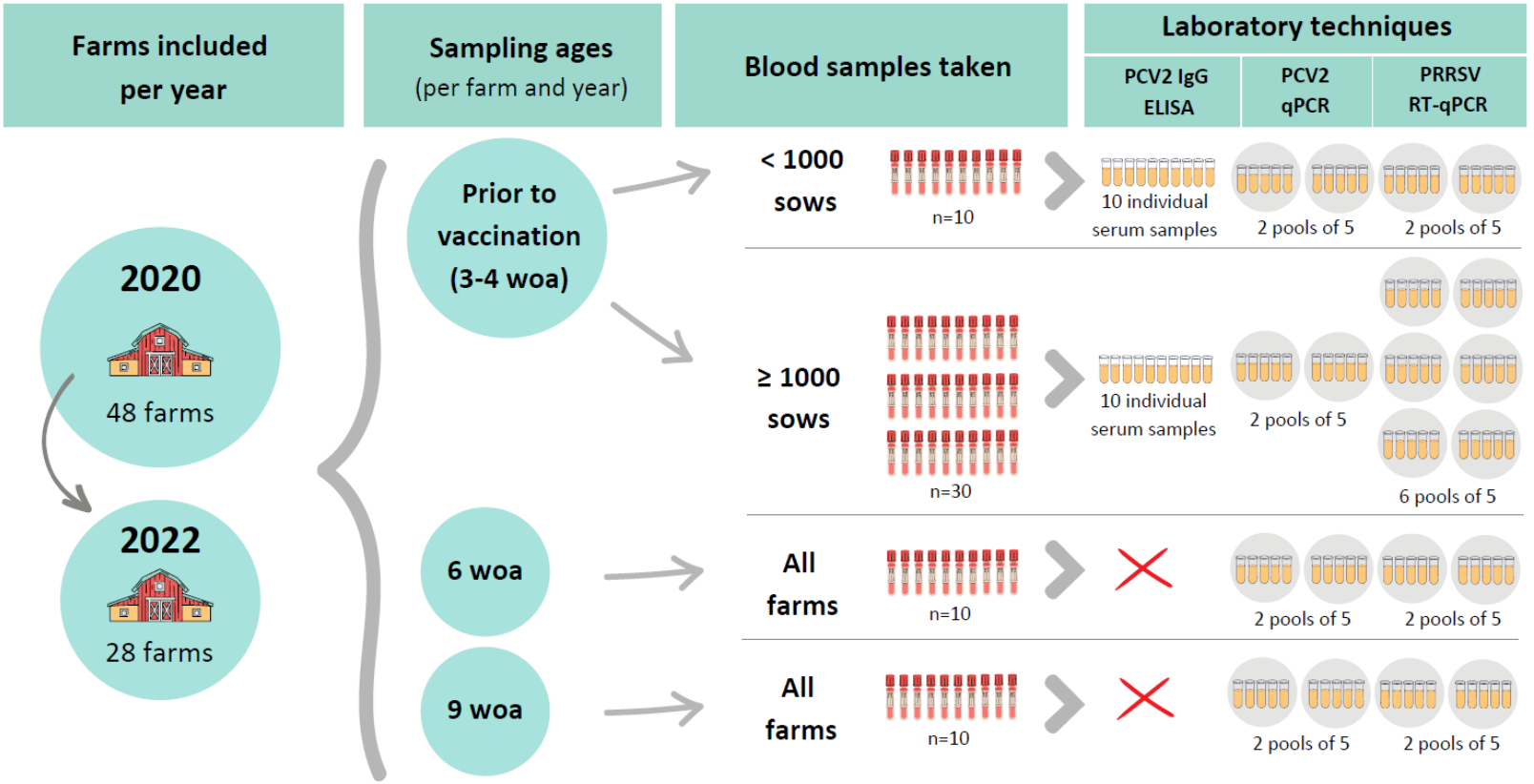

Figure 1.1. Scheme of the study design, showing the number of farms included in the study in 2020 and in 2022, the different sampled ages, the number of bled pigs depending on the farm size, the laboratory techniques performed (PCV2 IgG ELISA, qPCR for PCV2 and RT-qPCR for PRRSV), and the number of samples analysed per each technique. PCV2: porcine circovirus 2; PRRSV: porcine reproductive and respiratory syndrome virus; woa: weeks of age.

1.2.4. Statistical analyses

The normal distribution of the quantitative variables (PCV2 load and PCV2 ELISA S/P ratios) was checked by the Shapiro Wilk's test. PCV2 load values and PCV2 ELISA S/P ratios were analysed with the non-parametric Kruskal–Wallis test and Dunn's multiple comparison test (when comparisons were done between sampling points) or Mann–Whitney test (when comparisons were done between 2020 and 2022). Frequency of detection for both pathogens at different ages and between years were compared using Chi Square or Fisher's exact test. Statistical analyses and graphics were performed with Graphpad®. The significance level (*p*-value) was set at 0.05, and a trend towards statistical significance was set as 0.1.

1.3. Results

1.3.1. Forty-eight farms tested in 2020

Porcine circovirus 2 and PRRSV infection

Nine of the forty-eight (18.8%; CI: 7.7–29.8%) farms tested in 2020 had at least one pool positive by PCV2 qPCR. From the nine positive farms, one (11.1%; CI: 0.0–31.6%) had a qPCR positive pool prior vaccination, five showed positivity at 6 or at 9 woa (55.6%; CI: 23.1–88.0%), and finally three (33.3%; CI: 2.5–64.1%) had positive pools at both 6 and 9 woa (*Table 1.1* and *ST1.2 and ST1.3*). In these 9 farms, the PCV2 load ranged from 10^4 to 10^8 copies of PCV2/mL of pooled sera, being the highest ones detected in those farms where PCV2 infection was detected in two samplings points. Regarding PRRSV, 20 (41.7%; CI: 27.7–55.6%) of the 48 tested farms had at least one pool positive by RT-qPCR. From these 20 PRRSV positive farms, only 3 (15%; CI: 0.0–30.6%) were also PCV2 qPCR positive. Coinfection between PRRSV and PCV2 was detected in pools from sera collected at 6 woa (2 farms) or 9 woa (1 farm).

Table 1.1. PCV2 qPCR and PRRSV RT-qPCR results obtained prior to vaccination (3-4 woa), and at 6 and 9 woa in the 48 farms sampled in 2020, clustering the farms by their PCV2 qPCR results.

PCV2 qPCR result	Farms n (%)		Sampling points	Pools n (%)	PCV2 load*	PRRSV RT-qPCR Positive farms n (%)
Positive	9 (18.8%)	1 (2.1%)	Only at 3-4 woa	1 (0.3%)	$8.9x10^4$	0 (0.0%)
		3 (6.2%)	Only at 6 woa	5 (1.7%)	$2.6x10^7$ ($3.4x10^5 – 9.1x10^7$)	2 (66.7%)
		2 (4.2%)	Only at 9 woa	3 (1.0%)	$1.7x10^6$ ($1.0x10^4 – 4.99x10^6$)	1 (50.0%)
		3 (6.2%)	At 6 and 9 woa	9 (3.1%)	$1.1x10^8$ ($9.8x10^4 – 7.0x10^8$)	0 (0.0%)
Negative	39 (81.2 %)		At 3-4, at 6 and at 9 woa	270 (93.8%)	-	17 (43.6%)
Total	48 (100.0%)		-	288 (100.0%)	$6.1x10^7$ ($1.0x10^4 – 7.0x10^8$)	20 (41.7%)

PCV2: porcine circovirus 2; PRRSV: porcine reproductive and respiratory syndrome virus; woa: weeks of age; * Mean (Min-Max) of PCV2 load was calculated considering only positive (quantifiable and non-quantifiable) serum pools and are expressed in PCV2 genome copies/mL.

Porcine circovirus 2 IgG antibody levels in serum prior to PCV2 vaccination

Globally, farms with pools positive to PCV2 qPCR (n=9) showed lower ($p<0.1$) S/P levels (0.529 ± 0.287) than negatives ones (0.587 ± 0.287). Specifically, farms with serum pools positive by PCV2 qPCR at two sampling points (6 and 9) had statistically significant lower PCV2 IgG ELISA S/P ratios than those with pools positive only at 6 or 9 woa ($p<0.05$) (*Table 1.2*). However, the coefficient of variation (CV) of these S/P ratios was high in all groups (*Table 1.2*).

Table 1.2. PCV2 IgG ELISA mean S/P ratios obtained prior to vaccination (3-4 woa), grouped based on PCV2 qPCR results at 3-4, 6 and 9 woa in the 48 farms sampled in 2020.

PCV2 qPCR		PCV2 IgG ELISA prior to vaccination S/P ratio	
Result	**Sampling time-point**	**$\bar{X}$ ± SD**	**CV (%)**
Positive	Only at 3-4 woa	0.549 ± 0.203 [ab]	37.0%
	Only at 6 woa	0.622 ± 0.316 [a]	50.2%
	Only at 9 woa	0.631 ± 0.303 [a]	48.7%
	At 6 and 9 woa	0.361 ± 0.187 [b]	51.8%
Negative	At 3-4, at 6 and at 9 woa	0.587 ± 0.286 [a]	48.9%
Total		0.576 ± 0.259	47.3%

CV: coefficient of variation; ELISA: enzyme-linked immunosorbent assay; PCV2: porcine circovirus 2; woa: weeks of age; SD: standard deviation. Different superscript within each row indicates statistically significant differences between groups ($p<0.05$).

1.3.2. Twenty-eight farms tested in 2022

Porcine circovirus 2 and PRRSV infection

From the 28 farms tested in 2022, 12 (42.9%; CI: 24.5% - 61.2%) had at least one pool positive by PCV2 qPCR. Most of these farms (n=9, 75%; CI: 50.5% - 99.5%) had positive pools at 9 woa where the peak of viral load (approx. 10^8 genome copies of PCV2/mL of pooled sera) was also detected (*Table 1.3* and *ST1.2*).

Fifteen (53.6%; CI: 35.1% - 72.0%) out of these 28 re-sampled farms had at least one PRRSV RT-qPCR positive pool. From these 15, 9 (60%; CI: 35.2% - 84.8%) were also positive to PCV2.

Porcine circovirus 2 IgG antibody levels in serum prior to PCV2 vaccination

Porcine circovirus 2 IgG ELISA S/P ratios in farms with qPCR positive serum pools (0.517 ± 0.315) were lower than the ones obtained in the negative ones (0.541 ± 0.285), although not being statistically different. Within the PCV2 qPCR positive farms, those with pools positive at 3-4 woa had significantly lower S/P ratios than the ones positive only at 9 woa (*Table 1.4*). However, a high variability was observed in all groups as CV was higher than 50% in all groups (*Table 1.4*).

Table 1.3. PCV2 qPCR and PRRSV RT-qPCR results obtained prior to vaccination (3-4 woa), and at 6 and 9 woa in the 28 farms sampled in 2022, clustering the farms by their PCV2 qPCR results.

PCV2 qPCR result	Farms n (%)		Sampling points	Pools n (%)	PCV2 load*	PRRSV RT-qPCR Positive farms n (%)
Positive	12 (42.9%)	1 (3.6%)	Only at 6 woa	2 (1.2%)	$6.6x10^5$ ($1x10^4 – 1.3x10^6$)	1 (3.6%)
		5 (17.9%)	Only at 9 woa	8 (4.8%)	$1.1x10^8$ ($1x10^4 – 8.3x10^8$)	4 (14.3%)
		4 (14.3%)	At 6 and 9 woa	12 (7.1%)	$1.2x10^6$ ($1x10^4 – 9.5x10^6$)	3 (10.7%)
		1 (3.6%)	At 3-4 and 9 woa	3 (1.8%)	$8.3x10^7$ ($1.7x10^4 – 2.3x10^8$)	1 (3.6%)
		1 (3.6%)	At 3-4, at 6 and at 9 woa	4 (2.4%)	$3.4x10^6$ ($1x10^4 – 1.3x10^7$)	0 (0.0%)
Negative	16 (57.1 %)		At 3-4, 6 and 9 woa	139 (82.7%)	-	6 (21.4%)
Total	28 (100.0%)		-	168 (100.0%)	$4.1x10^7$ ($1x10^4 – 8.3x10^8$)	15 (53.6%)

PCV2: porcine circovirus 2; PRRSV: porcine reproductive and respiratory syndrome virus; woa: weeks of age; * Mean (Min-Max) of PCV2 load was calculated considering only positive (quantifiable and non-quantifiable) serum pools and are expressed in PCV2 genome copies/mL.

Table 1.4. PCV2 IgG ELISA mean S/P ratios obtained prior to vaccination (3-4 woa), grouped based on PCV2 qPCR results at 3-4, 6 and 9 woa in the 28 farms sampled in 2022.

PCV-2 qPCR		PCV-2 IgG ELISA prior to vaccination	
Result	Sampling time-point	S/P ratio	
		$\bar{X}$ ± SD	CV (%)
Positive	Only at 6 woa	0.432 ± 0.298 [ab]	69.0%
	Only at 9 woa	0.647 ± 0.345 [a]	53.4%
	At 3-4 and 9 woa	0.280 ± 0.205 [b]	73.3%
	At 6 and 9 woa	0.470 ± 0.260 [ab]	55.4%
	At 3-4, 6 and 9 woa	0.378 ± 0.204 [ab]	54.0%
Negative	At 3-4, 6 and 9 woa	0.541 ± 0.285 [ab]	52.7%
Total		**0.530 ± 0.298**	**56.1%**

CV: coefficient of variation; ELISA: enzyme-linked immunosorbent assay; PCV2: porcine circovirus 2; woa: weeks of age; SD: standard deviation. Different superscript within each row indicates statistically significant differences between groups ($p<0.05$).

1.3.3. Porcine circovirus 2 and PRRSV infection and PCV2 IgG antibodies in 28 farms in 2020 and 2022: definition of epidemiological scenarios

The 28 farms tested both years were classified considering the PCV2 virological results into four different epidemiological scenarios (*ST1.2* and *ST1.3*):

- POS20-POS22: farms PCV2 qPCR positive in both years (n=4, 14.3%). Although the four farms were positive in both years, two of these farms in 2022 had a lower frequency of detection and the PCV2 load was non-quantifiable (*ST1.4*). Regarding PRRSV viremia, three out of these four farms maintained the status

in both years and the remaining one (SP-19) changed from RT-qPCR negative to positive.

- POS20-NEG22: farms PCV2 qPCR positive in 2020 but negative in 2022 (n=1, 3.6%). This scenario was composed only by one farm that in 2020 had pools positive to PCV2 prior to vaccination, but in 2022 all the tested pools were negative. This farm was RT-qPCR negative for PRRSV in both years.
- NEG20-POS22: farms PCV2 qPCR negative in 2020 that turned to be positive in 2022 (n=8, 28.6%). In this scenario, a statistically significant increase of PCV2 detection frequency ($p<0.05$) was detected from 2020 to 2022, mainly among the 6 and 9 woa groups (*ST1.5*). Within this scenario, the number of PRRSV RT-qPCR positive farms was significantly higher ($p<0.05$) in 2022 (7 out of 8, 87.5%; CI: 64.6% - 100.0%) compared to 2020 (3 out of 8, 37.5%; 3.95% - 71.05%).
- NEG20-NEG22: farms that were negative to PCV2 qPCR both years (n=15, 53.6%). Nine of these farms were PRRSV RT-qPCR positive in 2020 and five of them tested positive again in 2022. An additional farm turned positive that year (*ST1.6*).

Regarding PCV2 IgG detection, the mean ELISA S/P ratios increased from 2020 to 2022 in scenarios POS20-POS22 and POS20-NEG22, while these decreased over the same period for scenarios NEG20-POS22 and NEG20-NEG22 (*Table 1.5*). These variations were only significant for NEG20-POS22 farms. When comparing scenarios within each year, only mean S/P ratios were significantly lower ($p<0.05$) in POS20-POS22 farms compared to NEG20-POS22 and NEG20-NEG22 herds in 2020.

Table 1.5. PCV2 IgG ELISA mean S/P ratios obtained prior to vaccination (3-4 woa) in the 28 farms sampled in 2020 and 2022, grouping the farms in the four stablished scenarios considering PCV2 detection in both studied years.

Defined scenarios	PCV2 IgG ELISA S/P ratio prior to vaccination (3-4 woa)			
	2020		2022	
	$\bar{X}$ ± SD	CV (%)	$\bar{X}$ ± SD	CV (%)
POS20-POS22	0.397 ± 0.197 $^{a\,A}$	49.1%	0.599 ± 0.372 $^{a\,A}$	62.0%
POS20-NEG22	0.549 ± 0.203 $^{a\,AB}$	37.0%	0.686 ± 0.263 $^{a\,A}$	38.4%
NEG20-POS22	0.628 ± 0.286 $^{a\,B}$	45.5%	0.476 ± 0.275 $^{b\,A}$	57.9%
NEG20-NEG22	0.639 ± 0.342 $^{a\,B}$	53.4%	0.531 ± 0.284 $^{a\,A}$	53.6%
Total	**0.598 ± 0.315**	**56.6%**	**0.530 ± 0.298**	**56.1%**

CV: coefficient of variation; ELISA: enzyme-linked immunosorbent assay; PCV2: porcine circovirus 2; woa: weeks of age; SD: standard deviation. Different superscript lowercase letters indicate statistically significant differences between years for each scenario, and the uppercase ones indicate statistically significant differences between scenarios in each year ($p<0.05$).

1.4. Discussion

The economic impact of PCV2-ADs has been considerably reduced since the advent of PCV2 vaccines (Young et al., 2011; Alarcon et al., 2013; Segalés. 2015). Such control of overt diseases associated with PCV2 lead to the fact that the most frequent presentation nowadays is PCV2-SI (Young et al., 2011; Segalés, 2012; Nielsen et al., 2017), which implies the interest to monitor the infection despite the lack of clinical signs. Therefore, the present study sought to determine the PCV2 and PRRSV frequency of detection in nursery pigs from 48 commercial farms in Spain in 2020 with no clinical signs associated to PCV2-ADs and to evaluate the epidemiological situation of these two pathogens in a proportion of these farms (n=28) two years later.

In both years, there were farms with PCV2 qPCR positive results in 3-to-9-week-old pigs with high PCV2 loads, up to $1.0x10^8$ copies/mL of pooled sera, which might potentially fit with a tentative diagnosis of PCV2-SD when following the different thresholds proposed (Harding et al., 2008; Grau-Roma et al., 2009; Segalés, 2012). However, these thresholds were established with different qPCR methods compared to those used nowadays (Segalés and Sibila, 2022); therefore, it is very likely that obtained values (from pools of five sera) could be indicative of subclinical infections considering the lack of overt clinical signs in the herd at the moment of sampling. It is important to remark that a final diagnosis of PCV2-SD must be established by means of histopathological lymphoid lesions and detection of PCV2 within these lesions (Segalés and Sibila, 2022); therefore, the unequivocal diagnosis of PCV2-SD could not be established based only on qPCR results.

In the 48 farms analysed in 2020, the frequency of PCV2 detection in pools of piglet sera was very low at vaccination age (3-4 woa, 2.1% of the farms, n=1/48) but increased up to 10.4% at the end of the nursery period (n=5/48). The obtained low prevalence in suckling pigs agrees with previous epidemiological studies performed in Europe that described PCV2 early viremia in piglets from endemically infected farms as fairly uncommon (Eddicks et al., 2016; Dieste-Pérez et al., 2018). Across the 28 farms analysed in 2022, the frequency of PCV2 detection was higher towards the end of the nursery period (n=11/28, 39.3% of the farms), despite being lower at vaccination age (n=2/28, 7.1% of the farms). Results from both years are relatively low compared with the ones obtained in other studies performed in North-America (Shen et al., 2010; Dvorak et al., 2013). However, these latter studies were published at a time when PCV2 vaccination was not as extensive as nowadays. Additionally, all these studies tested individual samples, whereas the present study is based on pooled samples to mimic usual field sampling and monitoring conditions used by

swine veterinarians in different parts of the world. Studies in pools likely imply a reduction in the observed PCV2 detection frequency (Rovira et al., 2007; Cortey et al., 2011b), but it was adopted to have the possibility to screen the higher number of farms/animals possible based on the epidemiological criteria set (theoretical frequency of infection of 25% for PCV2 at all tested ages, and 10% at weaning and 25% at 6 and 9 woa for PRRSV with 95% confidence). In turn, this represents one of the limitations of this study, since we would not be able to detect epidemiological situations in which lower percentages of infection with these pathogens may occur.

Maternally derived immunity transferred to piglets was evaluated in terms of antibody levels at vaccination age (3-4 weeks of age) both years, and antibody S/P values were moderate-to-low and highly variable (close to 50% CV). Specifically, in both years, the ELISA S/P ratio tended to be lower when PCV2 viremia was detected at more than one sampling time-point in a farm, than when viremia was not detected or was detected only in one sampling time-point. This could reinforce the statement that MDI has a protective effect against PCV2 infection, and the moderate-to-low values observed fit with the described epidemiological change of PCV2 infection due to vaccination pressure (Figueras-Gourgues et al., 2019; Segalés and Sibila, 2022). These results are also in line with a previous study indicating that protection conferred by MDA is titre dependent, so its presence does not guarantee full protection against the infection, as previously described (McKeown et al., 2005; Rodriguez-Arrioja et al., 2002; Larochelle et al., 2003).

Additionally, the number of farms positive to PRRSV increased from almost 42% in 2020 up to 54% in 2022. In January 2020, a PRRSV-1 strain of increased virulence (commonly known as Rosalia), which was characterised by high abortion rates and increase mortality rates in weaners, was reported in North-Eastern (NE) Spain (Martín-Valls et al., 2022, 2023). The NE and its surrounding regions concentrate

almost half of the Spanish pig farms and corresponds to the region from most of the farms tested in the present study (MAPA, 2022). This situation would probably explain the increase of farms positive to PRRSV, despite clinical signs due to this viral infection were not seen at the time when samplings were performed.

As previously mentioned, PRRSV and PCV2 target the host's immune cells by disrupting their function, and they have been detected coexisting in some PCV2-SD cases, emphasizing that such coinfection can be a main driver for overt PCVD expression (Ouyang et al.; 2019; Zhao et al., 2021). Therefore, it is not surprising to have cases of PCV2 and PRRSV coinfection in the nursery phase (in 15.0% and 60.0% of PRRSV positive farms in 2020 and 2022, respectively), as it has been already described (Zhao et al., 2021), despite not having clinical problems in analysed farms.

To further examine the PRRSV and PCV2 farm status co-evolution in more detail, the results from the 28 farms that were tested in 2020 and in 2022 were compared, classifying them into four epidemiological scenarios. In POS20-POS22 and NEG20-NEG22 ones, the total PRRSV detection frequency decreased (from 13.9 to 8.3%, and from 29.7 to 24.6%, respectively). The same happened with the PCV2 detection frequency (from 45.8 to 33.3%) and the PCV2 load (from the $8.76x10^7$ to $1.13x10^7$ copies of PCV2 DNA/mL of pooled sera) in the PCV2 POS20-POS22 scenario. Meanwhile, in the NEG20-POS22 scenario, PRRSV detection frequency statistically increased (from 22.1 to 50.0%), and the same happened with PCV2 detection frequency (from negative to 43.8%). In the POS20-POS22 scenario, the overall increase in the average ELISA S/P ratios, together with the reduction in PCV2 load and PCV2 and PRRSV frequencies of detection between years, could reinforce the previously mentioned suggestion of the protective effect of MDI. However, in the NEG20-POS22 scenario, lower levels of anti-PCV2 IgG antibodies from 2020 to 2022 could have been facilitated by the PRRSV infection in the farms, since it has a

suppressive effect of the innate immunity and might jeopardize the pig's immune response (Zhao et al., 2021, Park et al., 2014; Shi et al., 2008; Ouyang et al., 2019; Butler et al., 2014). PRRSV influences the activation of the specific immune response, and an early PRRSV infection could compromise the efficacy of PCV2 vaccines due to its detrimental effect on the development of naïve T cells, while it could negatively influence on the immune response to other pathogens (Canelli et al., 2016; Zhao et al., 2021).

A similar reduction in the ELISA S/P ratio between years was observed in NEG20-NEG22, which could be due to an overall reduction in herd immunity due to the high efficacy of PCV2 vaccines decreasing infection pressure in the farms. In such scenario, sow vaccination could be a good option to avoid the putative future occurrence of PCV2-SD in piglets (Segalés, 2015; Segalés and Sibila, 2022).

1.5. Supplementary material

Table ST1.1. Characteristics of farms included in this study based on herd size, type of production system, farrowing batches, and piglet PCV2 vaccination age in 2020. Those farms re-sampled two years later are also noted.

Farm information					Sampling repeated in 2022
Farm ID	Herd size	Production system	Farrowing batch	Age at PCV2 vaccination (woa)	
SP-1	2500	1 site	weekly	4 woa	Yes
SP-2	1000			4 woa	No
SP-3	2000			4 woa	Yes
SP-4	500			3 woa	Yes
SP-5	2500			4 woa	Yes
SP-6	1000			4 woa	Yes
SP-7	500			4 woa	No
SP-8	500			4 woa	No
SP-9	750			4 woa	No
SP-10	750			4 woa	Yes
SP-11	1100			3 woa	Yes
SP-12	800			4 woa	Yes
SP-13	3400		3-weeks	4 woa	Yes
SP-14	1650			3 woa	Yes
SP-15	300			4 woa	No
SP-16	550		5-weeks	4 woa	No
SP-17	1400			3 woa	No
SP-18	800			3 woa	No
SP-19	800	2 sites *	weekly	4 woa	Yes
SP-20	700			3 woa	No
SP-21	230			3 woa	No
SP-22	200			4 woa	No
SP-23	1000			3 woa	Yes
SP-24	800			4 woa	No
SP-25	900			3 woa	No
SP-26	900			4 woa	Yes
SP-27	900			4 woa	No
SP-28	1400			3 woa	Yes
SP-29	700			3 woa	Yes
SP-30	1663			4 woa	Yes
SP-31	1196			3 woa	Yes
SP-32	1100			4 woa	No
SP-33	700			3 woa	Yes
SP-34	531			3 woa	Yes

* 2 sites farm were considered those having sites I (gestation and farrowing) and II (nursery) in the same farm; woa: weeks of age; PCV2: porcine circovirus 2.

Table ST1.1 (cont.). Characteristics of farms included in this study based on herd size, type of production system, farrowing batches, and piglet PCV2 vaccination age in 2020. Those farms re-sampled two years later are also noted.

Farm information					Sampling repeated in 2022
Farm ID	Herd size	Production system	Farrowing batch	Age at PCV2 vaccination (woa)	
SP-35	1700	2 sites *	2-weeks	4 woa	No
SP-36	1000			4 woa	Yes
SP-37	1233			4 woa	Yes
SP-38	572			4 woa	Yes
SP-39	525			4 woa	Yes
SP-40	634			4 woa	Yes
SP-41	361		3-weeks	4 woa	No
SP-42	2134			3 woa	Yes
SP-43	1350			3 woa	No
SP-44	1000			3-4 woa	Yes
SP-45	500			3 woa	Yes
SP-46	1700		5-weeks	3-4 woa	No
SP-47	680			3-4 woa	Yes
SP-48	600			3 woa	No

* 2 sites farm were considered those having sites I (gestation and farrowing) and II (nursery) in the same farm; woa: weeks of age; PCV2: porcine circovirus 2.

Table ST1.2. PCV2 qPCR and PRRSV RT-qPCR results, and PCV2 IgG ELISA S/P ratios of the 28 farms analysed in 2020 and 2022, ordered by the proposed scenarios.

Farm ID	2020									2022									Scenario
	PCV2 qPCR (POS / NO QUANT POS / NEG) (copies/mL)			PRRSV RT-qPCR (POS / NEG)			PCV2 ELISA (3-4 woa, prior to vaccination)			PCV2 qPCR (POS / NO QUANT POS / NEG) (copies/mL)			PRRSV RT-qPCR (POS / NEG)			PCV2 ELISA (3-4 woa, prior to vaccination)		
	3-4 woa	6 woa	9 woa	3-4 woa	6 woa	9 woa	S/P $\bar{X} \pm SD$	CV (%)		3-4 woa	6 woa	9 woa	3-4 woa	6 woa	9 woa	S/P $\bar{X} \pm SD$	CV (%)	
SP-06			<1x10^4 - 4.9x10^5				0.505 ± 0.185	36.6				9.3x10^4 - 8.1x10^7				1.014 ± 0.312	30.8	POS20 - POS22
SP-14		5.4x10^4	9.8x10^4 - 1.4x10^6				0.500 ± 0.199	39.9				<1x10^4				0.547 ± 0.307	56.1	
SP-19		1.6x10^4	3.0x10^3 - 3.5x10^6				0.228 ± 0.086	37.6			<1x10^4	<1x10^4				0.407 ± 0.333	82.0	
SP-37		1.6x10^7	2.3x10^4 - 7.0x10^8				0.354 ± 0.157	44.3			1.1x10^6	3.0x10^4 - 9.5x10^6				0.430 ± 0.170	39.6	
SP-34	8.9x10^4						0.549 ± 0.203	37.0								0.686 ± 0.263	38.4	POS20 - NEG22
SP-05							0.766 ± 0.331	43.2			5.1x10^5 - 1.9x10^6	<1x10^4				0.549 ± 0.233	42.3	NEG20 - POS22
SP-10							0.451 ± 0.203	45.0				<1x10^4 - 1.2x10^4				0.398 ± 0.323	81.2	
SP-11							0.666 ± 0.254	38.2				3.6x10^5				0.693 ± 0.245	35.3	
SP-23							0.365 ± 0.137	37.6				2.6x10^6 - 8.3x10^8				0.581 ± 0.238	40.9	
SP-30							0.664 ± 0.259	39.0			<1x10^4 - 1.0x10^6	<1x10^4 - 2.2x10^6				0.493 ± 0.292	59.2	
SP-39							0.779 ± 0.350	44.9		<1x10^4	7.5x10^4	4.0x10^5 - 1.3x10^7				0.378 ± 0.204	54.0	
SP-44							0.693 ± 0.285	41.2			<1x10^4 - 1.3x10^6					0.432 ± 0.298	69.0	
SP-47							0.637 ± 0.211	33.2		1.7x10^4		1.8x10^7 - 2.3x10^8				0.280 ± 0.205	73.3	
SP-01							0.377 ± 0.218	57.9								0.802 ± 0.323	40.4	NEG20 - NEG22
SP-03							0.632 ± 0.266	42.1								0.519 ± 0.226	43.5	
SP-04							0.504 ± 0.244	48.4								0.392 ± 0.216	55.1	
SP-12							1.055 ± 0.385	36.5								0.403 ± 0.187	46.4	
SP-13							0.667 ± 0.289	43.3								0.817 ± 0.314	38.4	
SP-26							0.701 ± 0.365	52.0								0.749 ± 0.207	27.7	
SP-28							0.521 ± 0.277	53.2								0.504 ± 0.167	33.2	
SP-29							0.869 ± 0.283	32.6								0.544 ± 0.323	59.5	
SP-31							0.454 ± 0.206	45.3								0.566 ± 0.247	43.7	
SP-33							0.950 ± 0.436	45,9								0.709 ± 0.414	58.4	
SP-36							0.526 ± 0.176	33.4								0.338 ± 0.136	40.2	
SP-38							0.401 ± 0.225	56.1								0.521 ± 0.140	26.9	
SP-40							0.458 ± 0.268	58.4								0.368 ± 0.127	34.5	
SP-42							0.725 ± 0.301	41.6								0.201 ± 0.055	27.2	
SP-45							0.746 ± 0.328	43.9								0.531 ± 0.209	39.4	

PCV2: porcine circovirus 2; PRRSV: porcine reproductive and respiratory syndrome virus; woa: weeks of age; POS: positive; NO QUANT POS; non-quantifiable positive; NEG: negative; ELISA: enzyme-linked immunosorbent assay; SD: standard deviation; CV: coefficient of variation.

Table ST1.3. PCV2 qPCR and PRRSV RT-qPCR results, and PCV2 IgG ELISA S/P ratios of the 20 farms analysed exclusively in 2020.

Farm ID	2020								2022							Scenario
	PCV2 qPCR (POS / NO QUANT POS / NEG) (copies/mL)			PRRSV RT-qPCR (POS / NEG)			PCV2 ELISA (3-4 woa, prior to vaccination)		PCV2 qPCR (POS / NO QUANT POS / NEG) (copies/mL)			PRRSV RT-qPCR (POS / NEG)			PCV2 ELISA (3-4 woa, prior to vaccination)	
	3-4 woa	6 woa	9 woa	3-4 woa	6 woa	9 woa	S/P $\bar{X} \pm SD$	CV (%)	3-4 woa	6 woa	9 woa	3-4 woa	6 woa	9 woa	S/P $\bar{X} \pm SD$	CV (%)	
SP-02							0.359 ± 0.098	27.4	–			–			–		–
SP-07		$3.4x10^5$ – $9.1x10^7$					0.341 ± 0.201	58.9	–			–			–		
SP-08							0.513 ± 0.178	34.8	–			–			–		
SP-09		$1.1x10^6$ – $3.9x10^7$					0.935 ± 0.197	21.1	–			–			–		
SP-15							0.494 ± 0.296	60.0	–			–			–		
SP-16							0.548 ± 0.243	44.3	–			–			–		
SP-17							0.540 ± 0.164	30.3	–			–			–		
SP-18							0.372 ± 0.149	40.0	–			–			–		
SP-20							0.561 ± 0.188	33.6	–			–			–		
SP-21							0.610 ± 0.164	26.9	–			–			–		
SP-22							0.463 ± 0.128	27.8	–			–			–		–
SP-24		$5.3x10^5$					0.589 ± 0.216	36.7	–			–			–		
SP-25							0.506 ± 0.310	61.2	–			–			–		
SP-27							0.522 ± 0.199	38.2	–			–			–		
SP-32							0.599 ± 0.183	30.6	–			–			–		
SP-35							0.575 ± 0.135	23.5	–			–			–		
SP-41			$4.4x10^4$				0.758 ± 0.352	46.4	–			–			–		
SP-43							0.534 ± 0.286	53.5	–			–			–		
SP-46							0.405 ± 0.131	32.4	–			–			–		
SP-48							0.685 ± 0.186	27.2	–			–			–		

PCV2: porcine circovirus 2; PRRSV: porcine reproductive and respiratory syndrome virus; woa: weeks of age; POS: positive; NO QUANT POS; non-quantifiable positive; NEG: negative; ELISA: enzyme-linked immunosorbent assay; SD: standard deviation; CV: coefficient of variation.

Table ST1.4. PCV2 qPCR and PRRSV RT-qPCR detection frequency, and PCV2 load, at different time-points in 2020 and 2022 of the farms grouped in scenario POS20-POS22.

Age (woa)	Year	PCV2 qPCR				PRRSV RT-qPCR		
		Positive farms (n)	Positive pools (n)	Viral load PCV2 copies/mL of serum $\bar{X}$ (Min -Max)	Detection frequency	Positive farms (n)	Positive pools (n)	Detection frequency
3-4 woa	2020	0/4	0/8	-	0% [a]	1/4	1/20	5.0% (CI: 0.0% - 14.6%)
	2022	0/4	0/8	-	0% [a]	1/4	1/20	5.00% (CI: 0.0% - 14.6%)
6 woa	2020	3/4	3/8	$7.67x10^6$ ($1.60x10^6$ - $1.60x10^7$)	37.5% [a] (CI: 4.0% - 71.1%)	1/4	2/8	25.0% (CI: 0.0% - 55.0%)
	2022	2/4	2/8	$1.05 x10^4$ ($<1x10^4$ – $1.10x10^4$)	25.0% [a] (CI: 0.0% - 55.0%)	0/4	0/8	0%
9 woa	2020	4/4	8/8	$1.18x10^8$ ($<1x10^4$ – $7.00x10^8$)	100% [b]	1/4	2/8	25.0% (CI: 0.0% - 55.0%)
	2022	4/4	6/8	$1.51x10^7$ ($<1x10^4$ – $8.10x10^7$)	75.0% [b] (CI: 45.0% - 100.0%)	1/4	2/8	25.0% (CI: 0.0% - 55.0%)
Total 2020		4/4	11/24	$8.76x10^7$ * ($<1x10^4$ – $7.00x10^8$)	45.8% (CI: 25.9% - 65.8%)	1/4	5/36	13.9% (CI: 2.6% - 25.2%)
Total 2022		4/4	8/24	$1.13x10^7$ ** ($<1x10^4$ – $1.13x10^7$)	33.3% (CI: 14.5% - 52.2%)	2/4	3/36	8.3% (CI: 0.0% - 17.4%)

PCV2: porcine circovirus 2; PRRSV: porcine reproductive and respiratory virus; woa: weeks of age; CI: confidence interval. Ranges (Min-Max) have been calculated considering only positive pools of samples and those are expressed in PCV2 genome copies/mL. Different superscript letters indicate statistically significant differences in PCV2 prevalence within time-points and ages ($p<0.05$), and a statistically significant trend is marked with */** ($p<0.1$).

Table ST1.5. PCV2 qPCR and PRRSV RT-qPCR detection frequency, and PCV2 load, at different time-points in 2020 and 2022 of the farms grouped in scenario NEG20-POS22.

Age (woa)	Year	PCV2 qPCR Positive farms (n)	PCV2 qPCR Positive pools (n)	PCV2 qPCR Viral load PCV2 copies/mL of serum $\bar{X}$ (Min -Max)	PCV2 qPCR Detection frequency	PRRSV RT-qPCR Positive farms (n)	PRRSV RT-qPCR Positive pools (n)	PRRSV RT-qPCR Detection frequency
3-4 woa	2020	0/8	0/16	-	0% [a]	3/8	7/36	19.4% [A] (CI: 6.5% - 32.4%)
	2022	2/8	2/16	$1.35x10^4$ ($<1.0x10^4 – 1.70x10^4$)	12.5% [a] (CI: 0.0% - 28.7%)	3/8	12/36	33.3% [A] (CI: 17.9 % - 48.7%)
6 woa	2020	0/8	0/16	-	0 % [a]	2/8	4/16	25.00% [A*] (CI: 3.8% - 46.2%)
	2022	3/8	7/16	$6.21x10^5$ ($<1.0x10^4 – 1.90x10^6$)	43.8% [b] (CI: 19.4% - 68.1%)	5/8	10/16	62.50% [A**] (CI: 38.8% - 86.2%)
9 woa	2020	0/8	0/16	-	0% [a]	2/8	4/16	25.0% [A] (CI: 3.8% - 46.2%)
	2022	7/8	12/16	$9.14x10^7$ ($<1.0x10^4 – 8.30x10^8$)	75.0% [b] (CI: 53.8% - 96.2%)	6/8	12/16	75.0% [B] (CI: 53.8% - 96.2%)
Total 2020		0/8	0/48	-	0% [a]	3/8	15/68	22.1% [A] (CI: 12.2% - 31.9%)
Total 2022		8/8	21/48	$5.24x10^7$ ($<1.0x10^4 – 8.30x10^8$)	43.8% [b] (CI: 29.7% - 57.8%)	7/8	34/68	50.0% [B] (CI: 38.1% - 61.9%)

PCV2: porcine circovirus 2; PRRSV: porcine reproductive and respiratory virus; woa: weeks of age; CI: confidence interval. Ranges (Min-Max) have been calculated considering only positive pools of samples and those are expressed in PCV2 genome copies/mL. Different superscript lowercase letters within 2020 and 2022 and different ages rows indicate PCV2 detection frequency statistically significant differences ($p<0.05$). The uppercase ones indicate the PRRSV detection frequency statistically significant differences ($p<0.05$), and a statistically significant trend is marked with */** ($p<0.1$).

Table ST1.6. PCV2 qPCR and PRRSV RT-qPCR detection frequency, and PCV2 load, at different time-points in 2020 and 2022 of farms grouped in scenario NEG20-NEG22.

Age (woa)	Year	PCV2 qPCR				PRRSV qPCR		
		Positive farms (n)	Positive pools (n)	Viral load PCV2 copies/mL of serum $\bar{X}$ (Min -Max)	Detection frequency	Positive farms (n)	Positive pools (n)	Detection frequency
3-4 woa	2020	0/15	0/30	-	0%	6/15	13/58	22.4% [a] (CI: 11.7% - 33.2%)
	2022	0/15	0/30	-	0%	4/15	10/58	17.2% [a] (CI: 7.5% - 27.0%)
6 woa	2020	0/15	0/30	-	0%	5/15	9/30	30.0% [ab] (CI: 13.6% - 46.4%)
	2022	0/15	0/30	-	0%	5/15	8/30	26.7% [ab] (CI: 10.8% - 42.5%)
9 woa	2020	0/15	0/30	-	0%	7/15	13/30	43.3% [b] (CI: 25.6% - 61.1%)
	2022	0/15	0/30	-	0%	6/15	11/30	36.7% [b] (CI: 19.4% - 53.9%)
Total 2020		0/15	0/90	-	0%	9/15	35/118	29.7% (CI: 21.4% - 37.9%)
Total 2022		0/15	0/90	-	0%	6/15	29/118	24.6% (CI: 16.8% - 32.3%)

PCV2: porcine circovirus 2; PRRSV: porcine reproductive and respiratory virus; woa: weeks of age; CI: confidence interval. Different superscript letters within 2020 and 2022 and different ages rows indicate PRRSV frequency of detection statistically significant tendencies ($p<0.1$).

Chapter 2

Efficacy of a novel PCV2d and *Mycoplasma hyopneumoniae* combined vaccine in piglets with high and low levels of PCV2 maternally derived antibodies at vaccination

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2.1. Introduction

The most prevalent PCV2 genotype nowadays reported in different regions including Europe, Asia and North America is PCV2d, a (Xiao et al., 2015, 2016; Franzo and Segalés, 2018; Qu et al., 2018; Huang et al., 2021; Sibila et al., 2021; Dei Giudici et al., 2023; Gomes-Gonçalves et al., 2025). Although most of the PCV2 vaccines available in the market since 2008 have been traditionally constructed with a PCV2a genotype and consist of the capsid protein encoded by ORF2, cross-protection with PCV2b and PCV2d has been demonstrated (Opriessnig and Langohr, 2013; Cho et al., 2020; Yu et al., 2023). Additionally, PCV2 vaccines based on different technologies, combining more than one genotype, as well as ready-to-mix and RTU combined vaccines with other pathogens such as *M. hyopneumoniae* are now commercially available (Venegas-Vargas et al., 2021; Sipos and Sipos, 2022; Krejci et al., 2025; Pálmai et al., 2025).

Maternally derived antibodies protect piglets during early life but may interfere with vaccine-induced seroconversion when present at high levels (Haake et al., 2014; Seo et al., 2014b; Oh et al., 2014; Feng et al., 2016). Nevertheless, this interference rarely compromises vaccine efficacy in reducing viremia, as both humoral and cell-mediated responses contribute to protection (Chae, 2012; Poulsen Nautrup et al., 2021; Maity et al., 2023).

The objective of this study was to assess the effect of high and low PCV2 MDA on the efficacy against PCV2 of a new ready-to-use combined vaccine based on PCV2d genotype and *M. hyopneumoniae* in piglets experimentally inoculated with a heterologous genotype (PCV2b).

2.2. Materials and methods

2.2.1. Animal selection and housing

A total of 150 2-week-old piglets (Large White – Landrace and Duroc crossbreed coming from non-vaccinated sows) were screened with the objective of selecting enough animals with low and high PCV2 antibody levels. Piglets came from a farm negative for PRRSV and seropositive against *M. hyopneumoniae, Glaesserella parasuis* and *A. pleuropneumoniae*. Forty-eight clinically healthy piglets were taken to complete the experimental study. Selected piglets were negative to PRRSV and PCV2 by RT-qPCR or qPCR, respectively, and were distributed according to their PCV2 ELISA S/P values (Ingezim Circo IgG 11.PCV.K1®, Gold Standard Diagnostics, Madrid, Spain) into two experimental groups of 24 animals. Group L comprised pigs with the lowest ELISA S/P ratios (ranging between 0.189 and 0.435), and group H those with the highest S/P ratios (ranging between 1.073 and 1.511). Afterwards, groups L and H were further divided into non-vaccinated (H-NV and L-NV) and vaccinated (H-V and L-V) sub-groups (n=12 each). These piglets were transported to A.M. Animalia Bianya S.L. experimental facilities (Girona, Spain) at two weeks of age. Housing conditions, feeding system, feed characteristics and health management were the same for all groups. This study was approved by the Ethics Commission of Generalitat de Catalunya (Spain) through A.M. Animalia Bianya S.L. under the reference 028/23.

2.2.2. Experimental study design and sample collection

Experimental design is shown in *Figure 2.1*. After a one-week acclimatation period, at three weeks of age, H-V and L-V piglets received intramuscularly one dose of 2 mL of Cirbloc® M Hyo (Ceva Santé Animale, Libourne, France) (batch number 002NG1N) on the right side of the neck muscles following the manufacturer's

recommendations, while H-NV and L-NV were injected with 2 mL of phosphate buffered saline (PBS) in an equivalent manner. Five weeks post-vaccination or PBS injection (5 wpv), all animals were challenged intranasally with 3 mL of inoculum (1.5 mL in each nostril) containing $10^{4,73}$ $TCID_{50}$/mL of PCV2b strain Sp-6-11-49-16 (Genbank accession number: EF647673.1). The study finished with the euthanasia and necropsy of all animals three weeks post-challenge (3 wpc).

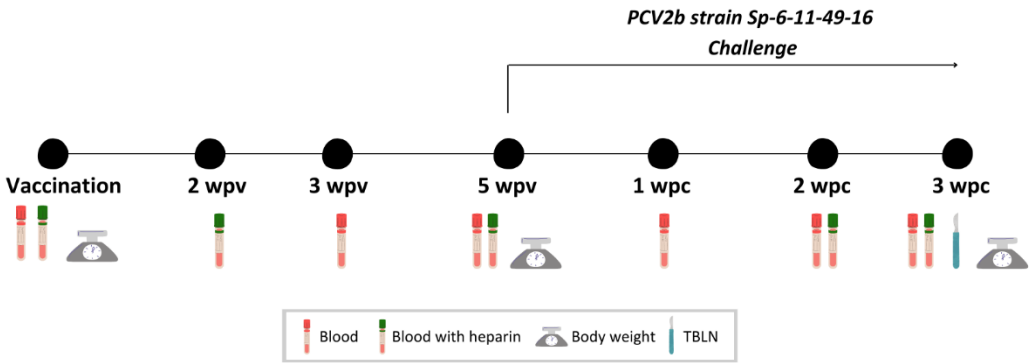


Figure 2.1. Scheme of the study design. TBLN: tracheobronchial lymph node; wpv: weeks post-vaccination; wpc: weeks post-challenge.

Blood samples were collected at vaccination, 3 and 5 wpv, and 1, 2 and 3 wpc to obtain serum (*Figure 2.1*). Heparinised blood samples were collected from half of the individuals of each group (n=24, being 6 per subgroup) at vaccination, 2 and 5 wpv, and 2 and 3 wpc to obtain PBMCs. Tracheobronchial lymph node (TBLN) was collected from each animal at necropsy (3 wpc), both fresh (to perform PCV2 qPCR) and fixed by immersion in 10% buffered formalin (for histopathology and *in situ* hybridisation (ISH) analyses).

Additionally, all animals were weighted at vaccination, 5 wpv and 3 wpc. The ADWG was calculated based on the body weight (BW) at three different periods: from vaccination to challenge, from challenge to necropsy, and from vaccination to necropsy.

2.2.3. DNA extraction and detection of PCV2 by qPCR

DNA extraction and PCV2 detection by qPCR were performed on serum samples and supernatant of macerated TBLN individually. DNA was extracted from 200 µL of serum or tissue supernatant using the MagMAX™ Pathogen RNA/DNA Kit (Applied Biosystems, Thermo Fisher Scientific, Waltham, Massachusetts, USA) according to the manufacturer's guidelines. Each extraction process and plate included negative controls to check for potential contamination.

To detect and quantify PCV2, the LSI VetMAX™ Porcine Circovirus Type 2 Quantification qPCR assay (Applied Biosystems, Thermo Fisher Scientific, Massachusetts, USA) was used. Every qPCR plate contained a standard curve, negative controls and an internal positive control (IPC) to ensure the reliability of extraction and amplification processes. The limit of quantification (LOQ) was set at $1.0x10^4$ genome copies/mL, and the limit of detection (LOD) was set at $4x10^3$ for both serum and tissue supernatant, as previously described (Pleguezuelos et al., 2022). qPCR results were then $\log_{10}$ transformed and classified in three categories: below LOD (which included from undetermined to <3.6 $\log_{10}$ PCV2 DNA copies/mL values), positive but not quantifiable results (between LOD and LOQ, namely from 3.6 to 4.0 $\log_{10}$ PCV2 DNA copies/mL), and positive and quantifiable results (>4.0 $\log_{10}$ PCV2 DNA copies/mL). For statistical purposes, the following assumptions were done based on a previous study (Pleguezuelos et al., 2022): undetermined and LOD results were assigned a value equivalent to half of the LOD (which corresponds to 3.3 $\log_{10}$) and positive but not quantifiable results were assigned the LOQ value (4.0 $\log_{10}$). Additionally, the area under the curve (AUC) of viral load was calculated for each animal from 5 wpv to 3 wpc.

2.2.4. Porcine circovirus 2 antibody levels measured by PCV2 IgG ELISA

PCV2 antibody levels were determined in serum samples as described in *Chapter 1*. Results were also reported as the mean S/P ratio per tested serum sample.

2.2.5. Histopathology and *in situ* hybridisation

Formalin fixed TBLN were dehydrated and embedded in paraffin. From each paraffin block, 4 µm thick sections were cut, stained with haematoxylin-eosin (HE) and examined for the presence of lesions indicative of PCV2 infection, such as LD and HR. Additionally, a contiguous section was prepared to detect PCV2 genome by ISH using RNAscope Technology (ACDBio, Newark, California, USA) according to manufacturer procedures and as previously described (Deleage et al., 2016; Cobos et al., 2024). Afterwards, LD, HR and the amount of PCV2 genome were scored from 0 (no lesions or no staining) to 3 (severe lesions or widespread antigen distribution) (Cobos et al., 2024).

2.2.6. Peripheral blood mononuclear cells isolation and stimulation

The PBMCs were extracted from blood samples collected in heparinized tubes using density gradient centrifugation with Histopaque® 1.077 (Sigma, Madrid, Spain). The isolated PBMCs were washed and resuspended in complete RPMI 1640 medium (cRPMI, Corning, New York, USA) supplemented with 10% foetal bovine serum (FBS) (Sigma, Madrid, Spain). Cell viability was determined using Trypan blue staining. The PBMCs were then plated in 96-well plates at a density of 1×10^6 cells per well and incubated with baculovirus-expressed PCV2 Cap protein (0.6 µg/mL final concentration per well, kindly provided by Gold Standard Diagnostics, Madrid, Spain), phytohemagglutinin (positive control, 10 µg/mL final concentration per well, Sigma, Madrid, Spain), or cRPMI with 10% FBS (negative control) for 24 hours at

37°C in a humidified 5% CO_2 atmosphere. After incubation, the plates were centrifuged, and the cell culture supernatants were collected and stored at -80°C for further analysis (Díaz and Mateu, 2005; Oliver-Ferrando et al., 2018; Díaz, 2022).

2.2.7. Multiplex immunoassay for the quantification of cytokines

Supernatant samples from PBMCs were analysed using the ProcartaPlex™ Porcine Cytokine & Chemokine Panel 1 (Invitrogen, Thermo Fisher Scientific, Waltham, Massachusetts, USA), following manufacturer's guidelines. This multiplex immunoassay uses Luminex® xMAP technology to measure nine cytokines: IFN-α, IFN-γ, IL-12, TNF-α, IL-1β, IL-8, IL-4, IL-6, and IL-10. The cytokine levels were quantified with a MAGPIX® analyser (Luminex Corporation, Austin, Texas, USA) and interpreted using xPONENT® 4.2 software (Luminex Corporation, Austin, Texas, USA), based on standard curves. For calculating PCV2-specific cytokine secretion (pg/mL), the cytokine concentrations in supernatants from PBMCs cultured with cRPMI (background) were subtracted from those in supernatants from PBMCs stimulated with the PCV2 Cap protein (Oliver-Ferrando et al., 2018).

2.2.8. Statistical analyses

The normal distribution of all studied quantitative variables (BW, ADWG, PCV2 ELISA S/P ratios, PCV2 $\log_{10}$ load in serum and tissue supernatant, AUC and cytokine amounts) was checked by the Shapiro Wilk's test. The different parameters were first analysed between V and NV to assess vaccination efficacy, and subsequently among H-V, H-NV, L-V, and L-NV to study the effect of MDA on this efficacy.

For the V and NV analyses, BW and ADWG differences for each time-point were analysed with t-test, and AUC differences with a Mann-Whitney test. The percentage of PCV2 qPCR positive serum samples (quantifiable and non-

quantifiable results) at 1, 2 and 3 wpc as well as PCV2 qPCR positive TBLN samples were compared using the Fisher test. PCV2 ELISA S/P ratio, and PCV2 $\log_{10}$ loads between groups and time-points were analysed with a two-way ANOVA test and Tukey's multiple comparison test. Additionally, the effect of MDA on the seroconversion of piglets was assessed by calculating the Pearson correlation coefficient of PCV2 S/P ratios at vaccination and the Delta value from the different periods: from vaccination to 3 wpv, from vaccination to 5 wpv and from 3 wpv to 5 wpv. Finally, the comparison of each cytokine concentration was done between groups and time-points using a mixed effects model and Tukey's multiple comparison test.

For all groups (H-V, H-NV, L-V and L-NV) comparison, BW and ADWG differences for each time-point were analysed with the one-way ANOVA test and Tukey's multiple comparison test, and AUC differences with a Kruskal-Wallis test and a Dunn's multiple comparisons test. The rest of the parameters were analysed as described for V and NV groups.

Statistical analyses and graphics were performed with Graphpad (La Jolla, California, USA). The significance level (*p-value*) was set at 0.05, and the trend towards statistical significance was set as 0.1.

2.3. Results

Results (except for clinical assessment, which are presented together) were first analysed comparing V and NV groups, and subsequently among H-V, H-NV, L-V, and L-NV ones.

2.3.1. Clinical assessment

During the study, four animals (three from H-NV group and one from L-V group) died due to causes unrelated to the vaccination or challenge procedures. One was due to an intestinal intussusception, another due to a haemorrhagic enteritis by *Escherichia coli*, and the other two died with signs and gross lesions compatible with septicaemia (cyanosis and petechial haemorrhages). The rest of the animals remained healthy during the whole experimental period, with not evident clinical signs after PCV2 challenge.

2.3.2. Comparison between V and NV groups

Body weight and average daily weight gain

Differences between V and NV groups on BW and ADWG were not statistically significant at any time point of the study (*Table 2.1*).

Porcine circovirus 2 IgG antibody levels dynamics

Vaccinated animals showed significantly higher PCV2 S/P ELISA values from 5 wpv to necropsy than NV ones. In fact, this latter group showed a statistically significant decline ($p<0.05$) in antibody levels over the time until 2wpc (*Figure 2.2A*). Significant ($p<0.05$) negative correlations were found between PCV2 ELISA S/P ratios at vaccination and the corresponding Delta values from vaccination to 3 wpv, and from vaccination to 5 wpv for both groups; from 3 to 5 wpv it was only significant for NV group *(Supplementary Figure* (SF) *2.1*). After 5 wpv, the V group exhibited relatively stable antibody levels.

Porcine circovirus 2 infection dynamics

Animals from both groups remained PCV2 qPCR negative during the period from vaccination to challenge. The V group showed significantly lower percentage PCV2 qPCR positive animals (*Figure 2.3A*) and lower mean viral load (*Figure 2.3B*) in serum at 2 and 3 wpc when compared to the NV one ($p<0.05$). These differences were coupled with a significantly lower AUC after challenge in the V group (10.2 ± 0.8) than in the NV group (10.9 ± 1.5) ($p<0.05$). The PCV2 load in TBLN was also significantly higher in the NV group compared to the V one ($p<0.05$), but all groups had a similar ($p>0.05$) percentage of qPCR PCV2 positivity of TBLN between groups (*Figure 2.4A*).

Lesion assessment and PCV2 antigen detection in TBLN

Three animals out of 21 from the NV group had lesions of HR (scores 1 or 2). The one with moderate HR had also LD score 1.

Examples of the ISH scoring values are depicted in *Figure 2.5*. Globally, the V group had a statistically higher percentage of animals scored as 0 (73.9%), compared to the NV group (33.3%) ($p<0.05$). Conversely, the NV group had a statistically higher percentage of animals scored as 3 (19.0%) compared to the V group (0.0%) ($p<0.05$) (*Table 2.2*).

Cytokine concentration

Vaccinated animals exhibited an ascending dynamic from vaccination to 5 wpv for IFN-α and IFN-γ ($p<0.05$), and for TNF-α and IL-12 ($p<0.1$), reaching a stability from 5 wpv onwards. However, for the NV group, a significant ascending dynamic relative to placebo administration was observed at 3 wpc for the three cytokines ($p<0.05$), except for TNF-α, which showed significance when comparing 2 wpv with 3 wpc

(*p<0.05*). The values of NV piglets were higher across the mentioned cytokines at 3 wpc compared to those of the V animals, showing significance for TNF-α (*p<0.05*) and a trend for IFN-α and IFN-γ (*p<0.1*) (*Figure 2.6A-D*).

IL-4 followed similar pattern as seen with the previous cytokines for V (ascending significantly from vaccination to 5 wpv, *p<0.05*) and NV (with an ascending dynamic from vaccination to 3 wpc -*p<0.05*-, and being also higher than V at that moment, *p<0.05*) piglets (*Figure 2.6E*). However, the IL-10 amount remained mostly stable in both V and NV groups from vaccination to 3 wpc, only being higher for V compared to NV at 5 wpv (*p<0.1*) (*Figure 2.6F*).

About the remaining cytokines, both V and NV animals showed significant increases in IL-1β from vaccination to 5 wpv and 3 wpc, with V pigs displaying higher concentrations at 5 wpv and 2-3 wpc (*p<0.05*) (*Figure 2.6G*). IL-6 increased significantly in V animals at 5 wpv (*p<0.05*) before declining, whereas NV pigs showed a trend towards an increase by 5 wpv, becoming significantly higher than V animal levels at 3 wpc (*p<0.05*) (*Figure 2.6H*).

Finally, IL-8 results could not be interpreted as these values were out of range for most of the samples.

Table 2.1. Comparison of the mean values of body weight (BW) and average daily weight gain (ADWG) at different time-points between V and NV and between the 4 subgroups. Values include standard deviation and the coefficient of variation in percentage (in brackets). Different superscript letters indicate statistically significant differences between groups for each time-point ($p<0.05$).

		BW (Kg) $\bar{X}$ ± SD (CV%)			ADWG (Kg) $\bar{X}$ ± SD (CV%)		
Group		**Vaccination**	**Challenge (5 wpv)**	**Necropsy (3 wpc)**	**(Vaccination – challenge)**	**(Challenge – necropsy)**	**(Vaccination – necropsy)**
V vs NV	**V**	5.72 ± 0.96 (16.8%)	23.80 ± 4.42 (18.6%)	42.70 ± 6.39 (15.0%)	0.463 ± 0.096 (20.7%)	0.840 ± 0.110 (13.0%)	0.602 ± 0.092 (15.3%)
	NV	5.58 ± 1.07 (19.1%)	23.90 ± 3.80 (15.9%)	43.00 ± 6.47 (15.0%)	0.469 ± 0.084 (17.9%)	0.851 ± 0.154 (18.1%)	0.609 ± 0.098 (16.1%)
All subgroups	**H-V**	5.91 ± 1.02 (17.3%)	24.81 ± 3.28 (13.2%)	44.45 ± 4.18 (9.4%)	0.485 ± 0.065 (13.4%)	0.873 ± 0.081 [a,b] (9.2%)	0.627 ± 0.653 (8.9%)
	H-NV	5.63 ± 1.08 (19.2%)	24.45 ± 3.73 (15.2%)	45.77 ± 5.49 (12.0%)	0.482 ± 0.090 (18.6%)	0.947 ± 0.116 [a] (12.3%)	0.653 ± 0.088 (13.4%)
	L-V	5.52 ± 0.89 (16.2%)	22.69 ± 5.33 (23.5%)	40.84 ± 7.94 (19.4%)	0.440 ± 0.120 (27.2%)	0.805 ± 0.129 [a,b] (16.0%)	0.575 ± 0.035 (20.4%)
	L-NV	5.55 ± 1.10 (19.8%)	23.46 ± 3.96 (16.9%)	40.96 ± 6.58 (16.1%)	0.459 ± 0.082 (17.9%)	0.779 ± 0.142 [b] (18.2%)	0.576 ± 0.095 (16.5%)

wpv: weeks post-vaccination; wpc: weeks post-challenge; H: high; L: Low; V: vaccinated; NV: not vaccinated.

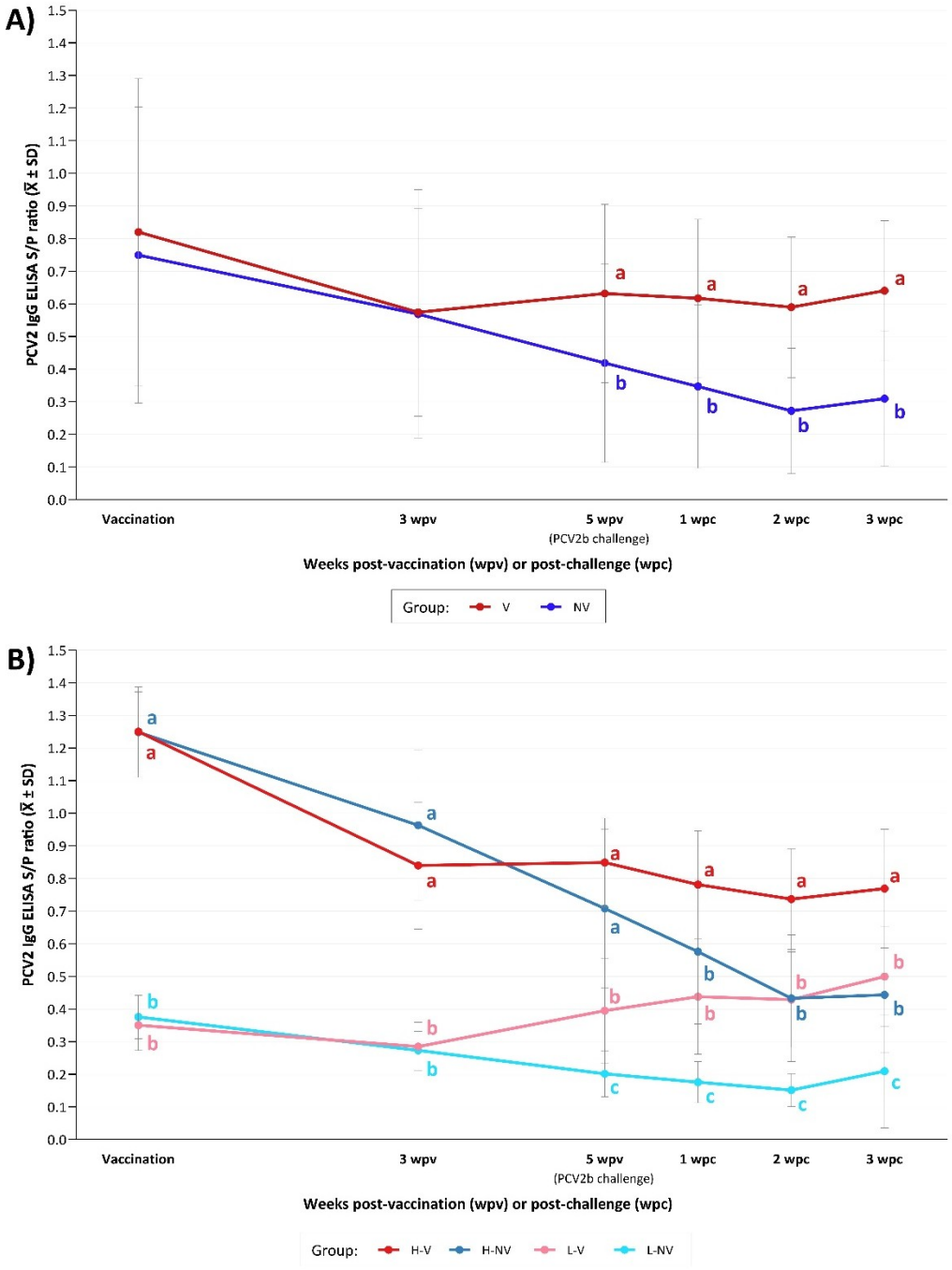


Figure 2.2. Mean (± SD) PCV2 IgG ELISA S/P ratio obtained at different time-points between V vs NV (**A**) and H-V, H-NV, L-V and L-NV (**B**) groups. Different letters in superscript indicate statistically significant differences between groups at each time point ($p<0.05$). H: high; L: low; V: vaccinated; NV: not vaccinated; wpv: weeks post-vaccination; wpc: weeks post-challenge.

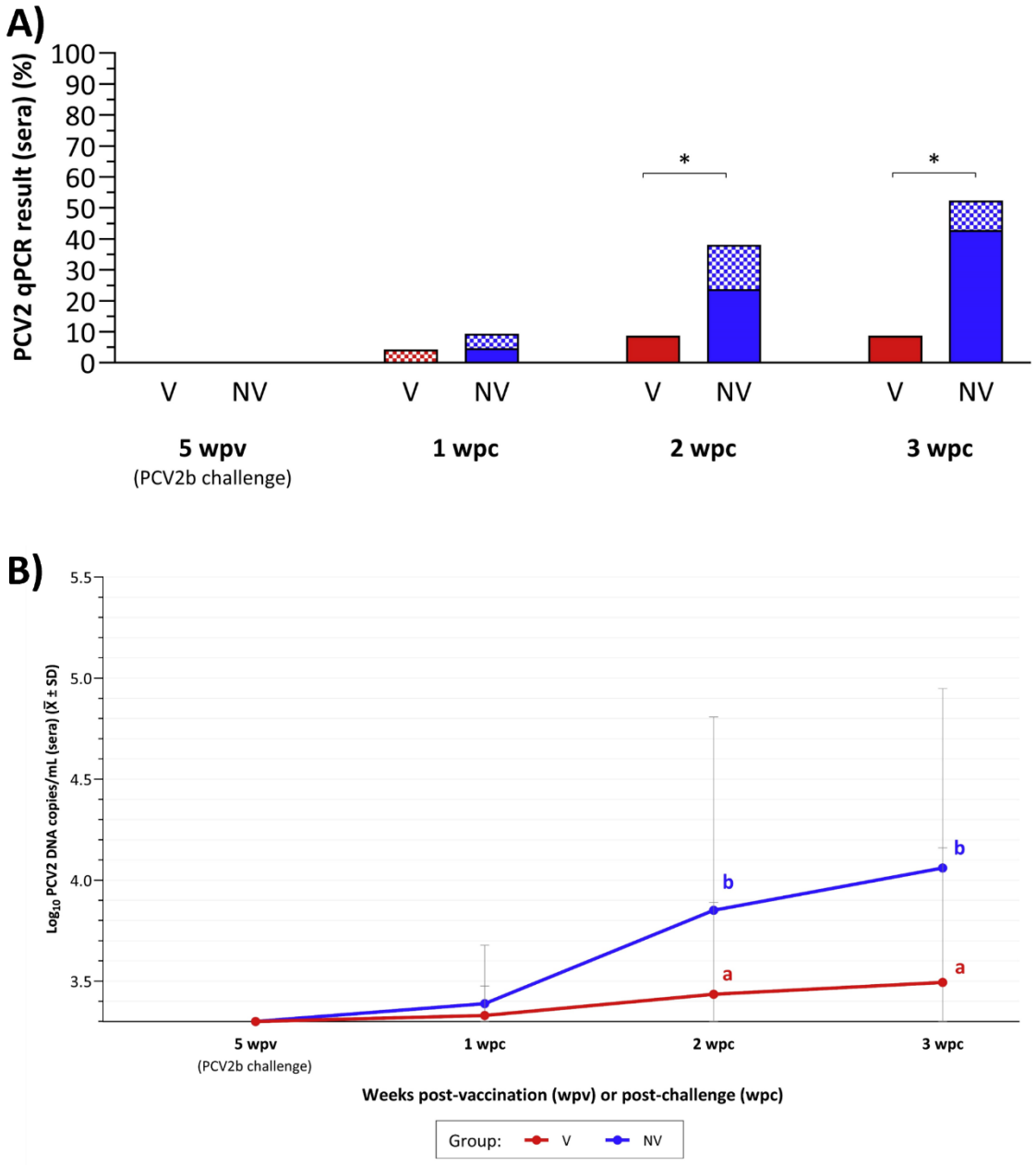
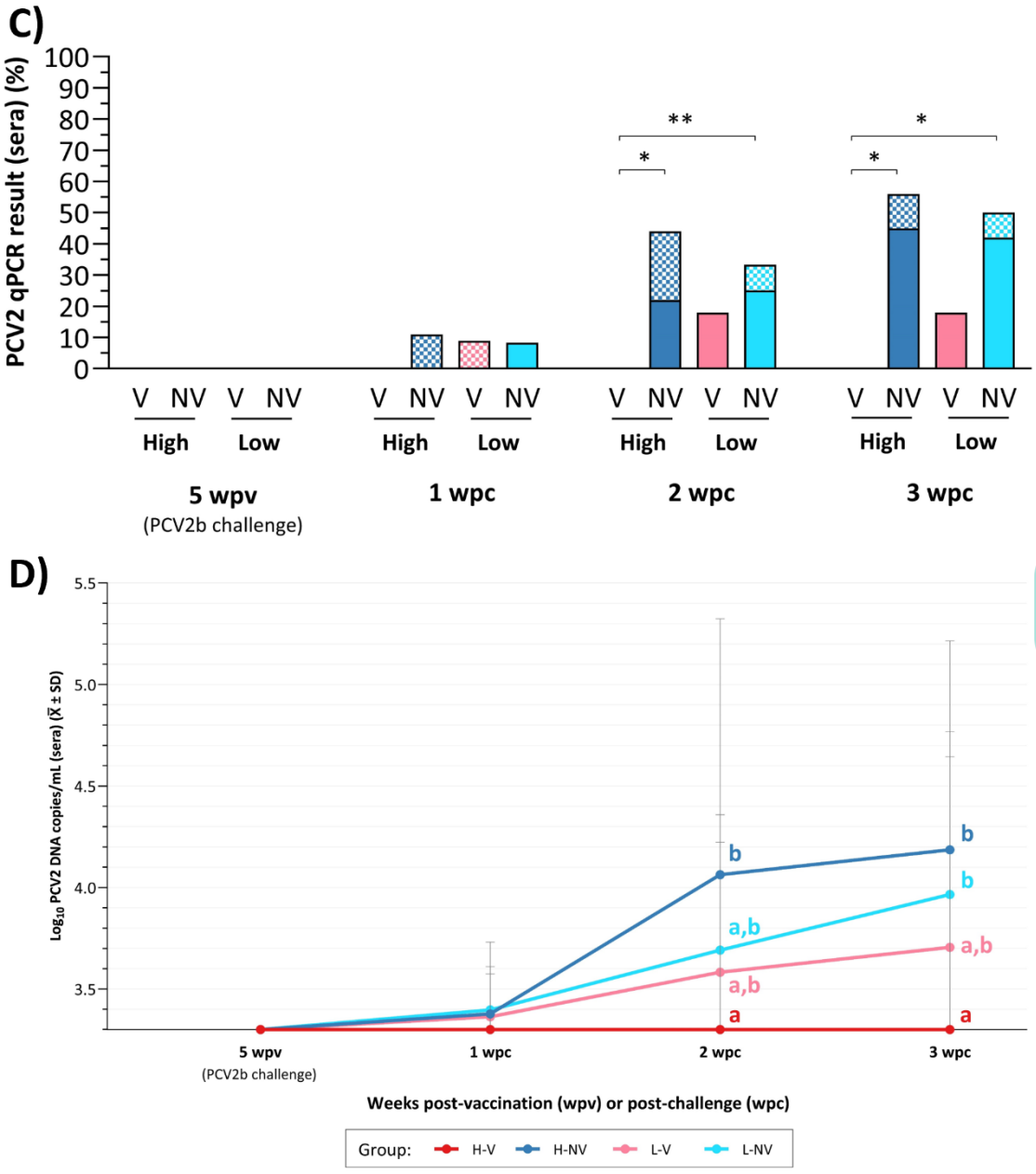


Figure 2.3. PCV2 qPCR results obtained in sera weekly after PCV2b challenge. **A)** Percentage of positive quantifiable (solid-coloured portion) and non-quantifiable below LOQ (checkered portion) samples in animals from V (in red) and NV (in blue) groups; **B)** Mean (± SD) log10 PCV2 DNA copies/mL of serum for V (in red) and NV (in blue) groups; **C)** Percentage of positive quantifiable (solid-coloured portion) and non-quantifiable and below LOQ (checkered portion) samples in animals from H-V (red), H-NV (dark blue), L-V (pink) and L-NV (light blue), and **D)** Mean (± SD) log_{10}…



…PCV2 DNA copies/mL of serum for H-V (red), H-NV (dark blue), L-V (pink) and L-NV (light blue) groups. In figures A and C, * indicates statistically significant differences between groups at each time-point (*p<0.05*) and ** indicate a trend towards statistical significance (*p<0.1*). In figures B and D, different superscript letters indicate statistically significant differences in mean log_{10} PCV2 copies/mL at each time-point between groups (*p<0.05*). PCV2: porcine circovirus 2; wpv: weeks post-vaccination; wpc: weeks post-challenge; H: high; L: low; NV: not vaccinated; V: vaccinated.

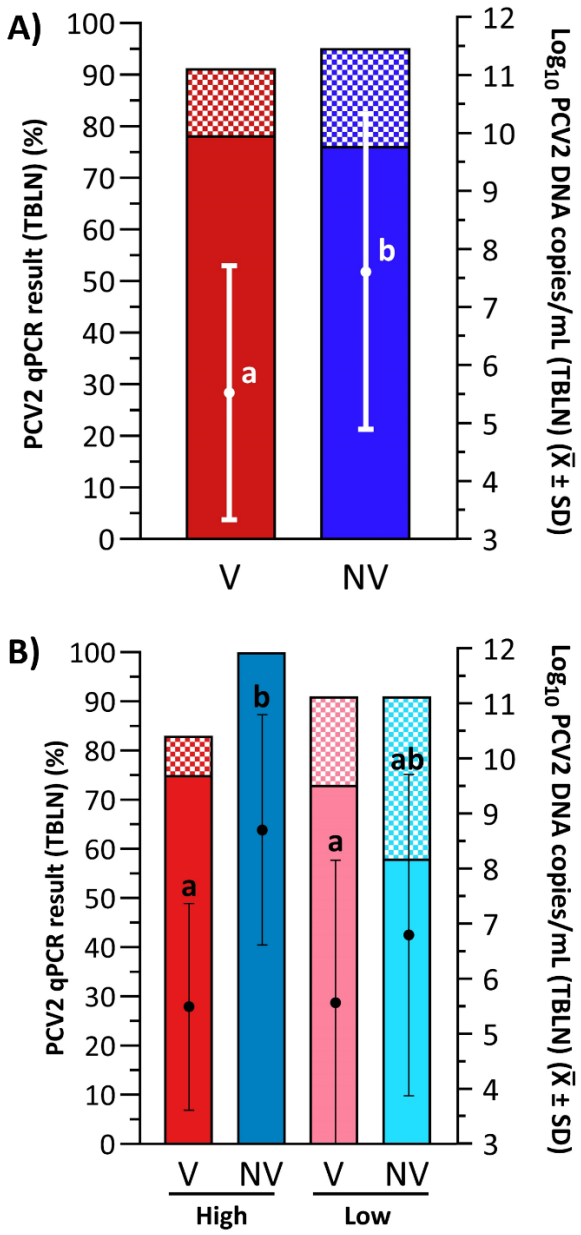


Figure 2.4. PCV2 qPCR results obtained in TBLN, for **A)** V vs NV, and **B)** H-V, H-NV, L-V and L-NV groups. Left Y axes indicate the percentage (in bars) of positive quantifiable (solid-coloured portion and non-quantifiable (checkered portion) samples. Right Y axes refer to the mean $\log_{10}$ PCV2 DNA copies/mL of tissue supernatant (± SD), displayed as dot. Different superscript letters indicate statistically significant differences in mean $\log_{10}$ PCV2 DNA copies/mL of tissue supernatant between groups ($p<0.05$). PCV2: porcine circovirus 2; TBLN: tracheobronchial lymph node; H: high; L: low; V: vaccinated; NV: not vaccinated.

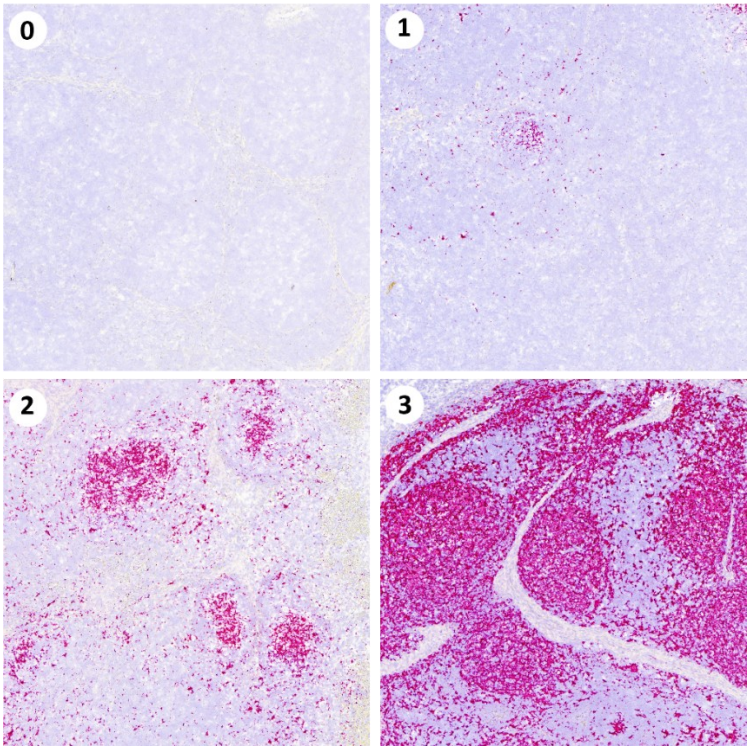


Figure 2.5. Representative images of the PCV2 *in situ* hybridisation (ISH) scoring system (0-3) from TBLN. Each red dot represents a hybridisation signal indicating the presence of PCV2 genome.

Table 2.2. ISH results obtained in TBLN for V vs. NV groups, as well as for all subgroups. The results are expressed as the number (%) of animals that received each score (0-3). Different superscript black letters indicate statistically significant differences between groups for each score ($p<0.05$), while blue letters indicate a trend towards statistical significance ($p<0.1$) between groups for each score.

		ISH (n/total pigs per group, %)			
Score (0-3)		0	1	2	3
V vs NV	V	17/23 [a] (73.9%)	3/23 [a] (13.0%)	3/23 [a] (13.0%)	0/23 [a] (0.0%)
	NV	7/21 [b] (33.3%)	3/21 [a] (14.3%)	7/21 [a] (33.3%)	4/21 [b] (19.0%)
All subgroups	H-V	8/12 [a] (66.7%)	3/12 [a] (25.0%)	1/12 [a] (8.3%)	0/12 [a] (0.0%)
	H-NV	1/9 [b] (11.1%)	2/9 [a] (22.2%)	3/9 [a] (33.3%)	3/9 [b] (33.3%)
	L-V	9/11 [a] (81.8%)	0/11 [a] (0.0%)	2/11 [a] (18.2%)	0/11 [a] (0.0%)
	L-NV	6/12 [a,b] (50.0%)	1/12 [a] (8.3%)	4/12 [a] (33.3%)	1/12 [a,b] (8.3%)

ISH*: in situ* hybridisation; NV: not vaccinated; V: vaccinated; H: high; L: low.

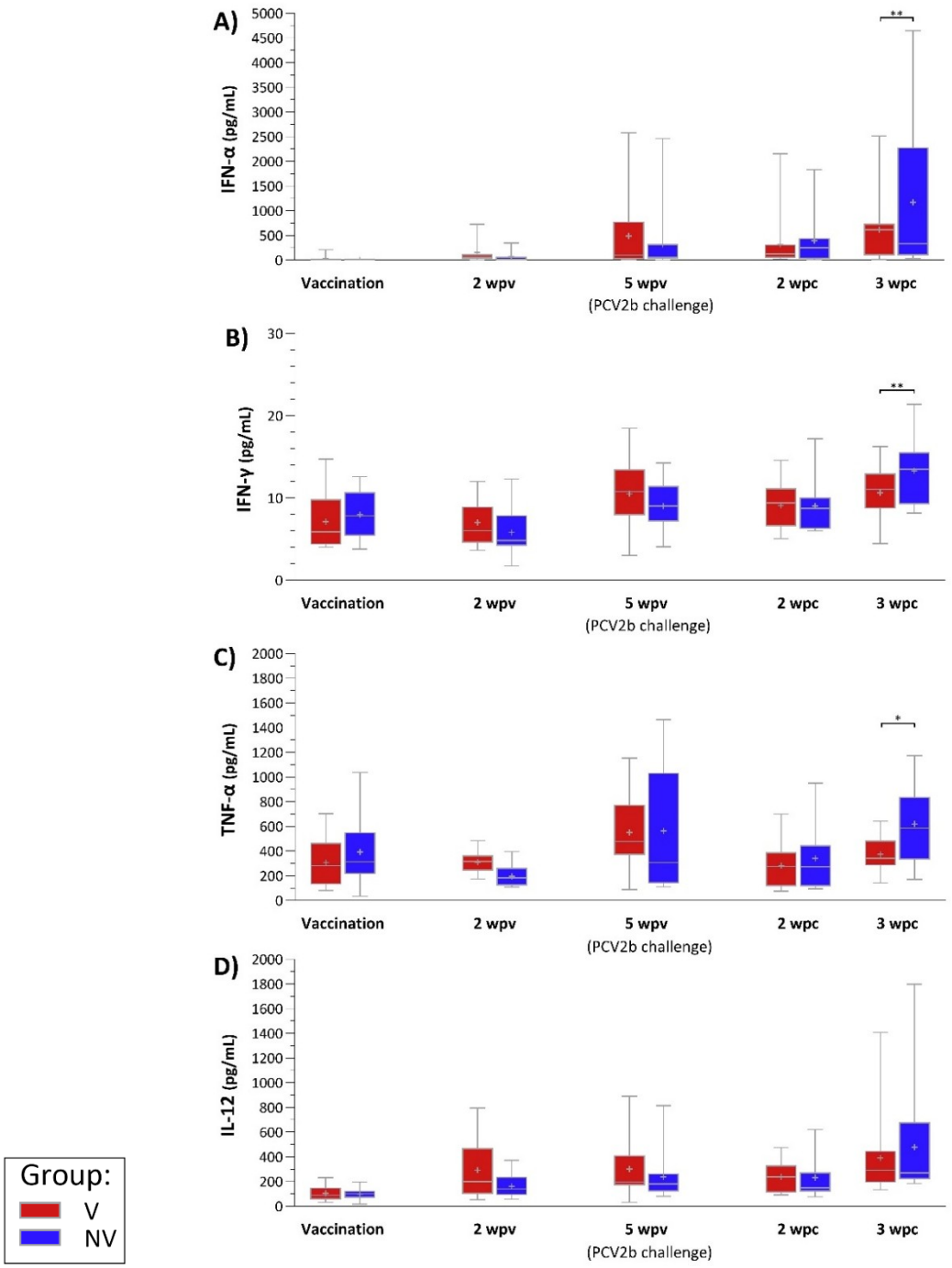
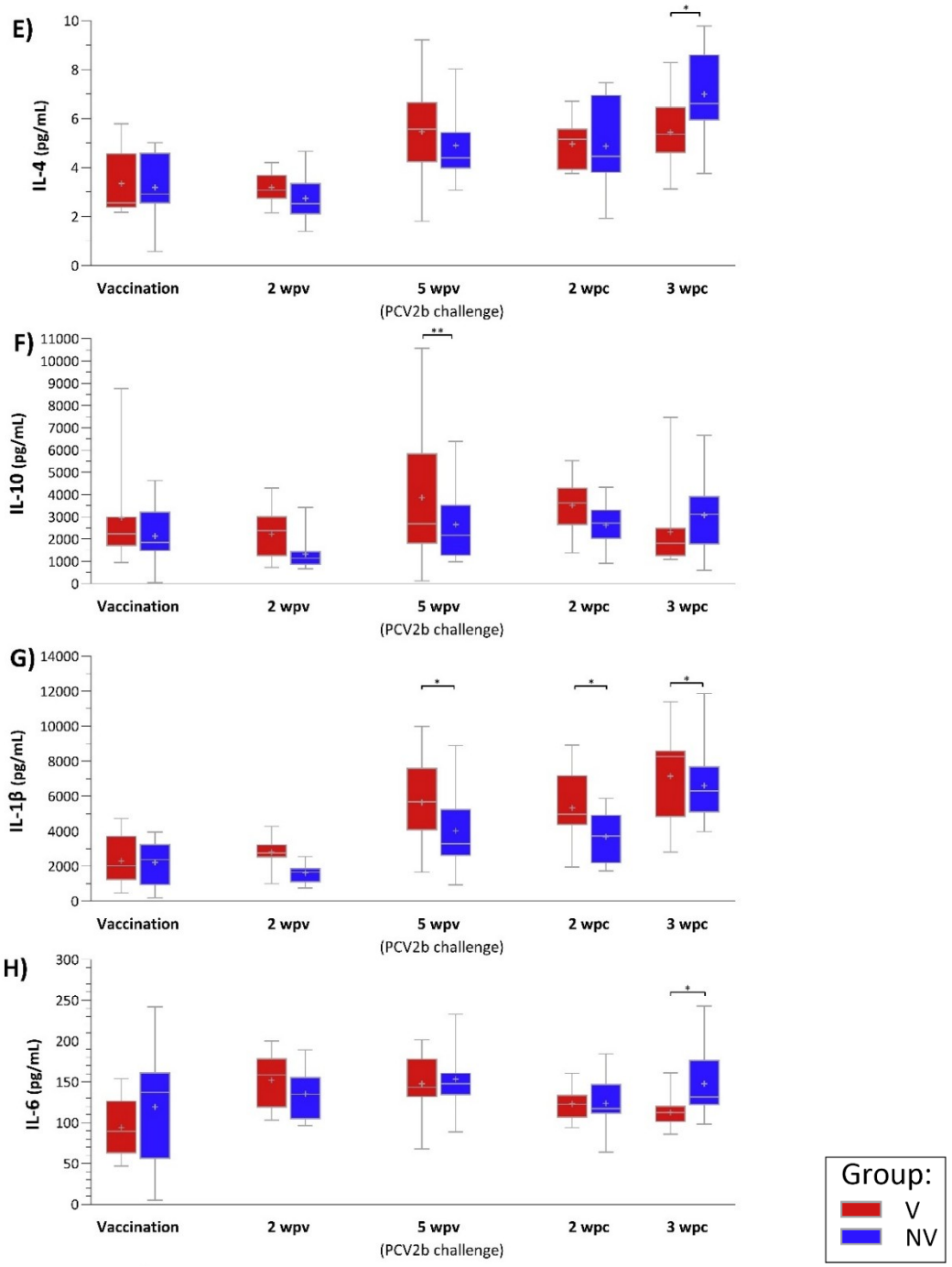


Figure 2.6. Boxplots of median (in line) and mean (cross) of PCV2-specific cytokine secretion (IFN-α, IFN-γ, TNF-α, IL-12, IL-4, IL-10, IL-1β and IL-6) (pg/mL) in PBMC supernatant samples from V and NV groups at different time-points. * indicates statistically significant differences between groups for…



... each time-point (*p<0.05*). ** indicates a trend towards statistical significance (*p<0.1*). IFN: interferon; IL: interleukin; TNF: tumour necrosis factor; PCV2: porcine circovirus 2; NV: not vaccinated; V: vaccinated.

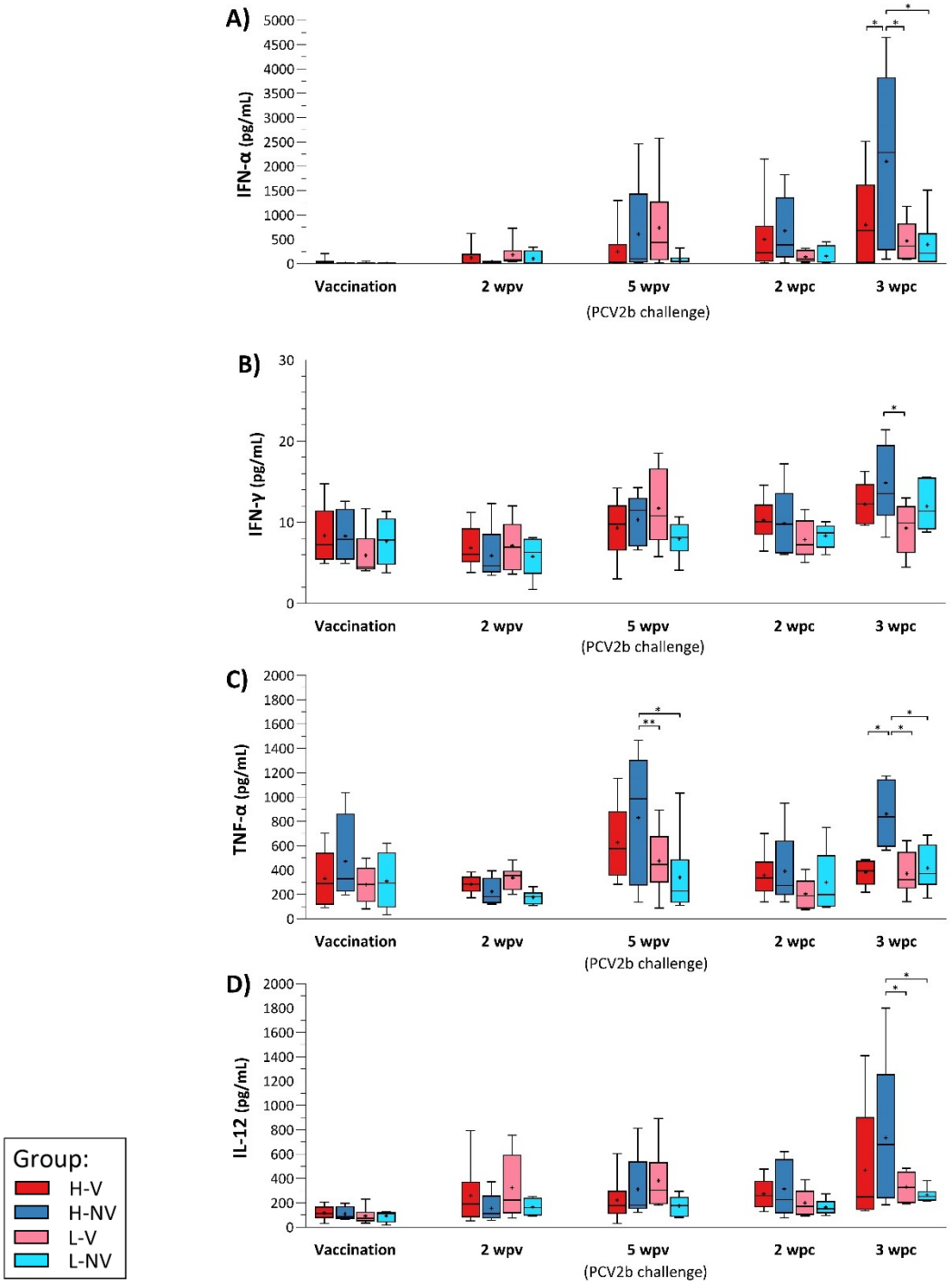
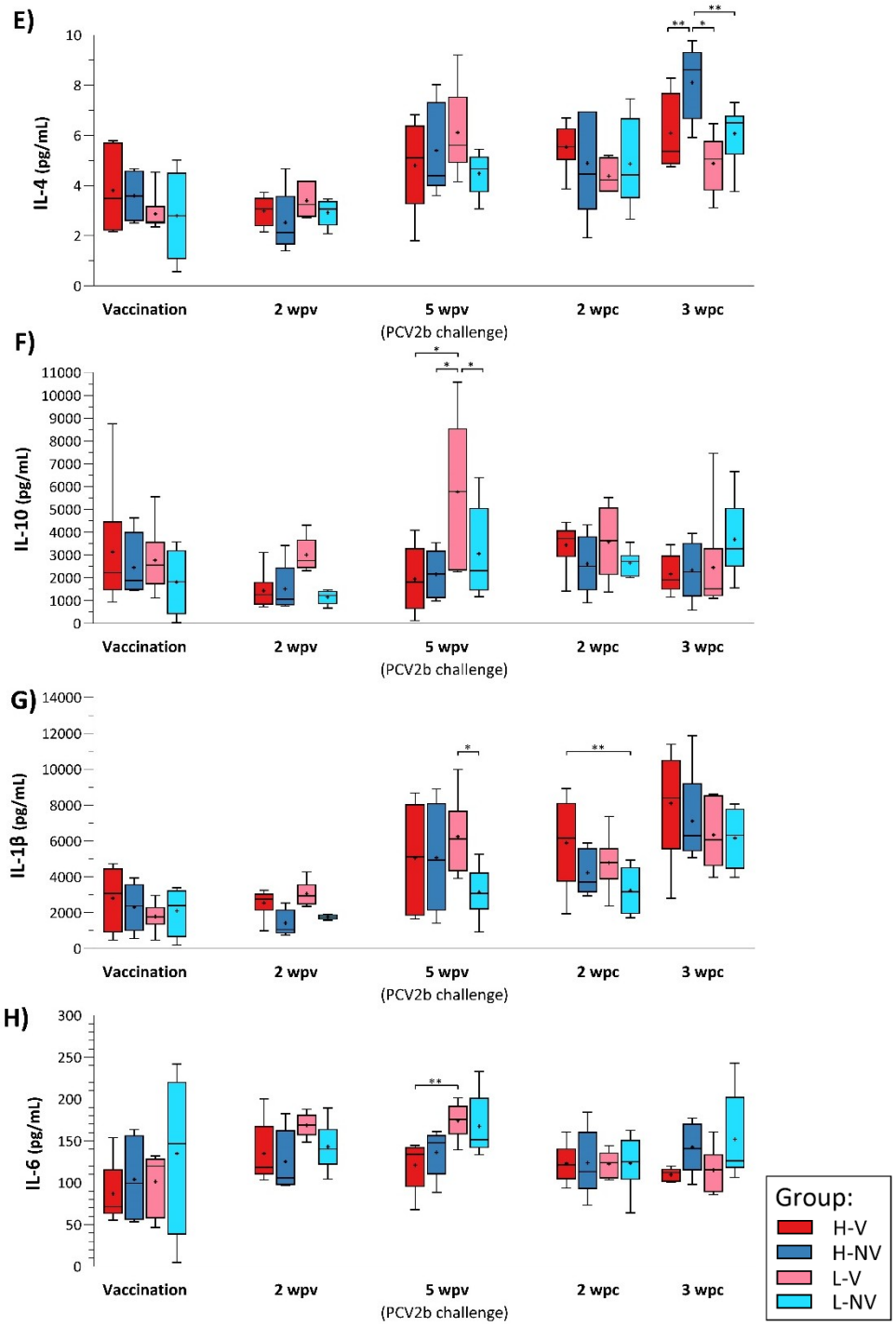


Figure 2.7. Boxplots of median (in line) and mean (cross) of PCV2-specific cytokine secretion (IFN-α, IFN-γ, TNF-α, IL-12, IL-4, IL-10, IL-1β and IL-6) (pg/mL) in PBMC supernatant samples from H-V, H-NV, L-V and L-NV groups at different time-points. * indicates statistically significant differences between…



…groups for each time-point (*p<0.05*). ** indicates a trend towards statistical significance (*p<0.1*). IFN: interferon; IL: interleukin; TNF: tumour necrosis factor; PCV2: porcine circovirus 2; H: high; L: low; NV: not vaccinated; V: vaccinated.

2.3.3. Comparison among H-V, H-NV, L-V and L-NV groups

Body weight and average daily weight gain

Differences in BW were not statistically significant between groups (*Table 2.1*). Animals from H-NV group showed significantly higher ADWG from challenge to necropsy when compared to L-NV counterpart ($p<0.05$) (*Table 2.1*).

Porcine circovirus 2 IgG antibody levels dynamics

H-V animals showed significantly higher values than the other three groups from 1 wpc onwards (*Figure 2.2B*). In fact, L-NV showed the lowest S/P ELISA values, being statistically significant compared to the other three groups from 5 wpv until necropsy ($p<0.05$). There were not statistically significant correlations between PCV2 ELISA S/P ratios at vaccination, and the corresponding Delta values from vaccination to 3 wpv, from vaccination to 5 wpv, neither from 3 to 5 wpv, for any of the groups. However, a trend towards significancy ($p<0.1$) from vaccination to 3 wpv for L-NV (r=-0.53), and from vaccination to 5 wpv for H-V (r=-0.53) group.

Porcine circovirus 2 infection dynamics

None of the animals from the H-V group had a viral load higher than the LOD throughout the study. In contrast, the group H-NV showed a significantly higher percentage of viraemic piglets when compared to the H-V group at 2 and 3 wpc ($p<0.05$) (*Figure 2.3C*). At necropsy (3 wpc), L-NV group had also a significantly higher percentage of viraemic piglets when compared to the H-V one ($p<0.05$). The same statistically significant differences were observed when the viral load was compared (*Figure 2.3D*). In terms of AUC, H-V pigs had statistically lower AUC (9.9 ± 0.0, as the undetermined values were given half of the LOD value) than H-NV (11.2 ± 1.8) ($p<0.05$) and L-NV (10.7 ± 1.2) ($p<0.1$) animals, but without differences with

L-V (10.5 ± 1.1). Notably, despite the lack of differences in positivity or viral load between the L-V and L-NV groups, the number of pigs ever viraemic (at least positive at one time-point) was lower in the L-V group (3/11, 27.3% compared to the L-NV group (6/12, 50%) (*ST2.1*).

The mean PCV2 load in TBLN was also significantly higher for H-NV than for both H-V and L-V groups ($p<0.05$), and no statistically significant differences in percentage of PCV2 positivity in TBLN was observed among groups (*Figure 2.4B*).

Lesion assessment and PCV2 antigen detection in TBLN

Two out of 12 animals from L-NV group (with a score of 1) and one out of 9 from H-NV group (with a score of 2) showed HR. The latter also showed a LD with a score of 1.

Regarding ISH, both vaccinated groups had a higher percentage of animals scored as 0 (H-V with 66.7% and L-V with 81.8%) ($p<0.05$) compared with H-NV (11.1%). The H-NV group also had 33.3% of the animals with score 3, showing a trend towards statistical significance ($p<0.1$) compared to V groups (*Table 2.2*).

Cytokine concentration

The H-NV group exhibited higher amounts of IFN-α, IFN-γ, TNF-α, and IL-12 at 3 wpc compared to the other groups, and was also higher for TNF-α at 5 wpv, with significant differences observed with the other groups ($p<0.05$) and from vaccination ($p<0.05$) (*Figure 2.7A-D*). In contrast, the L-NV group remained mostly stable for the mentioned cytokines, except for IFN-γ, which showed a significant increase from vaccination to 3 wpc ($p<0.05$). About L-V, a significant increase from vaccination to 5 wpv were observed with IFN-γ and IL-12 ($p<0.05$), while the

increase in the H-V group was significative from vaccination to 3 wpc for both cytokines ($p<0.05$).

About IL-4 and IL-10, the L-V group showed a significant increase of both from vaccination to 5 wpv ($p<0.05$) (*Figure 2.7E-F*). Similarly, H-NV pigs exhibited a significant rise in IL-4 from 5 wpv to 3 wpc ($p<0.05$), although all groups also showed significant increases from vaccination to challenge ($p<0.05$). In contrast, the amount of IL-10 remained stable throughout the study for H-V, H-NV and L-NV.

Finally, all groups, except the L-NV ones, showed significant increases in IL-1β from vaccination to 5 wpv and to 3 wpc ($p<0.05$) (*Figure 2.7G*). About IL-6, both H-V and L-V showed significant increases from vaccination to 2 wpv ($p<0.05$), and for L-V it was followed by a significant decrease at 3 wpc ($p<0.05$), while H-NV and L-NV remained mostly stable (*Figure 2.7H*).

2.4. Discussion

The current study aimed to evaluate how different levels of MDA (high and low) against PCV2 influence the efficacy of a new, ready-to-use combined vaccine containing the PCV2d genotype and *M. hyopneumoniae* in piglets that were vaccinated at 3 weeks of age and experimentally infected 5 wpv with a PCV2b strain. Specifically, this work was designed to study such efficacy based on several parameters including ADWG, virology, pathology as well as humoral and cellular immune responses, in a subclinically PCV2 infection scenario.

When assessing the humoral immune response, V group maintained significantly higher S/P ratio levels than NV group from 5 wpv to 3 wpc, results that were also consistent with the significantly lower PCV2 load in serum and in TBLN, a reduced AUC, as well as a lower percentage of qPCR positive animals in serum and absence

of animals scored 3 by ISH. Those results align with the efficacy seen in previous studies with other PCV2 combined ready-to-mix and/or ready-to-use vaccines (Bandrick et al., 2022; Pleguezuelos et al., 2022) and also with a recent study evaluating the same vaccine (Pálmai et al., 2025). The lack of differences in BW or ADWG among V and NV groups is probably not surprising considering that the number of animals tested under experimental conditions was limited, the infection outcome was subclinical, and the time evaluated (3 weeks from challenge to necropsy) was short (Opriessnig et al., 2008a, 2014). Noteworthy, ADWG numerical differences were observed among groups, and those were significant between H-NV and L-NV pigs. Such result reinforces the significant contribution of maternally derived immunity in the protective efficacy against PCV2 infection (McKeown et al., 2005). However, pathological and virological results among these two groups were not significantly different.

The present study demonstrated genotype cross-protection effect, since the challenge was conducted with PCV2b, and the tested vaccine contains PCV2d. This result is coherent with findings for other PCV2 vaccines with varying formulations, as summarized in a recent review (Guo et al., 2022), and further supported by a newly published study performed with the same vaccine (Krejci et al., 2025).

In the present study, PCV2 infection was monitored longitudinally in serum by qPCR, finding viral loads below LOQ at 1 to 3 wpc in several pigs. One possible explanation for this limited detection rate could be the lower viral dose used for inoculation (3 mL of $10^{4,73}$ $TCID_{50}$/mL) compared to previous studies in which a similar PCV2b isolates were used (Fort et al., 2012; Sibila et al., 2020). Another potential factor that influences the outcome of PCV2 infection is host genetics, i.e., Landrace pigs were found more susceptible than Large White or Duroc pigs (Opriessnig et al., 2006; Zhang et al., 2018; Ouyang and Nauwynck, 2024). Supporting this hypothesis,

another study (Li et al., 2016) also found genetic differences to be significant in determining susceptibility to PCV2 infection among pig breeds, further demonstrating this relationship (Maity et al., 2023). In contrast, TBLN tested positive for PCV2 by qPCR in almost all animals. The selection of the TBLN for viral load assessment stems from its role as a lymphoid tissue involved in PCV2 pathogenesis (Saril et al., 2021; Segalés and Sibila, 2022) and usually display the highest viral loads compared to other lymph nodes under experimental conditions (*Chapter 3*). Moreover, previous studies have shown a 2-3 $\log_{10}$ difference between viral loads in blood and lymph nodes (Segalés and Sibila, 2022). In fact, in the present study NV groups showed significantly higher viral loads in the TBLN compared to V animals, with some reaching 10–11 Log_{10} PCV2 DNA copies/mL.

Additionally, none of the animals displayed clinical signs compatible with PCV2-systemic disease, fact that correlates with serum viral loads below the classical threshold of 10^7 PCV2 copies/mL described in several works (Brunborg et al., 2004; Olvera et al., 2004), and with the limited number of animals with histopathological lesions (only three animals and with mild to moderate lesions). On the contrary, a high percentage of infected animals from NV groups showed abundant PCV2 antigen (ISH score 3) in TBLN by RNAscope technology. This discrepancy reflects the high sensitivity of this latter technique when compared with other techniques such as IHC or the traditional ISH (Cobos et al., 2024). These less sensitive techniques are typically able to confirm infection and disease in animals with moderate to high viral loads (Kim et al., 2009b; Sarli et al., 2021).

Maternally derived antibodies are known to provide piglet protection against PCV2 infection early in life; however, high or very high levels of antibodies at the moment of vaccination may interfere with the humoral immune response elicited by the vaccine and the ADWG (Feng et al., 2016). Notably, declining PCV2 antibody level

trends were observed across all groups from vaccination to 3 wpv, likely due to the natural waning of MDA. The phenomenon has been shown to occur between 4 and 12 weeks of age and has been reported in several studies examining PCV2 vaccination (Fachinger et al., 2008; Opriessnig et al., 2008a; Martelli et al., 2016; Kiss et al., 2021).

The L-V group showed seroconversion from 3 to 5 wpv, a response that was less pronounced in the H-V group, as expected, as high MDA levels jeopardize the vaccine-induced seroconversion in piglets (Poulsen Nautrup et al., 2021). However, a mild seroconversion after vaccination in the presence of MDA should not be considered as a negative indicator for the vaccination effectiveness (Tassis et al., 2017; Figueras-Gourgues et al., 2019).

In the present study, the H-V group exhibited the lowest viral loads, with all animals remaining below the LOQ across all time points in both sera and TBLN. This group also had a reduced viral AUC and no ISH score 3 by in TBLN, showing no significant differences compared to the L-V group. In contrast, the H-NV group showed the highest viral loads and AUC values, together with higher ISH scores (6 out of 9 animals had scores of 2 and 3), followed by the L-NV group. These findings highlight the importance of vaccination in reducing PCV2 viral replication (Segalés and Sibila, 2022; Maity et al., 2023) and indicate that vaccination efficacy was not jeopardized despite high values of MDA, as seen also in previous studies (Poulsen Nautrup et al., 2021; Kiss et al., 2022). In this regard, the reduction in viremia and tissue viral load observed in vaccinated animals supports the concept of virological protection, a term commonly used in vaccine efficacy studies to describe partial protection based on virological outcomes rather than on the complete prevention of infection (Kerkhofs et al., 2003; Roederer et al., 2014; Bonckaert et al., 2016; Tan et al., 2022).

Both cellular and humoral responses are considered of great importance for the PCV2 control, as PCV2 antibodies are not capable to confer full protection against this viral infection (Meerts et al., 2006; Trible and Rowland, 2012; Shi et al., 2021). Indeed, the level of neutralizing antibodies has been correlated with viral replication, lesion severity and disease development in PCV2-infected pigs (Meerts et al., 2006; Song et al., 2007). In addition, MDA have been shown to protect piglets by reducing the probability of viraemia (McKeown et al., 2005; Ostanello et al., 2005; Saha et al., 2014). Regarding cellular immunity, T helper (Th) 1 cytokines promote the ability to fight intracellular pathogens such as PCV2, and Th2 cytokines play a role in neutralizing viruses, but being more associated with B-cell proliferation and specific antibody formation (Raymond and Wilkie, 2004; Moss et al., 2004; Darwich and Mateu, 2012). Here, the cellular responses were studied using a multiplex immunoassay, which allowed simultaneous quantification of cytokines in PBMCs and allowed the assessment of IFN-α, IFN-γ, TNF-α, and IL-12, IL-4, IL-10, IL-1β and IL-6.

In terms of cytokine response, the NV group (especially H-NV) exhibited increases in IFN-γ, IFN-α, and TNF-α post-challenge, probably suggesting an inflammatory response in the absence of previous vaccination. In contrast, V animals displayed relatively stable or moderate increase of these cytokines after the PCV2b challenge, likely due to lower levels of viral replication. Such a response aligns with previous findings where vaccination triggered an early, strong IFN-γ-secreting cell response, while non-vaccinated animals exhibited higher IFN-γ amounts later (Ferrari et al., 2014). These results may indicate that vaccination fosters long-lasting immunity through memory T cells and IFN-γ-secreting cells, potentially preventing the onset of infection. The observed increase in IL-12 could further support IFN-γ production in NV animals by 3 wpc, reflecting a heightened immune activation following

infection (Muraille et al., 2014; Du et al., 2018). This amplified response of IL-12 was predominantly seen in H-NV animals, which also showed concurrent elevation of TNF-α alongside IFN-γ. Moreover, PCV2 infection *in vivo* has been shown to induce IFN-α secretion, establishing an antiviral state that may enhance the defence mechanism (Kekarainen and Segalés, 2015). In our study, the NV group showed higher amounts of IFN-α by 3 wpc, probably promoted by higher viral replication compared to V group.

Following a similar pattern, V pigs showed a significant increase in IL-4 by 5 wpv. This cytokine has been reported to be upregulated in PBMCs from vaccinated pigs (Quereda et al., 2013), potentially indicating that vaccination primed an effective humoral response. In contrast, NV ones exhibited IL-4 rise only by 3 wpc. This response, especially evident in H-NV pigs, may reflect a compensatory immune mechanism aimed at countering viral load post-infection, as this group exhibited the highest viral loads. On the other hand, IL-10 production was notably higher in the L-V group at 5 wpv. This cytokine is often linked to immune dysregulation in animals with PCV2-systemic disease (Kekarainen and Segalés, 2015; Du et al., 2016).

Although IL-1β had significant higher concentrations in the V group from 5 wpv onwards, when split for the subgroups, differences were not conclusive. Regarding IL-6, a well-known pro-inflammatory cytokine, it was significantly increased in NV animals at 3 wpc. This finding is in line with the higher viral load observed in this group and agrees with previous studies reporting that PCV2 infection can modulate IL-6 expression, with increased levels linked to enhanced inflammatory responses depending on infection stage (Zhu et al., 2016; Féher et al., 2023). However, when analysing the four subgroups of pigs, the differences were no longer significant, which can be explained by the smaller sample size per group and the consequent reduction in statistical power.

2.5. Supplementary material

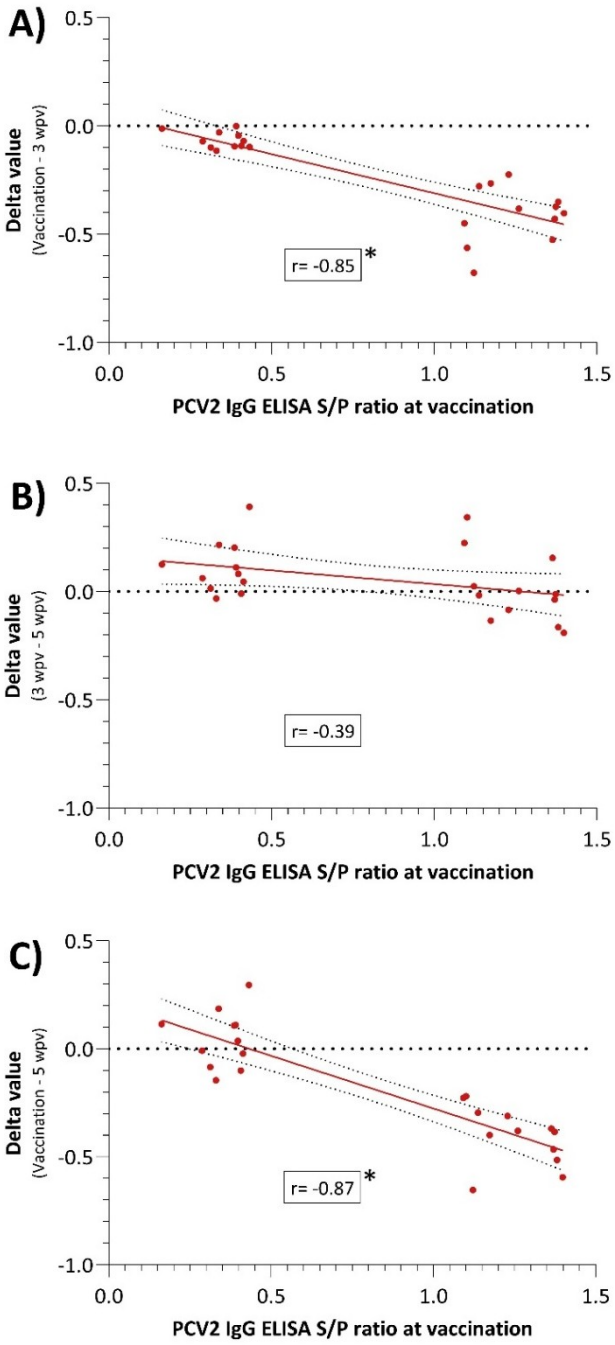
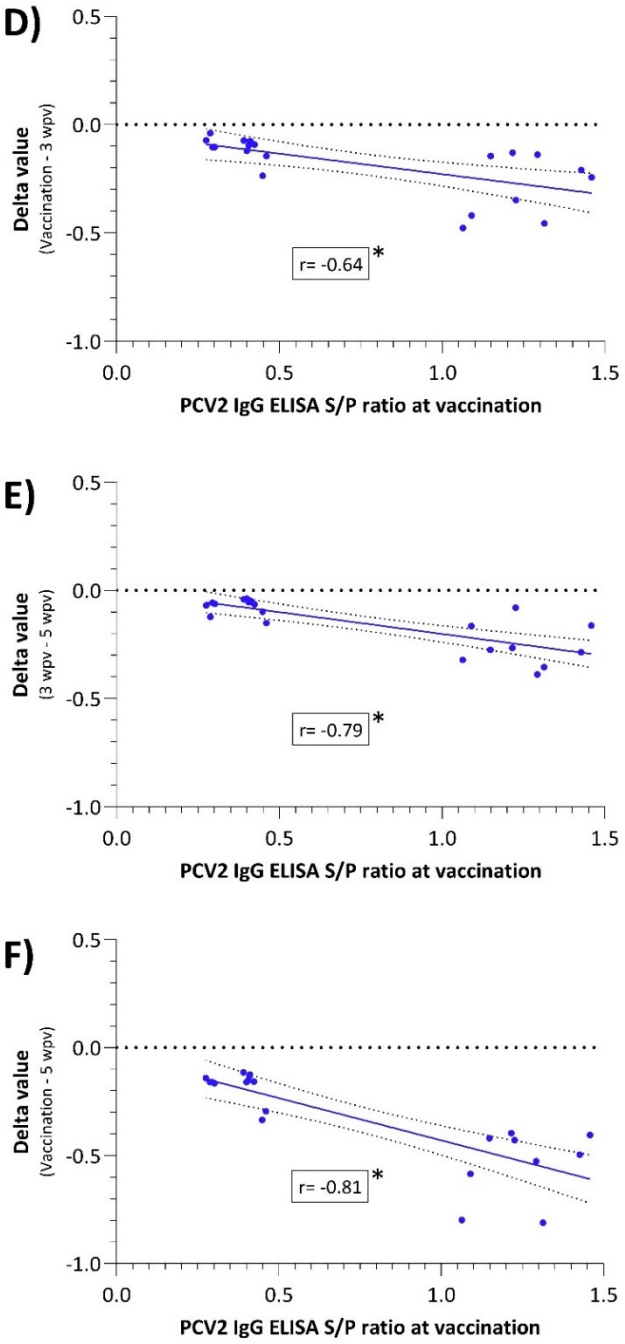
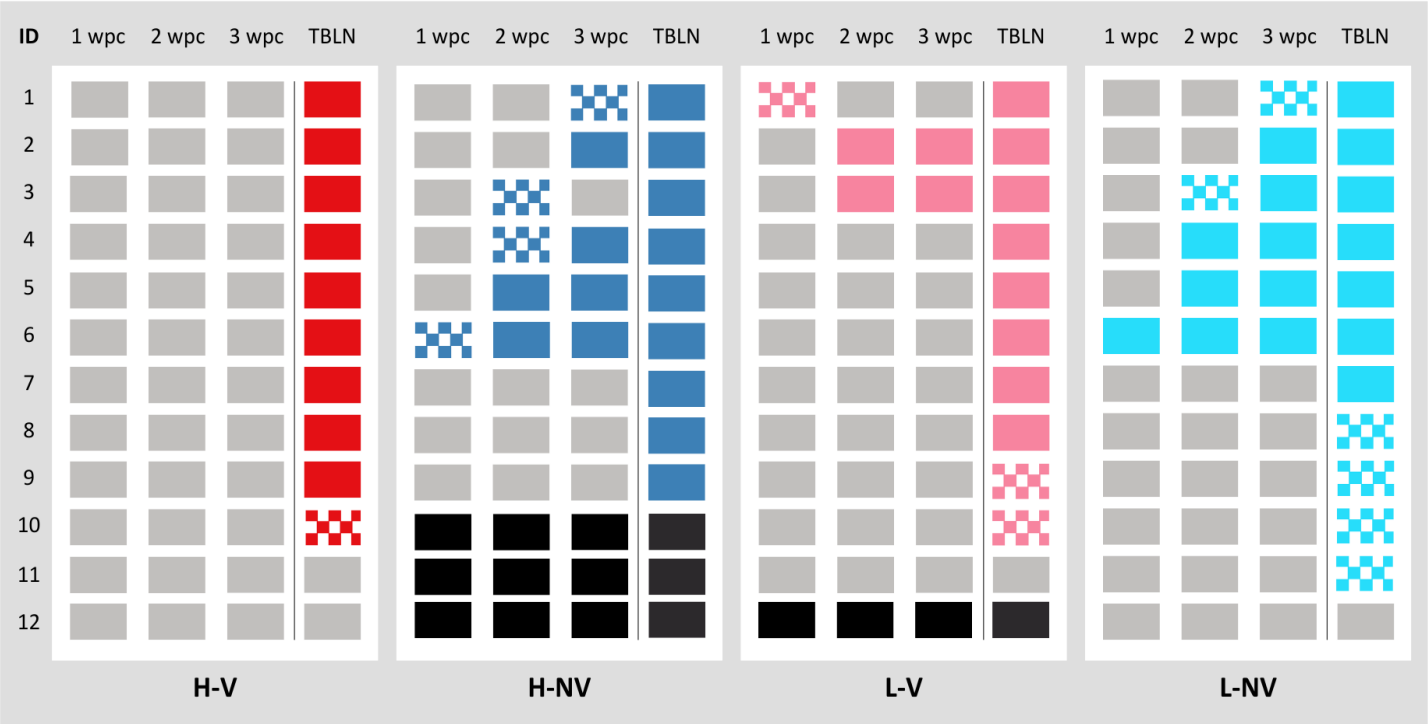


Figure SF2.1. Pearson correlation coefficient between vaccination PCV2 antibody levels and the Delta value from vaccination to 3 wpv (A and D), 3 to 5 wpv (B and E), vaccination to 5 wpv (challenge)…



… (C and F), in V in red (A, B and C), and NV in blue (D, E and F). * indicates a statistically significant correlation (*p<0.05*). wpv: weeks post-vaccination; NV: not vaccinated; V: vaccinated.

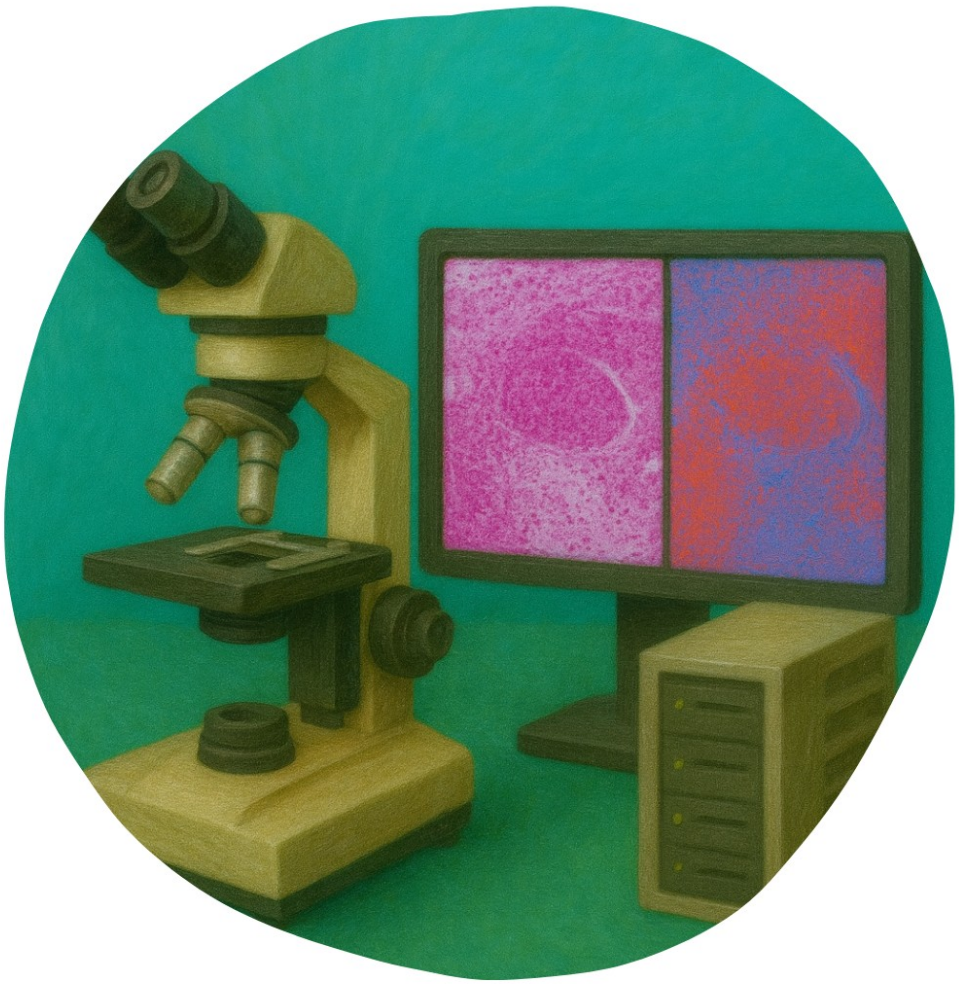
Table ST2.1. Distribution of PCV2 qPCR results for individual animals in each experimental group (H-V, H-NV, L-V, L-NV) at 1 to 3 wpc, and in serum and in tissue supernatant from TBLN. Each row represents an individual animal's qPCR result, with columns indicating time points and TBLN results. The results are interpreted as follows: positive quantifiable (solid coloured), below LOQ (checkered) and below LOD (white). Black boxes indicate missing data of animals that died during the study.



H: high; L: low; V: vaccinated; NV: not vaccinated; POS: positive; LOQ: limit of quantification; LOD: limit of detection; TBLN: tracheobronchial lymph node; wpc: weeks post-challenge.

Chapter 3

Automated pixel-based quantification of PCV2 genome in formalin-fixed, paraffin-embedded tissues using in situ hybridisation



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3.1. Introduction

Detection of PCV2 within tissues is commonly performed using IHC and conventional *in situ* hybridisation (C-ISH) to identify PCV2 antigens and genome, respectively (McNeilly et al., 1999; Rosell et al., 1999). Recently, RNAscope technology has been used for research purposes, offering higher sensitivity by detecting individual copies of single-stranded nucleic acids as distinct red dots in tissue sections (Deleage et al., 2016). Additionally, digital pathology tools such as QuPath (Bankhead et al., 2017) have improved the accuracy and reproducibility of viral quantification by enabling automated image analysis of IHC-stained sections (Horváth et al., 2025).

The present study aimed to evaluate a newly developed automated pixel classifier specifically designed for lymphoid tissue samples from experimentally infected animals using PCV2 ISH RNAscope technology, by correlating its results with visual scoring. Furthermore, the correlation between both visual and digital scores and the PCV2 load in tissue, quantified by qPCR, was also analysed.

3.2. Materials and methods

3.2.1 Experimental study design and sample collection

A total of 66 8-week-old pigs were included in the study. Forty-four animals corresponded to those described in *Chapter 2*, while samples from an additional 22 pigs from an independent study were also included. These animals had also been intranasally challenged with the same PCV2b isolate strain Sp-6-11-49-16 and euthanised 3 weeks post-challenge. From each animal, one sample of tonsils (TO) and TBLN, mesenteric (MSLN), and inguinal superficial (ISLN) lymph nodes was collected (being a total of 264 samples). All tissue samples were fixed by immersion

in 10% buffered formalin, dehydrated, and embedded in paraffin blocks, with all four tissue types from each animal contained within the same block. Fresh samples were also taken separately from each lymph node and stored at −80°C for subsequent PCV2 load investigations by qPCR, selecting the lymph node with the most extensive ISH labelling.

3.2.2 Histopathology and *in situ* hybridisation

From each paraffin block containing the four lymphoid tissues, 4-µm-thick sections were cut, stained with hematoxylin–eosin (HE), and used for examining the presence of lesions indicative of PCV2 infection, such as LD and HR. Another section of each block was prepared for detection of the PCV2 genome using ISH RNAscope, following the procedures described in *Chapter 2*. Moreover, digitalisation of the ISH sections was performed using the MoticEasyScan One® system (Motic, Hong Kong, China). The amount of PCV2-labelled area was analysed using QuPath 0.5.1. software (Bankhead et al., 2017). A pixel classifier was trained using QuPath to quantify the labelled area within tissues using a random tree classifier and very high resolution (0.26 µm/px). The trained classifier was then used to measure labelling in all lymph nodes and TO after manually delimiting the lymphoid tissue and excluding the adjacent tissue. The labelled area was recorded as a percentage of the area (labelled area/total area). Finally, all sections analysed by the pixel classifier were manually reviewed to identify potential preparation artifacts. If artefacts were detected, the sample was reanalysed with the appropriate filtering adjustments.

3.2.3 DNA extraction and detection of PCV2 by qPCR

DNA extraction from TBLN, PCV2 quantification by qPCR, and data handling (LOD/LOQ definition, categorisation, and value assignment for statistical analysis) were performed as described previously in *Chapter 2*.

3.2.4 Statistical analyses

The statistical analyses were performed using GraphPad (La Jolla, CA, USA) and R Studio (Boston, MA, USA). The normal distribution of the studied variables (histopathology LD and HR results, visual score, digital score, and PCV2 $\log_{10}$ load in tissue supernatant) was assessed using the Shapiro–Wilk test, and correlations between variables were evaluated using Spearman's rank correlation test. The percentage of each tissue classified within each visual and digital score category was compared using Fisher's exact test.

3.3. Results

None of the animals exhibited clinical signs compatible with PCV2-SD during the study, and no significant findings were observed at necropsy. Histopathological analysis revealed that only 7.6% of animals (5/66) had LD and/or HR lesions. Two of them had an HR score of 1 in the TBLN, while another two pigs showed the same score of 1 in 3 different lymph nodes (TBLN, MSLN, and ISLN). The fifth animal had a more extensive involvement, with an HR score of 2 and an LD score of 1 in the TBLN and ISLN, as well as an HR score of 1 in the MSLN and TO.

The visual scoring system ISH identified PCV2 labelling in 34.8% (23/66) of TO, 42.4% of TBLN (28/66), 33.3% of MSLN (22/66), and 28.8% of ISLN (19/66) samples (*Table 3.1*). Based on these findings, the following thresholds for the percentage of labelled area (digitally quantified) corresponding to the visual scores were established as follows: ≤0.0025% (score 0), 0.0026–1.0% (score 1), 1.01–5.0% (score 2), and >5.0% (score 3). A representative image for each of the score values of PCV2 ISH is provided in *Figure 3.1*.

Table 3.1. Percentage and number of animals per lymphoid tissue (TO, TBLN, MSLN, and ISLN) visual (scored 0-3) and digital (percentage of labelled area) scores obtained from the PCV2 *in situ* hybridisation. Different superscript letters indicate statistically significant differences between lymph nodes for each score value ($p<0.05$).

Lymphoid tissue	Visual score (0-3)				Digital score (%, labelled area)			
	0	1	2	3	≤0.0025%	0.0026 – 1.00%	1.00 – 5.00%	>5.00%
TO	65.2% (43/66)	24.3% (16/66)	6.1% [a] (4/66)	4.5% (3/66)	65.2% (43/66)	25.8% (17/66)	4.5% [a] (3/66)	4.5% (3/66)
TBLN	57.6% (38/66)	15.1% (10/66)	19.7% [b] (13/66)	7.6% (5/66)	57.6% (38/66)	15.1% (10/66)	19.7% [b] (13/66)	7.6% (5/66)
MSLN	66.7% (44/66)	21.2% (14/66)	7.6% [a,b] (5/66)	4.5% (3/66)	68.2% (45/66)	19.7% (13/66)	6.1% [a] (4/66)	6.1% (4/66)
ISLN	71.2% (47/66)	18.2% (12/66)	7.6% [a,b] (5/66)	3.0% (2/66)	69.7% (46/66)	19.7% (13/66)	7.6% [a,b] (5/66)	3.0% (2/66)

TO: tonsil; TBLN: tracheobronchial lymph node; MSLN: mesenteric lymph node; ISLN: inguinal superficial lymph node; PCV2: porcine circovirus 2.

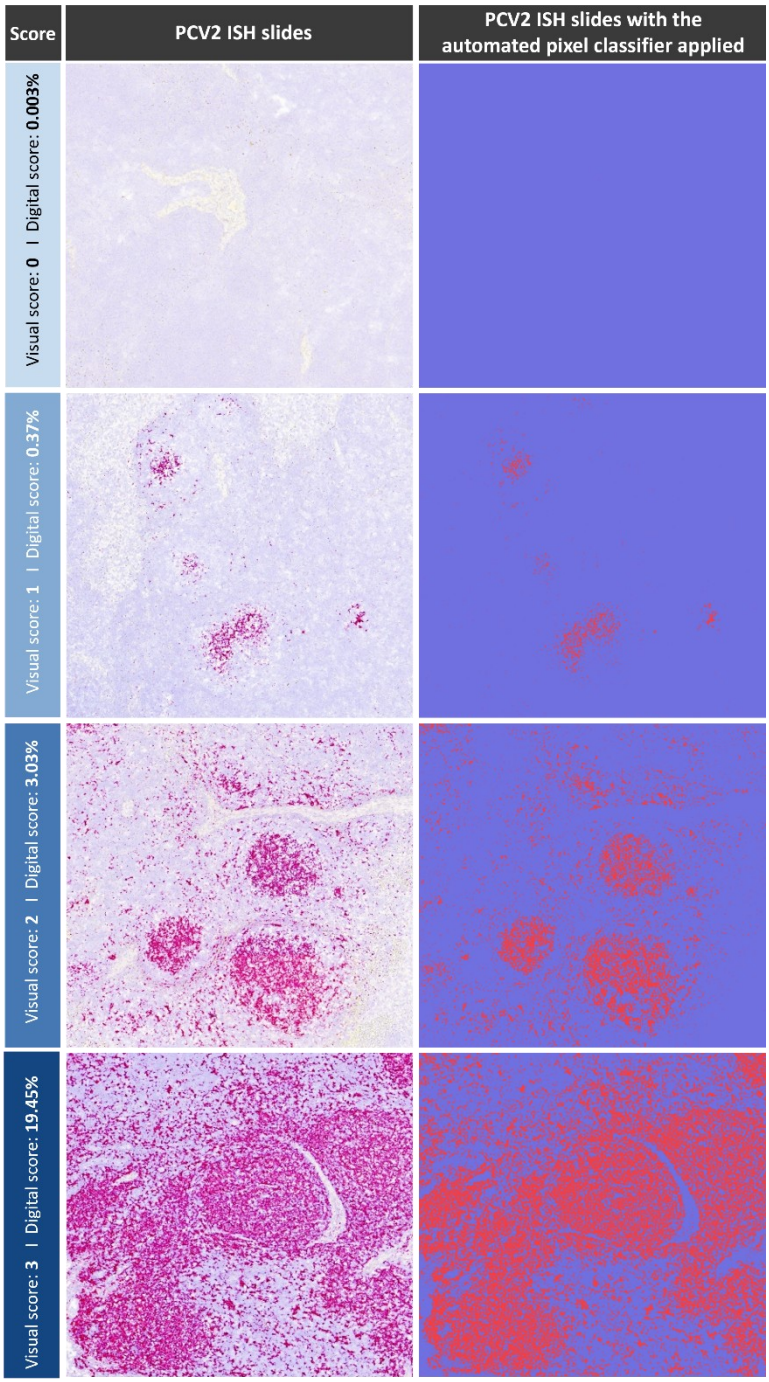


Figure 3.1. Representative images of PCV2 ISH staining (left column) and the corresponding automated pixel classification analysis (right column). Examples of ISH visual scores (0–3) are shown alongside their respective digital scores (% of labelled area). Each pink dot (left) and red dot (right) represents a PCV2 genomic copy. ISH: *in situ* hybridisation; PCV2: porcine circovirus 2.

The automated pixel classifier detected PCV2 in 34.8% (23/66) of TO, 42.4% of TBLN (28/66), 31.8% of MSLN (21/66), and 30.3% of ISLN (20/66) samples (*Table 3.1*). The comparison between the digital and visual PCV2 ISH scores across all tissues revealed a strong and statistically significant Spearman's correlation (ρ=0.96) (p<0.05). Conversely, when both scores were correlated with LD and HR lesion scoring obtained through histopathological analysis across all tissues, lower correlations were observed for both digital and visual scores with LD (ρ=0.16) and HR (ρ=0.38), which were statistically significant ($p<0.05$).

When digital and visual scoring were analysed separately for each tissue, the highest correlation was observed with TBLN (ρ=0.98), while the lowest correlation was observed with TO (ρ=0.94), and this difference was statistically significant in all cases ($p<0.05$) (*Figure 3.2*). Moreover, in both scoring systems, TBLN had a significantly higher number of animals with a visual score of 2 in TO ($p<0.05$), as well as in MSLN and TO for a digital score of 1.00–5.00% ($p<0.05$) (*Table 3.1*). Given these results, TBLN was the tissue of choice to be processed by qPCR.

When TBLN viral load was assessed with PCV2 qPCR, 74.2% (49/66) were positive. Out of these 49 qPCR-positive samples, 28 showed positive results for both ISH and qPCR (*Table 3.2*). The remaining 21 samples that were above the LOQ were classified as score 0 by the visual scoring of ISH and showed values between 0.0 and 0.0025% by the ISH digital scoring, and these samples corresponded mostly to the lowest viral loads within the positive range (*ST3.1*). Finally, a Spearman's correlation analysis between PCV2 qPCR results and both ISH visual and digital scoring classification revealed a strong and statistically significant correlation among the three techniques (ρ=0.85) ($p<0.05$).

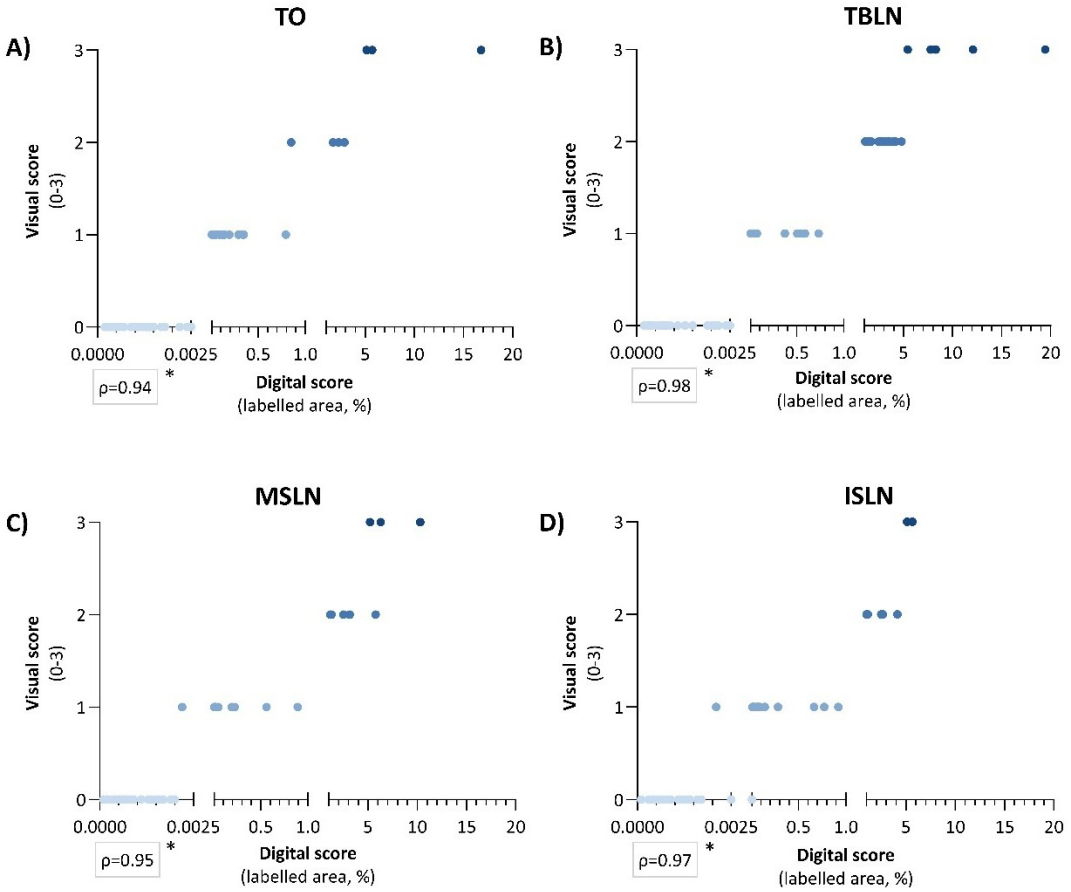


Figure 3.2. Spearman's rank correlation between PCV2 *in situ* hybridisation visual score (0–3) and digital score (labelled area expressed in percentage) for **(A)** tonsil (TO), **(B)** tracheobronchial lymph node (TBLN), **(C)** mesenteric lymph node (MSLN), and **(D)** inguinal superficial lymph node (ISLN). A single asterisk (*) indicates that Spearman's correlation coefficient is statistically significant ($p < 0.05$). The colour of the points corresponds to the visual score, ranging from light blue (0) to dark blue (3), to facilitate the visual interpretation of the graph. PCV2: porcine circovirus 2.

Table 3.2. Distribution of PCV2 qPCR results in TBLN according to both visual and digital ISH scores. The proportion of samples (%, n) falling below the qPCR LOD (3.3 log_{10} PCV2 DNA copies/mL), between LOD and LOQ (3.3 – 4.0 log_{10}) and above the LOQ (>4.0 log_{10}) is displayed. Mean viral load values (log_{10} PCV2 DNA copies/mL) and corresponding ranges (min–max) are provided for each ISH scoring system.

TBLN ISH score			**TBLN PCV2 qPCR**			
			Samples (%, n)			**Viral load** (log_{10} PCV2 DNA copies/mL)
Visual score (0-3)	**Digital score** (labelled area, %)	**Samples** (%, n)	**Below LOD**	**LOD - LOQ**	**Above LOQ**	**Mean ± SD** (Min – Max)
0	≤0.0025%	57.6% (38/66)	10.6% (7/66)	15.2% (10/66)	31.8% (21/66)	4.6 ± 1.2 (3.3 – 7.9)
1	0.0026 – 1.00%	15.1% (10/66)	0.0% (0/66)	0.0% (0/66)	15.1% (10/66)	7.6 ± 1.7 (5.3 – 10.7)
2	1.00 – 5.00%	19.7% (13/66)	0.0% (0/66)	0.0% (0/66)	19.7% (13/66)	9.6 ± 0.9 (8.5 – 11.2)
3	>5.00%	7.6% (5/66)	0.0% (0/66)	0.0% (0/66)	7.6% (5/66)	10.3 ± 0.8 (8.9 – 11.2)
	Total		10.6% (7/66)	15.2% (10/66)	74.2% (49/66)	6.5 ± 2.6 (3.3 – 11.2)

ISH: *in situ* hybridisation; LOD: limit of detection. LOQ: limit of quantification; PCV2: porcine circovirus 2; TBLN: tracheobronchial lymph node.

3.4. Discussion

Since PCV2-SD diagnosis rely on clinical signs, histopathological lymphoid lesions, and detection of the virus in these damaged tissues, the present study developed and evaluated an automated pixel classifier for quantifying PCV2 in lymphoid tissues using ISH RNAscope technology. Moreover, such automated measures were correlated with the traditional visual scoring (Rosell et al., 1999), LD and HR histopathology lesion scoring, and qPCR-based viral load (Olvera et al., 2004) quantification (in TBLN) in PCV2 experimentally infected pigs.

The automated pixel classifier showed a very strong concordance with visual scoring across all lymphoid tissues, suggesting that digital quantification may eventually replace optical evaluation with enhanced objectivity and reproducibility. These findings align with previous research that used digital quantification of IHC results in animals with different degrees of LD and HR (Horváth et al., 2025). Notably, in the present study, ISH RNAscope detected PCV2 loads as low as 5.3 $\log_{10}$ DNA copies/mL of lymph node supernatant as positive (score 1), outperforming C-ISH and IHC techniques, which typically require higher viral loads (two to three more $\log_{10}$ DNA copies) for reliable detection (Brunborg et al., 2004; Olvera et al., 2004). The results are consistent with a recently published study that also reported the superior sensitivity of ISH RNAscope to detect PCV2 when compared with C-ISH and IHC in porcine dermatitis nephropathy syndrome cases (Cobos et al., 2024), a PCV2-AD that typically displays lower viral loads compared to PCV2-SD.

Notably, the histopathological evaluation in this study confirmed that LD and HR were observed only in a subset of animals. Furthermore, no animal exhibited clinical signs compatible with PCV2-SD throughout the study, highlighting the subclinical nature of this experimental infection. These findings reinforce the relevance of using

highly sensitive molecular techniques, such as ISH RNAscope, for detecting PCV2 in subclinical infections, where traditional histopathological approaches may be insufficient. This is demonstrated in the present study by the low Spearman's correlation coefficients observed between histopathological LD and HR lesion scores and both digital and visual ISH scores, as well as by the lower number of animals with PCV2-compatible lesions detected through histopathology compared to those identified using ISH RNAscope.

A key aspect of the present study was the selection of the TBLN for viral load assessment, given its role as a primary site for PCV2 replication (Rosell et al., 1999; Segalés and Sibila, 2022). Under the present experimental conditions, TBLN consistently exhibited higher viral loads compared to other lymph nodes, probably due to the nature of the inoculation route, which was intranasal.

Although ISH RNAscope provides a highly sensitive method for PCV2 detection, its cost remains a limiting factor for routine diagnostic applications. However, its potential for research purposes is advisable, allowing for detailed characterisation of PCV2 distribution within lymphoid tissues. Future studies should explore the applicability of this technique across different PCV2-AD presentations and its potential for detecting early stages of infection before significant histopathological lesions develop, as well as other subclinical presentations (i.e., PCV2-reproductive disease). Such improved sensitivity could provide a deeper understanding of the disease and its dissemination through the organism.

Table ST3.1. PCV2 histopathology, ISH and qPCR results for all analysed lymphoid tissues. The table contains the complete dataset of PCV2 histopathology lesion scoring, visual and digital ISH scoring, and qPCR results. Data include all analysed lymphoid tissues (TO, TBLN, MSLN, and ISLN) from each of the 66 animals, with qPCR results specifically provided for TBLN samples.

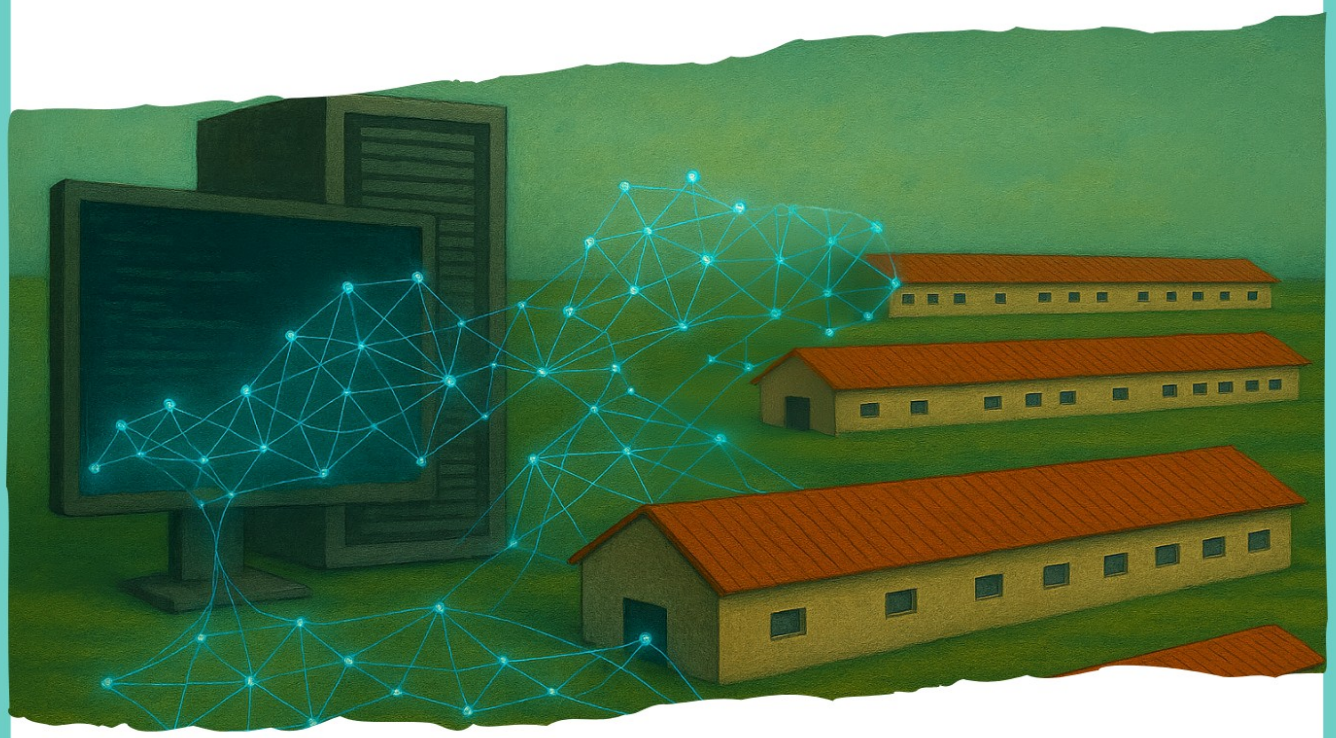
Animal	Histopathology								PCV2 ISH RNAscope								TBLN PCV2 qPCR
	Tonsil lesion score		TBLN lesion score		MSLN lesion score		ISLN lesion score		Tonsil		TBLN		MSLN		ISLN		
ID	LD (0-3)	HR (0-3)	LD (0-3)	HR (0-3)	LD (0-3)	HR (0-3)	LD (0-3)	HR (0-3)	Visual score (0-3)	Digital score (%)	Visual score (0-3)	Digital score (%)	Visual score (0-3)	Digital score (%)	Visual score (0-3)	Digital score (%)	PCV2 qPCR (log10 PCV-2 DNA copies/mL)
1	0	0	0	0	0	0	0	0	2	2,2886	3	7,7761	2	5,7711	2	1,1348	10,46
2	0	0	0	0	0	0	0	0	0	0,0005	0	0,0024	0	0,0013	0	0,0013	4,00
3	0	0	0	0	0	0	0	0	1	0,0423	1	0,0396	1	0,0469	1	0,0878	8,20
4	0	0	0	1	0	1	0	1	2	2,8852	2	3,5617	2	2,5061	2	2,6512	9,47
5	0	0	0	0	0	0	0	0	1	0,1394	1	0,3710	1	0,2216	1	0,1394	8,98
6	0	0	0	0	0	0	0	0	0	0,0009	0	0,0006	0	0,0003	0	0,0004	4,35
7	0	0	0	0	0	0	0	0	0	0,0004	0	0,0004	0	0,0002	0	0,0004	4,04
8	0	0	0	0	0	0	0	0	0	0,0007	0	0,0007	0	0,0001	0	0,0008	3,30
9	0	0	0	1	0	1	0	1	2	0,8513	2	3,3244	2	1,1737	2	1,0220	10,45
10	0	0	0	0	0	0	0	0	0	0,0005	0	0,0007	0	0,0004	0	0,0005	4,16
11	0	0	0	0	0	0	0	0	0	0,0024	0	0,0008	0	0,0016	0	0,0009	4,00
12	0	0	0	0	0	0	0	0	1	0,7950	2	4,1747	1	0,8925	1	0,7714	11,23
13	0	0	0	0	0	0	0	0	0	0,0005	0	0,0003	0	0,0005	0	0,0003	6,05
14	0	0	0	0	0	0	0	0	2	1,7148	2	4,7835	2	3,1530	2	4,1331	10,69
15	0	0	0	0	0	0	0	0	0	0,0002	0	0,0003	0	0,0005	0	0,0004	4,44
16	0	0	0	0	0	0	0	0	1	0,3426	2	1,4231	1	0,5593	1	0,6630	9,89
17	0	0	0	0	0	0	0	0	0	0,0002	0	0,0007	0	0,0008	0	0,0003	4,00
18	0	0	0	0	0	0	0	0	1	0,1137	3	19,4533	1	0,0022	1	0,0690	8,93
19	0	0	0	0	0	0	0	0	0	0,0002	0	0,0003	0	0,0008	0	0,0004	4,00
20	0	0	0	0	0	0	0	0	0	0,0003	0	0,0002	0	0,0004	0	0,0008	4,00
21	0	0	0	0	0	0	0	0	0	0,0009	0	0,0002	0	0,0002	0	0,0005	4,00
22	0	0	0	0	0	0	0	0	0	0,0015	0	0,0007	0	0,0020	0	0,0006	5,23
23	0	0	0	0	0	0	0	0	0	0,0009	0	0,0005	0	0,0002	0	0,0004	3,30
24	0	1	1	2	0	1	1	2	3	5,1355	3	12,0874	3	5,2005	2	2,4970	10,75
25	0	0	0	0	0	0	0	0	3	16,7845	3	8,2765	3	10,3015	3	5,1201	11,16
26	0	0	0	0	0	0	0	0	0	0,0002	1	0,0714	0	0,0002	0	0,0004	5,30
27	0	0	0	0	0	0	0	0	0	0,0003	1	0,5471	0	0,0005	0	0,0005	8,35
28	0	0	0	0	0	0	0	0	0	0,0002	0	0,0007	0	0,0002	0	0,0001	5,54
29	0	0	0	0	0	0	0	0	1	0,0326	2	3,0324	1	0,0185	1	0,0282	8,91
30	0	0	0	0	0	0	0	0	0	0,0011	0	0,0009	0	0,0011	0	0,0003	4,42
31	0	0	0	0	0	0	0	0	0	0,0001	0	0,0003	0	0,0001	0	0,0004	3,30
32	0	0	0	0	0	0	0	0	0	0,0012	0	0,0005	0	0,0005	0	0,0007	4,50
33	0	0	0	0	0	0	0	0	1	0,0119	2	1,0737	1	0,0120	1	0,0021	8,59
34	0	0	0	0	0	0	0	0	0	0,0011	1	0,0147	0	0,0004	0	0,0008	5,41

35	0	0	0	0	0	0	0	0	0	0,0002	0	0,0004	0	0,0002	0	0,0004	3,30
36	0	0	0	0	0	0	0	0	1	0,0208	2	1,6846	1	0,0063	1	0,0081	8,56
37	0	0	0	0	0	0	0	0	0	0,0013	0	0,0005	0	0,0006	0	0,0004	5,68
38	0	0	0	0	0	0	0	0	0	0,0007	0	0,0012	0	0,0011	0	0,0014	3,30
39	0	0	0	0	0	0	0	0	0	0,0025	1	0,0026	0	0,0011	0	0,0013	6,75
40	0	0	0	0	0	0	0	0	0	0,0014	0	0,0025	0	0,0017	0	0,0006	5,02
41	0	0	0	0	0	0	0	0	0	0,0007	0	0,0009	0	0,0015	0	0,0006	4,77
42	0	0	0	0	0	0	0	0	1	0,0856	2	2,4343	1	0,0080	0	0,0047	9,01
43	0	0	0	0	0	0	0	0	1	0,0044	2	2,6349	0	0,0019	0	0,0025	8,45
44	0	0	0	0	0	0	0	0	0	0,0017	0	0,0011	0	0,0009	0	0,0008	4,00
45	0	0	0	0	0	0	0	0	0	0,0013	0	0,0020	0	0,0017	0	0,0011	4,00
46	0	0	0	0	0	0	0	0	0	0,0018	0	0,0006	0	0,0005	0	0,0009	5,54
47	0	0	0	0	0	0	0	0	0	0,0014	0	0,0024	0	0,0008	0	0,0011	4,96
48	0	0	0	0	0	0	0	0	0	0,0014	0	0,0005	0	0,0005	0	0,0003	3,30
49	0	0	0	1	0	0	0	0	1	0,3338	2	3,9504	1	0,1967	1	0,9223	9,87
50	0	0	0	0	0	0	0	0	0	0,0010	0	0,0013	0	0,0016	0	0,0014	6,27
51	0	0	0	0	0	0	0	0	1	0,1902	2	4,0778	1	0,1907	1	0,0562	9,68
52	0	0	0	0	0	0	0	0	1	0,2883	2	2,7430	2	1,3001	1	0,2791	9,94
53	0	0	0	0	0	0	0	0	0	0,0014	1	0,5034	0	0,0006	0	0,0006	6,43
54	0	0	0	0	0	0	0	0	0	0,0010	0	0,0007	0	0,0009	0	0,0009	4,00
55	0	0	0	1	0	0	0	0	3	5,6961	3	5,4116	3	6,2868	3	5,6524	10,39
56	0	0	0	0	0	0	0	0	0	0,0024	0	0,0019	0	0,0008	0	0,0012	6,10
57	0	0	0	0	0	0	0	0	0	0,0022	0	0,0008	0	0,0016	0	0,0006	7,94
58	0	0	0	0	0	0	0	0	0	0,0010	0	0,0022	0	0,0009	0	0,0009	4,00
59	0	0	0	0	0	0	0	0	1	0,0031	1	0,7339	1	0,0031	0	0,0014	7,11
60	0	0	0	0	0	0	0	0	1	0,0506	1	0,5854	1	0,0184	0	0,0033	10,68
61	0	0	0	0	0	0	0	0	0	0,0010	0	0,0013	0	0,0014	0	0,0014	3,30
62	0	0	0	0	0	0	0	0	1	0,0057	1	0,5129	1	0,0091	1	0,0088	8,91
63	0	0	0	0	0	0	0	0	0	0,0022	0	0,0021	0	0,0011	0	0,0017	4,21
64	0	0	0	0	0	0	0	0	0	0,0012	0	0,0020	0	0,0007	0	0,0013	6,28
65	0	0	0	0	0	0	0	0	0	0,0011	0	0,0015	0	0,0013	0	0,0016	6,94
66	0	0	0	0	0	0	0	0	0	0,0025	0	0,0022	0	0,0015	0	0,0025	6,87

TO: tonsil; TBLN: tracheobronchial lymph node; MSLN: mesenteric lymph node; ISLN: inguinal superficial lymph node; PCV2: porcine circovirus 2.

Chapter 4

Data-driven strategies for optimizing the control of PCV2 infection by vaccination in swine: a modelling-based tool



Submitted for publication

4.1. Introduction

The multifactorial nature of PCV2-AD complicates the assessment of intervention strategies (Segalés et al., 2005; Rose et al., 2012). Epidemiological modelling provides an essential complementary tool to experimental approaches, integrating biological knowledge with production system features to evaluate control strategies under controlled and repeatable conditions (Andraud and Rose, 2020; Ezanno et al., 2020). Previous modelling studies characterised PCV2 transmission dynamics and demonstrated that husbandry practices and vaccination influence infection at the herd level (Andraud et al., 2008, 2009a,b). However, these models did not explicitly incorporate the variability in MDA levels, infection pressure, and performance outcomes that are known to modulate vaccine efficacy in commercial herds.

In this context, the present Chapter evaluated how PCV2 vaccination timing influences viral infection dynamics and batch performance under different initial levels of MDA and PCV2 prevalence at nursery-entry. A stochastic individual-based model was built using the EMULSION platform (Picault et al., 2019), integrating age-dependent MDA decay and infection pressure across a farrow-to-finish system.

4.2. Materials and methods

4.2.1. Model development

A stochastic, mechanistic, individual-based model was implemented using the EMULSION framework (Picault et al., 2019), version 1.2, to simulate PCV2 dynamics at both individual and batch levels in a farrow-to-finish farm (https://github.com/monicasagrera/PCV2-model.git). EMULSION, an open-source platform, converts human-readable configuration files into executable simulations through a Python-based engine, enabling researchers to adapt model assumptions

and parameters without programming skills. Epidemiological, biological or zootechnical processes were represented as "finite state machines", a structured approach from computer science that describes health states and their transitions over time (Picault et al., 2019). The model ran in daily time steps and tracked pigs from farrowing to slaughter. Five key processes were represented: *age group*, *life cycle*, *PCV2 vaccination*, *PCV2 MDA and PCV2 infection status*, as well as five interactions (*a-e*) linking them. These components and interactions are described in the following subsections.

Age group and life cycle

The age group state machine was designed as a simplified representation of the *life cycle* state machine, distinguishing only between piglets (juvenile individuals, corresponding to the suckling phase) and pigs (encompassing both the nursery and growing/finishing phases of the *life cycle* state machine). This categorization was introduced to allow, in future analyses, the aggregation of epidemiological and production-related outputs across the nursery and finishing phases, enabling results to be reported at a broader post-weaning level when required. The expected duration and mortality for each state of the *life cycle* were described in *Table 4.1*.

Beyond defining the temporal structure and baseline mortality, the *life cycle* state machine also supported the modelling of individual growth across production phases, implemented using a Gompertz growth function (*Equation 1*) (Besteiro et al., 2024). As detailed in *Table 4.2*, the model was parameterised using A=212 kg, B=5.035, and k=0.01025, which yields an initial BW of 1.38 kg, being consistent with an average biological farrowing weight reference (Moreira et al., 2020).

Table 4.1. Duration and baseline mortality parameters for each production phase in the *life cycle* state machine.

Parameter			Value	Description and justification
	State	Name		
Duration (days)	Suckling	*dur_suckling*	28	Number of days spent in the farrowing facilities during lactation, based on the minimum duration established by Spanish legislation (RD 1135/2002).
	Nursery	*dur_nursery*	42	Number of days spent in the nursery facilities. The literature value (44.48 days) was rounded to align with weekly management practices (Observatori del Porcí, 2023).
	Finishing	*dur_finishing*	140	Number of days spent in the finishing facilities. The literature value (138.5 days) was rounded to facilitate compatibility with week-based production scheduling (Observatori del Porcí, 2023).
Mortality (proportion)	Suckling	*base_mortality_suckling*	0.244	Proportion of piglets that die during the suckling (proportion relative to total born piglets), nursery and finishing periods, respectively, based on average values reported in Spain (Observatori del Porcí, 2023).
	Nursery	*base_mortality_nursery*	0.068	
	Finishing	*base_mortality_finishing*	0.059	

Table 4.2. Description of Gompertz growth function parameters.

Parameter	Units	Description
A (*gompertz_A*)	Kg	Represents the asymptotic or maximum body weight a pig can reach in the designed model. This parameter determines the upper limit of the growth trajectory.
B (*gompertz_B*)	-	Dimensionless scaling factor that defines the initial condition of the curve. It determines the starting fraction of A and influences the timing of the inflection point.
k (*gompertz_k*)	d^{-1}	Growth-rate coefficient that governs how rapidly animals approach their mature weight. Higher values of k correspond to steeper and earlier growth.
w_o (*default_initial_weight*)	Kg	Initial body weight at birth, which is not an independent parameter but is derived from A and B as $w_o = A \cdot e^{-B}$.
t_{inf}	d	Time at which growth rate is maximum (inflection point). Occurs when $W = A/e$, given by $t_{inf} = ln(B)/k$.

d = days.

The curve was fitted to reference weights of 5.3 kg at the end of lactation, 17.8 kg at the end of the nursery phase, and 118.0 kg at the end of the finishing phase, based on recent averages from Spanish pig farms (Observatori del Porcí, 2023). This function was used to estimate both BW and ADWG (*average_daily_weight_gain*) (*Equation 2*) over time.

$$W\ (t) = A \cdot e^{-B \cdot e^{-k \cdot t}} \qquad \textit{(Equation 1)}$$

$$ADWG = \frac{dW}{dt} = k \cdot W(t) \cdot \ln\left(\frac{A}{W(t)}\right) \qquad \textit{(Equation 2)}$$

Figure 4.1 displays the resulting growth profiles: the predicted BW trajectory (*Figure 4.1A*) and the ADWG curve (*Figure 4.1B*), which follows the typical bell-shaped pattern observed in swine production, with peak gain occurring during the mid-finishing phase. The model also produced average ADWG values of 0.32 kg/d during the nursery phase and 0.72 kg/d during the finishing phase, closely matching the benchmark values for Spanish herds (0.28 kg/d and 0.72 kg/d, respectively) (Observatori del Porcí, 2023), thereby reinforcing the biological consistency of the parametrisation.

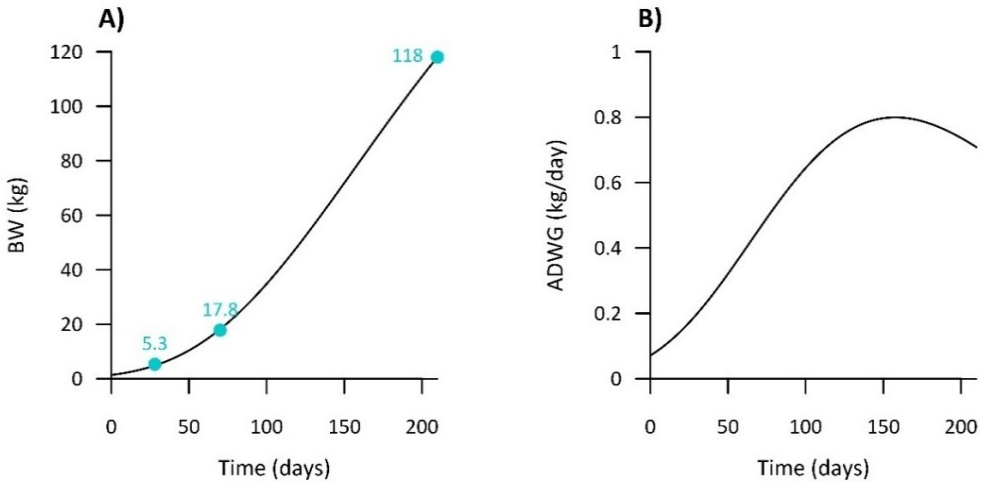


Figure 4.1. Growth dynamics of pigs predicted using the Gompertz growth function. **A)** Body weight trajectory from birth to 210 days, with reference weights at the end of lactation (5.3 kg), nursery (17.8 kg), and finishing (118.0 kg), and **B)** Average daily weight gain curve, which is the derivative of the Gompertz function, displaying the typical bell-shaped profile with maximal gain occurring during the mid-finishing phase. ADWG: average daily weight gain; BW: body weight.

PCV2 vaccination

The *PCV2 vaccination* state machine was structured with three states. The period immediately after vaccine administration is represented by the *onset_of_immunity* (*OOI*), during which the animal has received the vaccine product, but protective immunity has not yet developed. *Immunised (IMM)* corresponds to the effective immunised state, beginning 14 days after vaccination in accordance with the technical guidelines of commercial vaccines (European Medicines Agency, 2021, 2024, 2025b), and lasting until slaughter. Finally, the non-immunised state (*non_IMM)* represents the absence of effective vaccine-induced protection, regardless of its origin. Accordingly, animals may persist in the *non_IMM* state either because they were not vaccinated or because vaccination failed (e.g. due to administration errors or interference by very high levels of MDA). These situations are treated equivalently in the model, as they result in the same epidemiological behaviour.

The probability of successful immunisation in vaccination scenarios is governed by the parameter *default_proba_success_vacc*, set to 95% by default and modifiable to adapt simulations to farm-specific conditions. This probability is further modulated by MDA-related interference, with a reduced likelihood of effective immunisation in piglets with very high MDA levels (interaction “a” of *Interactions within the different processes* subsection).

PCV2 MDA

The state machine designed for PCV2 MDA was structured into four states: very high (VH), high (H), medium (M), and low (L). Thresholds between these states were defined based on S/P ratios obtained with the PCV2 Ingezim Circovirus IgG ELISA test (Gold Standard Diagnostics, Davis, CA, USA) (≤0.5 = L_MDA; 0.5–1.0 = M_MDA;

1.0–2.0 = H_MDA; >2.0 = VH_MDA). Piglets with VH MDA levels have previously shown reduced vaccine response and impaired growth performance (Feng et al., 2016), supporting the choice of >2.0 S/P as the cut-off for the VH category. The threshold between H and M MDA (S/P = 1.0) was based on three studies that used S/P values around 0.9–1.2 to discriminate between low and high MDA groups in vaccination trials (Martelli et al., 2016; Kiss et al., 2021, *Chapter 2*). The lower boundary for M MDA (S/P = 0.5) was set slightly above the positive cut-off provided by the manufacturer's instructions (0.3–0.4) to distinguish animals with detectable but low antibody levels from those with moderate passive immunity.

To represent the natural decline of MDA, internal observational data obtained with the aforementioned PCV2 IgG ELISA were used to characterise antibody waning during early life. Between 14 and 21 days of age, piglets with medium MDA levels showed an average decrease from 1.0 to 0.9 S/P, while piglets with very high MDA levels declined from 2.5 to 2.3 S/P over the same period. Assuming a constant proportional daily loss of antibodies, MDA decay was modelled with an exponential decay function (*Equation 3*), where MDA_{21} is the S/P ratio value at day 21 (commonly used as the reference time point because pre-vaccination serology is typically performed at this age), and *mda_waning* represents the daily decay rate (0.015).

$$MDA(t) = MDA_{21} \cdot e^{-mda\_waning \cdot t} \qquad (Equation\ 3)$$

Based on these observations, proportional decay across the remaining MDA ranges was inferred, allowing antibody waning to be simulated across the full range of initial conditions. By applying the decay function backwards in time, corresponding S/P values at day 0 were derived, as illustrated in the modelled decay curves (*Figure 4.2*).

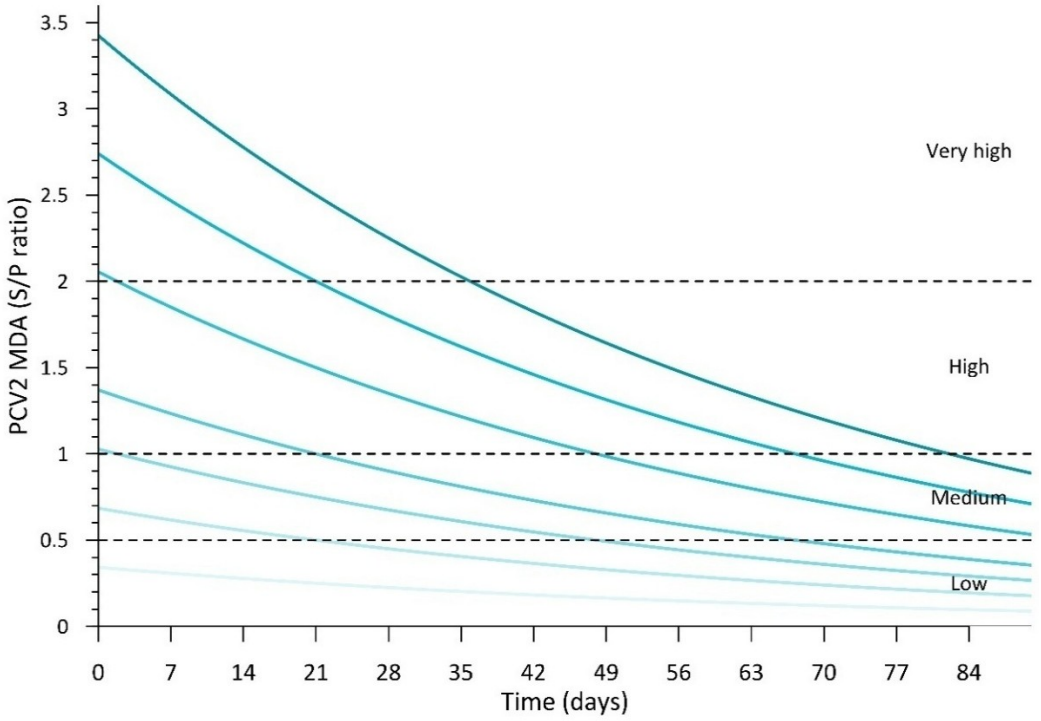


Figure 4.2. Decay curves of MDA expressed as S/P ratio over time. Different initial antibody levels were modelled using a decay rate of 0.015. Horizontal dashed lines represent cut-off thresholds at 0.5, 1.0, and 2.0 S/P, corresponding to the classification of piglets into Low, Medium, High, and Very high MDA categories, respectively. MDA: maternally derived antibodies.

PCV2 infection status

The *PCV2 infection status* state machine was designed by adapting a classic SEIR framework (Kermack and McKendrick, 1927) to the specific features of PCV2 infection. It comprises five states: susceptible (S), exposed (E), PCV2-SD, PCV2-SI, and recovered (R). Susceptible animals became E upon effective contact with infectious pigs (PCV2-SD or PCV2-SI). The transition rate is defined as *Equation 4*, where λ(t) represents the force of infection at time t, modelled as a frequency-dependent process driven by the number of infectious animals, and σ denotes the individual susceptibility, modelled as a function of vaccination status (σ_OOI and

σ_IMM) and MDA value (σ_L, σ_M, σ_H, σ_VH) ("b-c" interactions detailed in *Interactions within the different processes* subsection).

$$\text{Rate}(S \rightarrow E) = \lambda(t) \cdot \sigma \qquad (\textit{Equation 4})$$

After exposure, animals entered the E state, where they were infected but not yet infectious. Following this incubation period (*dur_E*), pigs transitioned either to PCV2-SI or to PCV2-SD, with the probabilities of developing systemic disease (*p_infection_PCV2SD*) or subclinical infection (*p_infection_PCV2SI*) specified in *Table 3*. Both infectious states were assigned the same duration (*dur_PCV2*) and contributed to transmission with different intensities, according to their respective transmission coefficients (*transmission_PCV2SI* and *transmission_PCV2SD*) (*Table 3*). During this period, pigs contributed to onward transmission and experienced a reduction in ADWG, with the magnitude differing between PCV2-SI and PCV2-SD and across production phases (interactions "d–e" of *Interactions within the different processes* subsection). Additionally, PCV2-SD was associated with lethality (*lethality_PCV2SD*) (*Table 4.3*).

At the end of *dur_PCV2*, animals recovered according to predefined daily rates (*recovery_PCV2SD* and *recovery_PCV2SI*), resulting in a gradual decline in infectious individuals (*Table 4.3*). Recovered pigs entered the R state and eventually returned to susceptibility as immunity waned (*waning_rate*). Although immunity waning has no practical impact within a single production cycle (since no further follow up is modelled in replacement stock), the mechanism was retained to ensure structural consistency and to facilitate future extensions of the model.

Table 4.3. Parameters used for the PCV2 infection status state machine.

State or transition	Parameter	Units	Value	Description and justification
S → E	*transmission_PCV2SI*	d^{-1}	0.35	Transmission parameters were informed directly by the within-pen estimates reported (Andraud et al., 2008), whose maximum-likelihood density curves for β show values concentrated around 0.3–0.4 day^{-1}, with upper density extending towards approximately 0.5–0.6 day^{-1}. The parameter for the PCV2-SI state was therefore anchored within this empirically supported range, whereas the PCV2-SD transmission rate was set approximately 1.5-fold higher to account for the assumption that animals showing more pronounced clinical signs would shed greater amounts of virus, while remaining consistent with the biologically plausible interval defined by these experimental data.
	transmission_PCV2SD	d^{-1}	0.55	
E	*dur_E*	d	18	Early viral detection may occur as soon as 4–7 days post-infection (dpi) (Ladekjaer-Mikkelsen et al., 2002), although substantial viraemia and widespread replication typically arise later. Marked increases in viral load are consistently reported around 14–21 dpi (Rovira et al., 2002; Resendes et al., 2004; Chung et al., 2005), while lesions consistent with PCV2-SD often appear between 21 and 30 dpi (Krakowka et al., 2000; Rovira et al., 2002). Caprioli et al. (2006) similarly observed first blood PCR positives around 9 dpi, with most pigs becoming PCR-positive from 12 to 21 dpi, indicating gradual systemic infection establishment. Transmission trials further show that pigs are not infectious before ~8 dpi and reach maximal infectiousness at 18 dpi (Andraud et al., 2009a). Altogether, these findings support an incubation interval of two to three weeks; therefore, the exposed period was set to 18 days as an evidence-based average aligned with virological and infectiousness data. To reflect natural variability in this interval, the model applies a gamma distribution centred on this mean duration, allowing realistic individual differences while avoiding a fixed incubation time and generating smoother, biologically coherent epidemic dynamics.
E → PCV2-SD	*p_infection_PCV2SD*	Prop.	0.10	Based on the morbidity ranges originally described for PCV2-SD, which typically affects 4-30% of pigs in unvaccinated or non-protected populations (Segalés and Domingo, 2002), the model assumed that 10% of infected animals progressed to the PCV2-SD state under non-vaccinated scenarios, while the remaining 90% developed subclinical infection (PCV2-SI).
E → PCV2-SI	*p_infection_PCV2SI*	Prop.	0.90	

PCV2-SD and PCV2-SI	*dur_PCV2*	d	60	Experimental and field studies demonstrate that PCV2 infection can persist for extended periods, with viral DNA detectable for 7-69 days under experimental settings (Resendes et al., 2004) and for much longer under natural conditions, including up to 209 days (Patterson et al., 2011b) and up to 25 weeks in commercial herds (Oliver-Ferrando et al., 2016). Longitudinal observations in farms with PCV2-SD cases also showed that some pigs may remain PCR-positive for 16-21 weeks despite the absence of clinical signs (Rodríguez-Arrioja et al., 2002), indicating that prolonged viral replication is common and not restricted to clinically affected animals. Transmission modelling further reveals an infectiousness peak around 18 dpi, followed by a gradual decline until approximately 39 dpi (Andraud et al., 2009a). Because no empirical evidence supports distinct virological durations for PCV2-SI and PCV2-SD, both states were modelled using a common infectious period as a biologically realistic average capturing the extended replication and waning infectiousness described across these studies. To represent natural variability in this duration rather than imposing a fixed value for all pigs, the model applies a gamma distribution centred on this mean. This approach generates a narrow yet biologically plausible spread of infectious periods, allowing some individuals to experience longer viraemic phases (as observed in field studies) while producing smoother and more realistic epidemic dynamics.
	lethality_PCV2SD	Prop.	0.70	The value reflects the high case fatality reported in clinically affected pigs, as PCV2-SD typically results in death in 70–80% of animals that develop the systemic form of the disease (Segalés and Domingo, 2002), consistent with earlier descriptions of its severe and progressive clinical course (Harding and Clark, 1997; Allan and Ellis, 2000).
PCV2-SD → R	*recovery_PCV2SI*	d^{-1}	0.015	Recovery rates for PCV2-SI and PCV2-SD were derived from the mean duration of infection established in *dur_PCV2*. Because PCV2 infection is known to persist for several weeks under both experimental and field conditions, recovery for PCV2-SI was defined as 1/60 d^{-1}, reflecting the persistence typically observed in subclinical carriers. For PCV2-SD, a slightly higher recovery rate was used to account for the fact that clinically affected pigs often leave the infectious compartment earlier due to disease progression and lethality rather than actual viral clearance. These values remain consistent with the overall infection duration justified for *dur_PCV2*, while allowing state-specific differences in the exit dynamics from the infectious compartments. A gamma distribution was applied to preserve natural variability in infectious durations.
PCV2-SI → R	*recovery_PCV2SD*	d^{-1}	0.02	
R → S	*immunity_waning_rate*	d^{-1}	1/180	Although PCV2 induces both humoral and cellular immune responses, the duration of immunity after natural infection is not well characterised, and available studies do not clearly define how long protection is maintained (Segalés, 2015; Mancera-Gracia et al., 2021; Segalés and Sibila, 2025). In contrast, vaccine-induced protection is better established, with commercial PCV2 vaccines providing immunity for approximately six months (EMA, 2021, 2024, 2025). Although immunity waning has minimal impact in the present model (where pigs are followed only until slaughter age), the structure was retained to ensure consistency with the model framework and to allow future extensions to longer production cycles or multi-batch systems, in which immunity dynamics may become more relevant.

S: susceptible; E: exposed; PCV2: porcine circovirus 2; PCV2-SD: systemic disease; PCV2-SI: subclinical infection; R: recovered; dpi: days post-infection; d: days; d^{-1}: per day; prop: proportion.

Interactions within the different processes

Beyond the core infection dynamics, the model incorporated a set of interaction parameters between processes, linking PCV2 with susceptibility to infection, vaccination outcomes and productive performance. Specifically, the model accounted for the interference of MDA with vaccination efficacy ("a"), the modulation of susceptibility to infection by vaccination and MDA level ("b–c"), and the effects of PCV2-SD and PCV2-SI on ADWG ("d–e"). The corresponding parameters and assumptions are summarised in *Table 4.4*.

4.2.2. Scenarios and outputs

To explore the impact of MDA on PCV2 dynamics, four baseline MDA distributions (A–D) were defined. Their temporal evolution, expressed as weekly values from 0 to 42 days of age (*Figure 4.3)*, illustrates the progressive decline in ELISA S/P ratios as MDA wane.

For each of these four distributions, six PCV2 single-dose vaccination strategies were evaluated: no vaccination and vaccination at 14, 21, 28, 35 or 42 days of age. Each intervention strategy was explored under three levels of PCV2 infection pressure at entry into the nursery unit (*PCV2_prevalence_nursery*), defined by initial prevalences of 1%, 5% and 10%. The full factorial structure of these scenarios (4 MDA distributions × 6 intervention strategies × 3 infection-pressure levels) is summarised in *Figure 4.4.* Their complete parameterisation is provided in *Supplementary File 4.1*.

Table 4.3. Parameters used for the *PCV2 infection status* state machine.

Interaction		Parameter	Units	Value or formula	Description and justification
a)	PCV2 MDA interference with vaccination efficacy	*proba_success_vacc_Piglet*	-	*default_proba_success_vacc* * (1 – 0.5 * is_VH_MDA)	A 50% reduction was applied to vaccination success in piglets with VH MDA titres. This assumption reflects the suggested VH MDA interference in the literature (Feng et al., 2016).
b)	Effect of PCV2 vaccination on susceptibility to PCV2 infection	σ_OOI (*Susceptibility_OOI_P*)	Prop.	0.1	Susceptibility for the vaccinated states was defined according to the expected progression of vaccine-induced protection. During the inset of immunity (OOI), individuals were assumed to be partially susceptible to infection. Susceptibility was progressively reduced to reflect the onset of vaccine-induced protection shortly after vaccination, thereby preventing unrealistically high infection rates during this transitional period. Once immunity is established (*IMM* state), susceptibility was further reduced to represent the strong, though not sterilizing, protection assumed in the model. In both states, susceptibility is expressed as a proportion between 0 (complete protection) and 1 (full susceptibility).
		σ_IMM (*Susceptibility_IMM_P*)	Prop.	0.005	
c)	Effect of PCV2 MDA on susceptibility to PCV2 infection (σ)	σ_L (*Susceptibility_L_MDA*)	Prop.	1.00	Values range between 0 (full protection) and 1 (full susceptibility). Values of susceptibility were assumed in a gradual manner, decreasing as MDA levels increased from low to very high. This structure reflects the progressive protective effect of MDA while also accounting for the potential interference with vaccine-induced seroconversion observed at exceptionally high titres (Feng et al., 2016).
		σ_M (*Susceptibility_M_MDA*)	Prop.	0.2	
		σ_H (*Susceptibility_H_MDA*)	Prop.	0.1	
		σ_VH (*Susceptibility_VH_MDA*)	Prop.	0.01	
d)	Effect of PCV2-SD on ADWG	*reduction_adwg_PCV2SD_nursery*	Kg/d	0.04	PCV2-SI was modelled to reduce ADWG by 10–40 g/day based on published estimates (Kristensen et al., 2011). Growth losses for PCV2-SD were set at roughly double this range to reflect the greater systemic impact of clinical disease, consistent with field observations showing that pigs with high or persistent viraemia (typical of PCV2-SD) exhibit ADWG reductions of around 60–70 g/day compared with animals infected showing lower viral loads (López-Soria et al., 2014).
		reduction_adwg_PCV2SD_finishing	Kg/d	0.08	
e)	Effect of PCV2-SI on ADWG	*reduction_adwg_PCV2SI_nursery*	Kg/d	0.02	
		reduction_adwg_PCV2SI_finishing	Kg/d	0.04	

ADWG: average daily weight gain; MDA: maternally derived antibodies; Prop.: proportion; d: days; PCV2: porcine circovirus 2; PCV2-SD: systemic disease; PCV2-SI: PCV2 subclinical infection; L: low; M: medium; H: high; VH: very high.

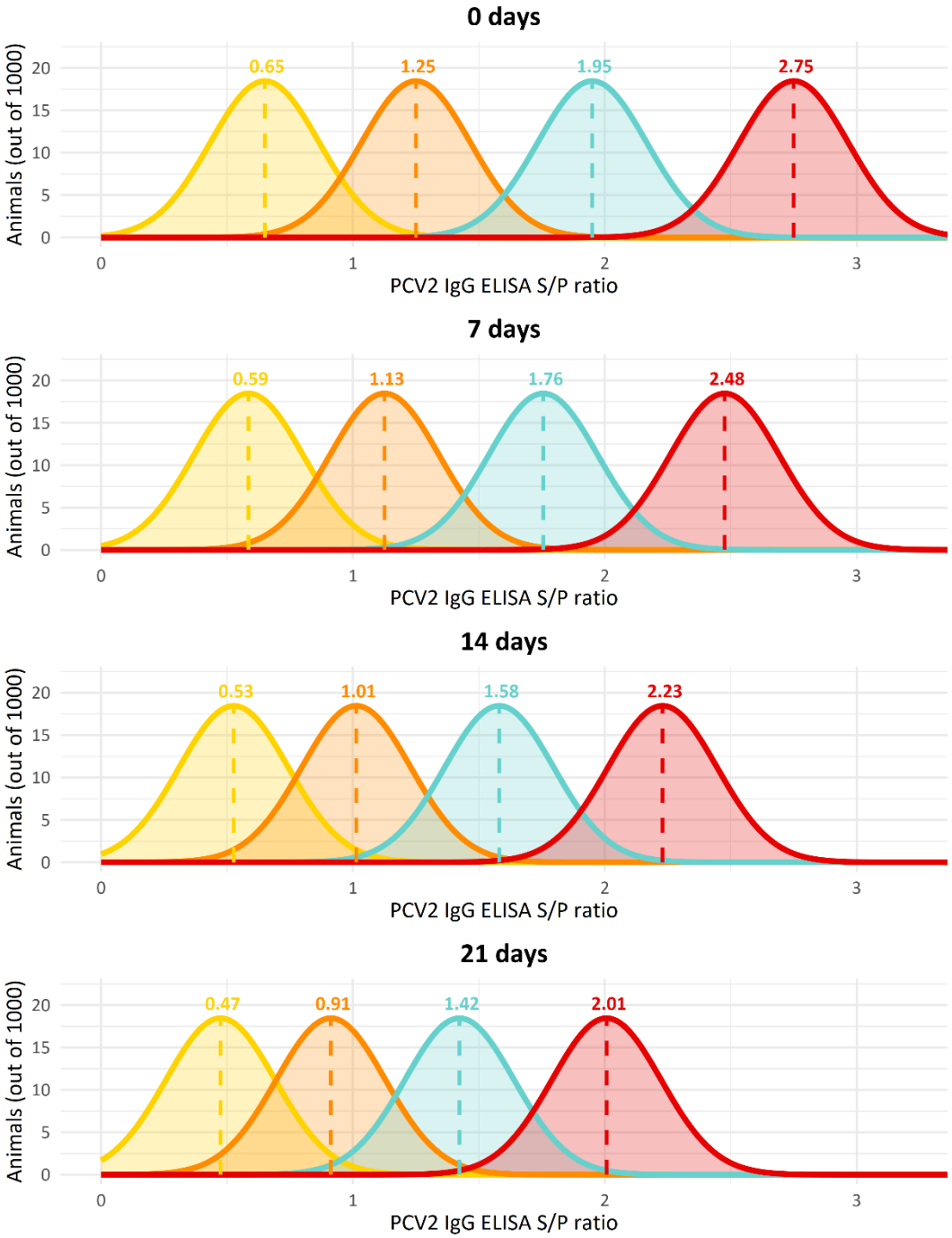
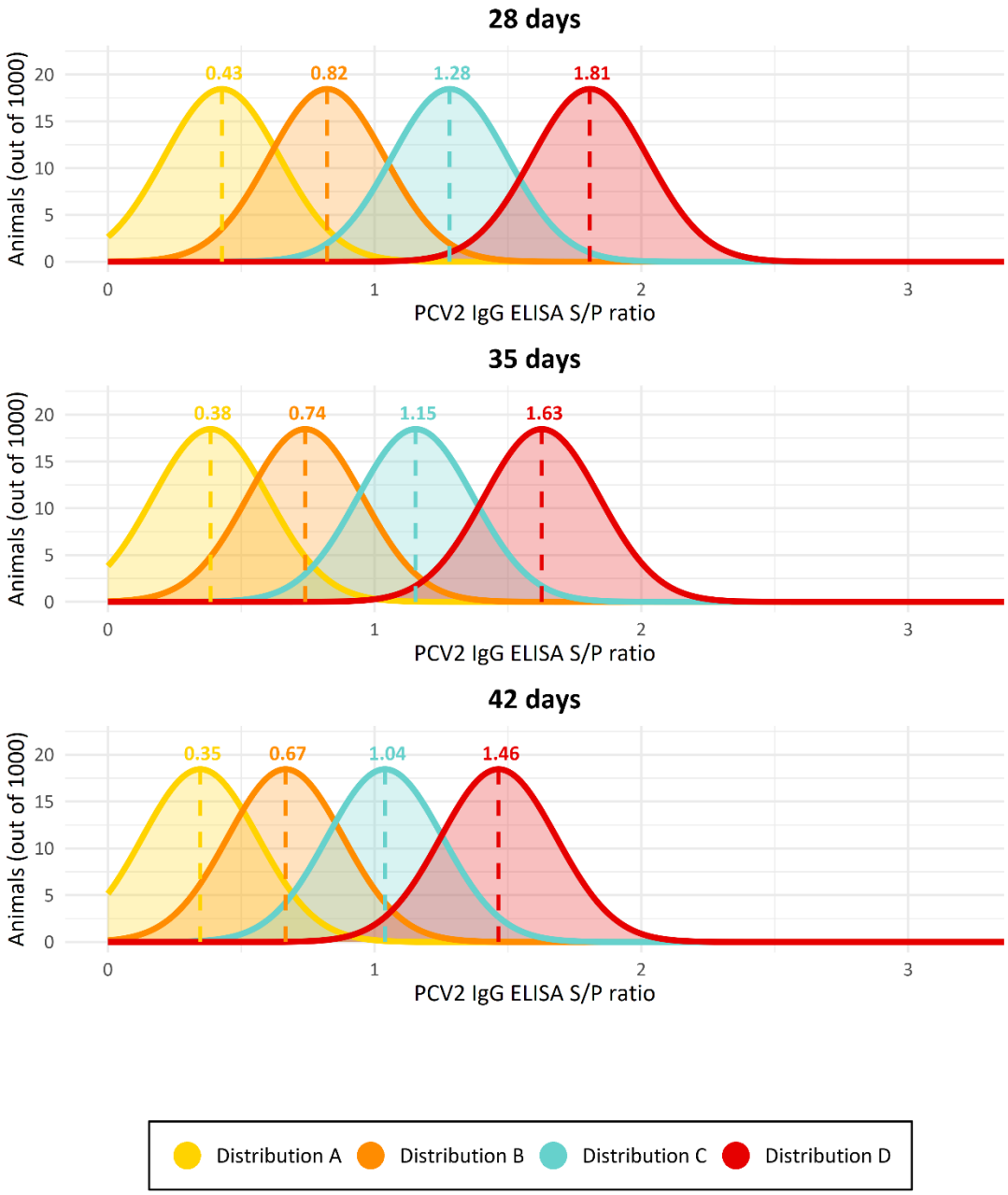


Figure 4.3. Temporal evolution of the four baseline MDA distributions (A to D) from day 0 to day 42. Curves represent the distribution of PCV2 IgG ELISA S/P ratios at weekly intervals, illustrating the progressive leftward shift caused by MDA waning over time. Dashed vertical lines mark the mean…



…S/P ratio of each distribution at each time point. MDA: maternally derived antibodies; PCV2: porcine circovirus 2.

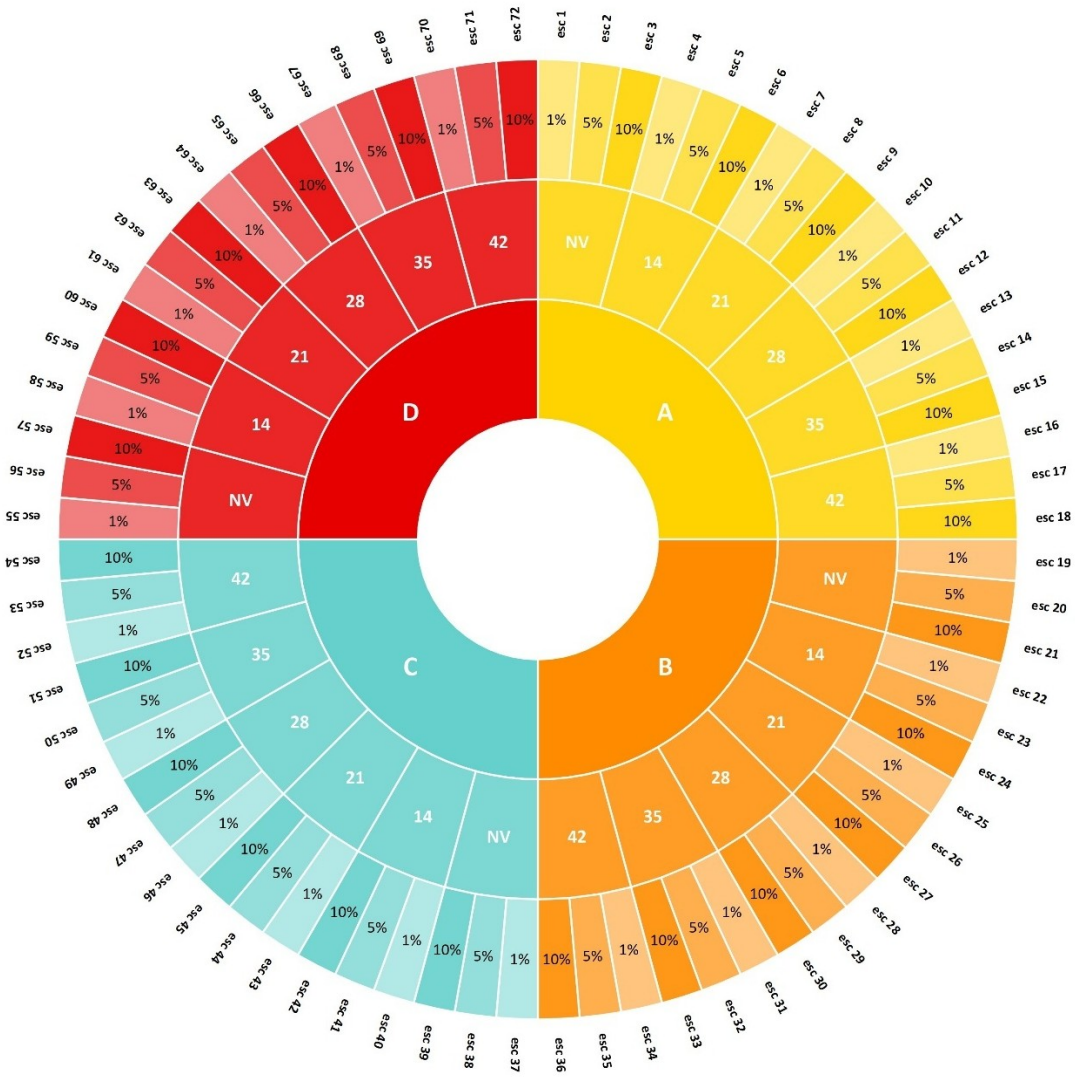


Figure 4.4. Scheme of all epidemiological scenarios included in the study. The inner ring shows the four baseline MDA distributions (A-D), represented in yellow, orange, blue and red, respectively. For each distribution, the middle ring displays the six vaccination strategies evaluated: no vaccination (NV) and vaccination at 14, 21, 28, 35 or 42 days after farrowing. The outer ring represents the three levels of PCV2 infection pressure at entry into the nursery unit, defined by initial prevalences of 1%, 5% and 10%. Each sector corresponds to one simulation scenario (esc_1, esc_2, …).

A baseline PCV2 prevalence of 1% was assumed at the start of farrowing for all scenarios (*initial_prevalence_PCV2*). This low-level background infection reflects an

endemic circulation and prevents stochastic extinction of the virus during the early stages of simulation, while allowing infection pressure to be modulated exclusively through the predefined nursery-entry prevalences. This baseline value is also fully modulable within the model structure and future applications may require alternative starting conditions, although it was not varied in the present analysis.

Each scenario started with 1000 animals and was simulated 10 times to account for stochastic variability inherent to the model. Output values were summarised across replicates, allowing an estimation of the central tendency and dispersion for each metric. Specifically, the analyses included daily and cumulative numbers of PCV2-SI and PCV2-SD cases, number of PCV2-SD–attributable deaths, total mortality, and two production indicators: BW and ADWG, at the end and during the finishing period, respectively.

4.2.3. Economic analysis per scenario

An economic assessment was performed to translate productive outcomes into financial value, using the 2025 Spanish average market price of 1.70 €/kg live weight (LW) (Mercolleida, 2025). Production costs were derived from phase-specific expenses (*Table 4.5*) (SIP Consultors, 2025). These costs reflect the different resource requirements of each production stage and were implemented as follows: 0.42 €/pig/day during suckling (days 0–28), 0.56 €/pig/day during the nursery period (days 28–70), and 0.74 €/pig/day during finishing (days 70–210). Fixed sow-related costs (21.6 €/piglet, corresponding to lactation feed and reproductive expenses) were allocated entirely to the suckling phase. Phase-specific feed, housing, health and reproduction costs were incorporated as detailed in *Table 4.5*, resulting in a total production cost of 159.6 €/pig for an animal reaching day 210 (slaughter age stablished in the model).

Table 5. Production cost estimation for the economic model adjusted to a 117.5 kg pig.

Cost item	€/Kg LW (SIP Consultors, 2025)	Suckling €/pig	Nursery €/pig	Finishing €/pig	Description and justification
Feed	0.896	16.1	7.4	81.8	• **Suckling:** value derived from 44 kg of lactation feed allocated per piglet (SIP Consultors, 2025), which considers sow feed consumption. Using a higher assumed LDF price (0.365 €/kg LDF), the resulting cost for the suckling period is 16.1 €/pig. • **Nursery**: value calculated from the nursery FCR (1.68) (SIP consultors, 2025), applied to the nursery weight gain, multiplied by the feed price (339 €/t) (SIP Consultors, 2025). • **Finishing:** value calculated from the finishing FCR (2.45) (SIP Consultors, 2025), applied to the finishing weight gain, and multiplied by the feed price (339 €/t) (SIP Consultors, 2025).
Housing and management	0.348	8.8	13.1	19.0	• **Suckling and nursery:** a single housing and management cost is available for pigs up to 19 kg (21.9 €) (SIP Consultors, 2025). This value was proportionally allocated to each phase according to its duration in the model, resulting in 8.8 € for the 28-day suckling period and 13.1 € for the 42-day nursery period. • **Finishing:** value corresponding to the phase-specific housing and management cost reported for the finishing period (SIP Consultors, 2025).
Pharmaceutical products	0.068	2.9	2.9	2.1	• **Suckling and nursery:** a single medication cost for pigs up to 19 kg (5.8 €/pig) (SIP Consultors, 2025). This value was divided equally between the suckling and nursery stages, assuming comparable expenditure on treatments and vaccinations across both phases. • **Finishing:** value corresponding to the phase-specific housing and management cost reported for the finishing period (SIP Consultors, 2025).
Reproduc-tion costs	0.047	5.5	-	-	• **Suckling:** value reflecting sow-related reproductive costs fully allocated to the piglet cost (SIP Consultors, 2025).
Total cost	1.359	33.3	23.4	102.9	As it is planned in a farrow-to-finish system, the final estimated costs are slightly lower than the 37 € for a 6-kg piglet and 59 € for a 20-kg pig (SIP Consultors, 2025). The resulting value of 1.359 €/kg live weight matches the published figure exactly (SIP Consultors, 2025).

FCR: feed conversion rate; LD: lactation diet feed.

4.2.4. Sensitivity analysis

A two-phase sensitivity analysis was performed to identify the parameters with the greatest influence on model outputs and to assess potential parameter interactions. In a first screening phase, a one-at-a-time (OAT) approach was applied to 13 model parameters, which were individually varied by ±20% around their baseline values while keeping all other parameters constant. This screening was conducted across four MDA distributions and two vaccination scenarios (vaccination at 21 days of age and non-vaccinated), with 10 stochastic replicates per scenario.

In a second phase, the most influential parameters identified in the screening step were further explored using a full factorial design with three levels (low, baseline, high). This factorial analysis was crossed with MDA distributions and vaccination scenarios, and model outputs were analysed using linear regression models including main effects and first-order interactions. The relative contribution of each parameter to output variability was quantified using variance decomposition. Full details on parameter ranges, experimental design, and analytical procedures are provided in *Supplementary File 4.2*.

4.2.5. Simulation infrastructure

All simulations were executed on the Migale bioinformatics platform (INRAE, France) due to the high computational demand. Using this infrastructure ensured stable performance, prevented local computing limitations from affecting execution, and guaranteed reproducibility across all scenario replicates.

4.2.6. Statistical analyses

All statistical analyses were performed in R version 4.3 (R Core Team, 2024). The outcomes of the 10 stochastic replicates of each scenario were summarized as

medians with interquartile ranges. Normality was assessed using Q–Q plots and the Shapiro–Wilk test. Epidemiological outcomes (PCV2-SI incidence, PCV2-SD incidence, PCV2-SD mortality, and PCV2-SI peak metrics) did not meet normality assumptions and contained ties; therefore, they were analysed using Kruskal–Wallis tests to compare initial prevalence levels (1%, 5%, 10%) within each MDA distribution and MDA distributions (A–D) within each prevalence level, followed by Dunn's post-hoc tests with Bonferroni correction (FSA package; Ogle et al., 2023). In contrast, production traits (finishing BW and ADWG) met normality assumptions within each MDA × prevalence combination, allowing the effect of vaccination age (14, 21, 28, 35, 42 days) to be evaluated using one-way ANOVA with Tukey's HSD post-hoc tests. However, comparisons across MDA distributions or prevalence levels involved mixtures of groups with different means and variances, which did not satisfy normality assumptions; therefore, these between-group contrasts for BW and ADWG were analysed using Kruskal–Wallis tests with Dunn's post-hoc correction. Finishing BW was recorded at day 210, and ADWG during the finishing period was computed as (BW210 – BW70)/140 days, with Δ vs NV calculated for both traits. A significance threshold of $p<0.05$ was used for all analyses.

In V scenarios, 5% of pigs were designated *Non_IMM* (*default_proba_success_vacc*). To quantify the contribution of this fraction to residual PCV2-SD incidence, observed PCV2-SD cases in V populations were compared with expected values calculated as: (median number of *Non_IMM* pigs per scenario) × (PCV2-SD incidence observed in *Non_IMM* populations). The ratio of observed to expected cases was computed for each scenario to assess whether clinical outcomes were consistent with the *Non_IMM* fraction. Ratios close to 1.0 indicated that observed PCV2-SD cases matched the expected number based solely on *Non_IMM* pigs; values below 0.8 suggested possible indirect protection through herd immunity; and values above

1.5 combined with high absolute case numbers indicated additional disease beyond what would be expected from *Non_IMM* animals alone.

About the economic outcomes, income at slaughter was calculated as finishing BW × market price. Production costs were classified as: (i) fixed costs (sow-related: lactation feed and reproduction), applied to all 1000 initial animals regardless of survivability; and (ii) variable costs (housing, health, and post-weaning feed), accumulated proportionally according to days lived. For dead animals, costs were calculated by accumulating fixed costs plus variable costs from each phase until the day of death; these costs were then allocated to the batch and assigned to sold animals. Total batch costs therefore comprised: costs of sold pigs (fixed + full variable cycle) plus costs of dead pigs (fixed + variable accumulated until death). Revenue was computed as survivors × mean finishing BW × market price. Net profit was calculated as total revenue minus total costs, expressed per initial animal (net profit/pig = batch profit / 1000 animals) to represent the actual economic return per pig placed. Finally, two cost perspectives were compared: (i) real margin, where mortality costs were allocated to sold animals; and (ii) theoretical margin, considering only production costs of survivors without mortality allocation. The theoretical margin per kg LW served as validation against the industry reference of ~€0.34/kg LW (SIP Consultors, 2025). Optimal vaccination age was determined for each MDA distribution × prevalence combination based on maximum net profit per pig, and vaccination ages were ranked (1^{st}–5^{th}) within each scenario. Additionally, optimal vaccination timing was identified under theoretical cost perspectives to assess whether mortality cost allocation altered the recommended vaccination strategy. All figures were produced using ggplot2 (Wickham, 2016) and supporting packages for multipanel layouts (patchwork, cowplot, ggh4x) and statistical annotations (emmeans, multcomp).

4.3. Results

4.3.1. Baseline dynamics: model behaviour in the absence of vaccination

Under NV conditions, the model reproduced the expected progression through PCV2 infection states, with *Figure 4.5* illustrating the sequential transitions between states using distribution B as an example. While PCV2-SI cases increased and declined over time, the number of active PCV2-SD cases remained low throughout the simulation (peaking at 29.6 cases around day 127) consistent with the inclusion of disease-associated lethality for this state in the model (*Figure 4.5*). Building on this baseline behaviour, the timing of PCV2-SI peak infection differed significantly across MDA distributions and prevalence levels under NV conditions ($p<0.05$) (*Figure 4.6*). As MDA levels increased from Distribution A to D, PCV2-SI peaks shifted progressively later in time. Distribution A showed the earliest peaks (days 116–120), while Distribution D peaked much later (days 157–183). These differences were significant across all prevalence levels ($p<0.05$), with post-hoc tests confirming that peaks occurred earlier in A than C, A than D, and B than D. Higher initial prevalence consistently advanced the peak within each distribution ($p<0.05$). The peak number of PCV2-SI cases remained broadly similar across scenarios (≈604–675 cases per 1000 pigs). Between distributions, A and B reached higher peaks than D at 5% and 10% prevalence ($p<0.05$), whereas no differences appeared at 1% prevalence.

Regarding cumulative PCV2-SI incidence, significant within-distribution effects of initial prevalence were detected in B, C and D distributions, whereas no prevalence effect was observed in Distribution A (*Figure 4.7*). In addition, cumulative PCV2-SI differed significantly between MDA distributions at all prevalence levels: Distribution A showed higher incidence than Distribution C at 1% and 5%, and higher than Distribution D at 1%, 5% and 10%; Distribution B also exceeded Distribution D at 5% and 10% ($p<0.05$). By contrast, cumulative PCV2-SD incidence

and PCV2-SD mortality only showed significant differences within Distribution A, where cumulative values were higher at 10% than at 1% prevalence ($p<0.05$) (*Figure 4.7*).

Taken together, these patterns resulted in total cumulative mortality that remained remarkably stable across scenarios, with a median of 300 deaths per 1000 animals (27-32% of cumulative mortality) from farrowing to the slaughter age. Of these, approximately 22–24% were attributable to background (non-PCV2-related) mortality, while 6–7.5% corresponded to PCV2-SD-associated deaths. No significant differences in either total or background mortality were detected between MDA distributions or between prevalence levels within distributions.

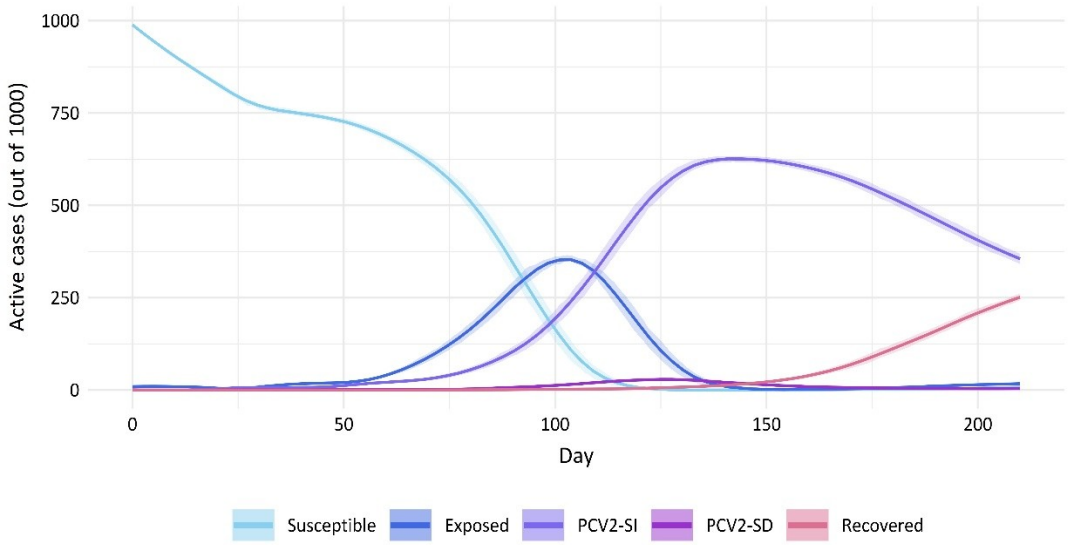
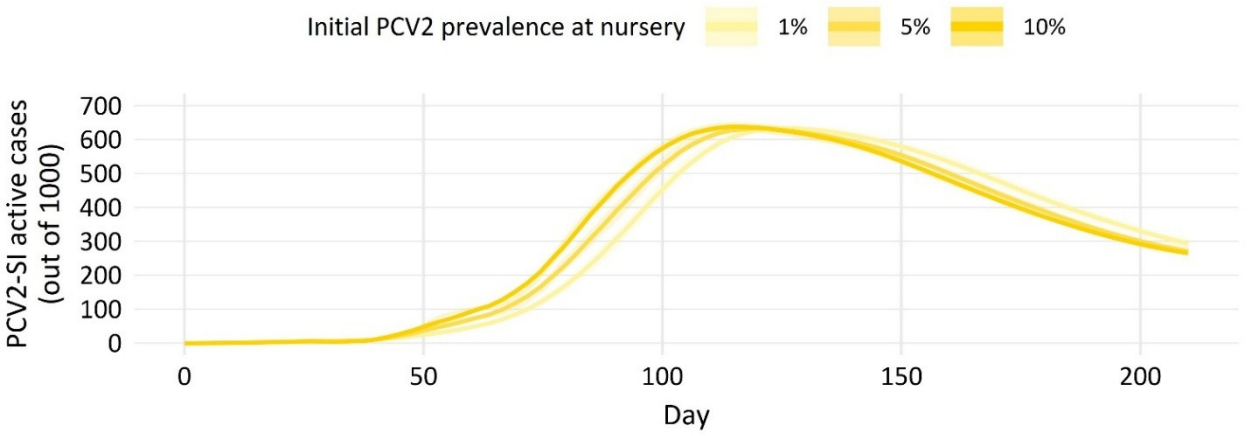
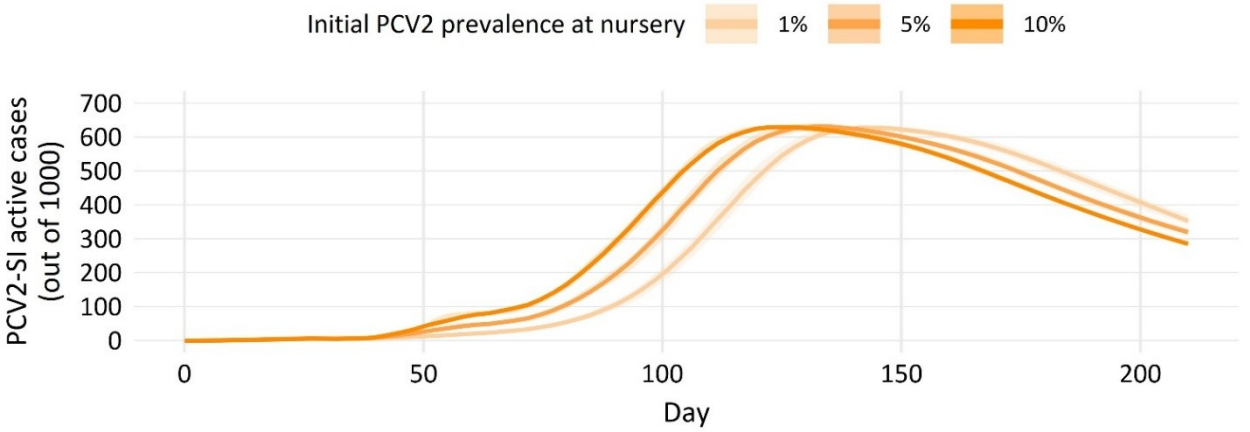


Figure 4.5. Temporal dynamics of PCV2 infection states in NV pigs. Simulation of disease progression showing susceptible, exposed, PCV2-SI, PCV2-SD, and recovered states over 210 days in a cohort of 1000 pigs with MDA distribution B and 1% initial prevalence at the nursery entry. Lines represent means across 10 replicates with 95% confidence intervals (shaded areas). PCV2-SD: porcine circovirus 2 systemic disease. MDA: maternally derived antibodies; PCV2-SI: porcine circovirus 2 subclinical infection.

Distribution A



Distribution B



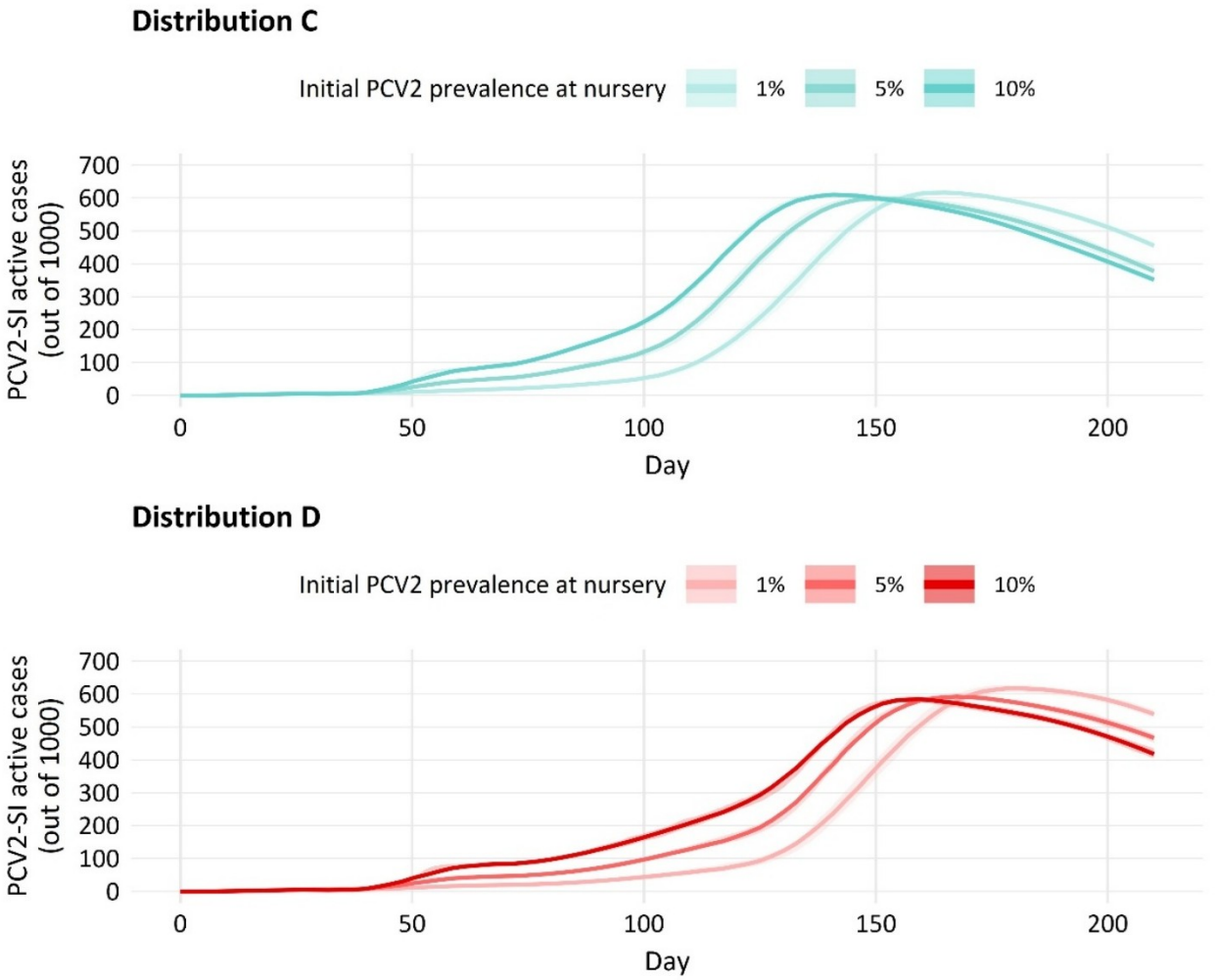
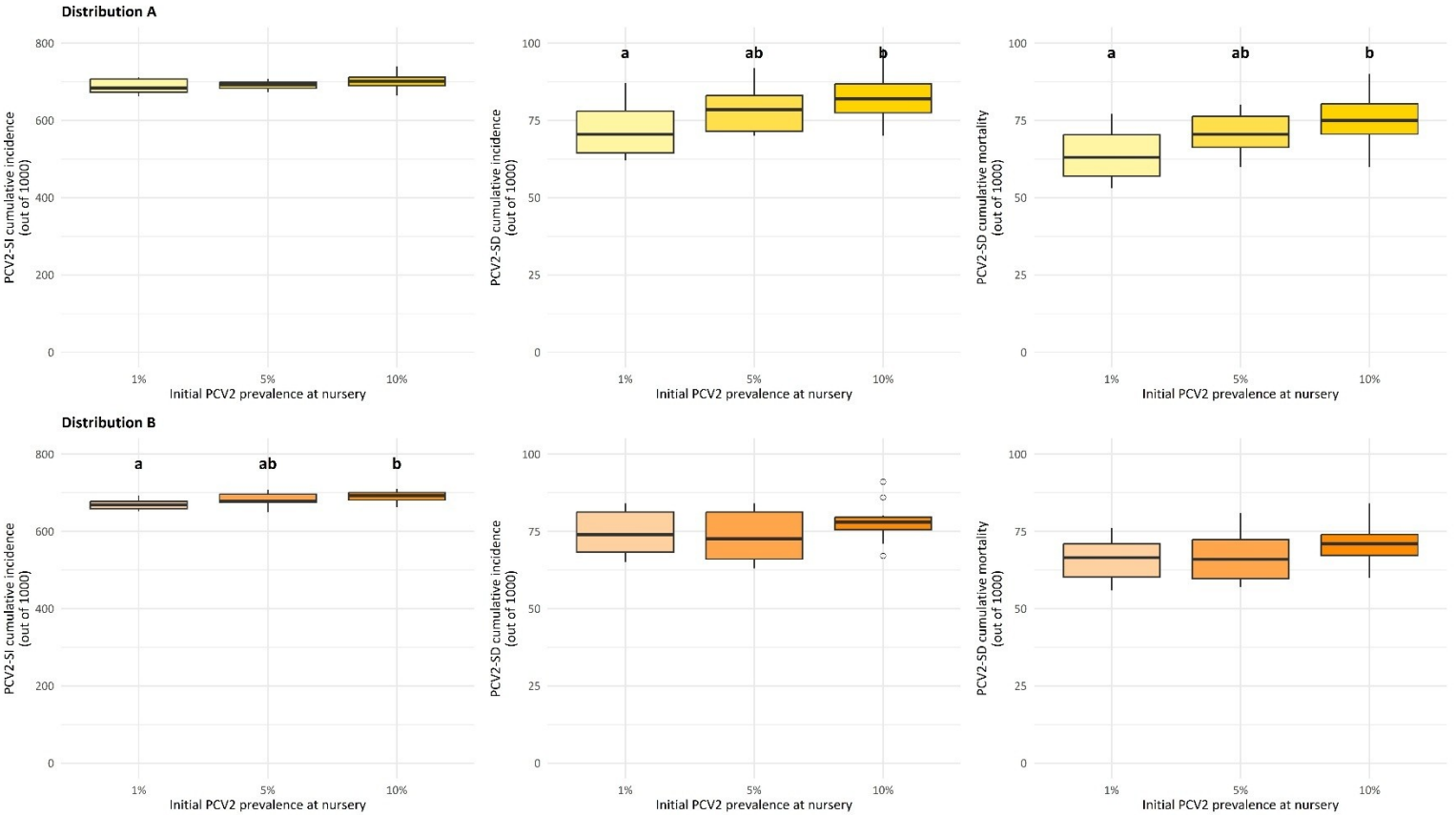


Figure 4.6. Daily number of active PCV2-SI cases (per 1000 animals) in the absence of vaccination under the four baseline MDA distributions (A-D). Within each distribution, curves represent simulations with three levels of PCV2 prevalence at entry into the nursery unit (1%, 5% and 10%). Shaded areas represent variability across 10 simulation replicates.


Distribution A
PCV2-SI cumulative incidence (out of 1000)
800
600
400
200
0
1%
5%
10%
Initial PCV2 prevalence at nursery
PCV2-SD cumulative incidence (out of 1000)
100
75
50
25
0
a
ab
b
1%
5%
10%
Initial PCV2 prevalence at nursery
PCV2-SD cumulative mortality (out of 1000)
100
75
50
25
0
a
ab
b
1%
5%
10%
Initial PCV2 prevalence at nursery
Distribution B
PCV2-SI cumulative incidence (out of 1000)
800
600
400
200
0
a
ab
b
1%
5%
10%
Initial PCV2 prevalence at nursery
PCV2-SD cumulative incidence (out of 1000)
100
75
50
25
0
1%
5%
10%
Initial PCV2 prevalence at nursery
PCV2-SD cumulative mortality (out of 1000)
100
75
50
25
0
1%
5%
10%
Initial PCV2 prevalence at nursery

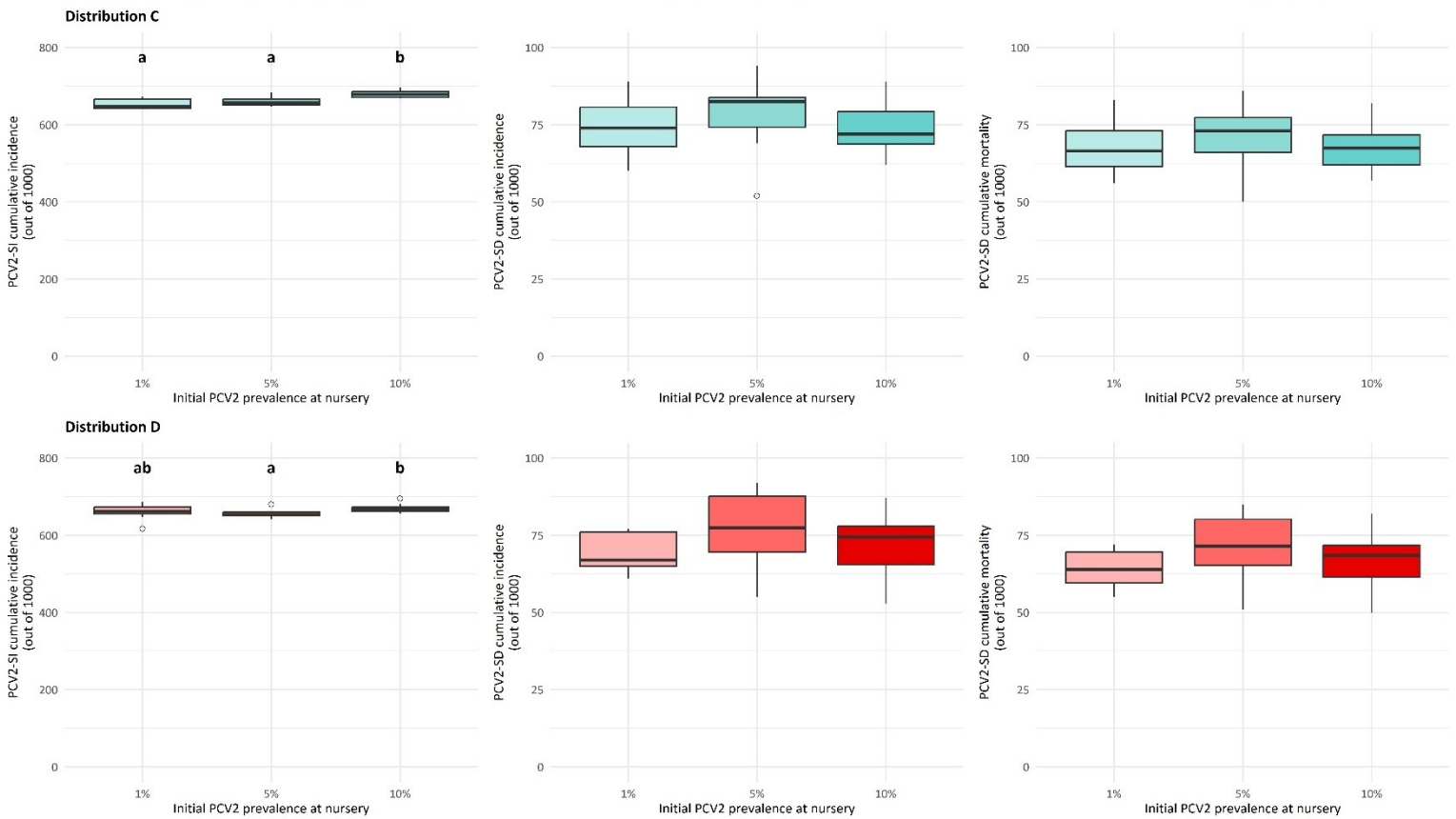


Figure 4.7. Cumulative incidence of PCV2-SI (left), PCV2-SD (middle), and PCV2-SD associated mortality (right) at the end of the finishing period across four MDA distributions (A to D) and three initial nursery prevalence levels (1%, 5%, 10%) under NV scenario. Boxplots show median, quartiles, and outliers from 10 replicates. Letters indicate significant differences within each distribution ($p<0.05$).

4.3.2. Impact of PCV2 vaccination at different ages

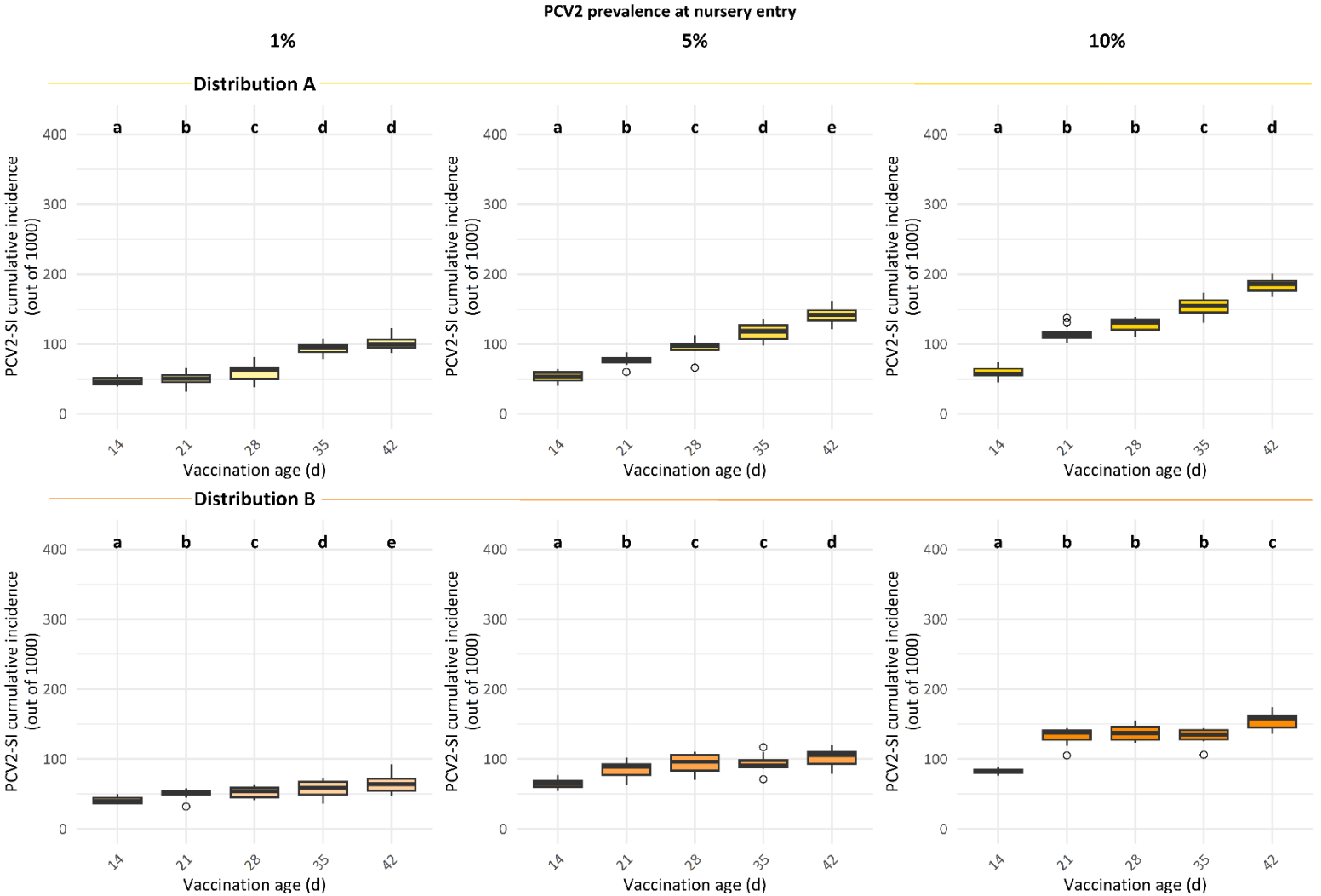
Vaccination age significantly affected cumulative PCV2-SI incidence at the end of the finishing period (*Figure 4.8*). In Distributions A, B and C, early vaccination (14–21 days) consistently produced the lowest incidence, whereas in Distribution D the best outcomes were achieved with later vaccination (28–42 days) ($p<0.05$). Significant differences between distributions were also observed at several vaccination ages: at 14 days, Distribution D showed higher incidence than A, B and C ($p<0.05$), whereas at 35–42 days the reverse pattern emerged ($p<0.05$). Initial prevalence had an impact across all distributions and vaccination ages ($p<0.05$), with 10% prevalence consistently yielding higher cumulative incidence than 1%. However, contrasts between adjacent prevalence levels (1% vs. 5% or 5% vs. 10%) were not uniformly significant, particularly when vaccination takes place at 14 days in Distributions A and D (*Figure 4.8*).

Optimal vaccination timing (14–21 days for Distributions A–C; 28–42 days for Distribution D) reduced PCV2-SI incidence by 80–94% and PCV2-SD incidence by 93–97% compared with NV scenarios (*Supplementary File 4.3*). Under these optimal vaccination conditions, median PCV2-SD incidence ranged from 2 to 5 cases per 1000 animals. Across these vaccination scenarios, the proportion of *Non_IMM* ranged from 4.5–5.0%, depending on vaccination age (*Supplementary File 4.3*).

The contribution of the *Non_IMM* fraction to residual PCV2-SD cases was assessed by calculating observed-to-expected ratios, which ranged from 0.79 to 2.52 (median ~1.4) (*SF4.1*). In Distributions A, B, and C, where vaccination achieved high coverage (~95%), residual PCV2-SD cases (median 2–6 per 1000 animals) were attributable to the *Non_IMM* fraction (*SF4.1*). In Distribution D with early vaccination (14–21 days), ratios of 1.58–1.72 combined with higher case counts (16.5–36.5 per 1000)

indicated disease beyond the *Non_IMM* fraction, consistent with MDA interference preventing effective immunization (*Supplementary File 4.3 and SF4.1*).

Vaccination age did not significantly affect PCV2-SD cumulative incidence in Distributions A, B, and C, where clinical disease remained low (median 2–6 cases per 1000 animals). In Distribution D, vaccination age had a significant effect on PCV2-SD incidence ($p<0.05$): vaccination at 14 days resulted in 34–36.5 cases per 1000 animals, whereas vaccination at 35 days reduced incidence to 2–4.5 cases per 1000 (*Supplementary File 4.3*).


PCV2 prevalence at nursery entry
1%
5%
10%
Distribution A
Distribution B
PCV2-SI cumulative incidence (out of 1000)
Vaccination age (d)
0
100
200
300
400
14
21
28
35
42
a b c d d
a b c d e
a b c d e
a b b c d
a b c c d
a b b b c

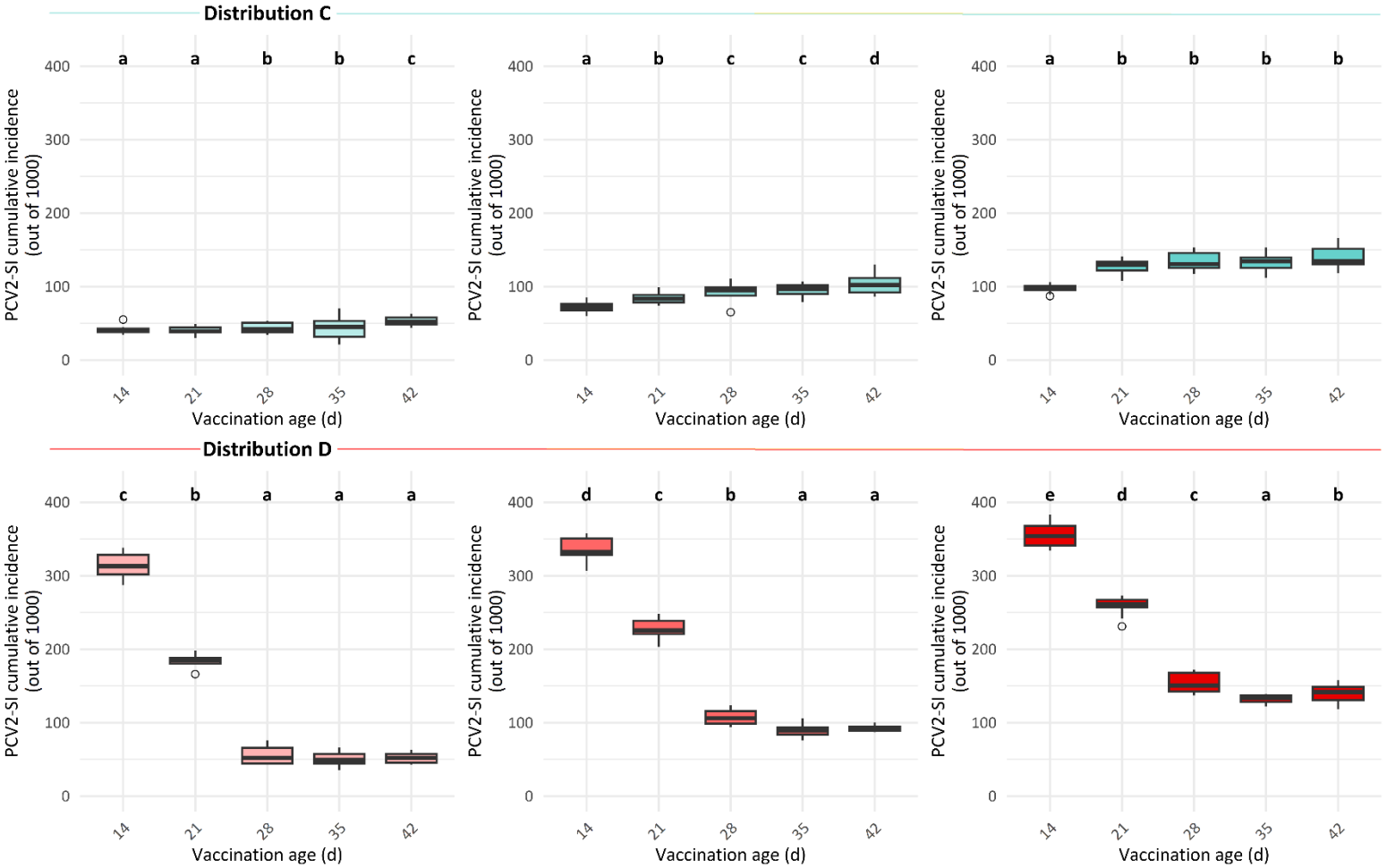


Figure 4.8. Cumulative incidence of PCV2-SI at the end of the finishing period across four MDA distributions (A to D) and three initial nursery prevalence levels (1%, 5%, 10%) under 5 different vaccination ages (14, 21, 28, 35 and 42 days). Boxplots show median, quartiles, and outliers from 10 replicates. Letters indicate significant differences within each distribution ($p<0.05$).

4.3.3. Impact of PCV2 vaccination on production performance and economic outcomes

Vaccination significantly improved finishing BW and ADWG compared with NV populations across all scenarios ($p<0.05$) (*Figures 4.9 and 4.10*). In Distributions A-C, vaccination at 14-21 days resulted in the highest BW and ADWG values, while for Distribution D was at 28-42 days ($p<0.05$). A higher initial PCV2 prevalence at nursery significantly reduced both production parameters across all vaccination ages and distributions ($p<0.05$) (*Figures 4.9 and 4.10*).

Income at slaughter ranged from 187.37 to 198.95 €/pig across all scenarios (*Figure 4.11*). Vaccinated groups consistently achieved higher income (196.64–198.95 €/pig) compared to NV groups (187.37–191.76 €/pig). In Distributions A, B, and C, income differences among vaccination ages were <2 €/pig, whereas in Distribution D, early vaccination (14–21 days) resulted in reduced income (194.89–197.85 €/pig), representing a 2–4 €/pig decrease compared with vaccination at 28–42 days (*Figure 4.11*).

Economic margin per kg LW followed a similar pattern (*Figure 4.12*). Vaccinated scenarios achieved higher margins (0.134–0.184 €/kg LW), while NV scenarios showed lower values (0.049–0.097 €/kg LW). The economically optimal vaccination window ranged from 14–28 days in Distributions A–C and from 28–42 days in Distribution D. Margins remained stable across vaccination ages in Distributions A, B, and C (0.160–0.184 €/kg LW). In Distribution D, vaccination at 35–42 days maximized economic margin (0.164–0.181 €/kg LW), whereas early vaccination at 14 days reduced margins to 0.134–0.150 €/kg LW. Detailed economic parameters including costs breakdown, net profit, and profitability indicators are provided in *Supplementary File 4.4*.

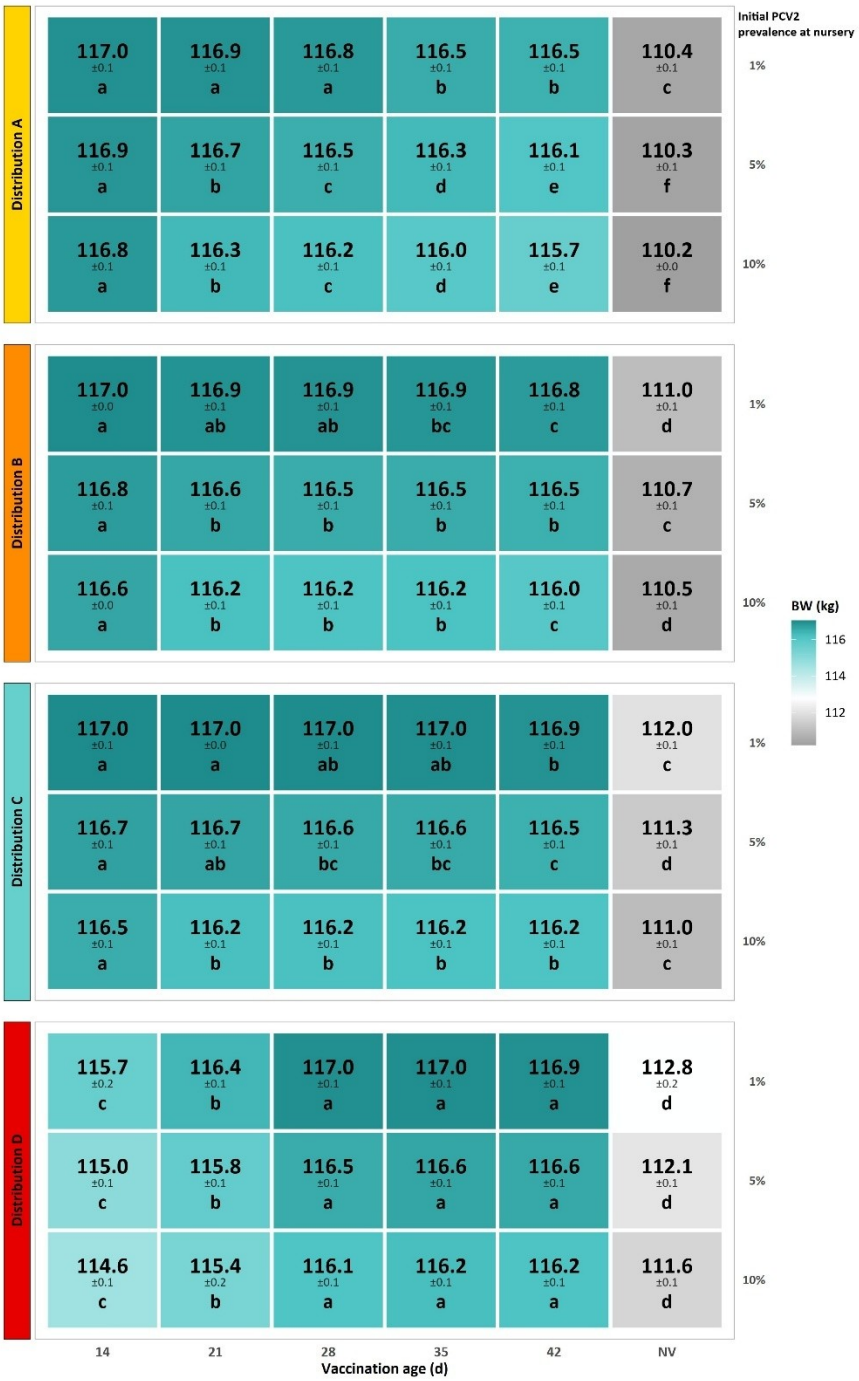


Figure 4.9. Average final BW (± SD) at slaughter across four MDA distributions (A to D) and three initial nursery prevalence levels (1%, 5%, 10%) under five vaccination ages (14, 21, 28, 35 and 42 days) and NV scenario (right column). Heatmap show mean BW ± SD from 10 replicates. Letters indicate significant differences within each distribution and initial PCV2 prevalence at nursery ($p<0.05$). BW: body weight; NV: not vaccinated; PCV2: porcine circovirus 2.

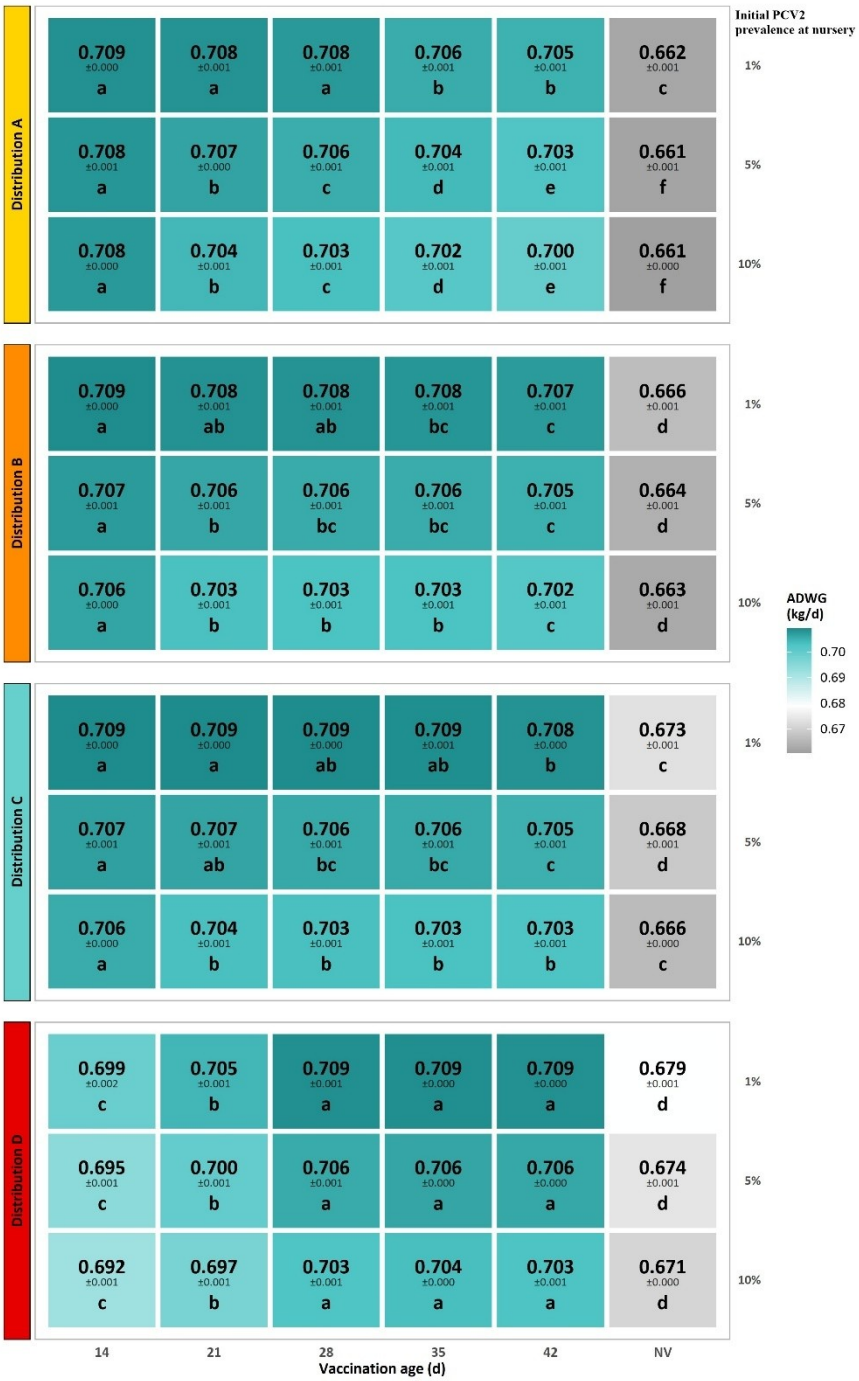


Figure 4.10. Average ADWG (± SD) across four MDA distributions (A to D) and three initial nursery prevalence levels (1%, 5%, 10%) under NV and five vaccination ages (14, 21, 28, 35 and 42 days) and NV scenario (right column). Heatmap shows mean ADWG ± SD from 10 replicates. Letters indicate significant differences within each distribution and initial PCV2 prevalence at nursery ($p<0.05$). ADWG: average daily weight gain; NV: not vaccinated; PCV2: porcine circovirus 2.

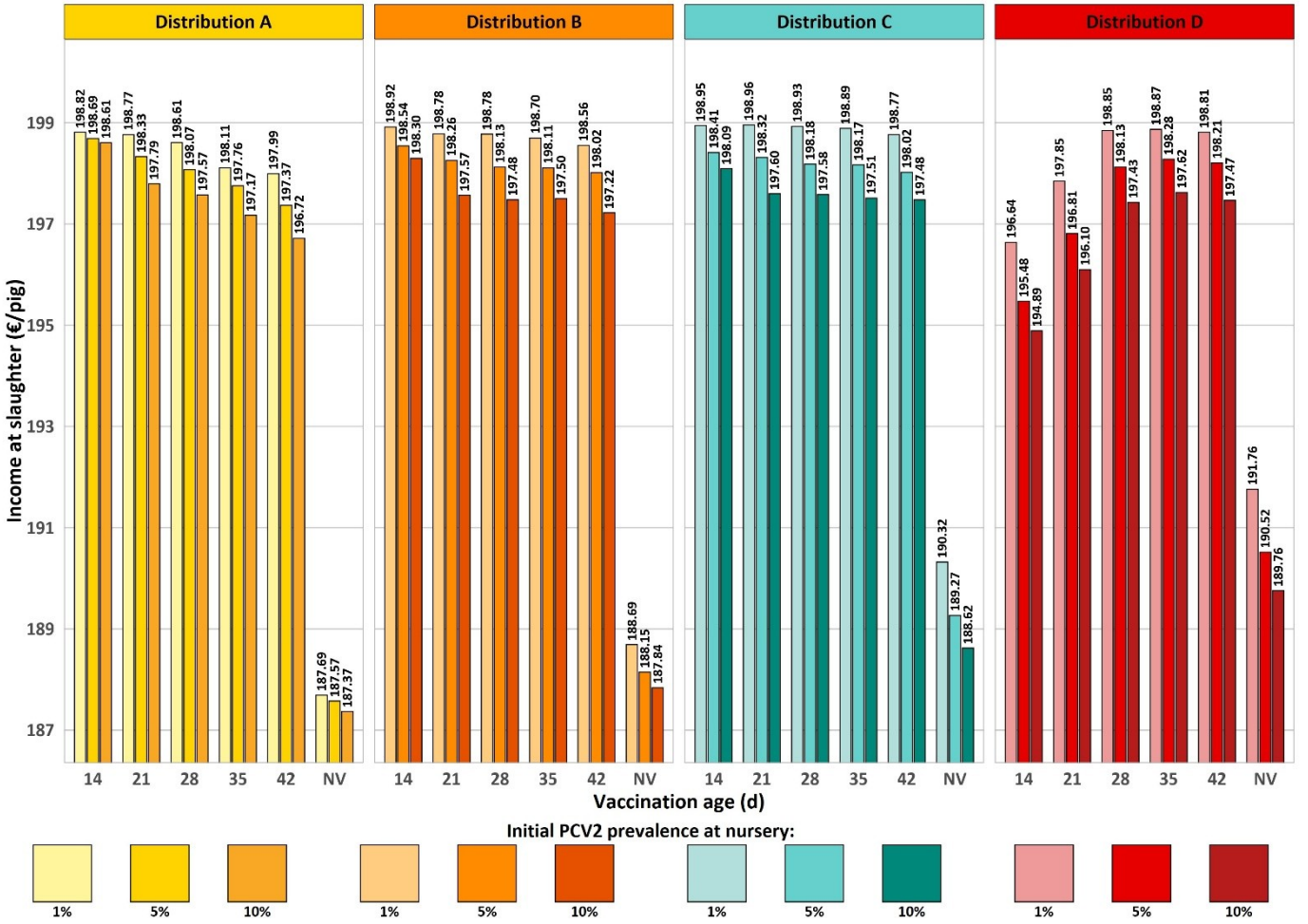


Figure 4.11. Income at slaughter (€/pig) across four MDA distributions (A to D) and three initial PCV2 nursery prevalence levels (1%, 5%, 10%) under five vaccination ages (14, 21, 28, 35 and 42 days of age) and NV scenario. Values were calculated based on mean finishing body weight at day 210 and a market price of 1.70 €/kg live weight. Colour intensity indicates initial prevalence level (light to dark: 1%, 5%, 10%). MDA: maternally derived antibodies; NV: not vaccinated; PCV2: porcine circovirus 2.

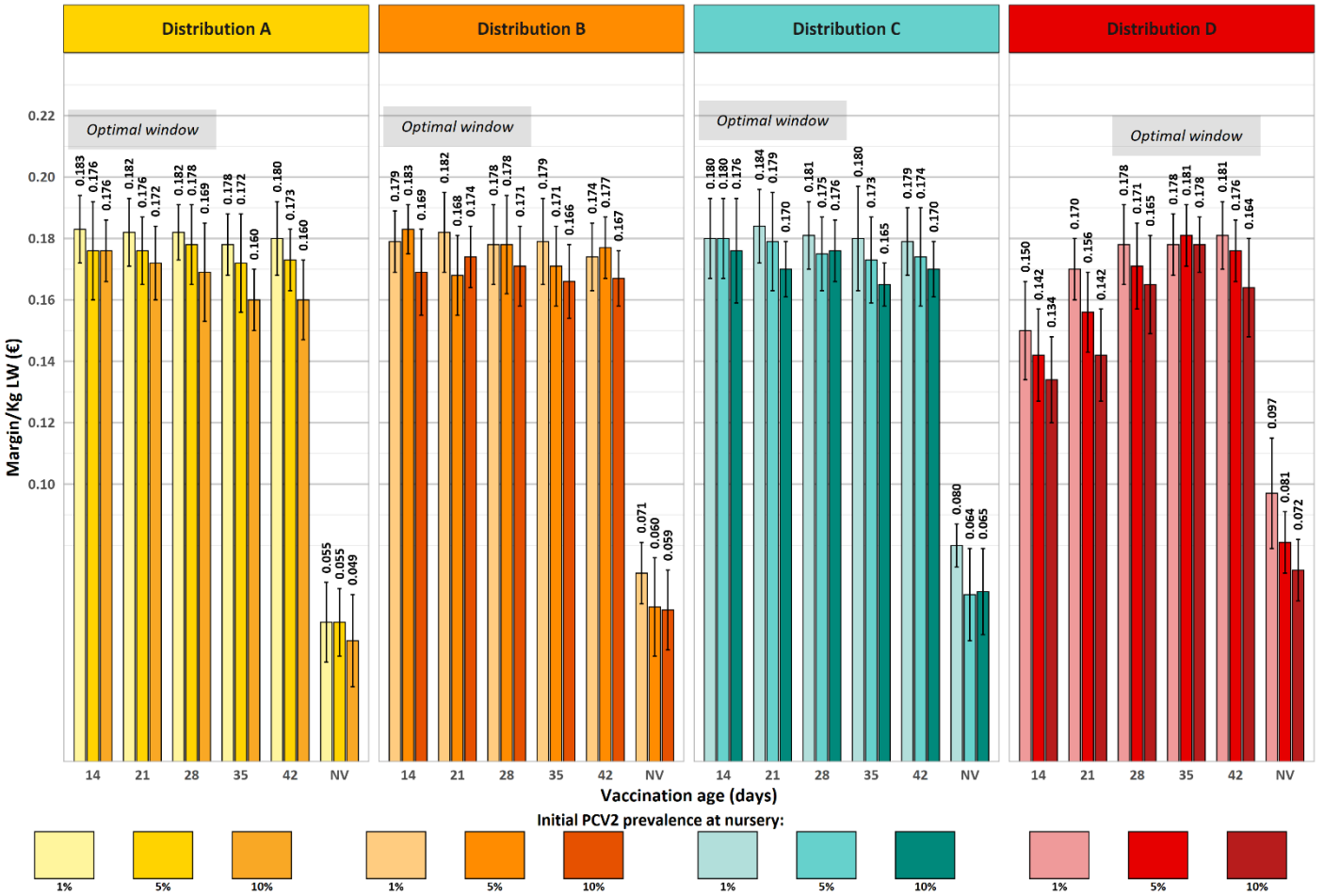


Figure 4.12. Margin/kg LW (€) across four MDA distributions (A to D) and three initial PCV2 nursery prevalence levels (1%, 5%, 10%) under five vaccination ages (14, 21, 28, 35 and 42 days of age) or NV scenario. Bars show the mean margin/kg LW from 10 replicates, with error bars representing ±SD. Colour intensity reflects the initial PCV2 prevalence at nursery (light to dark: 1%, 5%, 10%). Shaded boxes indicate the optimal vaccination window, defined as the range of vaccination ages yielding the highest economic margin within each MDA distribution. LW: live weight; MDA: maternally derived antibodies; NV: not vaccinated; PCV2: porcine circovirus 2; SD: standard deviation.

4.4. Discussion

Porcine circovirus 2 infection dynamics in post-weaning pigs are known to be shaped by the interplay between MDA, infection pressure, and management conditions (Rose et al., 2012). Experimental and field evidence shows that MDA attenuate early PCV2 replication and reduce the likelihood of infection during the first weeks of life (Fort et al., 2009a,b; Oh et al., 2012; Figueras-Gourgues et al., 2019). Very high MDA titers can further extend this protective window and delay susceptibility to infection, although these studies also highlight that such high MDA levels may interfere with vaccine-induced immunity (Haake et al., 2014; Feng et al., 2016). Building on this biological framework, the present model based on a farrow-to-finish farm production system was used to disentangle how contrasting MDA landscapes and infection pressures jointly determine PCV2 transmission dynamics and to evaluate vaccination strategies under these immunological/infection conditions.

Consistent with these expectations, when examining PCV2 dynamics in the absence of vaccination, the model showed that infection patterns were primarily governed by the timing at which pigs became susceptible rather than by differences in the final proportion of infected animals. Higher MDA levels postponed susceptibility to infection and shifted PCV2-SI peaks to later ages, mirroring experimental evidence that MDA delay early replication and modulate infection onset (Fort et al., 2009a,b; Oh et al., 2012; Figueras-Gourgues et al., 2019). In contrast, the model showed that the higher the initial PCV2 prevalence, the sooner the PCV2-SI cases take place. This temporal spacing between MDA distributions was substantial, with peak timing differing by almost two months between the lowest (A) and highest (D) MDA distributions at low prevalence, illustrating the protective window conferred by maternally derived immunity (McKeown et al., 2005). Beyond delaying infection,

high MDA levels also attenuated the peak number of PCV2-SI cases in several scenarios (particularly in the highest-MDA levels, distribution D, at 5% and 10% PCV2 prevalence). This finding indicates that higher MDA do not merely postpone infection but also limit early PCV2-SI case accumulation resulting in lower epidemic peaks, as expected (Poulsen Nautrup et al., 2021). The cumulative PCV2-SI incidence remained consistently high across all scenarios, in line with longitudinal observations from unvaccinated herds where nearly all pigs become infected at some point post-weaning (Rodríguez-Arrioja et al., 2002). In such NV scenario, animals that did not enter PCV2-SI progressed instead to PCV2-SD according to the modelled probability of systemic disease, and the high lethality obtained for PCV2-SD reflects classical descriptions reporting 70–80% case mortality (Segalés and Domingo, 2002). As a result, the proportion of deaths attributable to PCV2-SD remained low compared with total mortality, which was dominated by background losses consistent with reference data for Spanish herds (Observatori del Porcí, 2023).

In addition to MDA, early PCV2 infection pressure at nursery entry is a key determinant of post-weaning infection dynamics, as it conditions the timing and intensity of viral circulation once piglets become susceptible. Previous field studies indicated that PCV2 viremia at or shortly after weaning is highly variable across farms and production contexts, ranging from very low prevalence levels to values exceeding 10% in some epidemiological situations, particularly in herds experiencing concomitant health challenges (Shen et al., 2010; Dvorak et al., 2013). Indeed, recent data from Spanish commercial farms reported detectable PCV2 infection in a minority of animals at weaning, with prevalence increasing at the end of the nursery phase (*Chapter 1*), supporting the use of 1, 5 and 10% prevalence scenarios to represent realistic gradients of infection pressure at nursery entry.

Having established the baseline behaviour of the system under NV conditions, the model allowed the assessment of how vaccination performance shifts across contrasting MDA distributions and PCV2 prevalence at nursery entry. The model indicates that the effectiveness of PCV2 vaccination is conditioned by the interaction between MDA levels and vaccination age. In low-MDA populations (Distribution A), the model reproduces a clear benefit of early vaccination at 14-21 days, which minimizes cumulative PCV2-SI because piglets become susceptible soon after weaning and can rapidly mount active immunity elicited by vaccination, an outcome consistent with empirical studies showing efficient vaccine intake at low MDA titres (Fort et al., 2009a; Seo et al., 2014; Kiss et al., 2021, *Chapter 2*). Conversely, the model shows that in populations with very high MDA levels (Distribution D), early vaccination at 14 days becomes counterproductive: vaccine antigen is neutralised by circulating MDA, leading to insufficient number of pigs seropositive and higher PCV2-SI and PCV2-SD incidence. This behaviour reproduces the interference mechanisms described for VH MDA titres when vaccination is applied between 14-28 days of age (Feng et al., 2016). According to the model, delaying vaccination to 35 days of age in these high-MDA herds could recover vaccine efficacy rates by allowing MDA to decline to permissive levels, thereby sharply reducing both subclinical and clinical diseases.

The model also indicates that patterns of clinical disease are closely tied to vaccination timing relative to MDA decay. When timing is optimal for each MDA profile, PCV2-SD cases in vaccinated populations match the expected contribution from the putative 5% non-immunised fraction, suggesting that residual disease stems primarily from the 5% established management-related non-immunised animals rather than vaccine failure. Observed-to-expected PCV2-SD case ratios below 1.0 in some scenarios reflect herd immunity effects, whereby reduced viral

circulation indirectly protects non-immunised pigs, an effect most pronounced in VH MDA populations vaccinated at 35 days. In contrast, the model shows that vaccinating VH MDA piglets at 14 days produces a tenfold excess of PCV2-SD cases relative to the expected non-immunised contribution, indicating MDA-driven vaccination failure. Overall, the outputs of this model highlight that optimal vaccination is not universal but MDA-dependent: early vaccination benefits low-MDA herds, while VH MDA populations require delayed vaccination. Practically, the model supports MDA-informed vaccination strategies and reinforces the value of serological monitoring and vaccination audits to optimise both timing and coverage to minimise PCV2-AD.

The sensitivity analysis further supports the biological coherence and robustness of the modelling framework. In NV scenarios, infection pressure emerged as the dominant driver of disease-related outcomes, whereas in V populations, vaccine efficacy accounted for most of the variability in epidemiological and productive outputs. In addition, the rate of MDA waning consistently influenced outcomes, particularly in NV animals and in V populations with low initial MDA levels, reinforcing the importance of aligning vaccination timing with declining maternally derived immunity. The limited contribution of parameter interactions indicates that the effects of the main drivers act largely independently, rather than synergistically, thereby facilitating the interpretation of model outputs and increasing confidence in the robustness of the simulated vaccination scenarios.

When production indicators were examined, the model reproduced patterns consistent with published evidence showing that subclinical PCV2-SI impairs growth performance (Kristensen et al., 2011; da Silva et al., 2014). A field study indicated that higher PCV2 viraemia loads were associated with reduced ADWG (≈60–70 g/day in pigs with high PCV2 viral loads) (López-Soria et al., 2014), in line with

previously reported growth penalties of 10–40 g/day associated with PCV2-SI in NV farms (Kristensen et al., 2011). This pattern was reflected in the NV scenarios of this model, where extensive subclinical circulation resulted in a 3-7 kg reduction in finishing BW, with the corresponding decrease in ADWG. In contrast, the impact of PCV2-SD on productive outcomes was minimal because most clinically affected pigs did not survive long enough to reach the end of the finishing period, consistent with the high case lethality described for PCV2-SD. Importantly, the model also reproduced that inappropriate vaccination timing (particularly vaccinating VH MDA populations too early) generated detectable growth penalties of around 1-2 kg in V groups as well. In these scenarios, interference between MDA and vaccine antigen resulted in residual subclinical infection, leading to BW and ADWG values that were significantly lower than those of optimally vaccinated pigs and, in some cases, only marginally better than NV controls. Together, these dynamics indicate that productive losses arise predominantly from subclinical infection, and they emphasize that vaccination timing must be aligned with MDA levels to fully realise its growth-promoting benefits (Kristensen et al., 2011; Poulsen Nautrup et al., 2021).

The economic evaluation consolidated the epidemiological trends observed in the model. In Distributions A–C, early vaccination not only minimized PCV2-SI and improved growth but also translated into the highest net profit per pig and the most favourable margin per kg LW. Conversely, in VH MDA herds (Distribution D), vaccinating too early generated measurable economic losses due to persistent subclinical infection and reduced growth performance. These model-derived margins (0.13–0.18 €/kg LW in vaccinated batches) were consistent with sectoral estimates for Spanish farrow-to-finish systems (SIP Consultors, 2025), although slightly lower in absolute terms because the model allocates mortality-related costs

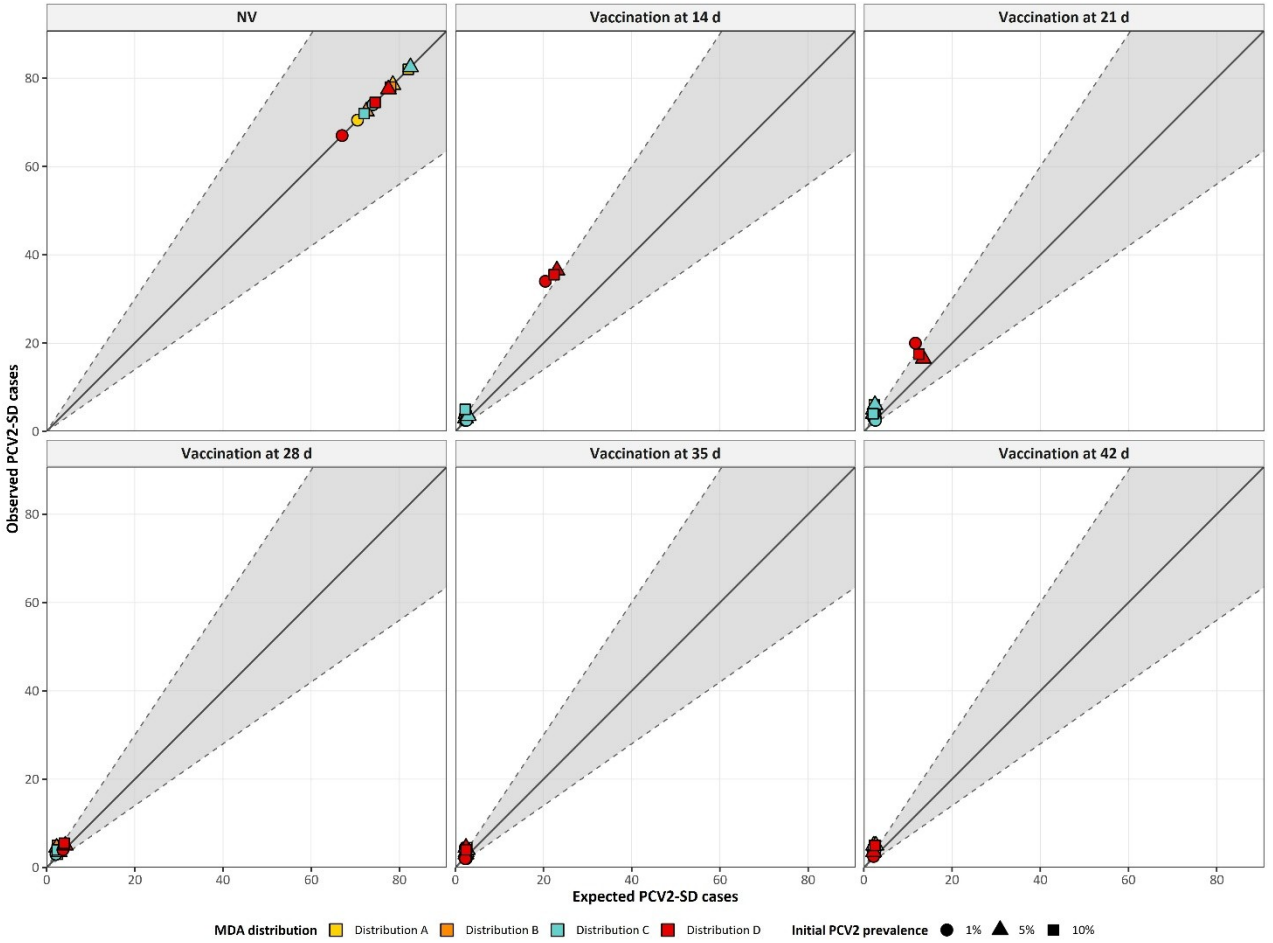
to the income received from animals that reached slaughter. This reduction reflects the high cumulative mortality observed across scenarios, a pattern that mirrors the concerningly elevated death losses currently reported in the Spanish swine sector (Observatori del Porcí, 2023), which have been partly associated with the emergence of highly pathogenic porcine reproductive and respiratory syndrome virus (PRRSV) strains in the same region (Martín-Valls et al., 2023). Such mortality levels increase production costs, compress margins, and highlight the growing economic relevance of improving herd health and optimising vaccination timing. Importantly, although income differences across vaccination ages were modest (<2 €/pig) in Distributions A–C, early vaccination in Distribution D resulted in a 2–4 €/pig loss compared with optimal timing, demonstrating that inappropriate scheduling carries tangible economic penalties despite similar vaccine costs. These results reinforce that vaccination strategy is an economic decision as much as a biological one: aligning timing with the prevailing MDA profile maximises biological protection while improving financial return per pig placed.

Several limitations should be considered when interpreting the outcomes of this modelling framework. First, the model represents a single farrow-to-finish batch and does not capture continuous multi-batch production systems, where overlapping age groups, persistent viral reservoirs, and cross-batch transmission may substantially alter PCV2 dynamics. Thus, the extension of this framework to consecutive batches would allow incorporation of long-term circulation and reinfection processes. Second, co-infections known to potentiate PCV2 replication and disease expression, such PRRSV, PPV, *M. hyopneumoniae*, and SIV, were not included. Indeed, experimental evidence shows that PRRSV and PPV exacerbate PCV2-associated lesions and clinical outcomes (Allan et al., 1999; Rovira et al., 2002; Shi et al., 2008; Butler et al., 2014; Ouyang et al., 2019; Zhao et al., 2021), *M.*

hyopneumoniae enhances viral replication and increases PCV2-SD risk (Saade et al., 2020), and SIV may increase susceptibility through epithelial damage and cytokine imbalance (Montoya et al., 2017; Bakre et al., 2021). Thus, the exclusion of such co-infections likely underestimates the impact of PCV2-AD, but robust parameterisation of these interactions remains unfeasible due to fragmented quantitative evidence. Third, vaccine responses were modelled using a generic efficacy profile, as product-specific data on interactions with MDA decay are scarce and heterogeneous, with only a limited number of studies explicitly comparing vaccine performance across MDA levels (Oh et al., 2012; Feng et al., 2016; Kiss et al., 2021, *Chapter 2*), and substantial variability in qPCR methods, ELISA kits, S/P thresholds, and study designs, precluding quantitative consensus. Accordingly, vaccine–MDA interactions were simplified as a reduced probability of successful immunisation in piglets with VH MDA levels, acknowledging that real-world responses may be more nuanced and product-dependent. Finally, although biological processes were parameterised from peer-reviewed evidence, several numerical inputs are inherently farm-specific such as baseline prevalence, infection pressure at nursery entry, MDA distributions, probability of successful vaccine administration, and growth-related variability. Therefore, their adaptability is a strength of the framework, which has been explicitly designed to incorporate herd-specific ELISA (MDA quantification) and qPCR prevalence (infection pressure) as external inputs when applied in commercial settings.

Future developments should prioritise the incorporation of multi-batch farm structure and the direct integration of herd-level serological and virological data into a decision-support system capable of generating tailored vaccination recommendations. In this envisioned framework, PCV2 ELISA at weaning (MDA assessment) and qPCR prevalence at nursery entry (infection pressure) would serve

as key inputs to simulate PCV2 dynamics. The resulting simulation would enable the comparison of vaccination strategies and the selection of the most appropriate schedule according to each farm's specific epidemiological and serological context, as well as the estimation of the associated economic impact. These extensions should not replace the current findings but should broaden their applicability, allowing the framework to evolve from a validated conceptual model into an operational, data-driven tool for PCV2 control. The consistency observed across epidemiological, productive, and economic outputs further supports this transition and underscores the model's value as a foundation for practical implementation under commercial conditions.



SF4.1. Comparison of observed versus expected PCV2-SD cases across intervention scenarios. Each panel represents a different intervention strategy (NV or vaccination at 14, 21, 28, 35, and 42 days of age). Expected cases (total number of cases) were calculated based on the *default_proba_success_vacc* and the PCV2-SD incidence rate observed in NV populations. The solid diagonal line indicates perfect agreement (ratio = 1.0), where observed cases equal expected cases. The shaded area represents the range where observed/expected ratio falls between 0.7 and 1.5, indicating that PCV2-SD cases may be attributable to the *Non_IMM* fraction (*default_proba_success_vacc*). Points below this area suggest possible herd immunity effects, while points above indicate disease occurrence beyond what would be expected from *Non_IMM* animals alone. Colours represent MDA distributions (A–D); shapes represent initial PCV2 prevalence levels (1%, 5%, 10%). *Non_IMM*: non immunised; MDA: maternally derived antibodies; PCV2-SD: porcine circovirus 2 systemic disease.

The *Supplementary Files 4.1, 4.2, 4.3, and 4.4* are available in the following public repository: https://github.com/monicasagrera/PCV2-model.git. Descriptions and content are:

- *Supplementary File 4.1.* Initial PCV2 scenario conditions of the analysed simulation. This file contains the full parameterisation of all simulated scenarios, including MDA distributions (A–D), intervention strategies (NV, 14, 21, 28, 35, 42 days), infection-pressure levels at nursery entry (1%, 5%, 10%), and baseline PCV2 prevalence at farrowing. It provides the complete factorial structure of the experiments, and all model inputs used to initialise the simulations.
- *Supplementary File 4.2.* Sensitivity analyses of the model. This file presents the sensitivity analyses performed to evaluate the robustness of model outputs. It includes parameter ranges, methodological approach, and resulting variations in PCV2-SI, PCV2-SD, mortality, and growth performance.
- *Supplementary File 4.3.* Vaccination coverage, NV fraction, and PCV2-SD infection outcomes in vaccinated populations. This file reports detailed epidemiological outputs for all vaccination scenarios, each simulated with 10 stochastic replicates, including the proportion of NV pigs in vaccinated scenarios due to *default_proba_success_vacc*, cumulative PCV2-SI and PCV2-SD incidence, and PCV2-SD–associated mortality across all combinations of MDA distribution, vaccination age, and initial prevalence. For each scenario, the file provides the observed number of PCV2-SD cases in V populations and compares them with the expected values derived from the NV fraction and the corresponding incidence observed under fully NV conditions. Observed-to-expected ratios are presented to identify whether residual PCV2-SD cases are fully explained by the NV fraction, indicate potential indirect protection, or reflect additional disease due to MDA interference.

- *Supplementary File 4.4.* Economic outputs per scenario. This file includes the full economic breakdown for each scenario: cost structure by production phase, accumulated costs for survivors and dead pigs, revenue at slaughter, net profit per initial pig placed, real and theoretical margins per kg LW, and ranking of optimal vaccination ages under both cost perspectives. For each combination of initial prevalence and MDA distribution, the vaccination age yielding the highest net profit is highlighted in green.

General discussion

Despite nearly two decades of widespread vaccination against PCV2, this virus remains a globally ubiquitous pathogen in modern swine production, where it continues circulating (Guo et al., 2022; Segalés and Sibila, 2025). Although current vaccines have significantly reduced the incidence of PCV2-SD, they do not provide sterilising immunity, and viral persistence and transmission still occur within herds (Segalés, 2015; Segalés and Sibila, 2022). Under these conditions, the epidemiological and immunological landscape of PCV2 infection is less characterised by overt outbreaks and more by PCV2-SI. However, such subclinical scenarios, at least in unvaccinated populations, have been repeatedly shown to impair growth performance and productive economic efficiency (Segalés et al., 2005a; Alarcon et al., 2013a,b; López-Soria et al., 2014; Boeters et al., 2023).

The persistence of PCV2 in the population is favoured by its high transmission rate and remarkable environmental stability, which enable sustained viral presence also in vaccinated herds (Rose et al., 2012; Dvorak et al., 2013; López-Lorenzo et al., 2019, 2022). Moreover, the frequent co-circulation of immunomodulatory pathogens such as PRRSV, PPV and *M. hyopneumoniae* can exacerbate PCV2 infection dynamics and clinical expression, often acting as disease-enhancing cofactors (Holtkamp et al., 2013; Segalés, 2012; Boeters et al., 2023). This multi-pathogen context is central to PCV2 epidemiology in contemporary production systems.

Within this scenario, MDA provides partial protection against early PCV2 infection and contributes delaying viral circulation to older piglets (Rodriguez-Arrioja et al., 2002; McKeown et al., 2005; Larochelle et al., 2003; Figueras-Gourgues et al., 2019). However, high MDA levels at the time of vaccination may interfere with the induction of active immunity, particularly affecting vaccine-induced seroconversion (Haake et al., 2014; Feng et al., 2016). Therefore, the balance between protection

and interference makes the dynamics of passive immunity a central parameter when designing and optimising PCV2 vaccination programmes (Martínez-Boixaderas et al., 2022).

The data presented in *Chapter 1* demonstrated that under field conditions, the level of MDA at weaning is not uniform across monitored farms. This variability is influenced by herd immunity, vaccination practices, and the concurrent presence of immunomodulatory pathogens such as PRRSV, as specifically addressed in that study. However, a limitation of the obtained field data is that detailed information on sow vaccination protocols and immunisation history was not available, which would have provided additional insight into the determinants of MDA heterogeneity at weaning. Herds showing lower MDA levels in piglets prior to PCV2 vaccination exhibited a higher frequency of early-life PCV2 viremia, indicating an increased window of susceptibility before effective active immunity could be induced. In contrast, herds with higher MDA levels tended to show delayed onset of infection, although this may also influence the response to vaccination depending on the timing of application (Segalés and Sibila, 2022).

Moreover, farms with active PRRSV circulation showed a greater likelihood of early PCV2 infection. This finding reinforced the concept that PRRSV acts as a potent immunomodulatory cofactor, capable of impairing innate immune responses and altering lymphoid microenvironments, eventually facilitating PCV2 replication and persistence. This interaction between PCV2 and PRRSV is consistent with previous experimental and field studies describing synergistic interaction between these pathogens, where coinfected pigs exhibit increased PCV2 viremia, more severe lymphoid lesions, and reduced growth performance compared to single infections (Allan et al., 1999; Allan et al., 2000b; Rovira et al., 2002; Shi et al., 2008; Opriessnig and Halbur, 2012; Park et al., 2014; Ouyang et al., 2019; Zhao et al., 2021).

Collectively, these findings indicated that PCV2 infection dynamics arise from herd-level processes, where the interplay between MDA profiles, vaccination practices and coinfection pressure determine the age at which pigs become susceptible to PCV2 and the extent of viral circulation (Holtkamp et al., 2013; Segalés, 2015). Obtained results reinforce the importance of adapting vaccination strategies to the immunological context of each herd, particularly through monitoring MDA to identify the optimal immunisation window (Segalés, 2015; Segalés and Sibila, 2025). In this framework, piglet vaccination acts as a targeted intervention to reshape susceptibility over time, aiming to induce active immunity as passive protection declines (Martínez-Boixaderas et al., 2022). Whether vaccination can achieve this objective in the presence of variable MDA levels was the central question addressed experimentally in *Chapter 2*.

The experimental study described in *Chapter 2* supports the notion that piglet vaccination remains effective even in the presence of high levels of MDA under a subclinical infection scenario. Piglets vaccinated at three weeks of age with the combined PCV2d and *M. hyopneumoniae* vaccine exhibited, regardless the level of MDA, significantly lower PCV2 loads in serum and lymphoid tissues, together with reduced distribution of PCV2 genome in TBLN quantified by RNAscope ISH, compared to NV animals. Under the subclinical infection conditions of this study, LD and HR were infrequent in both groups. Therefore, the main effect of vaccination was expressed as restriction of viral replication and tissue dissemination, rather than as overt lesion prevention. These observations align with the concept of virological protection, whereby vaccination reduces viral burden and limits lesion progression, also when infection is not completely prevented (Kerkhofs et al., 2003; Bonckaert et al., 2016; Tan et al., 2022).

From an immunological perspective, the experimental results are consistent with previous studies in which PCV2 protection is understood as the result of coordinated humoral and cellular immune mechanisms rather than antibody titres alone (Meerts et al., 2006; Trible and Rowland, 2012; Shi et al., 2021). While PCV2-specific antibodies are important, they do not fully avoid viral replication (Meerts et al., 2006; Trible and Rowland, 2012), reinforcing the relevance of CMI mediated by IFN-γ-secreting Th1 cells (Fort et al., 2009a; Martelli et al., 2011; Ferrari et al., 2014).

The cytokine profiles further support the interpretation that vaccination modifies the quality rather than the magnitude of the immune response to PCV2. Vaccinated pigs displayed an earlier and more coordinated Th1-oriented activation, reflected in controlled induction of IFN-γ, IL-12 and IFN-α, which aligns with the establishment of memory T cell responses capable of limiting viral replication upon exposure (Fort et al., 2009b; Martelli et al., 2011; Ferrari et al., 2014). In contrast, NV pigs exhibited a delayed, virus-driven inflammatory response following infection, particularly in animals experiencing higher PCV2 replication (H-NV group), a pattern that could reflect immune dysregulation associated with insufficient viral control (Darwich et al., 2003b; Segalés, 2012; Kekarainen and Segalés, 2015; Du et al., 2018; Fehér et al., 2023).

The dynamics of IL-4 and IL-10 further illustrate the functional divergence between vaccine-primed and infection-driven responses. The earlier IL-4 increase in vaccinated pigs may be compatible with B-cell proliferation and the establishment of a sustained humoral response, while the later IL-4 rise in NV animals likely reflects compensatory immunoregulation in response to higher viral loads (Quereda et al., 2013). Similarly, the moderate IL-10 elevation observed in V pigs may represent controlled regulatory feedback that tempers inflammation while preserving antiviral control (Du et al., 2016; Kekarainen and Segalés, 2015), whereas NV

animals IL-10 is classically associated with dysregulated immune environments in the context of persistent viral replication (Darwich et al., 2003b; Fehér et al., 2023).

Importantly, when comparing groups stratified by both vaccination status and MDA levels, the H-V group exhibited the most controlled immune profile with the lowest viral loads and minimal inflammatory cytokine elevation, despite having the highest initial MDA. This contrasts with H-NV animals, which showed the highest viral loads and most pronounced inflammatory responses (IFN-γ, TNF-α and IL-12), suggesting that high MDA alone, without vaccination, do not confer adequate protection. The L-V group achieved effective viral control with a balanced cytokine response, whereas L-NV animals displayed intermediate outcomes. These group-level patterns reinforce that vaccination efficacy was not compromised by high MDA levels, and that the combination of vaccination and MDA provides additive rather than interfering protection. Taken together, the immunological patterns observed in *Chapter 2* reinforce the idea that the primary effect of piglet vaccination under contemporary PCV2 circulation is not the prevention of infection *per se*, but the reprogramming of the host response towards early, controlled antiviral activation, limiting viral amplification and preserving immune function and performance. It should be noted that these observations were obtained under a subclinical infection scenario characterised by relatively low viral loads. Under conditions of higher infection pressure or more pronounced viraemia, differences between groups (particularly regarding the interaction between vaccination and MDA levels) might be expected to become more evident.

An intriguing observation from *Chapter 2* was the absence of detectable interference between MDA and vaccine-induced humoral immunity, in contrast to previous studies reporting impaired seroconversion when piglets with high MDA levels are vaccinated at 3-4 weeks of age (Haake et al., 2014; Feng et al., 2016).

Several factors may explain this discrepancy. First, the MDA levels in our study population, although stratified into high and low categories based on S/P ratios, may not have reached the very high threshold at which interference has been described (Feng et al., 2016). Second, the ELISA used to measure antibody responses (Ingenzim, based on PCV2a antigen) may underestimate the seroconversion induced by a PCV2d-based vaccine due to partial antigenic mismatch. Thus, the use of a homologous PCV2d-based ELISA (such as Biochek) might have revealed more nuanced patterns of immune kinetics. Importantly, the model presented in *Chapter 4* predicts that interference would become more pronounced if sow vaccination protocols were intensified, resulting in overall higher piglet MDA levels. Under such conditions, early piglet vaccination could be expected to fail more frequently, reinforcing the need to monitor MDA levels and adjust vaccination timing accordingly.

Because vaccination limits PCV2 replication without fully preventing infection, characterising low-grade viral persistence in tissues becomes essential, particularly under the subclinical conditions that dominate in contemporary herds (Segalés and Sibila, 2025). In *Chapter 2*, the TBLN was selected for virological and pathological evaluation. This selection was based on the longstanding empirical experience of the research group, which had consistently observed that TBLN tends to harbour higher PCV2 loads than other lymphoid tissues following PCV2 oronasal infection. However, despite this practical recognition, this tissue-specific predominance had not yet been formally demonstrated in a comparative manner, a gap that *Chapter 3* was specifically designed to address.

Moreover, the findings from *Chapter 2* highlighted a noticeable discrepancy between relatively high viral loads and RNAscope ISH signal in TBLN, and the minimal or absent lymphoid lesions detected by classical histopathology. This

mismatch reinforces the notion that lesion-based diagnostic criteria may underestimate evidence of infection under subclinical conditions and raises an important question: if viral replication can persist in lymphoid tissues without producing detectable lesions, PCV2-SI may be considerably more prevalent in vaccinated herds than currently recognised. The true impact of such widespread low-grade infection remains poorly understood; in case that may have modest effects on growth efficiency, when scaled across large populations, such low-grade infection could translate into substantial economic losses that go undetected by conventional diagnostic approaches.

Both gaps (the lack of formal tissue comparison and the need to better characterise low-grade viral persistence) provided the motivation for *Chapter 3*, which was specifically designed to address these questions through comparative quantification across TBLN, ISLN, MSLN and TO tissues of animals included in the study of *Chapter 2*. The investigation was further expanded by the inclusion of samples from an additional 22 animals with comparable characteristics to increase the number of samples available for lesion assessment. Notably, RNAscope ISH has been shown to offer higher sensitivity than conventional ISH or IHC for detecting PCV2 genome within lymphoid tissues (Cobos et al., 2024), supporting its use when evaluating viral persistence in the absence of marked lesions. This work demonstrated for the first time that TBLN consistently exhibits the highest PCV2 genomic burden under the studied conditions, thereby providing a formal biological basis for its routine selection as a sentinel tissue in experimental and diagnostic settings. Although the exact mechanisms underlying this tissue distribution remain unclear, the higher PCV2 genomic burden observed in the TBLN may be explained by anatomical and functional factors. The TBLN drains a large portion of the respiratory tract, which represents a major interface for PCV2 exposure under field conditions, potentially

resulting in sustained antigenic input and viral accumulation. In contrast, tonsils (while recognised as key sites of PCV2 persistence) are thought to represent a distinct immunological niche that may favour long-term viral maintenance rather than consistently high genomic loads (Darwich et al., 2003b; Segalés, 2012).

The evaluation of PCV2 signal in TBLN in *Chapter 2* was based on visual scoring (0-3), a widely used but semi-quantitative and partly subjective method that limits the ability to detect subtle or intermediate differences in viral presence (Rosell et al., 1999; Chianini et al., 2003; Szczotka et al., 2011). To overcome this limitation and to obtain continuous, reproducible measurements, *Chapter 3* implemented a digital score ISH quantification approach, expressing PCV2 signal as the percentage of labelled area within lymphoid tissue. This was supported by the development of a pixel-based classifier that allowed automated and standardised identification of PCV2-positive signal across samples. This method provided a more precise and objective characterisation of tissue viral burden than categorical scoring alone, particularly under subclinical infection conditions where classical lesions may be minimal.

Although this digital RNAscope ISH quantification provides a highly sensitive and objective means of detecting low-grade viral persistence, it remains a laboratory-intensive and costly approach, requiring specialised reagents, equipment, and image analysis infrastructure. For this reason, it is not intended for routine diagnostic use at the farm or field level. Instead, its value is conceptual rather than operational: it helps to clarify the biological substrate of PCV2-SI, demonstrating how viral replication can persist despite minimal lesions and in the presence of vaccine-induced immunity. This improved understanding does not translate into routine diagnostic work and direct on-farm decision-making, but it provides a more

robust interpretative framework for understanding infection dynamics under current vaccination conditions.

While these findings deepen the understanding of PCV2 replication and immune control at the individual and tissue levels, the practical challenge in the field arises at the herd scale, where the timing of exposure, the decay of MDA, and the scheduling of vaccination interact to shape the window of susceptibility. Importantly, this window is not fixed but shifts according to the immunological status of the breeding herd and the infection pressure in the farm environment.

As a result, piglet vaccination cannot be understood as a single-age intervention, but rather as a measure that must be positioned within the dynamic landscape of MDA decline and expected exposure timing. In practice, vaccination programmes can be adapted at the sow and/or piglet level at the herd scale, particularly in production systems with established sow vaccination schemes. In these settings, MDA monitoring can be used to inform the alignment of immunisation timing with the window of susceptibility in each herd (Segalés, 2015; Segalés and Sibila, 2022). This population-level perspective provides the conceptual basis for *Chapter 4*, in which a stochastic individual-based model was developed to represent and quantify PCV2 infection dynamics and its consequences at productive and economic levels.

The designed model integrates MDA decay, PCV2 transmission, and vaccination response, and simulates how different baseline MDA levels (resulting from sow vaccination or natural exposure) interact with piglet vaccination timing and herd infection pressure. In doing so, it generates epidemiological outcomes with varying prevalence of PCV2-SI and PCV2-SD and estimates effects on BW and expected vaccine performance. By moving from qualitative reasoning to mechanistic

simulation, this framework provides a quantitative basis for adapting vaccination to herd-specific conditions.

The model demonstrated that, in the absence of vaccination, infection patterns were governed primarily by the timing at which pigs became susceptible rather than by differences in the proportion of animals eventually infected. Higher MDA levels postponed susceptibility and shifted PCV2-SI peaks to later ages, whereas higher initial PCV2 prevalence advanced the timing of infection. These patterns are consistent with experimental evidence showing that MDA delay viral replication and modulate infection onset (Fort et al., 2009; Oh et al., 2012; Figueras-Gourgues et al., 2019), while increased infection pressure accelerates transmission dynamics. Beyond delaying infection, high MDA levels also attenuated peak case numbers, indicating that maternal immunity not only postpones but also limits early PCV2-SI accumulation, resulting in lower epidemic peaks (Poulsen Nautrup et al., 2021). However, cumulative PCV2-SI incidence remained consistently high across all NV scenarios, in line with longitudinal observations from unvaccinated herds where nearly all pigs become infected post-weaning (Rodríguez-Arrioja et al., 2002).

Vaccination performance was highly dependent on the interaction between MDA levels and vaccination age. In low-MDA populations, early vaccination at 14-21 days minimised cumulative PCV2-SI because piglets became susceptible soon after weaning and could mount active immunity rapidly, consistent with empirical studies showing efficient vaccine uptake at low MDA titres (Fort et al., 2009; Seo et al., 2014; Kiss et al., 2021). In contrast, in the experimental setting of *Chapter 2*, piglets classified as having high MDA levels at vaccination exhibited effective virological protection when vaccinated at three weeks of age, with reduced viral loads and tissue dissemination compared to non-vaccinated animals. These results indicate that the high MDA titres observed in *Chapter 2* remained within a permissive range

that did not compromise the efficacy of the tested vaccine, in line with the model predictions for high (but not extreme or very high) MDA scenarios.

Conversely, in very high MDA populations, early vaccination at 14 days became counterproductive: vaccine antigen was neutralised by circulating MDA, leading to insufficient seroconversion and higher PCV2-SI and PCV2-SD incidence, reproducing the interference mechanisms described for very high MDA titres (Feng et al., 2016). According to the model, delaying vaccination to 35 days of age in these high-MDA herds recovered vaccine efficacy by allowing MDA to decline to permissive levels.

Based on these results, observed findings in *Chapter 2* indicate that the experimental outcomes observed are consistent with the modelled dynamics in *Chapter 4*. Vaccine interference was predicted under very high MDA levels, corresponding to titres substantially higher than those observed in the experimental study. Although piglets in *Chapter 2* were classified as having high MDA levels, their antibody titres did not reach the range at which interference is anticipated in the model. Therefore, the absence of detectable interference in the experimental setting aligns with the model predictions. These results reinforce the central message that optimal vaccination timing is not universal but MDA-dependent.

Production indicators reproduced patterns consistent with published evidence showing that subclinical PCV2-SI impairs growth performance (Kristensen et al., 2011; López-Soria et al., 2014). The model estimated 3-7 kg reductions in finishing BW in NV scenarios with extensive subclinical circulation, and notably, inappropriate vaccination timing in very high MDA populations also generated detectable growth penalties of around 1-2 kg. In contrast, in the experimental setting of *Chapter 2*, no statistically significant differences in BW or ADWG were detected between

vaccinated and non-vaccinated groups. This apparent discrepancy may be explained by the short observation period, which constrains the ability to detect modest production effects at the individual level.

The modelling approach in *Chapter 4* extends these experimental observations by extrapolating such subtle individual-level effects to the population scale, demonstrating how small, early differences in viral replication and timing of infection (similar to those observed in *Chapter 2*) can accumulate over time and translate into meaningful losses in finishing BW and economic performance at the herd level. This interpretation is further supported by the field data from *Chapter 1*, where piglets were vaccinated at 3-4 weeks of age under moderate-to-low and highly variable MDA levels, and where subclinical PCV2 circulation was frequently detected later in the nursery phase without overt clinical disease.

The economic evaluation consolidated these trends: early vaccination in low-to-moderate MDA herds translated into the highest net profit per pig, whereas vaccinating too early in very high MDA herds generated measurable losses due to persistent subclinical infection and reduced growth. Thus, these findings indicate that the absence of significant production differences in controlled experimental settings does not contradict the biological relevance of PCV2-SI, but rather highlights the added value of population-level modelling to capture cumulative productive and economic impacts that are difficult to quantify experimentally. In this context, vaccination strategy emerges as an economic decision as much as a biological one, and aligning vaccination timing with the prevailing MDA profile maximises both biological protection and financial return.

The model therefore acts as a bridge between the biological insights from *Chapters 1-3* and practical decision-making. It translates patterns of MDA decline and viral

circulation into predictions of when pigs become susceptible and how vaccination can most effectively shift this window. Importantly, it is grounded in field-accessible inputs: MDA levels could be assessed by PCV2 ELISA at approximately two weeks of age (before PCV2 vaccination), and herd infection pressure by PCV2 qPCR on pooled sera.

From a practical field perspective, when the objective is to detect PCV2 circulation under subclinical conditions, a unified sampling approach can be applied for both MDA profiling and virological surveillance. Based on calculations using standard epidemiological tools (de Blas et al., 2006), testing approximately 30 piglets per batch at around 3–4 weeks of age enables the detection of a theoretical 10% prevalence with 95% confidence. This sampling size can be adjusted according to the expected prevalence in the target population: for instance, detecting a 25% prevalence requires only 10–12 samples, whereas detecting lower prevalences (5–10%) demands larger sample sizes (30 or more). Individual sera should be analysed by PCV2 ELISA to characterise both the level and variability of passive immunity within the batch. For PCV2 qPCR, these same samples can be processed in pools of five sera each, as this pool size has been shown to maintain adequate diagnostic sensitivity for herd-level surveillance (Cortey et al., 2011b); in contrast, larger pool sizes (≥20 samples) markedly compromise detection capacity.

As an example, the epidemiological study described in *Chapter 1* applied a flexible sampling strategy adapted to herd size, with 10 samples collected in farms with fewer than 1,000 sows and 30 samples in larger production systems. This approach was designed to detect a theoretical 25% seroprevalence of PCV2 and 10–25% frequency of infection for PRRSV at different ages with 95% confidence. In practice, the exact sampling intensity should be defined on a case-by-case basis, considering herd size, batch structure, and the expected prevalence to be detected. Combining

virological and serological measures from the same sampling enables estimation of the expected susceptibility window for each batch, allowing vaccination strategies to be tailored to the immunological context of individual farms.

The model's outputs must be interpreted within the assumptions underlying its construction. The current version focuses on PCV2 as a primary agent and does not yet include the immunomodulatory influence of coinfections such as PRRSV or *M. hyopneumoniae*, nor the effects of host genetics or environmental stressors, which are simplified for tractability. As such, the predictions it generates are probabilistic guides rather than prescriptive rules and should be integrated with diagnostic monitoring and expert veterinary assessment.

Future developments will focus on incorporating coinfection dynamics, particularly the synergistic and regulatory interactions associated with PRRSV and *M. hyopneumoniae*, as well as calibrating the model with longitudinal field data to refine parameter estimates and improve predictive accuracy across different production systems. Ultimately, this evolution may allow the model to function as a dynamic decision-support tool, where routine ELISA- and qPCR-based monitoring informs adaptive, farm-specific (and ideally batch-specific) vaccination strategies. Such an approach would operationalise precision vaccination, aligning immune protection with the critical window of susceptibility and reducing the risk of PCV2 infection and its associated productive impacts.

Conclusions

1. Porcine circovirus 2 maternally derived antibodies showed high variability across commercial herds. This heterogeneity was associated with differences in PCV2 circulation, highlighting the dynamic interplay between herd immunity and viral pressure.
2. A higher frequency of PRRSV and PCV2 detection occurred in 2022 compared to 2020, including a higher incidence of coinfections (60% of PRRSV-positive farms were also PCV2-positive in 2022 versus 15% in 2020). These findings highlight the relevance of PRRSV circulation as a factor associated with PCV2 infection and underscore the need for continuous health monitoring to detect changes in infection dynamics and guide timely interventions.
3. Vaccination of piglets with a PCV2d-based vaccine provided effective virological protection under subclinical PCV2 infection conditions, significantly reducing PCV2 replication in serum and TBLN compared to NV animals, in piglets with low or high MDA levels at vaccination age.
4. Vaccination efficacy was not compromised by high MDA levels. The H-V group exhibited the lowest viral loads in serum and TBLN throughout the study and the lowest viral loads were detected in TBLN. In contrast, H-NV animals showed the highest viral burden and the highest pro-inflammatory cytokine concentrations (IFN-α, IFN-γ, TNF-α, and IL-12) at 3 wpc, despite similar initial MDA levels at vaccination.
5. Vaccinated animals displayed earlier Th1-oriented immune activation, with progressive increases in IFN-α, IFN-γ, TNF-α and IL-12 from vaccination to 5 wpv, followed by stabilisation after challenge. In contrast, NV pigs exhibited a delayed but more intense inflammatory response, with significantly higher peak values at 3 wpc.

6. Among lymphoid tissues, the TBLN consistently exhibited the highest PCV2 genomic load, confirming its value as a sentinel tissue for assessing viral replication following oronasal infection.
7. The digital quantification of PCV2 by RNAscope ISH combined with a pixel-based classifier enabled precise and objective measurement of viral distribution within lymphoid tissue samples. This methodology minimizes potential observer bias and facilitates reproducible quantification of PCV2 viral load.
8. The stochastic individual-based model demonstrated that PCV2 infection dynamics is primarily governed by the timing at which pigs become susceptible rather than by the proportion of animals eventually infected. Both MDA levels and infection pressure significantly influenced this timing but in opposite directions: higher MDA levels delayed the loss of passive protection and shifted PCV2-SI peaks to later, whereas higher initial prevalence at nursery entry advanced infection peaks within each MDA distribution.
9. The model indicated that optimal vaccination timing is MDA-dependent rather than universal: in low to high MDA populations, early vaccination at 14–21 days maximised protection by immunising piglets before the loss of passive immunity, whereas in very high-MDA populations, delaying vaccination to 28–42 days allowed MDA to decline to permissive levels, thereby recovering vaccine efficacy and minimising PCV2-SI and PCV2-SD incidence.
10. Optimal vaccination timing yielded consistent economic benefits: in low-to-high MDA populations, vaccination at 14–28 days achieved margins of 0.16–0.18 €/kg LW, while in very high-MDA populations, delaying vaccination to 35–42 days was required to obtain comparable economic outcomes. Conversely, early vaccination (14 days) in very high-MDA populations reduced margins to 0.13–0.15 €/kg LW, representing an economic penalty of 2–4 €/pig compared with vaccination at 28–42 days.

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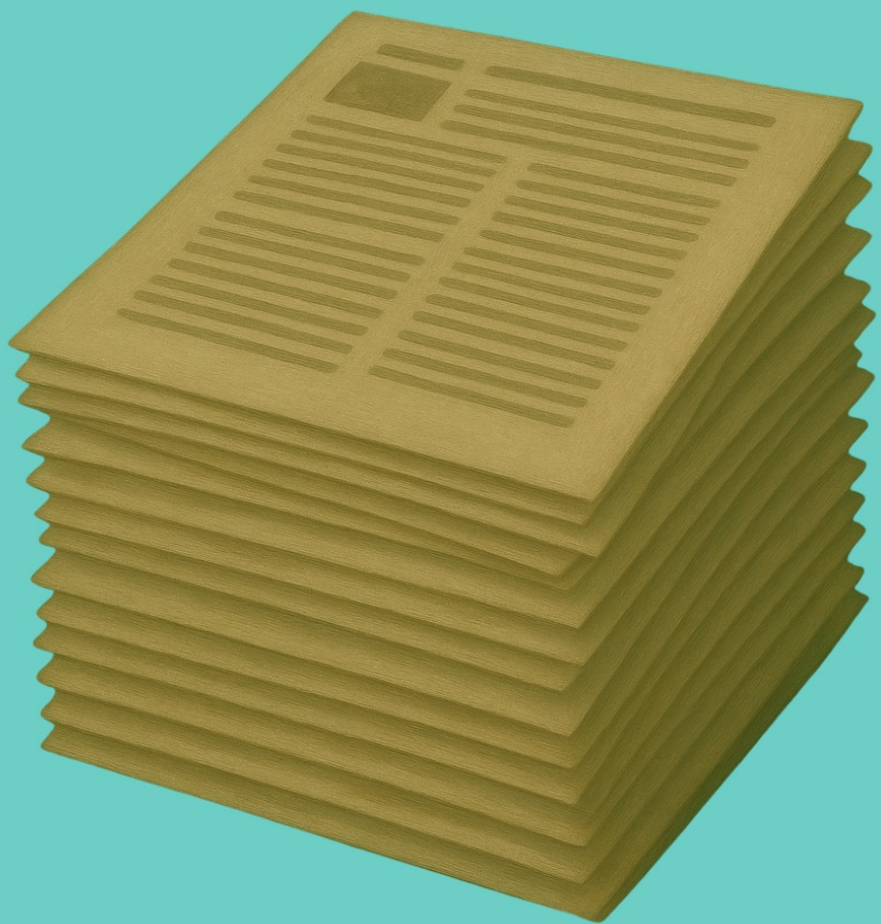

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EPILOGUE: REFLECTIONS ON THE DOCTORAL JOURNEY

This epilogue is originally written in Catalan and is followed by Spanish and English translations.

[Catalan]

Ara que el camí arriba al seu final és inevitable mirar enrere. Aquesta tesi és molt més que una successió de capítols, experiments i resultats; és un recorregut vital fet de decisions, d'esforç sostingut, de dubtes, d'aprenentatges i, sobretot, de persones. No tot allò que defineix un camí com aquest es pot explicar amb dades, figures o conclusions. Algunes coses només es poden comprendre (i compartir) a través de contes, com els que li llegim a la Blanca, recollits al llibre *Avui seré millor que ahir: contes per educar el caràcter* (Ballaz, 2021).

Al primer, que es diu "Els dos alpinistes", la Yang, una jove valenta i decidida, vol escalar el Teng-Su tota sola per demostrar la seva força. El seu oncle Yu, un alpinista experimentat però ja gran, li proposa un repte senzill: ell pujarà primer i ella sortirà després. Tot i l'entusiasme i la rapidesa de la Yang, l'esforç excessiu la deixa exhausta, i arriba al cim defallent, on l'oncle l'espera. Aquella experiència li fa entendre que la determinació no és suficient sense preparació i constància. Com recorda Júlia Navarro, «hi ha un moment a la vida en què hem de buscar dins nostre per decidir». Decidir és el primer pas, però només l'esforç sostingut, la perseverança i el temps permeten escalar una muntanya.

Una altra història, la del conte "La crida d'una cançó", ens parla de la Irina, que viu amb la seva àvia prop d'un bosc on té prohibit entrar, perquè temps enrere el seu germà Yossi s'hi va perdre. Un dia, guiada per una intuïció més forta que la por, s'endinsa al bosc. Per apaivagar el silenci i la incertesa, comença a cantarellar una melodia que la seva mare li havia ensenyat. La cançó és contestada per una altra veu, que la guia fins a un llenyataire, gràcies al qual pot retrobar el seu germà. Sense aquesta ajuda, i sense la complicitat creada a través de la cançó, el retrobament no hauria estat possible. Tal com va dir Henry Ford, «arribar junts és el principi, romandre junts és el progrés, treballar junts és l'èxit».

Aquestes històries, tot i néixer de contextos diferents, comparteixen una mateixa essència: els camins exigents no es recorren només amb empenta, ni tampoc en solitud. Avançar implica aprendre a respectar el ritme, a confiar en l'experiència (pròpia i aliena) i reconèixer el valor de caminar acompanyats.

I ara que arribem al final del camí és moment d'aturar-se un instant i mirar enrere. Aquesta tesi ha estat una decisió, sostinguda en el temps per la constància, el coratge i la paciència. Ha estat també un exercici d'aprendre a escoltar, a demanar ajuda i a reconèixer les mans que han acompanyat cada passa, fins i tot en aquells moments en què semblava que el pes era massa gran.

Si alguna cosa dona sentit a aquest viatge, és haver entès que arribar al cim no era l'únic objectiu. El veritable valor ha estat aprendre a caminar: amb fermesa, amb humilitat i amb companyia.

Aquí es tanca aquesta etapa. No com un punt final, sinó com el moment en què tot allò après es transforma en ales.

[Spanish]

Ahora que el camino llega a su fin, es inevitable mirar atrás. Esta tesis es mucho más que una sucesión de capítulos, experimentos y resultados; es un recorrido vital hecho de decisiones, de esfuerzo sostenido, de dudas, de aprendizajes y, sobre todo, de personas. No todo aquello que define un camino como este puede explicarse con datos, figuras o conclusiones. Algunas cosas solo pueden comprenderse (y compartirse) a través de historias, como las que le leemos a Blanca, recogidas en el libro *Avui seré millor que ahir: contes per educar el caràcter* (Ballaz, 2021).

La primera, titulada "Los dos alpinistas", cuenta cómo Yang, una joven valiente y decidida, quiere escalar el Teng-Su en solitario para demostrar su fortaleza. Su tío Yu, un alpinista experimentado pero ya mayor, le propone un reto sencillo: él subirá primero y ella partirá después. A pesar del entusiasmo y la rapidez de Yang, el esfuerzo excesivo la deja exhausta, y llega a la cima desfallecida, donde su tío la espera. Aquella experiencia le hace comprender que la determinación no es suficiente sin preparación y constancia. Como recuerda Júlia Navarro, «hay un momento en la vida en el que debemos buscar dentro de nosotros para decidir». Decidir es el primer paso, pero solo el esfuerzo sostenido, la perseverancia y el tiempo permiten escalar una montaña.

Otra historia, la del cuento "La llamada de una canción", nos habla de Irina, que vive con su abuela cerca de un bosque al que tiene prohibido entrar, porque tiempo atrás su hermano Yossi se perdió en él. Un día, guiada por una intuición más fuerte que el miedo, se adentra en el bosque. Para apaciguar el silencio y la incertidumbre, empieza a tararear una melodía que su madre le había enseñado. La canción es respondida por otra voz, que la guía hasta un leñador, gracias al cual puede reencontrarse con su hermano. Sin esa ayuda, y sin la complicidad creada a través de la canción, el reencuentro no habría sido posible. Tal como dijo Henry Ford, «llegar juntos es el principio, permanecer juntos es el progreso, trabajar juntos es el éxito».

Estas historias, aunque nacen de contextos distintos, comparten una misma esencia: los caminos exigentes no se recorren solo con empuje, ni tampoco en soledad. Avanzar implica aprender a respetar el ritmo, a confiar en la experiencia (propia y ajena) y a reconocer el valor de caminar acompañados.

Y ahora que llegamos al final del camino, es momento de detenerse un instante y mirar atrás. Esta tesis ha sido una decisión, sostenida en el tiempo por la constancia, el coraje y la paciencia. Ha sido también un ejercicio de aprender a escuchar, a pedir ayuda y a reconocer las manos que han acompañado cada paso, incluso en aquellos momentos en los que parecía que el peso era demasiado grande.

Si algo da sentido a este viaje, es haber entendido que llegar a la cima no era el único objetivo. El verdadero valor ha sido aprender a caminar: con firmeza, con humildad y en compañía.

Aquí se cierra esta etapa. No como un punto final, sino como el momento en que todo lo aprendido se transforma en alas.

[English]

Now that the path has reached its end, looking back becomes inevitable. This thesis is much more than a succession of chapters, experiments, and results; it represents a life journey shaped by decisions, sustained effort, doubts, learning processes, and, above all, by people. Not everything that defines a path such as this can be explained through data, figures, or conclusions. Some aspects can only be understood (and shared) through stories, such as those we read to Blanca, collected in the book *Avui seré millor que ahir: contes per educar el caràcter* (Ballaz, 2021).

In the first story, entitled *"The Two Mountaineers"*, Yang, a brave and determined young woman, sets out to climb Mount Teng-Su alone to prove her strength. Her uncle Yu, an experienced mountaineer, proposes a simple challenge: he will start climbing first, and she will follow later. Despite Yang's enthusiasm and speed, excessive effort leaves her

exhausted, and she reaches the summit weakened, where her uncle is waiting for her. Through this experience, she comes to understand that determination alone is not sufficient without preparation and consistency. As Júlia Navarro reminds us, “there comes a moment in life when we must look within ourselves to decide.” Deciding is the first step, but only sustained effort, perseverance, and time allow a mountain to be climbed.

Another story, *“The Call of a Song”*, tells of Irina, who lives with her grandmother near a forest she is forbidden to enter, because years earlier her brother Yossi disappeared there. One day, guided by an intuition stronger than fear, she ventures into the forest. To ease the silence and uncertainty, she begins to hum a melody her mother once taught her. The song is answered by another voice, which leads her to a woodcutter, through whom she can find her brother again. Without this help, and without the connection created through the song, the reunion would not have been possible. As Henry Ford once said, “coming together is a beginning; staying together is progress; working together is success”.

Although these stories arise from different contexts, they share the same essence: demanding paths are not travelled through momentum alone, nor in solitude. Progress requires learning to respect one’s pace, trusting experience (both one’s own and that of others) and recognising the value of walking alongside others.

And now, as this path reaches its end, it is time to pause for a moment and look back. This thesis has been a decision sustained over time through perseverance, courage, and patience. It has also been an exercise in learning to listen, to ask for help, and to acknowledge the hands that have accompanied each step, even in those moments when the weight seemed too heavy to bear.

If anything gives meaning to this journey, it is the understanding that reaching the summit was not the only goal. The true value has been learning how to walk: with determination, with humility, and in the company of others.

This stage now comes to an end. Not as a final full stop, but as the moment when what has been learned begins to open new possibilities.

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