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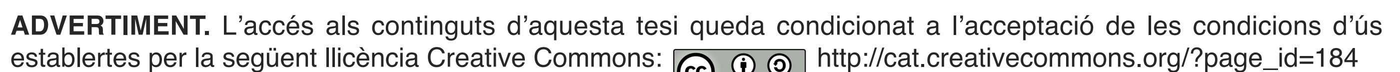
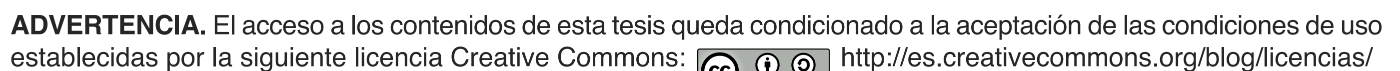
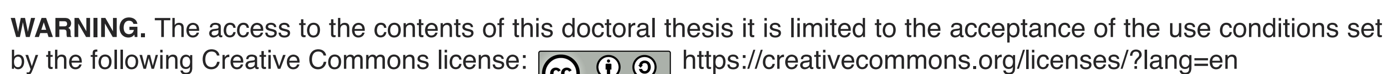
ADVERTIMENT. L'accés als continguts d'aquesta tesi queda condicionat a l'acceptació de les condicions d'ús establertes per la següent llicència Creative Commons:  http://cat.creativecommons.org/?page_id=184

ADVERTENCIA. El acceso a los contenidos de esta tesis queda condicionado a la aceptación de las condiciones de uso establecidas por la siguiente licencia Creative Commons:  http://es.creativecommons.org/blog/licencias/

WARNING. The access to the contents of this doctoral thesis it is limited to the acceptance of the use conditions set by the following Creative Commons license:  https://creativecommons.org/licenses/?lang=en

Epidemiological and Pathological Studies for *Streptococcus suis*

Carlos María Neila Ibáñez

PhD Thesis

Bellaterra, 2022

UAB Universitat Autònoma de Barcelona

IRTA

CReSA Centre de Recerca en Sanitat Animal

Epidemiological and Pathological Studies for *Streptococcus suis*

Tesis doctoral presentada por **Carlos María Neila Ibáñez** para acceder al grado de Doctor en el marco del programa de Doctorado en *Medicina i Sanitat Animals de la Facultat de Veterinària de la Universitat Autònoma de Barcelona*, bajo la dirección de **Virginia Aragón Fernández**, **Jordi Casal i Fàbrega** y **Ernesto Sebastian Napp Avelli**.

Bellaterra, 2022

La Dra. **Virginia Aragón Fernández** y el Dr. **Ernesto Sebastian Napp Avelli**, investigadores del *Institut de Recerca i Tecnologia Agroalimentàries - Centre de Recerca en Sanitat Animal* (IRTA-CReSA) y el Dr. **Jordi Casal i Fàbrega**, catedrático del *Departament de Sanitat i d'Anatomia Animals de la Facultat de Veterinària de la Universidad Autónoma de Barcelona*.

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Que los trabajos de investigación desarrollados en la memoria de tesis doctoral "*Epidemiological and Pathological Studies for Streptococcus suis*", presentados por el licenciado **Carlos María Neila Ibáñez** para la obtención del Grado de Doctor en *Medicina i Sanitat Animals* se ha realizado bajo la dirección y tutoría, y autorizan su presentación a fin de ser evaluada por la comisión correspondiente.

Y porque así conste y tenga los efectos que correspondan, firman el presente certificado.

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Diseño de portada: Pablo María Neila Ibáñez

“No hay enseñanza sin investigación,
ni investigación sin enseñanza.”

Paulo Freire

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Abbreviations

ACTB: Beta-Actin;
AMR: Antimicrobial Resistances;
AMU: Antimicrobial Use;
ANOVA: Analysis of Variance;
AP: Alkaline Phosphatase;
APOA1: Apolipoprotein A1;
APP: Acute Phase Proteins;
ASV: Amplicon Sequence Variant;
B2M: Beta-2 Microglobulin;
BALF: Bronchoalveolar Lavage Fluid;
BCL2: B-cell Lymphoma 2;
BSL3: Biosafety Level 3;
C3: Complement Component 3;
CASP1: Caspase 1;
CCL2: C-C Motif Chemokine, Ligand 2;
CCL3: C-C Motif Chemokine, Ligand 3;
CCL4: C-C Motif Chemokine, Ligand 4;
CCL5: C-C Motif Chemokine, Ligand 5;
CCL5.RANTES: C-C Motif Chemokine, Ligand 6;
CCs: Clonal Complexes;
CD14: Cluster of Differentiation 14;
CD163: Cluster of Differentiation 163;
CDCD: Cesarean-Derived Colostrum-Deprived;
CFU: Colony Forming Unit;
CI: Confidence Interval;
COX-2: Cyclooxygenase-2;
CPS: Capsular Polysaccharides;
Cq: Quantification Cycle;
CXCL10: C-X-C Motif Chemokine, Ligand 10;
CXCL8: IL8 encoded gen;
CXCR2: C-X-C Motif Chemokine, Receptor 2;
CXCR3: C-X-C Motif Chemokine, Receptor 3;
DNA: Deoxyribonucleic Acid;
dpi: Day post-inoculation;
EF: Extracellular Factor;
EMA: European Medicines Agency;
ERIC-PCR: Enterobacterial Repetitive Intergenic Consensus - PCR;
FAS: Fas Receptor;
FOS: Proto-oncogene c-Fos;
GAPDH: Glyceraldehyde 3-Phosphate Dehydrogenase;
HP: Haptoglobin;
HPRT1: Hypoxanthine-Guanine Phosphoribosyltransferase;
HSD: Honest Significant Difference;
HSPA14: Heat Shock 70 kDa Protein 14;

ICAM1: Intercellular Adhesion Molecule 1;
ICAM2: Intercellular Adhesion Molecule 2;
IFITM1: Interferon-Induced Transmembrane Protein 1;
IFN: Interferon;
IFNA1: Interferon Alpha 1;
IFNG: Interferon Gamma;
IHC: Immunohistochemistry;
IKBKB: Inhibitor of Nuclear Factor Kappa-B Kinase, Subunit Beta, IKK-β;
IL-1: Interleukin 1;
IL10: Interleukin 10;
IL12B: Interleukin 12, p40;
IL15: Interleukin 15;
IL18: Interleukin 18;
IL1A: Interleukin 1, Alpha;
IL1B: Interleukin 1, Beta;
IL1B 1: Interleukin 1, Beta. Assay 1;
IL1B 2: Interleukin 1, Beta. Assay 2;
IL1R1: Interleukin 1, Receptor Type 1;
IL-1RA: Interleukin 1, Receptor Antagonist;
IL1RN: Interleukin 1, Receptor Antagonist encoded gene;
IL23: Interleukin 23;
IL6: Interleukin 6;
IL8: Interleukin 8;
IRF1: Interferon Regulatory, Factor 1;
IRF3: Interferon Regulatory, Factor 3;
IRF7: Interferon Regulatory, Factor 7;
IRFs: Interferon Regulatory Factors;
ITIH4: Inter-Alpha-Trypsin Inhibitor Heavy Chain Family Member 4;
JAK1: Janus Kinase 1;
JAK2: Janus Kinase 2;
JUN: c-Jun;
low-EDTA TE buffer: Low-(Ethylenediaminetetraacetic acid) Tris-EDTA buffer;
LY96.MD2: Lymphocyte Antigen 96 (MD2);
MCFA: medium-chain fatty acids;
MCL1: MCL1, BCL2 Family Apoptosis Regulator;
MLST: Multilocus Sequence Typing;
MRP: Muramidase-Released Protein;
MUC5AC: Mucin 5AC;
MYD88: Myeloid Differentiation Primary Response Protein 88;
NCL: Novel Capsule Loci;
NFKB1: Nuclear Factor Kappa B Subunit 1;
NFKBIA: Nuclear Factor of Kappa Light Polypeptide Gene Enhancer in B-Cells Inhibitor, Alpha;
OIE: Office International des Epizooties;
OR: Odds Ratio;
PBS: Phosphate Buffered Saline;
PCR: Polymerase Chain Reaction;
PCR-RFLP: polymerase chain reaction-restriction fragment length polymorphism;

PCV-2: Porcine Circovirus 2;
pig-MAP: Major Acute Phase Protein;
PPIA: Peptidylprolyl, Isomerase A;
PRRs: Pattern Recognition Receptors;
PRRSV: Porcine Reproductive and Respiratory Syndrome Virus;
PTGS2: Prostaglandin-Endoperoxide Synthase 2, COX2;
PTX3: Pentraxin 3;
RIN: Ribonucleic acid Integrity Number;
RNA: Ribonucleic Acid;
Rnase: Ribonuclease ;
RPL13A: 60S Ribosomal Protein L13a;
RT-qPCR: quantitative Reverse Transcription-PCR;
SA: Sensitivity Analysis;
SAA: Serum Amyloid A;
SARS-CoV-2: Severe Acute Respiratory Syndrome Coronavirus 2;
SELL: Selectin L;
SELP: Selectin P;
SIV: Swine Influenza Virus;
ST: Sequence Types;
STAT1: Signal Transducer and Activator of Transcription 1;
STAT2: Signal Transducer and Activator of Transcription 2;
STATs: Signal Transducer and Activator of Transcription Proteins;
TF: Transferrin;
THI: Temperature-Humidity Index;
TICAM1.TRIF: Toll Like Receptor, Adaptor Molecule 1;
TLR1: Toll Like Receptor, 1;
TLR2: Toll Like Receptor, 2;
TLR4: Toll Like Receptor, 4;
TLR6: Toll Like Receptor, 6;
TNF: Tumor Necrosis Factor;
TNFRSF10B: Tumor Necrosis Factor, Receptor Superfamily Member 10B (TRAIL-R);
TNFRSF1A: Tumor Necrosis Factor, Receptor 1;
TNFSF10: Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand (TRAIL short);
TP53: Tumor Protein p53;
TREM1: Triggering Receptor Expressed on Myeloid Cells 1;
VCAM1: Vascular Cell Adhesion Molecule 1;
WGS: Whole-Genome Sequencing;
YWHAZ: 14-3-3 Protein Zeta/Delta

Abstract

Streptococcus suis is a major swine pathogen with a worldwide distribution. Despite being a natural inhabitant of the pig respiratory tract, it can sometimes cause disease in weaning piglets, characterized by arthritis, meningitis, and/or sudden death. It is also a zoonotic pathogen, particularly important in Southeastern Asian countries.

The impact of the disease in porcine production seems to be significant, but there are no accurate estimations. In this thesis the prevalence and cost of the *S. suis*-associated disease were estimated for three of the main pig-producing countries in Europe: Germany, the Netherlands, and Spain (Study I). Our results showed that the disease is highly prevalent in pig farms in these countries, with substantial differences in prevalence and mortality between phases, and higher prevalence in the post-weaning period. Costs were higher in Germany, followed by the Netherlands and Spain, and they were essentially explained by the measures applied to prevent or control *S. suis* (autovaccines and antimicrobials), although the value of the animals that die because of the pathogen was also important in the estimation, especially in the post-weaning period.

Pigs in commercial farms get colonized by *S. suis* early in life, but only a fraction of farms and pigs develop the disease later. Because of the endemic aspect of *S. suis* and the estimated prevalence, different factors that have traditionally been associated with the appearance of *S. suis* disease were studied in two Catalan farms (Study II). Results showed that animal stress and higher humidity before weaning, presence of porcine respiratory and reproductive syndrome virus, lower temperature after weaning, and lower parity of the dams increased the risk of developing clinical signs associated with the disease.

Host susceptibility and the virulence of the strains present in the farms also play an important role in the development of the disease. The pig response against this early colonizer was studied in cesarean-derived colostrum-deprived piglets intranasally inoculated either with a virulent or a non-virulent *S. suis* strain (Study III). This infection allowed the

observation for the first time of the innate immune response against the colonization in nasal mucosa, blood, lung, trachea, liver, and spleen by transcriptional analysis. On the one hand, the host response to the non-virulent *S. suis* strain was characterized by rapid control at the site of inoculation. On the other hand, the piglet local inflammatory response was maintained longer in nasal mucosa for the virulent strain, demonstrating that the host response depends on the virulence of the strain. In addition, the virulent strain was detected deep in the cribriform plate of the ethmoid bone, suggesting that this may be an alternative route of invasion for *S. suis*.

As indicated above, antimicrobials are one of the tools used for control of *S. suis* disease, with the consequent problems in the emergence and spread of resistances. Recently, the microbiota was identified as a factor involved in health. Thus, in order to face the current challenge of reducing the use of antimicrobials in livestock, the protective efficacy of a mixture of nasal probiotics was tested against a lethal challenge with a virulent *S. suis* strain (Study IV). Commensals, belonging to the genera *Rothia*, *Moraxella* and a non-virulent *S. suis* strain, were selected based on different criteria, such as relative abundant in healthy piglets, *in vitro* *S. suis* inhibition, or adherence ability. Despite the lack of significant differences between groups, piglets inoculated with a combination of two *Rothia* and one *Moraxella* showed the best survival rate and less severe clinical signs and lesions after challenge. This study lays the groundwork for future studies with porcine nasal probiotics, an alternative to prevent the diseases caused by pathogens that are early colonizers of the upper respiratory tract.

Resumen

Streptococcus suis es un importante patógeno porcino con una distribución mundial. A pesar de ser un habitante natural de las vías respiratorias de los cerdos, en ocasiones puede causar en los lechones una enfermedad caracterizada por artritis, meningitis y/o muerte súbita. Es también un patógeno zoonótico, particularmente importante en los países del sudeste asiático.

Aunque el impacto de la enfermedad en la producción porcina es importante, no existen estimaciones precisas. En la presente tesis se ha estimado la prevalencia y el coste de la enfermedad asociada a *S. suis* para tres de los principales países productores de cerdos de Europa: Alemania, Países Bajos y España (Estudio I). Nuestros resultados mostraron que la enfermedad tiene una alta incidencia en las explotaciones porcinas de estos países, con diferencias sustanciales en la prevalencia y mortalidad entre fases productivas, y una mayor prevalencia en la transición. Los costes fueron mayores en Alemania, seguido de Países Bajos y España, y se explicaron fundamentalmente por las medidas aplicadas para prevenir o controlar *S. suis* (autovacunas y antimicrobianos), aunque fue también importante en la transición el valor de los animales que mueren a causa del patógeno.

A pesar de que los lechones son colonizados por *S. suis* en el momento del nacimiento, solo una parte desarrollan la enfermedad más tarde. Debido al carácter endémico de *S. suis* y a su prevalencia, se estudiaron diferentes factores que tradicionalmente se han asociado a la aparición de la enfermedad en dos explotaciones porcinas catalanas (Estudio II). Los resultados mostraron que el estrés de los animales y la humedad más elevada en maternidad, la presencia del virus del síndrome respiratorio y reproductivo porcino, la temperatura más baja en transición, así como la menor paridad de las madres, aumentaron el riesgo de desarrollar signos clínicos asociados con la enfermedad.

La susceptibilidad del hospedador y la virulencia de las cepas de *S. suis* presentes en las granjas juegan también un papel importante en el desarrollo de la enfermedad. Se estudió la respuesta inmune innata contra *S. suis* en lechones nacidos por cesárea y privados de calostro mediante el desafío por vía intranasal con una cepa virulenta y otra no virulenta de *S. suis* (Estudio III). Esta infección permitió observar por primera vez la respuesta frente a la colonización en mucosa nasal, sangre, pulmón, tráquea, hígado y bazo mediante análisis transcriptómico. La respuesta a la cepa no virulenta de *S. suis* se caracterizó por un rápido control en el sitio de inoculación. Sin embargo, para la cepa virulenta, la respuesta inflamatoria local se mantuvo más tiempo en la mucosa nasal, lo que demuestra que la respuesta del hospedador depende de la virulencia de la cepa. Además, la cepa virulenta se detectó más profundamente en el interior de la lámina cribosa del hueso etmoides, lo que sugiere una ruta alternativa de invasión para *S. suis*.

Los antimicrobianos han sido una de las herramientas utilizadas para el control de la enfermedad provocada por *S. suis*, no obstante, su uso conlleva la aparición y propagación de resistencias. La microbiota ha sido identificada recientemente como un factor involucrado en la salud. Para afrontar el reto de reducir el uso de antimicrobianos en el ganado, se probó la eficacia protectora de una mezcla de probióticos nasales frente a un desafío letal con una cepa virulenta de S. suis (Estudio IV). Los comensales, pertenecientes a los géneros *Rothia* y *Moraxella*, y una cepa no virulenta de *S. suis*, se seleccionaron en función de diferentes criterios, como la abundancia relativa en lechones sanos, la inhibición *in vitro* de *S. suis* o su capacidad de adherencia. A pesar de la falta de diferencias significativas entre los grupos, los lechones inoculados con una combinación de dos cepas de *Rothia* y otra de *Moraxella* mostraron la mejor tasa de supervivencia y menor gravedad de signos clínicos y lesiones después del desafío. Estos resultados sientan las bases para futuros estudios con probióticos nasales porcinos, una alternativa en la prevención de enfermedades causadas por patógenos que son colonizadores tempranos de las vías respiratorias.

Resum

Streptococcus suis és un important patogen porcí amb distribució mundial. Tot i habitar de manera natural a les vies respiratòries dels porcs, a vegades pot produir malaltia als garrins en el moment del deslletament, caracteritzada per artritis, meningitis i/o mort sobtada. És a més un patogen zoonòtic, particularment rellevant als països del sud-est asiàtic.

Tot i que l'impacte de la malaltia en la producció porcina sembla rellevant, encara no s'ha estimat amb precisió. En aquesta tesis s'ha fet una estimació de la prevalença i el cost de la malaltia associats a *S. suis* per a tres dels principals països productors de porc a Europa: Alemanya, Països Baixos i Espanya (Estudi I). Els nostres resultats mostren que la malaltia té una alta incidència en les explotacions porcines d'aquests països, amb diferències substancials en la prevalença i la mortalitat entre fases, i una major prevalença en la transició. Els costos van ser més elevats a Alemanya, seguit de Països Baixos i Espanya, i s'expliquen fonamentalment per les mesures aplicades per a prevenir o controlar *S. suis* (autovacunes i antimicrobians), encara que a les transicions també va ser rellevant el valor dels animals morts a causa del patogen.

Malgrat que els garrins són colonitzats per *S. suis* en el moment del naixement, només una part desenvolupa la malaltia posteriorment. A causa del caràcter endèmic de *S. suis* i de la seva prevalença, s'han estudiat diferents factors tradicionalment associats amb l'aparició de la malaltia en dues explotacions porcines catalanes (Estudi II). Els resultats mostren que l'estrès dels animals i una elevada humitat en maternitat, la presència del virus de la síndrome respiratòria i reproductiva porcina, la temperatura més baixa en transició, així com la menor paritat de les truges augmenten el risc de desenvolupar signes clínics associats a la malaltia.

La susceptibilitat de l'hoste i la virulència de les soques de *S. suis* presents a les granges també juguen un paper molt important en

l'aparició de la malaltia. S'ha estudiat la resposta immune innata contra *S. suis* en garrins nascuts per cesària i privats de calostre amb un desafiament per via intranasal amb una soca virulenta i una altra no virulenta de *S. suis* (Estudi III). Aquesta infecció va permetre observar per primera vegada la resposta davant la colonització en mucosa nasal, sang, pulmó, tràquea, fetge i melsa mitjançant anàlisi transcriptòmic. La resposta a la soca no virulenta de *S. suis* es caracteritzà per un control ràpid en el lloc d'inoculació. Per altra banda, la resposta inflamatòria local de la soca virulenta es va mantenir més temps en la mucosa nasal, demostrant que la resposta de l'hoste depèn de la virulència de la soca. Addicionalment, la soca virulenta es va detectar més profundament a l'interior de la làmina cribosa de l'os etmoides, observació que suggereix una ruta alternativa d'invasió per a *S. suis.*

Els antimicrobians s'han fet servir com a eines per al control de la malaltia causada per *S. suis,* tanmateix, el seu ús comporta l'aparició i propagació de resistències. Recentment, la microbiota ha sigut identificada com un factor involucrat en la salut. Per tal d'afrontar el repte de reduir l'ús d'antimicrobians en el bestiar, es va provar l'eficàcia protectora d'una barreja de probiòtics nasals davant un desafiament letal amb una soca virulenta de *S. suis* (Estudi IV). Els comensals, pertanyents als gèneres *Rothia*, *Moraxella* i una soca no virulenta de *S. suis*, es van seleccionar en funció de diferents criteris, com l'abundància relativa en garrins sans, la inhibició *in vitro* de *S. suis* o la capacitat d'adherència. Tot i la falta de diferències significatives entre els grups, els garrins inoculats amb una combinació de dues soques de *Rothia* i una altra de *Moraxella* mostraren la millor taxa de supervivència i menor gravetat de signes clínics i lesions després del desafiament. Aquests resultats estableixen les bases per a futurs estudis amb probiòtics nasals porcins, una alternativa en la prevenció de malalties degudes a patògens colonitzadors primerencs de les vies respiratòries.

GENERAL INTRODUCTION

1. *Streptococcus suis*

Streptococcus suis is a Gram-positive coccus considered the most important streptococcal swine pathogen due to its impact in both traditional and intensive swine farms worldwide (Gottschalk and Segura, 2019). It can cause systemic disease in piglets, including meningitis, polyarthritis, polyserositis, valvular endocarditis, septicemia, and acute death. Even though the disease affects mostly post-weaning piglets (between 5- to 10-week-old), suckers and fatteners can also be affected (Gottschalk and Segura, 2019).

The first *S. suis* cases were reported in the early 1950s in the Netherlands and United Kingdom in piglets affected by meningitis (Jansen and Dorseen, 1951; Field et al., 1954), but it was not until 1987 when the bacterium was described as a novel specie (Kilpper-Balz and Schleifer, 1987).

Taxonomically, *S. suis* is part of the *Firmicutes* phylum, *Bacilli* class, *Lactobacillales* order, *Streptococcaceae* family, and *Streptococcus* genus. This genus cover more than 100 species and subspecies (de la Maza et al., 2020). Based on the antigenicity of the capsular polysaccharides (CPS), 35 *S. suis* serotypes were described (serotypes 1–34 and serotype 1/2 that reacts antisera of both 1 and 2 serotypes) (Higgins et al., 1995). However, some serotypes were later reclassified as new *Streptococcus* species based on different phylogenetic analyses (Okura et al., 2016). Serotype 32 and 34 were reclassified as *Streptococcus orisratti* (Hill et al., 2005), serotypes 20, 22, and 26 were reclassified as the novel species *Streptococcus parasuis* (Nomoto et al., 2015), and more recently, serotype 33 was reclassified as *Streptococcus ruminantium* due to its isolation only in ruminants, both domestic and wild (Tohya et al., 2017; Okura et al., 2019; Neila-Ibáñez et al., 2022). Studies on non-typeable strains have proposed 27 new serotypes based on novel capsule loci (NCL), designated as serotypes *Chz* and NCL1 to 26 (Pan et al., 2015; Zheng et al., 2015; Qiu et al., 2016; Huang et al., 2019).

Due to the high *S. suis* diversity within and between serotypes, Multilocus Sequence Typing (MLST) is the most widely used method to classify *S. suis* and to study the genetic diversity among isolates (Hatrongjit et al., 2020). This technique uses the genetic variation that accumulates very slowly in seven different housekeeping genes (*cpn60*, *dpr*, *recA*, *aroA*, *thrA*, *gki*, and *mutS*) and classifies the isolates into different sequence types (ST) and clonal complexes (CCs) (King et al., 2002). More than 1800 ST have been reported (May 2022, https://pubmlst.org/ssuis/), and the CCs analyses revealed that most of the pig and human infections are linked to only a few CCs, some of which have a global distribution (Goyette-Desjardins et al., 2014; Hatrongjit et al., 2020). Recently, it was demonstrated that strains from the same CC can switch the capsule between various serotypes, which adds difficulty to study *S. suis* epidemiology (Zhu et al., 2021).

S. suis is considered an early colonizer and is part of the natural microbiota from the swine upper respiratory tract, particularly tonsils and nasal cavities, but it is also found in the genital tract (Robertson and Blackmore, 1989; Galina et al., 1994; Alves et al., 2022) (Figure 1.1). Its natural presence in the respiratory system and the possibility of triggering disease led some authors to classify *S. suis* as a pathobiont (Vötsch et al., 2018). The bacterium is detected in almost all farms and pigs of any age, with close to 100% of the animals being carriers (Brisebois et al., 1990; MacInnes et al., 2008; Segura et al., 2017; Werinder et al., 2020). However, disease only develops in a few farms, as a result of the interplay between the host, the environment and the pathogen (Gottschalk and Segura, 2019).

Piglets are exposed to *S. suis* the moment they pass through the birth canal and later by transmission from the maternal upper respiratory tract (Amass et al., 1997; Berthelot-Hérault et al., 2001). Transmission between animals occurs by direct contact or aerosol, especially with animals clinically affected during outbreaks due to the increased number of bacteria shed (Berthelot-Hérault et al., 2001; Cloutier et al., 2003). In spite of *S. suis* isolation from feed troughs and its survival for several

days in water and feces, the importance of environmental contamination in the transmission is uncertain (Robertson et al., 1991; Gottschalk and Segura, 2019). It remains to be elucidated whether the oro-gastrointestinal infection route occurs in pigs as it seems to occur in humans (Ferrando and Schultsz, 2016; Segura et al., 2016). Experimental infection with *S. suis* administered orally in gastric-acid resistant capsules resulted in some piglets with clinical symptoms compatible with *S. suis* and bacteria in the mesenteric lymph nodes (Ferrando et al., 2015). However, the results published by Warneboldt and collaborators (2016) suggest that *S. suis* is not able to survive in the stomach.

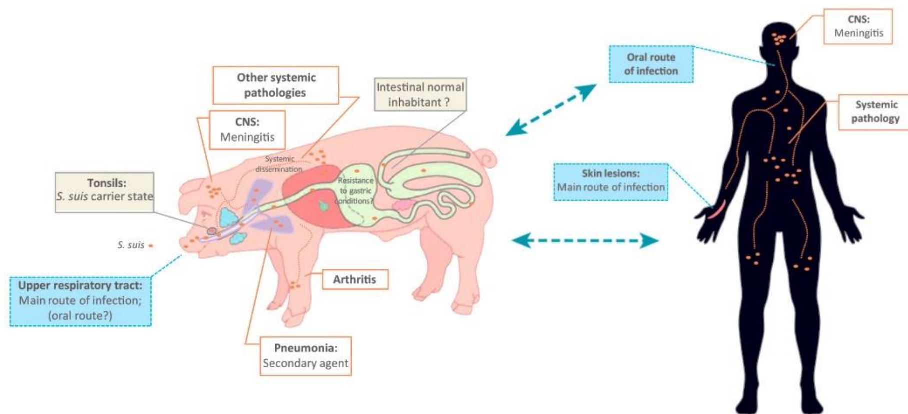

Figure 1.1 | Pathogenesis and epidemiological features of *S. suis*-associated disease in piglets and humans. Figure adapted from Segura and collaborators (2017).

The virulence of *S. suis* is variable, with more than 100 putative virulence factors or traits described, of which at least 37 have been claimed as being critical for virulence (Segura et al., 2017). Yet it is still unknown whether any of those factors is absolutely necessary for disease development (Tram et al., 2021). The majority of the virulence studies have focused in the muramidase-released protein (*mrp*), the hemolysin suilysin (*sly*), the extracellular factor protein (*ef*), and the CPS, still, strains that do not have any of those factors or are unencapsulated can develop disease (Fittipaldi et al., 2009; Lakkitjaroen et al., 2011; Segura et al., 2017; Petrocchi-Rilo et al., 2021). Recently, genes encoding the opacity factor of serum (*ofs*) and the sortase F (*srtF*) have been proposed

as predictors of pathogenicity, but, more studies with isolates from different geographical locations are needed since only strains recovered in the USA were used (Estrada et al., 2021).

2. Disease impact

Despite *S. suis* being a normal inhabitant of the upper respiratory tract, carrier piglets may develop disease (Goyette-Desjardins et al., 2014). Pigs are usually colonized by more than one serotype, but only a few virulent strains can cause disease, and usually a single strain is the cause of an outbreak (Flores et al., 1993; Cloutier et al., 2003; Marois et al., 2007). The isolation of different virulent strains within the same herd, and the possibility that lesions can be produced by more than one strain, reflect the complex epidemiology of the infection by *S. suis* and its associated disease (Vela et al., 2003).

The serotypes most frequently reported worldwide are 1 through 7, 1/2, 9, and 14, although their distribution is not homogeneous and some geographical differences are observed (Goyette-Desjardins et al., 2014). While serotype 2 is the most frequently isolated from lesions in most parts of the world, both in pigs and humans, in Europe the serotype 9 is also highly prevalent in swine (Goyette-Desjardins et al., 2014).

In spite of the importance of *S. suis* in swine production, there have only been a few attempts to estimate its economic impact. Because reporting of *S. suis* is not required by law, there is a lack of data on its incidence, the control measures implemented, or the losses it causes. Bennett and collaborators (1999) estimated that the annual losses caused by *S. suis* type II meningitis in 1996 in Great Britain were between 25,000 and 2 million pounds. This wide range was updated by the same authors a few years later to be between 100,000 and 1.3 million pounds (Bennett and IJpelaar, 2005).

S. suis is also a public health concern because of its role as zoonotic agent, which, together with its importance for swine production, results in *S. suis* being included among the top ten swine pathogens of

scientific interest (VanderWaal and Deen, 2018). Though the first human case was reported in Denmark in 1968 (Perch et al., 1968), its importance has increased in the last twenty years, becoming an emerging zoonotic pathogen (Wertheim et al., 2009; Gottschalk et al., 2010), particularly in Southeast Asia, where *S. suis* has caused important outbreaks in China in 1998 and 2005 (Tang et al., 2006; Yu et al., 2006). Phylogenomic analyses of isolates recovered during 36 years in different countries identified three main *S. suis* clades. One of those clades, associated to human cases, originated in Europe between the 1960s and the 1970s and spread worldwide through the export of European swine breeds (Dong et al., 2021).

Workers who are in contact with pigs and raw meat, especially those who work in intensive production systems, such as farmers, abattoir workers, butchers, or veterinarians, are considered particularly at risk (Walsh et al., 1992; Huang et al., 2005; Tang et al., 2006; Guo et al., 2022). Moreover, in some Southeast Asian countries it is also a foodborne disease due to the practice of consuming uncooked pork (Segura et al., 2016). The symptomatology in humans is similar to that presented in pigs, with mortalities that have reached 50% in Europe and 73% in Asia (Walsh et al., 1992; Navacharoen et al., 2009) (Figure 1.1).

In regard to the economic impact as zoonosis, the cost varies depending on the country incidence, with higher costs in countries where human cases are more frequent (e.g. Southeast Asian countries). There is only one recent study, that was carried out in Vietnam, and the annual cost of human cases was calculated to be between 2.64 and 3.38 million US$ (Huong et al., 2019).

3. Immune response and pathogenesis

After upper respiratory tract colonization, virulent strains can remain as part of the natural microbiota or disseminate through haematogenous and/or lymphogenous routes, causing systemic disease (Madsen et al., 2002; Segura et al., 2016). The mechanisms that allow bacteria to breach

the mucosal barrier and reach the bloodstream are unknown (Bleuzé et al., 2021) (Figure 1.2).

In the bloodstream, *S. suis* attaches to the surface of monocytes or circulates freely (Gottschalk and Segura, 2000; Fittipaldi et al., 2012), and activates the mobilization of neutrophils, which are usually able to clear minor infections (Bleuzé et al., 2021). When present in blood, *S. suis* expresses different genes from those expressed when present in organs such as brain or heart (Arenas et al., 2019).

In response to *S. suis* infection, neutrophils can initiate and maintain inflammation (Kolaczkowska and Kubes, 2013), participating in the immunomodulation among other immune cells such as monocytes and macrophages (Tecchio et al., 2014). Despite the limited knowledge on the early immune response in swine, it is well known that an exacerbated response leads to tissue damage, which contributes to increased clinical manifestations (Domínguez-Punaro et al., 2007; Ye et al., 2009). *S. suis* is able to resist the attack of the host innate immune system by reducing cell activation, avoiding phagocytosis, and/or inducing the death of leukocytes (Gottschalk and Segura, 2019). In this sense, the peptidoglycan and teichoic and lipoteichoic acid, together with the CPS, play an important role in the bacterial resistance to phagocytosis (Fittipaldi et al., 2008a, 2008b; Gottschalk and Segura, 2019).

Numerous *in vitro* studies have addressed the role of cytokines produced by *S. suis* infection, but the extrapolation to the *in vivo* situation is difficult because of the complexity of the immune system. There are a few studies characterizing the swine immune response but they use different *S. suis* reference strains, which makes the results difficult to compare (Sorensen et al., 2006; Li et al., 2010; Liu et al., 2011; Lin et al., 2015).

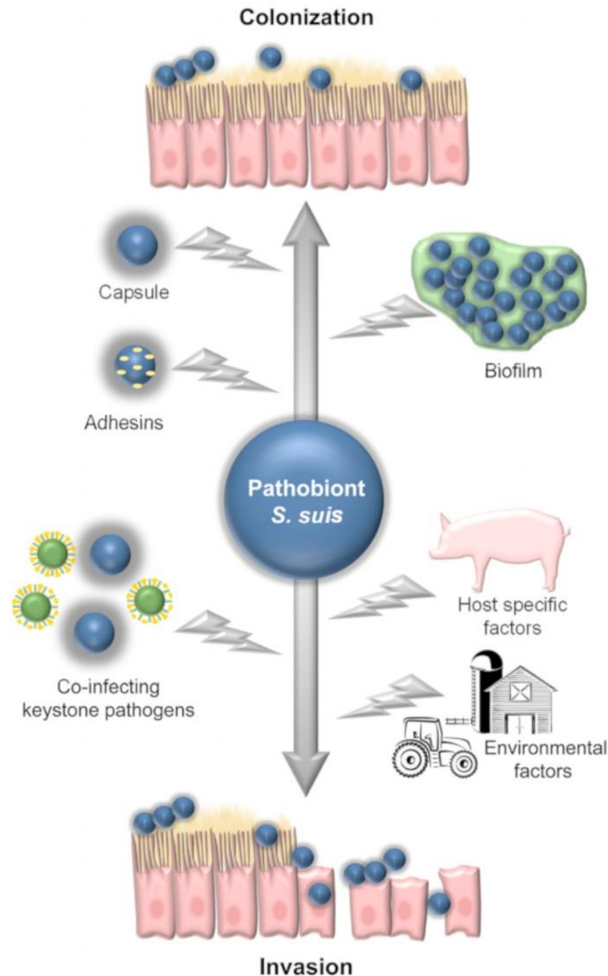

Figure 1.2 | Representation of the evolution of *S. suis* from commensal to pathogen in the pig respiratory tract. Adapted from Vötsch and collaborators (2018).

4. Risk factors

4.1. Management factors

There are factors related to the management of pigs that can contribute to the appearance of *S. suis*-associated disease because of their stressful effect (Obradovic et al., 2021a). Thus, environmental factors such as poor ventilation, excessive temperature fluctuations, overcrowding, or mixing of pigs of different ages or origins could increase the

susceptibility of the weakest pigs to *S. suis* (Dee et al., 1993; Staats et al., 1997) (Figure 1.2).

Other factors that can contribute to the development of the disease caused by *S. suis* are those related to the sow. Piglets from sows with a history of *S. suis*-associated disease in previous litters were less likely to develop disease (Hopkins et al., 2018). Besides, piglets that have an inadequate colostrum intake seem more likely to develop the associated disease because they receive less protective antibodies (Zoric et al., 2004).

4.1. Mixed infections with viruses

There is a debate about the opportunistic nature of *S. suis*, as this pathogen can be isolated with other bacteria (e.g. *Actinobacillus pleuropneumoniae*, *Mycoplasma hyopneumoniae*, or *Bordetella bronchiseptica*), especially in respiratory infections (Saade et al., 2020; Obradovic et al., 2021a; Hennig-Pauka et al., 2022). The possibility that infections caused by *S. suis* may increase when a viral episode is taking place on a farm, has also been proposed. However, the experimental demonstration of this hypothesis is difficult, and only few *in vivo* studies have been carried out to test this interaction (Obradovic et al., 2021a). The number of different viruses that may play a role in *S. suis* infection is large, but considering their important for the swine industry, the list may be reduced to three: porcine reproductive and respiratory syndrome virus (PRRSV), swine influenza virus (SIV), and porcine circovirus 2 (PCV-2).

PRRSV, an enveloped single-stranded positive-sense RNA virus, was first isolated in Europe and North America in 1991 and 1992, respectively (Wensvoort et al., 1991; Collins et al., 1992). PRRSV is considered one of the most important pathogens that affect the swine industry because it causes reproductive failure in sows and respiratory symptoms in weaned and growing pigs, which result in significant economic costs (Nathues et al., 2017). Even though it is widely believed, especially by veterinary practitioners, that farms that are PRRSV positive

unstable are more prone to have severe *S. suis*-associated disease, the scientific literature is scarce, especially for studies in field conditions. This synergy between PRRSV and *S. suis* has been demonstrated in *in vivo* studies by infecting animals with the virus and later on with a *S. suis* strain (Thanawongnuwech et al., 2000; Feng et al., 2001; Xu et al., 2010), or by infecting piglets first with *S. suis* and later with the virus (Brockmeier et al., 2017; Sun et al., 2020). In all these coinfections, piglets presented more severe clinical signs and higher mortality. In addition, it has been shown that the efficacy of antimicrobials, specifically ceftiofur, was diminished in those animals infected with both PRRSV and *S. suis*, indicating that the coinfection has an implication not only in the severity of disease, but also in the efficacy of the treatment (Day et al., 2015).

PCV-2 is a single-stranded DNA virus, isolated for the first time in Europe in 1999 (Allan et al., 1999). In unvaccinated animals, PVC-2 can cause a post-weaning multisystemic wasting syndrome (PMWS), affecting mainly post-weaning and fattening pigs in which the major clinical sign is wasting (Segalés et al., 2005). The only *in vivo* study using *S. suis* and PCV-2 found in the literature shows that pigs coinfected with both pathogens develop more severe lesions and clinical signs (Wang et al., 2020). In field conditions, although there are studies that correlated the presence of *S. suis* in septicemia cases and PCV-2 isolation (Pallarés et al., 2002; Opriessnig and Halbur, 2012), the epidemiological link between the two pathogens is not clear (Obradovic et al., 2021a). Nowadays, PVC-2 infections are usually kept at a subclinical level by vaccination.

Another important swine virus with possible interaction with *S. suis* is SIV. The first reports of influenza-like disease in swine, presumably caused by this single-stranded RNA virus, date back to 1918 in the United States and Europe, in conjunction with the human influenza pandemic (Taubenberger and Palese, 2016). Its worldwide distribution, associated mortality, which can reach up to 15%, and the economic burden due to the weight loss, make it an important pathogen for the

swine industry (Ma, 2020). Coinfections of SIV with other viruses or bacteria have shown to increase the clinical signs of SIV (Jung et al., 2005; Ma, 2020). There is only one *in vivo* study of *S. suis*-SIV coinfection, which resulted in an increase of clinical signs in animals inoculated with both pathogens (Lin et al., 2015). In addition, *Streptococcus pneumoniae* secondary infections in humans are an important cause of excess mortality during influenza pandemics or epidemics (Alicino et al., 2011).

Suid herpesvirus 1 (SuHV-1), also known as pseudorabies virus, responsible of the Aujeszky's disease, has also been used in coinfections with *S. suis* (Obradovic et al., 2021a). Even though the pseudorabies virus is currently eradicated in domestic pigs in most of the main pig producing countries, it has been a major pathogen for the industry (Mettenleiter et al., 2019). Experimental coinfection of *S. suis* with pseudorabies virus produced an increase in the frequency and severity of clinical signs and lesions observed (Iglesias et al., 1992).

5. Diagnosis

S. suis diagnosis is generally based on the clinical signs, the age of the animal and the macroscopic lesions (Gottschalk and Segura, 2019). One of the main problems for identifying an outbreak is the lack of pathognomonic lesions. Therefore, necropsy findings must be confirmed in the laboratory using bacterial culture and molecular techniques to avoid misclassifications, as other agents such as *Glaesserella (Haemophilus) parasuis* cause similar lesions (Aragon et al., 2019).

5.1. Clinical signs and lesions

The first clinical sign of the infection caused by *S. suis* is fever, which can result in a rise of the rectal temperature from 40.0 to 42.5ºC (Clifton-Hadley et al., 1986; MacInnes and Desrosiers, 1999). After the fever, piglets can develop different clinical signs. Neurological signs, associated with meningitis, are frequently observed, and include paddling, head tilt, inability to stand, or convulsions; but lameness due

to arthritis is also an important finding (Sanford and Tilker, 1982; Gottschalk and Segura, 2019).

Sudden death has also been reported in *S. suis* infections, but in a farm setting, the cause of a sudden death is difficult to know unless samples are taken at necropsy, which is seldom if only a few animals in a batch die (Gottschalk and Segura, 2019). Mortality during an outbreak is usually less than 5% (Clifton-Hadley et al., 1986), but it can reach values up to 20 or 30% if no treatment is applied (Cloutier et al., 2003; Hopkins et al., 2018).

At necropsy, pathological findings depend mainly on the duration of the infection, and they are not serotype dependent (Reams et al., 1994; Staats et al., 1997). In sudden death cases, in which infection occurs acutely or peracutely, usually no gross lesions are observed (Power, 1978). Lack of gross lesions can also occur in pigs presenting arthritis or meningitis (Clifton-Hadley and Alexander, 1988). The presence of fibrin deposits and an increase in the amount of fluids are often findings in the abdominal and/or thoracic cavities (Power, 1978). In the heart, it is possible to observe fibrinopurulent pericarditis or vegetative valvular endocarditis (Sanford and Tilker, 1982). The polyserositis produced in those cavities among with the polyarthritis, meningitis, and sudden death, are the reasons why the clinical diagnosis *S. suis* may be confused with *G. parasuis* (Kang et al., 2012; Aragon et al., 2019).

Regarding the joints, the most affected are the carpus and tarsus, and fibrinous arthritis can be observed in them (Windsor and Elliott, 1975). It is also frequent to observe an increase in synovial fluid, which may become purulent, fibrinous, or fibrinopurulent (Sanford and Tilker, 1982; Clifton-Hadley and Alexander, 1988).

It may be argued that *S. suis* is a respiratory and systemic pathogen, due to the importance of the respiratory route and the nervous symptomatology (Obradovic et al., 2021a). In the respiratory context, *S. suis* has historically been considered a secondary pathogen within the Porcine Respiratory Disease Complex (PRDC), among others such as

Actinobacillus suis, *G. parasuis*, *Pasteurella multocida*, or low virulent strains of *A. pleuropneumoniae* (Saade et al., 2020). The pulmonary lesions may include fibrinohaemorrhagic pneumonia or suppurative bronchopneumonia among others (Clifton-Hadley and Alexander, 1988; Reams et al., 1995). However, the presence of lesions in the lower respiratory tract with positive isolation of *S. suis* must be treated with caution, especially in finisher pigs, because *S. suis* strains may be present as part of the natural microbiota or reach the lung from the upper respiratory tract during agony or by gravity after death (Gottschalk and Segura, 2019; Ruggeri et al., 2020; Obradovic et al., 2021a). Other lesions such as interstitial pneumonia can be observed, but they are considered secondary to septicemia (Reams et al., 1994).

Congestion of the lymph nodes and meninges, as well as an increase of the cerebrospinal fluid with or without purulent components, may be observed in animals with nervous symptomatology (Windsor and Elliott, 1975). The most frequent microscopic lesions in animals with neurologic clinical signs are non-suppurative, suppurative, fibrinopurulent, or lymphocytic meningitis, which may be accompanied with encephalitis, oedema, and congestion (Sanford and Tilker, 1982; Reams et al., 1994, 1996). In meningeal lesions, *S. suis* has been detected in the cytoplasm of neutrophils and in macrophages by immunohistochemistry (IHC) (Zheng et al., 2009).

5.2. Laboratorial identification

S. suis is an ovoid-shaped Gram-positive bacteria, that can form short chains, but is usually found in pairs or as a single organism. In sheep blood agar it forms transparent to grey 1-2 mm diameter slightly mucoid hemolytic colonies; while in chocolate agar, colonies are brownish green. The hemolysis produced depends on the strain, and it can be partial (α-hemolysis) or complete (β-hemolysis) in sheep and horse blood agar plates (Vecht et al., 1985; Staats et al., 1997).

S. suis is a non-motile bacterium, facultative anaerobic and catalase negative, with a low G-C content in the genome (38-42 %). It

can grow at temperatures from 20 to 40°C, with 37°C and 5% CO_2 being optimal for growth (Hommez et al., 1986; Kilpper-Balz and Schleifer, 1987). The absence of growth in 6.5% NaCl, the negative result to the Voges-Proskauer test, and the production of acid in trehalose and salicin broths have been used as methods to differentiate *S. suis* from other bacteria within the same genus (Higgins and Gottschalk, 1990), however, a misidentification is possible using these tests for some *S. suis*-like isolates (Okura et al., 2016).

In the diagnostic laboratory, traditionally the phenotypically identification was performed with the commercial API® multitest systems. However, the wide phenotypic variation displayed by the *Streptococcus* genus makes species-level identification difficult and may be misleading (Janda, 2014). Moreover, strains belonging to serotypes 9 to 22 are usually misidentified in these commercial multitests (Higgins and Gottschalk, 1990).

The polymerase chain reaction (PCR) assay is one of the most widely used methods to identify *S. suis* (Okura et al., 2016). The PCR that targets the housekeeping gene *gdh* (glutamate dehydrogenase), developed by Okwumabua and collaborators (2003), has been used in reference laboratories because it detects the reference strains of the 35 *S. suis* serotypes originally described by Higgins and collaborators (1995). However, another method is needed to discriminate serotypes that no longer belong to *S. suis* from true *S. suis*. Thus, Ishida and collaborators (2014) developed a new PCR that targets the *recN* gene, which encodes a recombination/repair protein. This PCR creates a specific product of 336 base pair for all the *S. suis* strains tested, but not for strains that belonged to serotypes 20, 22, and 33, as well as for other species that were positive for the *gdh* PCR, such as *S. gallinaceus*, *S. ovis*, or *S. ruminantium* (Ishida et al., 2014; Okura et al., 2019). With these results, *recN* PCR became the best molecular test for *S. suis* diagnosis, although alternative tools such as matrix-assisted laser desorption/ionization time-of-flight mass spectrophotometry (MALDI-TOF MS) has showed good results (Matajira et al., 2017).

As previously mentioned, MLST is a useful technique to differentiate *S. suis* strains for epidemiological studies, but it is not used routinely for the identification of the agent involved in an outbreak (Hatrongjit et al., 2020). Other molecular typing techniques that have been used to study the genetic diversity are pulse-field gel electrophoresis (PFGE) (Berthelot-Hérault et al., 2002; Vela et al., 2003), randomly amplified polymorphic DNA (RAPD) (Chatellier et al., 1999; Cloutier et al., 2003), amplified fragment length polymorphism (AFLP) (Rehm et al., 2007), polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) (Marois et al., 2006), ribotyping (Harel et al., 1994; Okwumabua et al., 1995), or multilocus variable number tandem repeat analysis (MLVA) (Li et al., 2010).

Advances in sequencing technologies, such as whole-genome sequencing (WGS), have allowed a more precise characterization, with greater reproducibility and discrimination power. This characterization has made possible to differentiate between epidemic/highly virulent, virulent, or intermediately/weakly virulent isolates, or, more recently, to predict the potential virulence of an isolate (Zheng et al., 2014; Wileman et al., 2019). The WGS approach allows the differentiation of pathogenic strains and has been useful in demonstrating that a *S. suis* genome reduction is correlated with the strain pathogenicity, which may help predict and prevent future outbreaks (Murray et al., 2021). However, the price is still high compared to other characterization methods, such as Multiplex PCR or PCR-RFLP (Hatrongjit et al., 2020).

6. Disease control

6.1. Management factors and biosecurity measures

Besides a prompt parenteral treatment of diseased animals, the actions aimed at minimizing the spread of the disease are essential (MacInnes and Desrosiers, 1999; Varela et al., 2013). In any case, the best option to reduce the effect of a *S. suis* outbreak is its prevention, which can be done using different tools (Gottschalk and Segura, 2019).

Among those actions, there are some basic biosecurity measures that should be applied in every farm, such as an all-in/all-out pigs flow, avoiding overcrowding, mixing animals of different ages and improving the ventilation, as well as segregating animals with clinical signs compatible with *S. suis*-associated disease to nursing pens for better observation and follow-up (Sanford, 1989; Akkermans and Vecht, 1994; Blackwell, 2005). Measures focused on getting *S. suis*-free herds, such as getting piglets born by cesarean section or separated from mothers at birth, have been ineffective or impractical in a field context (Amass et al., 1996, 1997).

6.2. Antimicrobials

Antimicrobials have been used to increase the health of the livestock, for preventing and controlling diseases, but also as growth promoters (Casal et al., 2007). However, there is a global concern about the widespread use of antimicrobials in animals due to the emergence of antimicrobial resistances (AMR) in pathogenic bacteria, which is one of the major global health challenges of this century (Marshall and Levy, 2011; Michael et al., 2014). For this reason, their use as growth promoters was banned in the European Union in 2006 (Dibner and Richards, 2005), and the USA and China have applied restrictions in 2017 and 2020, respectively (Centner, 2016; Hu and Cowling, 2020). Nevertheless, other countries still allow the use of antimicrobials as growth promoters (Muurinen et al., 2021).

Despite having a common policy, there are great differences in the sale of antimicrobials for animals among countries of the European Union (EMA, 2021). In the last years there has been a clear reduction in the sales of antimicrobials, which may be further reduced due to the implementation of the new EU regulations on the prophylactic and metaphylactic use of veterinary medicinal products, which have come into force in January 2022 (EU, 2019a, 2019b). In fact, *S. suis* is one of the swine pathogenic bacteria selected by the European Antimicrobial Resistance Surveillance network in Veterinary medicine (EARS-Vet) to

strengthen the European One Health antimicrobial resistance surveillance approach (Mader et al., 2022). Novel approaches combining epidemiological information along with antimicrobial susceptibility are being used to reduce the probability of inducing AMRs and increase treatment success (Vilaró et al., 2020).

The only way to achieve the recovery of animals that presented clinical signs associated with *S. suis* is through antimicrobial treatment. Although a wide range of antimicrobials have been suggested for *S. suis* treatment, including β-lactams, aminoglycosides, and phenicols, the choice should be based on the specific sensitivity of the isolate causing disease (Gottschalk et al., 1991; Marie et al., 2002). The selected antimicrobial should be applied via parenteral and could be accompanied by an anti-inflammatory product, such as dexamethasone, which can control the inflammation and therefore maximize pig survival (MacInnes and Desrosiers, 1999). The antimicrobial can also be administered orally, in medicated feed or added to drinking water, with the in-water administration being preferred over in-feed because sick animals are more likely to drink than to eat (Varela et al., 2013). However, this is a practice more linked to prophylactic and metaphylactic measures than to the direct treatment of diseased animals.

Prophylactic and metaphylactic use of antimicrobials is widespread in many countries due to its easy application and low costs (Varela et al., 2013; Seitz et al., 2016), but that has led to the emergence of resistances (Yongkiettrakul et al., 2019). Moreover, the massive and unspecific antimicrobial use may result in an imbalance of the microbiota diversity (Schokker et al., 2014; Correa-Fiz et al., 2016, 2019), which can favor the infectivity of colonizing pathogens (Thomason et al., 2017).

Due to its low resistance reported against *S. suis*, amoxicillin is the antimicrobial most used worldwide (Marie et al., 2002; Burch and Sperling, 2018; EFSA, 2021). Despite the antimicrobial treatment, the prognosis of sick animals is often poor (Seitz et al., 2016). For that reason

piglets should be monitored daily during an outbreak in order to start the treatment of new cases as soon as possible (Varela et al., 2013).

Referring to the *S. suis* AMRs, the transfer of AMRs genes between strains has been demonstrated *in vitro* (Palmieri et al., 2011; Huang et al., 2016). Although historically low resistance has been reported to penicillin, recent studies have indicated the needed to limit the use of this antimicrobial due to the presence of penicillin-resistant *S. suis* (Varela et al., 2013; van Hout et al., 2016; Cucco et al., 2022). Moreover, Libante and collaborators (2019), reported almost 400 AMR genes in 214 *S. suis* genomes, and Hadjirin and collaborators (2021) found disease-causing strains resistant to antimicrobials that are not typically used to treat *S. suis* infection.

The difficulty in preventing the disease caused by *S. suis*, together with the limitations in the use of antimicrobials, make essential the development of effective alternative tools to control the disease (Segura, 2020; Segura et al., 2020). One alternative to improve the effectiveness of antimicrobials is their combination with other substances, such as essential oils or solid lipid nanoparticles, but, their efficacy against *S. suis* has only been demonstrated *in vitro* and not *in vivo* (Ling et al., 2018; de Aguiar et al., 2019).

6.3. Vaccines

The use of vaccines to prevent *S. suis*-associated disease is controversial. Two different kinds of vaccines are used in the field: autogenous bacterin vaccines (autovaccines), elaborated from strains isolated from the clinical cases in the farm; and commercial vaccines, available in some countries, but none approved by the European Medicines Agency (EMA) (Segura, 2015; Rieckmann et al., 2020).

Despite its use, the suitability of autovaccines to prevent *S. suis*-associated disease is in question due to the contradictory results reported (Rieckmann et al., 2020). A large number of factors have been proposed for the failure of autovaccines. They include the possibility of the disease

being associated to multiple strains, which may belong or not to the same serotype. The presence of concomitant diseases that hinder the immune response, especially PRRSV infection, is also important. Another possibility is an inhibitory interference of the maternal antibodies. Similarly, the incorrect or incomplete sampling of diseased animals, which compromises the isolation of the disease-causing strain, may limit the efficacy of the autovaccine. Because of that, it is recommended to avoid taking samples from the upper respiratory tract and to sample at least 4 animals per farm (Baums et al., 2010; Rieckmann et al., 2020).

The development of new vaccine technologies, as well as the new information obtained from bioinformatic analyses, provide insights for the design of a vaccine to prevent *S. suis*-associated disease in the era of antimicrobial restriction (Segura, 2020). In this sense, recent studies have demonstrated the importance of a proper selection of the adjuvant, given that for the same bacterin, there may be differences in piglet survival and immune responses (Obradovic et al., 2021b). Also, the inclusion of biopolymer particles in conserved *S. suis* antigens helped to improve the antigen-specific humoral immune response (Gonzaga et al., 2021). The combination of novel tools and the identification of conditionally meningeal infection essential genes as the reported by Arenas and collaborators (2020) add a wide testing options in this field.

6.4. Probiotics

A new tool that may represent an important change in the paradigm of antimicrobial use for disease control is the use of probiotics, not only in swine but also in other livestock (Costa-Hurtado et al., 2020). Probiotics are defined by the Food and Agricultural Organization (FAO) and the World Health Organization (WHO) as “live microorganisms that when administered in adequate amounts confer a health benefit on the host” (WHO, 2001).

The goal when administering probiotics is to get a more favorable microbiota environment (Hill et al., 2014). Achieving a stable microbiota has demonstrated to provide a wide array of health effects such as

pathogen inhibitions, even exclusion (Lebeer et al., 2008, 2010), local and systemic immune response modulation (Wells, 2011), or enhancement of the epithelial barrier (Mack, 2003; Rao and Samak, 2013). Those effects are not provided by all probiotics and vary depending on the probiotic strain and the administration route (Martens et al., 2018).

Even though most studies on probiotics, both in humans and livestock, refer to gut microbiota, nasal microbiota has also been associated with diseases (Man et al., 2017). The development of respiratory disease in swine has been linked with different oropharyngeal microbiota (Wang et al., 2018). The composition of the nasal microbiota plays a role in the development of Glässer’s disease, caused by *G. parasuis*, and *Mycoplasma hyorhinis*-associated disease (Correa-Fiz et al., 2016; Blanco-Fuertes et al., 2021). In the case of *S. suis*, some bacterial groups from the tonsillar microbiota were associated with health, as detected when comparing the bacterial composition in apparently healthy piglets and piglets with confirmed *S. suis* infection (Niazy et al., 2022).

Similar to other putative pathogens found naturally in the upper respiratory tract, such as *G. parasuis*, *M. hyorhinis*, or *A. suis*, *S. suis* is an early colonizer (Cerdà-Cuéllar et al., 2010; Brockmeier et al., 2017; Roos et al., 2019). The use of selected putative probiotics that act as early colonizers and niche competitors could result in a healthier animal status. Although there are evidences of the efficacy of nasal probiotics in humans (Dimitri-Pinheiro et al., 2020), it is a field in which more studies are necessary.

There are only two in vitro studies that identified potential probiotics with a *S. suis* inhibitory effect and potential to reduce *S. suis*-associated disease in pigs (Gu et al., 2015; Sirichokchatchawan et al., 2018), and only one was tested *in vivo*, showing the reduction of *S. suis* in the hindgut at 35 days of age (Su et al., 2008). Recently, Vaillancourt and collaborators (2022) reported two bacteriocins produced by

Streptococcus pluranimalium which can be used as a therapeutic agent for controlling *S. suis* infections. However, their activity was only tested *in vitro* and the use of this *S. pluranimalium* strain as probiotic needs further study.

6.5. Prebiotics

The use of feeds with added feed additives or prebiotics, which are defined by Gibson and collaborators (2017) as "a substrate that is selectively utilized by host microorganisms conferring a health benefit", may be another way to avoid the massive use of antimicrobials. Prebiotics can benefit piglets by improving microbiota diversity, inhibiting pathogens, or stimulating the immune system (Gibson et al., 2017). In fact, feed additives based on a combination of encapsulated short-chain fatty acids, medium-chain fatty acids (MCFA), and protected essential oils increased the digestive bacterial diversity in post-weaned piglets, which is linked to health (Soler et al., 2018). In the case of *S. suis*, clinical signs compatible with *S. suis* were reduced with the addition of MCFA and a natural anti-inflammatory (Correa-Fiz et al., 2020).

OBJECTIVES

S. suis disease is considered one of the main causes for use of antimicrobials in pig production. However, the impact of *S. suis* disease in pig production is not totally known. The real incidence and mortality of the disease associated to *S. suis* and the cost that it entails for the swine industry need to be known to stablish a baseline information and to monitor changes in trends in the future. Furthermore, if the factors involved in the appearance of *S. suis* disease are identified, prevention and control of the pathogen would be facilitated.

Prevention measures must take into account the current framework in the use of antimicrobials, developing new alternatives to reduce their use as far as possible. Those new measures must consider the host-pathogen interplay, including the immune response and the colonization status of the host, not only for *S. suis*, but also for other microbiota components.

With those premises, the specific objectives of the thesis were as follows:

- To estimate the prevalence and mortality of the disease associated to *S. suis* infections, as well as quantifying the associated costs in three of the main swine-producing countries of Europe: Germany, the Netherlands and Spain (Study I).
- To assess the risk factors involved in the development of clinical disease caused by *S. suis* in Spanish swine farms (Study II).
- The description of the piglet early immune response to an intranasal challenge with either virulent or non-virulent *S. suis* strain (Study III).
- To study the protective effect of selected nasal commensals against a *S. suis* after virulent strain challenge of piglets (Study IV).

STUDY I

Stochastic assessment of the economic impact of *Streptococcus suis*-associated disease in German, Dutch and Spanish swine farms

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ABSTRACT

The economic assessment of animal diseases is essential for decision-making, including the allocation of resources for disease control. However, that assessment is usually hampered by the lack of reliable data on disease incidence, or treatment and control measures, and that is particularly true for swine production diseases, such as infections caused by *Streptococcus suis*. Therefore, we deployed a questionnaire survey of clinical swine veterinarians to obtain the input data needed for a stochastic model to calculate the costs caused by *S. suis*, which was implemented in three of the main swine producing countries in Europe: Germany, the Netherlands and Spain. *S. suis*-associated disease is endemic in those countries in all production phases, though nursery was the phase most severely impacted. In affected nursery units, between 3.3 and 4.0% of pigs had *S. suis*-associated disease and the mortalities ranged from 0.5 to 0.9%. In Germany, the average cost of *S. suis* per pig (summed across all production phases) was 1.30 euros (90% CI: 0.53-2.28), in the Netherlands 0.96 euros (90% CI: 0.27-1.54), and in Spain 0.60 euros (90% CI: 0.29-0.96). In Germany, that cost was essentially influenced by the expenditure in early metaphylaxis in nursery and in autogenous vaccines in sows and nursery pigs; in the Netherlands, by expenditure on autogenous vaccines in sows and nursery pigs; and in Spain, by the expenditures in early metaphylaxis and to a lesser extent by the mortality in nursery pigs. Therefore, the differences in costs between countries can be explained to a great extent by the measures to control *S. suis* implemented in each country. In Spain and in Germany, use of antimicrobials, predominantly beta-lactams, is still crucial for the control of the disease.

Keywords: *Streptococcus suis*, antimicrobials, questionnaires, economic assessment, swine production disease, incidence, stochastic model, cost of disease.

INTRODUCTION

Streptococcus suis is an encapsulated Gram-positive bacterium naturally present in the upper respiratory tract of healthy pigs, mainly in saliva, tonsils and nasal cavities (O'Dea et al., 2018). Pigs are usually colonized by more than one serotype, but only a few virulent strains are responsible for the disease (Goyette-Desjardins et al., 2014). *S. suis* can cause disease in suckling piglets and fattening pigs, but most frequently in nursery pigs. The most common clinical signs are meningitis, polyarthritis and acute death (Gottschalk and Segura, 2019). *S. suis* is also a zoonotic agent that may cause severe disease in humans, characterized by meningitis, but also sepsis, arthritis or endocarditis (Huong et al., 2014). Human *S. suis* infections were considered rare in the past, but the number of cases reported has increased considerably in recent years (Goyette-Desjardins et al., 2014).

For the last 30 years, *S. suis* infections have been considered a major problem in the swine industry worldwide, in particular in intensive pig production systems (Gottschalk et al., 2010). *S. suis* is among the pathogens for which scientific interest has increased faster in recent years, and it is currently included among the top ten swine pathogens worldwide (VanderWaal and Deen, 2018). Despite this, estimations of its economic impact are lacking. *S. suis* belongs to the group of pathogens that cause production diseases (i.e., diseases not notifiable, but with significant negative impacts on mortality, morbidity, reproduction or growth), and which include for example porcine reproductive and respiratory syndrome virus (PRRSV) (VanderWaal and Deen, 2018). As for production diseases reporting is not required by law, data on their frequency of infection in farms are seldom recorded, or if recorded, results are not comparable due to the absence of a common case definition. Another problem is the lack of documented information on the costs associated to the disease (e.g., treatments). Therefore, novel approaches need to be developed for the estimation of the economic impact of swine production diseases. In data-scarce situations, such as in

countries with inadequate disease surveillance infrastructures or in species for which reporting is not compulsory, questionnaire-based surveys, collecting the information directly from the people able to provide the data, may be the only alternative. Examples of the use of this methodology include the estimation of the incidence of foot-and-mouth disease in Asia, Africa and South America (Sumption et al., 2008) or the incidence of leishmaniosis in dogs from south-eastern Spain (Ruiz de Ybáñez et al., 2009).

An added difficulty in the case of *S. suis* infections is that presumptive diagnosis is often based on clinical signs without laboratory confirmation, although other diseases (e.g., *G. parasuis* infections) may give a similar clinical picture (Gottschalk and Segura, 2019). A further complication for measuring the real impact of *S. suis* infections is that, in order to control the disease, a wide range of antimicrobial agents are sometimes used in farms both prophylactically and metaphylactically (Varela et al., 2013; Seitz et al., 2016). Despite this antimicrobial use (AMU), some animals become diseased and the prognosis of these animals is often poor (Seitz et al., 2016). Moreover, widespread use of antimicrobials may result in the emergence of resistances (Yongkiettrakul et al., 2019). As a result, the AMU is increasingly being restricted, which has contributed a 34.6% decrease in the sales of antimicrobial agents in the 25 reporting EU countries between 2011 and 2018 (EMA, 2020). Pressure to reduce AMU in livestock hinders the control of *S. suis* (Segura et al., 2020), and further restrictions in AMU in coming years may result in an increase of the morbidity due to *S. suis* if not compensated by other measures.

The main objectives of this study were to estimate the frequency of disease associated with the presence of *S. suis* infections in pig farms, as well as quantify the main costs associated with the disease in three of the main pig-producing countries of Europe: Germany, the Netherlands and Spain. Such baseline information is essential to detect changes in the patterns (e.g., an increase of incidence) of *S. suis*-associated disease, to make sensible decisions on whether to allocate resources for their

control, or to evaluate the efficacy of possible interventions. In order to fulfil those objectives, questionnaire-based surveys of clinical swine veterinarians were carried out to obtain input data, that were later fed to mathematical models for the calculation of the costs of disease. To allow the incorporation of variability and/or uncertainty associated with many of its inputs, a stochastic model was developed. Models for the calculation of the cost of animal diseases are commonly stochastic (e.g., (Onono et al., 2014; Wang et al., 2018; Lyons et al., 2019)).

MATERIAL AND METHODS

Selection of study areas

In order to estimate the frequency and costs of *S. suis* infections in Europe, the main pig-producing areas in three of the countries with the largest pig populations within the European Union (EU), namely Germany, the Netherlands and Spain, were selected. Germany had the largest pig production of the EU in 2019 with 22.5% of the total pigs produced (EU, 2020a). Within Germany, most of the data was obtained from Lower Saxony, the region with the highest pig density (Gilbert et al., 2018). Spain had the second largest number of pigs produced in the EU in 2019 with 21.6% of the total production (EU, 2020a). Within Spain, the areas selected were Aragon and Catalonia, which represented 51.6% of the total pig population in Spain, according to the Spanish agricultural census (MAPA, 2019). Finally, the Netherlands had the sixth largest pig production of the EU in 2019, 6.8% (EU, 2020a), and has one of the highest density of pigs in the continent.

Questionnaires for *S. suis*-disease

The majority of the data needed for the model was obtained through a comprehensive questionnaire, which was administered (throughout 2019) to a group of swine clinical veterinarians aimed to be representative of the different types of pig production present in the areas of study. An initial version of the questionnaire was drafted by a panel of experts, then tested with several clinical veterinarians, and

deficiencies were corrected (a copy of the final questionnaire is included as Supplementary Data S2.1). To allow the veterinarians to collect the data requested from all the farms for which they had information, questionnaires were sent a few days in advance, and then the interview was carried out by phone to facilitate clarification of any questions. Because of the complexity of the questionnaire, the final interview took about 1 h. In total, 12 clinical veterinarians were interviewed in Spain, 10 in Germany and 11 in the Netherlands. To avoid confidentiality issues, the names of the veterinarians and the companies they worked for were not recorded.

The questionnaire for veterinarians included questions in relation to several parameters:

a) number of farms of the different types (e.g., farrowing, finishing or farrow to finish) of which they were in charge, as well as the mean number of animals of the different types within them.

b) for each production phase (i.e., suckling piglets, nursery pigs and fatteners) in those farms, the proportion of times in the last year those phases were affected by *S. suis* clinical disease, the proportion of batches affected within those phases, the proportion of animals affected within those batches, and the proportion of deaths. Because diagnosis of *S. suis* infection is usually based on clinical signs without laboratory confirmation, our case definition for the questionnaires was based on the presence of signs compatible with *S. suis* infection (i.e., arthritis, incoordination or paddling). A case-farm was a farm with at least one animal with clinical disease caused by *S. suis* infection in the last 12 months.

c) classes of the antimicrobial products, route of administration and duration of treatments in each production phase. Three types of treatments were considered: early metaphylactic, late metaphylactic and therapeutic. The term early metaphylaxis referred to the administration of antimicrobials to healthy animals in farms endemically affected by *S. suis* disease; late metaphylaxis was when the treatment was applied

also to healthy animals, but there were already sick animals in the group; and therapeutic was the treatment of only sick animals.

d) proportion of farms in which autogenous vaccines were applied.

e) proportion of farms in which samples from suspected cases of *S. suis* disease were sent to a laboratory for confirmation, and proportion of times those suspected cases were actually confirmed.

The reason for requesting information independently for each production phase was that several parameters (e.g., prevalence or treatments) varied significantly between phases. Therefore, throughout the text we use the terms production phases to refer to the phases of suckling piglets, nursery pigs and fatteners; and the term production units to refer to the sites where those phases took place.

Within each country, we wanted to account for the fact that the veterinarians providing information on more farms should have more weight, but at the same time, we wanted to avoid the parameters being essentially determined just by a few veterinarians with the most farms. Therefore, we restricted the weights of the veterinarians to between 1 and 20% depending on the number of farms they provided information for (see Supplementary Data S2.2 for a detailed explanation of the calculation of weights).

Questionnaires were completed in Excel, then data extraction was implemented within the R environment version 4.0.2 (R Core Team, 2020).

Quantifying the costs associated with *S. suis* infection

Based on the methodological framework proposed by Rushton (2009), the cost of disease was the sum of the losses caused directly by the disease, and the expenditures as a result of responding to the disease. For quantifying the cost of *S. suis*-associated disease, only visible losses caused by weight loss and mortality were included (Figure 2.1); invisible losses such as public health costs, were not quantified. On the other hand, expenditures comprised additional costs as a result of antimicrobial

treatments (early metaphylactic, late metaphylactic and therapeutic), and the expenditure on autogenous vaccines and on laboratory analyses (Figure 2.1). The expense of revenue forgone when denied access to better markets (Rushton, 2009) for example, was not considered.

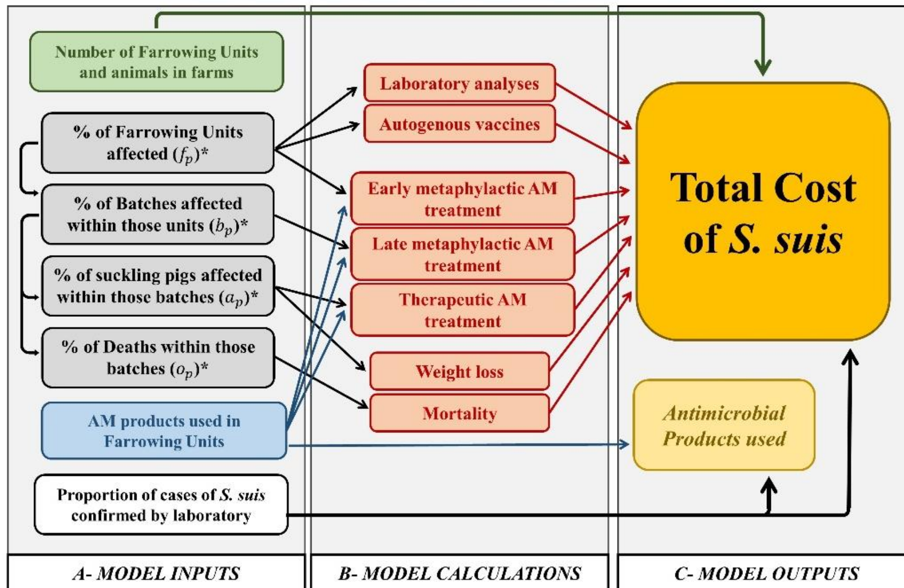

Figure 2.1 | Diagram of the quantification of costs associated with *S. suis* infection and antimicrobial use in farrowing units. Gray area represents data obtained from the questionnaire for veterinarians. Red area represents estimates of the different costs associated with *S. suis* infection. Yellow area represents the outputs of calculations. *Subindex $\boldsymbol{p}$ refers to suckling piglets (i.e., farrowing units), similar calculations were carried out for nursery pigs in weaning units (subindex $\boldsymbol{n}$) and fatteners in fattening units (subindex $\boldsymbol{f}$), which when summed result in the calculation of the total cost at the end of the production cycle.

In order to capture the different sources of variability and uncertainty associated with the data on *S. suis*, a stochastic model was developed. The model was built so that each of the losses and expenditures considered was defined by a probability distribution. In particular, discrete distributions were used to incorporate the weights of the different questionnaires/veterinarians (Vose, 2008).

Given the differences between production phases, the costs of the disease were calculated independently for suckling piglets, nursery pigs and fatteners. Sub-index $\boldsymbol{p}$ was used for suckling piglets in farrowing

units, $\boldsymbol{n}$ for nursery pigs in nursery units, and $\boldsymbol{f}$ for fatteners in fattening units. Besides, the costs of *S. suis* were calculated separately for Germany, the Netherlands and Spain, since for example the measures to control the disease and their associated costs were different.

Model calculations: First, for each country, we calculated the average costs of *S. suis* per animal (regardless of the health status) in each phase, given some level of infection in the corresponding production units (e.g., mean cost per suckling piglet in affected farrowing units in Spain). Second, the mean annual costs for each of those production units were calculated (e.g., mean annual cost per affected nursery unit in Germany). Third, the average costs per animal are summed across the three production phases to estimate total cost per pig produced in each country (e.g., mean cost of *S. suis* per pig, at the end of the production cycle, produced in the Netherlands in 2019).

<u>Estimation of the costs per animal in affected production phases</u>

a) <u>Losses due to weight loss</u>

First, for each questionnaire, we calculated the proportion of diseased suckling piglets in the farrowing units affected by *S. suis* ($\boldsymbol{d_{p,i}}$) as:

$$\boldsymbol{d_{p,i}} = \boldsymbol{b_{p,i}} \times \boldsymbol{a_{p,i}} \times \boldsymbol{p_p}$$

Where $\boldsymbol{b_{p,i}}$ was the proportion of batches with clinical disease in affected units according to questionnaire $\boldsymbol{i}$, $\boldsymbol{a_{p,i}}$ the proportion of animals with clinical disease within those batches according to questionnaire $\boldsymbol{i}$, and $\boldsymbol{p_p}$ was the proportion of clinical cases confirmed by the laboratory as caused by *S. suis* (Figure 2.1). Since the data obtained through the questionnaires was based on clinical diagnosis and there are other pathogens that may give a similar clinical picture, we had to account for that fact to obtain the real number of animals with disease caused by *S. suis*. That proportion ($\boldsymbol{p}$) varied between countries and between production phases (Supplementary Table S2.1).

Then, the average cost per suckling piglet due to weight loss for questionnaire $\boldsymbol{i}$ ($\boldsymbol{c_{p_{weight},i}}$) was calculated as:

$$\boldsymbol{c_{p_{weight},i} = d_{p,i} \times x_{p,i} \times v_p}$$

Where $\boldsymbol{x_{p,i}}$ was the proportion of weight loss in questionnaire $\boldsymbol{i}$, and $\boldsymbol{v_p}$ the average value of suckling piglets.

Finally, we defined the distribution for the losses due to weight loss per suckling piglet in affected farrowing units ($\boldsymbol{c_{p_{weight}}}$) based on the weights of the different questionnaires ($\boldsymbol{w_{p,i}}$) as:

$$\boldsymbol{c_{p_{weight}} = Discrete(\{c_{p_{weight},i}\},\{w_{p,i}\})}$$

b) Losses due to mortality

Similarly, for each questionnaire, we calculated the proportion of suckling piglets that died in farrowing units affected by *S. suis* ($\boldsymbol{m_{p,i}}$) as (Figure 2.1):

$$\boldsymbol{m_{p,i} = b_{p,i} \times o_{p,i} \times p_p}$$

Where $\boldsymbol{o_{p,i}}$ was the proportion of suckling piglets that died within batches affected with *S. suis* infection in questionnaire $\boldsymbol{i}$.

Then, the average loss per suckling piglet due to mortality in affected farrowing units for questionnaire $\boldsymbol{i}$ ($\boldsymbol{c_{p_{mort,i}}}$) was calculated as:

$$\boldsymbol{c_{p_{mort,i}} = m_{p,i} \times v_p}$$

And we defined the distribution for the loss per suckling piglet due to mortality in affected farrowing units ($\boldsymbol{c_{p_{mort}}}$) depending on the weights of the questionnaires as:

$$\boldsymbol{c_{p_{mort}} = Discrete(\{c_{p_{mort,i}}\},\{w_{p,i}\})}$$

c) <u>Expenditure on early metaphylactic antimicrobial treatment</u>

Different types of antimicrobials may be used as early metaphylactic treatment, so there were significant variations in the products and/or the routes of administration used, which have different costs. Therefore, for each questionnaire, and for each combination of product and route, we calculated the average expenditure per suckling piglet of that treatment (e.g., treatment number 1) ($\boldsymbol{c_{p_{E1,i}}}$) as:

$$\boldsymbol{c_{p_{E1,i}} = g_{p_{E1,i}} \times r_{p_{E1}} \times t_{p_{E1,i}} \times p_p}$$

Where $\boldsymbol{g_{p_{E1,i}}}$ was the mean proportion of affected farrowing units in which early metaphylactic treatment number 1 (i.e., $\boldsymbol{E_1}$) was applied according to questionnaire $\boldsymbol{i}$, $\boldsymbol{r_{pE1}}$ was the daily cost of that treatment per suckling piglet, $\boldsymbol{t_{p_{E1,i}}}$ the number of days of application according to questionnaire $\boldsymbol{i}$, and $\boldsymbol{p_p}$ was the proportion of clinical cases confirmed. The data on the costs of the different antimicrobial treatments used in each of the countries were obtained from clinical swine veterinarians. Given the variation in prices depending on factors such as the brand or the quantity bought, an average cost was calculated for each antimicrobial and each route of administration for each country (Supplementary Table S2.1).

Then, we added the different early metaphylactic antimicrobial treatments (represented by sub-index $\boldsymbol{j}$) to obtain the total expenditure per suckling piglet in farrowing units according to questionnaire $\boldsymbol{i}$ ($\boldsymbol{c_{p_{earlymeta,i}}}$) as:

$$\boldsymbol{c_{p_{earlymeta,i}} = \sum_{j=1}^{n} c_{p_{Ej,i}}}$$

Finally, we defined the distribution for the total expenditure in early metaphylactic antimicrobial treatments per suckling piglet in farrowing units ($\boldsymbol{c_{p_{earlymeta}}}$) depending on the weights as:

$$c_{p_{earlymeta}} = \boldsymbol{Discrete}(\{c_{p_{earlymeta,i}}\}, \{w_{p,i}\})$$

Similarly, for each questionnaire, and for each combination of product and route, we calculated the average expenditure of late metaphylactic treatment number 1 per suckling piglet ($c_{p_{L1,i}}$) as:

$$c_{p_{L1,i}} = g_{p_{L1,i}} \times r_{p_{L1}} \times t_{p_{L1,i}} \times p_p$$

Where $g_{p_{L1,i}}$ was the mean proportion of affected farrowing units in which late metaphylactic treatment number 1 (i.e. L_1) was applied according to questionnaire i, r_{pL1} was the daily cost of that treatment per suckling piglet, $t_{p_{L1,i}}$ the number of days of application according to questionnaire i, and p_p was the proportion of clinical cases confirmed.

And the same for therapeutic treatment number 1 per suckling piglet ($c_{p_{L1,i}}$):

$$c_{p_{T1,i}} = g_{p_{T1,i}} \times r_{p_{T1}} \times t_{p_{T1,i}} \times p_p$$

Where $g_{p_{T1,i}}$ was the mean proportion of affected farrowing units in which therapeutic treatment number 1 (i.e. T_1) was applied according to questionnaire i, r_{pT1} was the daily cost of that treatment per suckling piglet, $t_{p_{T1,i}}$ the number of days of application according to questionnaire i, and p_p was the proportion of clinical cases confirmed.

Then, we added the different late metaphylactic antimicrobial treatments and the different therapeutic antimicrobial treatments. Finally, we defined the distribution for the total expenditure in late metaphylactic and therapeutic antimicrobial treatments per suckling piglet in farrowing units ($c_{p_{latemeta}}$ and $c_{p_{therapeutic}}$, respectively) depending on the weights.

d) Expenditure on autogenous vaccines

First, for each questionnaire, we calculated the average expenditure per suckling piglet due to the use of autogenous vaccines ($c_{p_{autovac,i}}$) as:

$$\boldsymbol{c_{p_{autovac,i}}} = \boldsymbol{h_{p,i}} \times \boldsymbol{k_p}$$

Where $\boldsymbol{h_{p,i}}$ was the proportion of affected farrowing farms in which autogenous vaccines were used according to questionnaire $\boldsymbol{i}$, and $\boldsymbol{k_p}$ was the vaccination cost per animal. In farrowing units, passive immunization of suckling piglets relies on the vaccination of sows, although for simplification purposes, the costs were recalculated per piglet.

Then, we defined the distribution for the total expenditure on autogenous vaccines per suckling piglet ($\boldsymbol{c_{p_{autovac}}}$) depending on the weights as:

$$\boldsymbol{c_{p_{autovac}}} = \boldsymbol{Discrete}(\{\boldsymbol{c_{p_{autovac,i}}}\}, \{\boldsymbol{w_{p,i}}\})$$

e) Expenditure on analyses

First, for questionnaire $\boldsymbol{i}$, we calculated the average expenditure on analyses per suckling piglet ($\boldsymbol{c_{p_{analyses,i}}}$) as:

$$\boldsymbol{c_{p_{analyses,i}}} = \boldsymbol{l_{p,i}} \times \left(\frac{\boldsymbol{s}}{\boldsymbol{N_{p,i}}}\right)$$

Where, $\boldsymbol{l_{p,i}}$ was the proportion of affected farrowing farms that sent samples to the laboratory for confirmation according to questionnaire $\boldsymbol{i}$, $\boldsymbol{s}$ was the average cost of analysis including the shipping of samples and the laboratory costs and $\boldsymbol{N_{p,i}}$ was the average number of piglets produced per year per farrowing farm according to questionnaire $\boldsymbol{i}$. That way, costs per farm are transformed into costs per animal.

Finally, we defined the distribution for the total expenditure for analyses per suckling piglet ($\boldsymbol{c_{p_{analyses}}}$) depending on the weights as:

$$\boldsymbol{c_{p_{analyses}}} = \boldsymbol{Discrete}(\{\boldsymbol{c_{p_{analyses,i}}}\}, \{\boldsymbol{w_{p,i}}\})$$

<u>Estimation of the annual costs per affected production unit</u>

For quantifying the annual costs in affected production units, we first had to calculate the average number of animals produced in those units per year (Supplementary Data S2.3). By considering that, and the different losses and expenditures per animal according to questionnaire $\boldsymbol{i}$, we obtained the distributions per affected production phase per year. For example, the distribution for the total annual cost due to weight loss in suckling piglets in affected farrowing units ($\boldsymbol{C_{p_{weight}}}$) was defined as:

$$\boldsymbol{C_{p_{weight}}} = \boldsymbol{Discrete}\left(\left\{\boldsymbol{c_{p_{weight},i}} \times \boldsymbol{N_{p,i}}\right\}, \left\{\boldsymbol{w_{p,i}}\right\}\right)$$

Where $\boldsymbol{c_{p_{weight},i}}$ was the average cost per suckling piglet due to weight loss for questionnaire $\boldsymbol{i}$, $\boldsymbol{N_{p,i}}$ was the average number of suckling piglets produced per year per farrowing unit for questionnaire $\boldsymbol{i}$, and $\boldsymbol{w_i}$ was the weight of the questionnaire $\boldsymbol{i}$. Upper case "$\boldsymbol{C}$" was used for the costs per unit per year, and lower case "$\boldsymbol{c}$" for the costs per animal. Similarly, the distributions for the total annual losses due to mortality ($\boldsymbol{C_{p_{mort}}}$), and total annual expenditures in early metaphylactic antimicrobial treatments ($\boldsymbol{C_{p_{earlymeta}}}$), late metaphylactic antimicrobial treatments ($\boldsymbol{C_{p_{latemeta}}}$), therapeutic antimicrobial treatments ($\boldsymbol{C_{p_{therapeutic}}}$), autogenous vaccines ($\boldsymbol{C_{p_{autovac}}}$), and analyses ($\boldsymbol{C_{p_{analyses}}}$) in affected farrowing units, were also obtained.

Finally, the total cost per affected farrowing unit per year was calculated as:

$$\boldsymbol{C_{p_{total}}} = \boldsymbol{C_{p_{weight}}} + \boldsymbol{C_{p_{mort}}} + \boldsymbol{C_{p_{earlymeta}}} + \boldsymbol{C_{p_{latemeta}}}$$
$$+\boldsymbol{C_{p_{therapeutic}}} + \boldsymbol{C_{p_{autovac}}} + \boldsymbol{C_{p_{analyses}}}$$

<u>Estimation of the cost per animal by country, summed across all production phases</u>

Finally, in a given country, the average cost due to *S. suis* for each pig at the end of the production cycle (i.e., end of fattening), was estimated. In

order to do that, first the average cost per suckling piglet ($\boldsymbol{a_{p_{total}}}$), the average cost per nursery pig ($\boldsymbol{a_{n_{total}}}$), and the average cost per fattener ($\boldsymbol{a_{f_{total}}}$) was calculated as:

$$\boldsymbol{a_{p_{total}}} = \boldsymbol{c_{p_{total}}} \times \boldsymbol{f_p}$$

$$\boldsymbol{a_{n_{total}}} = \boldsymbol{c_{n_{total}}} \times \boldsymbol{f_n}$$

$$\boldsymbol{a_{f_{total}}} = \boldsymbol{c_{f_{total}}} \times \boldsymbol{f_f}$$

Where, $\boldsymbol{c_{p_{total}}}$, $\boldsymbol{c_{n_{total}}}$, and $\boldsymbol{c_{f_{total}}}$ were the average costs of *S. suis* in affected units per suckling piglet, nursery pig and fattener, respectively; and $\boldsymbol{f_p}$, $\boldsymbol{f_n}$, and $\boldsymbol{f_f}$ were the proportions of farrowing, nursery and fattening units affected by *S. suis*-disease, respectively.

Then, for a given country, e.g., Spain ($\boldsymbol{c_{Spain}}$), by adding the average costs for the different phases of production, the average cost due to *S. suis* for each pig at the end of the production cycle was calculated:

$$\boldsymbol{c_{Spain}} = \boldsymbol{a_{p_{total}}} + \boldsymbol{a_{n_{total}}} + \boldsymbol{a_{f_{total}}}$$

A numerical example of the calculation of the average cost due to *S. suis* for each pig at the end of the production cycle is included as Supplementary Data S2.4.

Sensitivity analysis

A sensitivity analysis (SA) was used to quantify the influence of the different losses and expenditures in the different production phases on the total costs of *S. suis* per pig at the end of the production cycle by country (i.e., $\boldsymbol{c_{Germany}}$, $\boldsymbol{c_{Netherlands}}$, and $\boldsymbol{c_{Spain}}$). Rank order correlation was used as recommended by the Office International des Epizooties (OIE) (Murray, 2004).

Modelling software

The spreadsheet model was constructed in Microsoft Excel (Microsoft® Office Professional Edition, 2013), and run for 150,000 iterations using

Latin Hypercube sampling as recommended (Murray, 2004) in @Risk version 6.1.1 (© Palisade Corporation). Such number of iterations was selected to ensure the convergence of all output parameters considering a convergence tolerance of only 1% with a confidence level of 95% for their mean values.

Costs of antimicrobials by family

Besides considering the costs of antimicrobials by types of treatment (i.e., early metaphylactic, late metaphylactic, and therapeutic), the costs of antimicrobials were also calculated by antimicrobial families. Antimicrobials were grouped in the following families: beta-lactams, cephalosporins, macrolides, sulphonamides, tetracyclines, and others.

Source of data

In addition to all the information obtained from the questionnaires, the model for the calculation of the costs of *S. suis* required many other input parameters, which were obtained from a variety of sources. A complete list of input parameters with their values, units and sources are detailed in Supplementary Table S2.1.

RESULTS

Questionnaires and the occurrence of *S. suis* infections in the countries of study

The clinical veterinarians interviewed were able to provide data from 1,652 production units in Germany, 480 in the Netherlands, and 1,583 in Spain (Table 2.1).

Differences between phases

S. suis-associated disease is endemic in Germany, the Netherlands and Spain in all production phases, although with differences between countries and phases (Table 2.1). In the three countries, the phase most severely affected was nursery with high proportions of units affected (62.0–82.9%) and batches within those units (64.1–66.5%). However, as those estimates were based on clinical diagnosis, we evaluated the

proportion of suspected clinical cases confirmed by the laboratory. That proportion varied between countries and particularly between production phases. In Germany, the Netherlands and Spain, the proportions of confirmation in suckling piglets were 75, 81, and 86%, respectively; in nursery 77, 91, and 76%, respectively; and in fattening 46, 81, and 50%, respectively.

Taking the probability of confirmation into account, in affected nursery units in those countries, between 3.3 and 4.0% of nursery pigs had *S. suis*-associated disease, with a mortality between 0.5 and 0.9% (Table 2.1). Morbidity and mortality were lower in affected farrowing units and much lower in affected fattening units (Table 2.1).

Differences between countries

The proportions of animals affected by *S. suis* and the mortalities in the different phases were similar between the countries (Table 2.1). The main discrepancies were in the proportion of animals affected in farrowing, which ranged between 1.2% in Spain and 2.2% in Germany.

Questionnaires of the costs associated with *S. suis* infection

Cost per animal in affected production phases

The mean total costs per suckling piglet in affected farrowing units were 0.86 euros in Germany, 0.61 in the Netherlands and 0.11 in Spain. In affected nursery units, the costs were higher, 1.06 euros per nursery pig in Germany, 0.73 in the Netherlands and 0.57 in Spain. In affected fattening units, the costs were much lower, 0.22, 0.11, and 0.07 euros per fattener, respectively in the three countries. The mean values for the different types of losses and expenditures and their 90% confidence intervals (CI), are shown in Table 2.2. The wide CI for some of the values obtained are indicative of significant variations between the costs, even within a country and a production phase.

Weight losses due to *S. suis* were considered negligible in the three phases. The mortality losses per nursery pig ranged from 0.19 euros

in Spain to 0.24 in Germany and the Netherlands, while values for suckling piglets and fatteners were much lower.

Substantial differences were observed between countries and phases in the expenditure of early metaphylactic treatments (Table 2.2). The highest expenditures were in Germany, in particular in nursery pigs (0.44 euros per animal), but also in suckling piglets (0.15 euros) and fatteners (0.14 euros). In Spain, the expenditure of early metaphylaxis was important only in nursery pigs (0.29 euros per animal), while in the Netherlands it was almost negligible in all phases. The expenditure of late metaphylactic treatments were consistently low, except for nursery pigs in Germany (0.17 euros per animal). The expenditure of therapeutic treatments was even lower (Table 2.2).

There were important differences in relation to the expenditure on autogenous vaccines. The costs of vaccination were high in sows (included in the costs of suckling piglets) in Germany and in the Netherlands (0.59 and 0.57 euros per piglet, respectively), and in nursery pigs in the Netherlands (0.44 euros). In contrast, in Spain, spending on autogenous vaccines was low in all phases. There were even substantial discrepancies in the expenditure on autogenous vaccines within a country (as shown by the wide CI).

Finally, the costs of laboratory analyses were almost negligible in all the countries.

Table 2.1 | Frequency of *S. suis* infections in the countries of study, including % of units clinically affected, % of batches clinically affected within affected units, % of animals with *S. suis* disease and mortality (%) caused by *S. suis* disease.

Phase	Country	Total units from which data was collected	Mean number of animals produced per unit per year	% of units clinically affected (f)*	% of batches clinically affected within affected units (b)*	% of animals with *S. suis* disease within affected units (d)*	Mortality (%) caused by *S. suis* disease within affected units (m)*
Suckling piglets	Germany	510	10,725	64.5%	52.9%	2.2%	0.4%
	The Netherlands	157	17,614	66.7%	43.3%	1.6%	0.3%
	Spain	437	25,780	80.4%	36.1%	1.2%	0.4%
Nursery pigs	Germany	468	10,620	62.0%	64.1%	3.3%	0.5%
	The Netherlands	171	16,423	68.0%	65.2%	4.0%	0.9%
	Spain	370	22,665	82.9%	66.5%	3.3%	0.7%
Fatteners	Germany	674	8,173	39.8%	19.8%	0.2%	0.0%
	The Netherlands	152	9,119	58.2%	28.3%	0.3%	0.1%
	Spain	776	7,774	47.1%	31.8%	0.3%	0.1%

* *Values weighted by questionnaires.*

Table 2.2 | Mean losses, expenditures and total cost per animal in affected production units (in euros) for the different production phases in the countries of study.

Phase	Country	Mortality	Early metaphylactic	Late metaphylactic	Therapeutic	Autogenous vaccines	Analyses	Total
Suckling piglets	Germany	0.05 (0.00-0.15)	0.15 (0.00-0.50)	0.06 (0.00-0.20)	0.01 (0.00-0.02)	0.59 (0.23-0.92)	0.01 (0.00-0.02)	0.86 (0.31-1.39)
	The Netherlands	0.03 (0.00-0.06)	0.00 (0.00-0.02)	-	0.00 (0.00-0.02)	0.57 (0.00-0.91)	0.00 (0.00-0.01)	0.61 (0.03-0.96)
	Spain	0.05 (0.00-0.10)	0.02 (0.00-0.12)	0.01 (0.00-0.04)	-	0.03 (0.00-0.36)	0.00 (0.00-0.01)	0.11 (0.01-0.40)
Nursery pigs	Germany	0.24 (0.05-0.70)	0.44 (0.00-1.34)	0.17 (0.02-1.81)	0.04 (0.00-0.24)	0.17 (0.00-0.92)	0.01 (0.00-0.02)	1.06 (0.19-2.49)
	The Netherlands	0.24 (0.05-0.38)	0.01 (0.00-0.04)	0.02 (0.00-0.10)	0.02 (0.00-0.07)	0.44 (0.00-0.91)	0.00 (0.00-0.01)	0.73 (0.15-1.32)
	Spain	0.19 (0.03-0.38)	0.29 (0.04-0.67)	0.05 (0.00-0.08)	0.01 (0.00-0.01)	0.04 (0.00-0.22)	0.00 (0.00-0.01)	0.57 (0.24-0.94)
Fatteners	Germany	0.02 (0.00-0.05)	0.14 (0.00-0.41)	0.05 (0.00-0.53)	0.01 (0.00-0.03)	-	0.00 (0.00-0.02)	0.22 (0.01-0.54)
	The Netherlands	0.05 (0.00-0.11)	0.00 (0.00-0.02)	0.00 (0.00-0.01)	0.00 (0.00-0.01)	0.04 (0.00-0.52)	0.00 (0.00-0.01)	0.11 (0.01-0.54)
	Spain	0.04 (0.01-0.17)	0.02 (0.00-0.07)	0.01 (0.00-0.05)	-	-	-	0.07 (0.01-0.23)

In brackets, 90% confidence interval (CI) of the corresponding cost.

Annual cost per affected production units

By considering the average number of animals produced per year in each type of production unit, and the associated costs per animal in affected units, we calculated the average costs per affected production unit in the three countries of study. The main economic costs occurred in affected nursery units, with an average annual cost per affected unit of 9.9 thousand euros in Germany, 11.2 in the Netherlands and 14.1 in Spain. The costs were also substantial in affected farrowing units in Germany (8.7 thousand euros per affected unit) and the Netherlands (10.2 thousand euros), and much lower in Spain. In affected fattening units, the annual costs were considerably lower. The costs (mean values and 90% CI) per affected production unit in the countries of study are shown in Supplementary Table S2.2.

Cost per animal by country, summed across all production phases

For a given country, taking into account the average costs per animal in affected farrowing, nursery and fattening units, and the proportions of those units affected, the average cost due to *S. suis* for each pig at the end of the production cycle was calculated. By considering the proportions of units affected, the value obtained is an average cost of *S. suis* for each of the pigs produced in the country.

In Germany, the mean cost of *S. suis* per pig at the end of the production cycle was 1.30 euros (90% CI: 0.53–2.28; in the Netherlands, 0.96 euros (90% CI: 0.27–1.54); and in Spain, 0.60 euros, (90% CI: 0.29–0.96). The probability distributions for the mean cost of *S. suis* per pig at the end of the production cycle for the countries of study are shown in Figure 2.2. The distribution for the Netherlands had a trimodal shape, while for Germany and Spain the distributions were bell-shaped.

The sensitivity analysis (Figure 2.2) showed that the cost in Germany was mainly influenced by the expenditures in early metaphylaxis in nursery and in autogenous vaccines in farrowing and nursery. In the Netherlands, the expenditures on autogenous vaccines in

sows and farrowing were the most influential. In Spain, the cost of *S. suis* was mainly influenced by the expenditures in early metaphylaxis and to a lesser extent by the mortality in nursery.

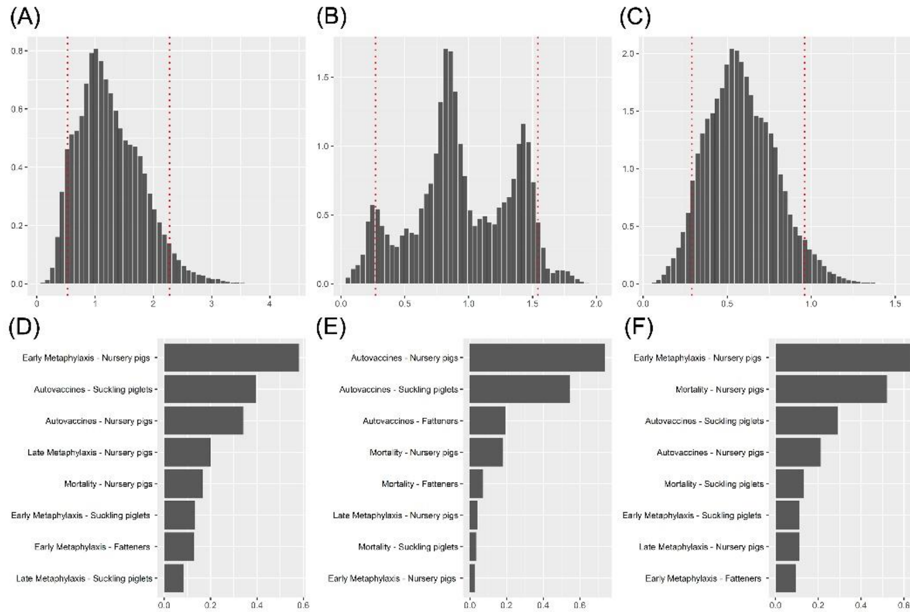

Figure 2.2 | Probability distributions for the mean cost of *S. suis* (summed across all production phases) per pig obtained at the end of the production cycle in Germany (A), the Netherlands (B), and Spain (C), and results of the sensitivity analysis of those costs (by rank order correlation) in Germany (D), the Netherlands (E), and Spain (F).

<u>Cost of antimicrobials by family</u>

The costs of antimicrobials (mean and 90% CI) per animal in affected production units in the countries of study, by antimicrobial families, are shown in Table 2.3. Beta-lactams represented a significant part of the cost of antimicrobials for the control of *S. suis*, in particular in nurseries in Germany and Spain (56.9 and 30.9 cents of euros per nursery pig, respectively). Cephalosporins, macrolides, sulphonamides, tetracyclines and other antimicrobials were used only occasionally in some phases and countries (Table 2.3; Figure 2.3).

Table 2.3 | The costs of antimicrobials (mean and 90% CI) per animal in affected production units by antimicrobial families for the countries (cost in cents of euros).

Phase	Country	Beta-lactams	Cephalosporins	Macrolides	Sulphonamides	Tetracyclines	Others	Total *S. suis*
Suckling piglets	Germany	13.9 (0.1-46.0)	0.4 (0.0-1.6)	0.5 (0.0-4.2)	0.4 (0.0-4.6)	<0.1 (0.0-<0.1)	6.6 (0.0-22.8)	21.7 (5.5-46.8)
	The Netherlands	0.6 (0.1-2.2)	-	-	0.1 (0.0-0.2)	<0.1 (0.0-<0.1)	-	0.6 (0.1-2.2)
	Spain	1.3 (0.0-7.6)	1.5 (0.0-4.3)	0.1 (0.0-1.8)	-	-	0.1 (0.0-1.2)	2.9 (0.0-8.3)
Nursery pigs	Germany	56.9 (4.1-181.9)	<0.1 (0.0-0.2)	1.9 (0.0-29.9)	1.4 (0.0-16.0)	0.3 (0.0-4.7)	1.6 (0.0-27.3)	64.9 (4.1-181.9)
	The Netherlands	3.5 (0.2-13.6)	-	-	0.8 (0.0-2.3)	-	0.2 (0.0-2.0)	4.5 (0.8-14.4)
	Spain	30.9 (8.3-53.0)	0.6 (0.0-4.8)	-	0.9 (0.0-9.4)	1.5 (0.0-20.7)	0.2 (0.0-0.9)	34.1 (8.3-56.9)
Fatteners	Germany	12.2 (0.0-101.2)	<0.1 (0.0-<0.1)	<0.1 (0.0-0.3)	-	0.5 (0.0-5.3)	1.0 (0.0-13.9)	13.7 (0.0-101.2)
	The Netherlands	0.3 (0.0-1.4)	-	-	0.1 (0.0-0.7)	-	<0.1 (0.0-<0.1)	0.5 (0.0-1.4)
	Spain	2.5 (0.0-7.3)	<0.1 (0.0-<0.1)	-	<0.1 (0.0-<0.1)	<0.1 (0.0-<0.1)	<0.1 (0.0-<0.1)	2.7 (0.0-7.3)

The value <0.1 is used for values below 0.1 but different from zero.

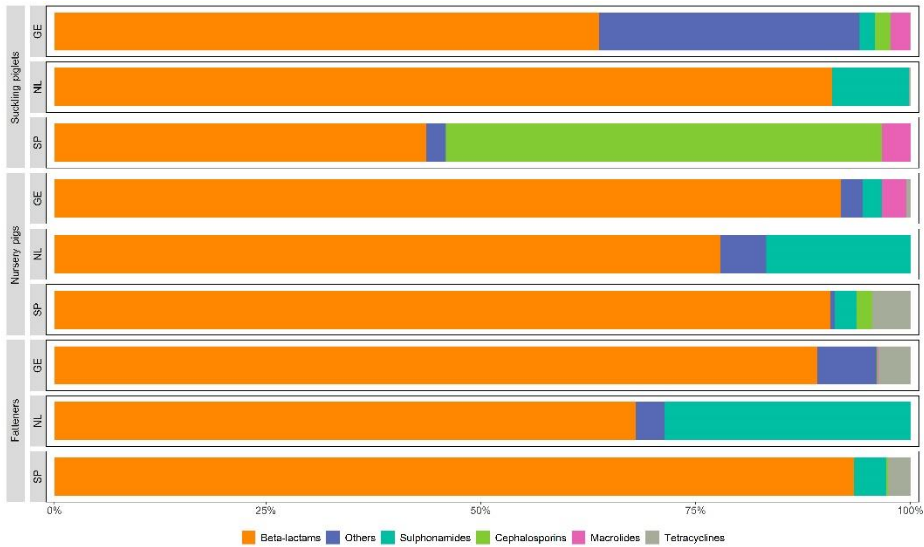

Figure 2.3 | Proportions of the costs of antimicrobials per animal in affected production units by antimicrobial families for the countries of study.

Discussion

S. suis-associated disease is regarded as one of the main diseases in the swine industry, in particular in intensive pig production systems (Gottschalk et al., 2010). However, as with other production diseases such as PRRS or swine influenza, because reporting is not compulsory, there is almost no data on their occurrence, the measures by which they are currently controlled in the field, and most importantly, the losses and expenditures they cause. *S. suis*-associated disease is considered endemic in the majority of countries of the world, although studies on its frequency are lacking. Our results show that the disease is highly prevalent in German, Dutch and Spanish pig farms. The proportions of animals affected and the mortalities in the different phases were quite similar in the three countries despite differences in the proportions of units and batches affected or in the measures applied to control the disease.

There were substantial differences in the frequency of disease between phases. Nursery was the phase most frequently affected, and also where morbidity and mortality were highest, confirming previous

observations (Gottschalk and Segura, 2019). The mortality of *S. suis*-disease reported is usually lower than 5% (Clifton-Hadley et al., 1986), although in absence of treatment outbreaks could reach mortalities of 20-30% (Cloutier et al., 2003; Hopkins et al., 2018). Decrease of maternally-derived antibodies during the nursery phase, or stress due to the movement of animals to the nursery units, or the mixing of animals from different litters, may explain why the disease is more frequent in nurseries (Rooke and Bland, 2002; Goyette-Desjardins et al., 2014; Corsaut et al., 2020).

Our study showed that *S. suis* also causes losses in suckling piglets, as indicated by the morbidity and mortality reported by the veterinarians we surveyed. In some farms the amount of colostrum ingested by piglets may not be adequate, which may compromise the passive maternal immunity in piglets. *S. suis*-associated disease may also occur in suckling piglets from gilts due to lower levels of antibodies (Gottschalk and Segura, 2019). In contrast, *S. suis*-disease was much less of a problem for fatteners, which coincides with previous knowledge that *S. suis*-disease rarely occurred in pigs 10 weeks of age or older (Segura, 2020). It is believed that older animals are resistant to the disease due to the presence of high levels of antibodies (Gottschalk and Segura, 2019; Corsaut et al., 2020).

The causes of *S. suis*-associated disease endemicity in Germany, the Netherlands and Spain are not well-known. Intensification of pig production resulted in a shift of the relative importance of swine pathogens, with parasites becoming less common and bacterial diseases more frequent (with *S. suis* among the group that has increased faster) (VanderWaal and Deen, 2018). Coinfection with viruses such as PRRSv or swine influenza virus, which are prevalent in the countries of study, results in a higher incidence of *S. suis*-disease and more severe lesions (Schmitt et al., 2001; Lin et al., 2015).

As diagnosis of *S. suis* infection in the field is essentially clinical, but there are other pathogens that may give similar signs, we obtained

information on the proportion of suspected cases that were actually confirmed by the laboratory. According to our results, the majority (>75%) of the clinical cases in suckling piglets and nursery pigs were confirmed, while in fattening the proportion was lower.

S. suis has important consequences for swine production, although with differences between the countries of study. In Germany, *S. suis*-disease primarily affected suckling piglets and nursery pigs, and to a lesser extent fatteners; in the Netherlands it largely affected suckling piglets and nursery pigs; while in Spain, *S. suis*-disease affected mainly nursery pigs. Direct losses were almost exclusively caused by mortality, as weight loss in affected animals was considered insignificant because they recovered and regained their normal weight before the end of the production cycle. The costs due to mortality were relatively similar between countries, but there were differences between phases, with much higher losses in nursery than in suckling piglets (where the mortality and the values of the animals were lower) and fattening (where the values of the animals was higher, but the mortality much lower). In Spain, mortality in nursery pigs was particularly influential on the total cost per pig at the end of the production cycle (as shown in the SA).

Antimicrobial costs of *S. suis* were considerable in Germany and Spain as a result of early metaphlyactic treatment (also revealed in the SA). Use of antimicrobials in swine production has traditionally been considered a cost-effective tool to control diseases (Moreno, 2014); some farmers have the perception that they contribute to increased profits (Stevens et al., 2007).

The pattern of AMU in each country was likely dependent on how restrictive the national legislation was in that regard. In Germany, AMU is only justified if confirmed by laboratory diagnosis or if there are epidemiological evidences it is caused by a specific pathogen. Late metaphylactic treatment is allowed, while early metaphylaxis is only justifiable in exceptional cases. In the Netherlands, early metaphylactic treatment is forbidden but late metaphylaxis is allowed, and there are

further restrictions in relation to the types of antimicrobials that can be used on animals. That was reflected in our results on the expenditure in antimicrobials in the Netherlands, which was extremely low. In contrast, in Spain early metaphylaxis is not forbidden by law (Cameron-Veas et al., 2018). However, since the introduction of the electronic prescription in 2019, and the new EU regulation on medicated feed, justifying this preventive treatment has become very difficult, and a reduction on AMU is likely to occur in the following years. In fact, Spain has reduced 45% the sales of antimicrobials for food-producing animals from 2014 to 2018 (EMA, 2020). Widespread AMU in Spain was probably influenced also by the lower prices compared to Germany and the Netherlands.

In contrast, the unit cost of autogenous vaccines was similar in the three countries, and therefore the differences in expenditure on autovaccines were determined by how often they were applied. Frequency of use was highest in the Netherlands, then in Germany, and lowest in Spain (data not shown). In fact, the SA indicated that expenditure on autogenous vaccines in farrowing and in nursery were highly influential on the total cost per pig at the end of the production cycle in Germany and the Netherlands. In the Netherlands, two very distinctive patterns in the use of autogenous vaccines occurred in the country, with many farms spending very little and many others spending quite a lot, and that was responsible for the trimodal profile of the distribution in the SA. The use of autogenous vaccines is still controversial due to the limited scientific evidence validating their efficacy and their contradictory results. Failure of autogenous vaccines has been attributed to loss of antigenicity because of the killing of the bacteria, failure in the diagnosis or selection of the strain included, or even differences between laboratories in the production process (Rieckmann et al., 2020; Segura, 2020).

The estimation of the annual cost of *S. suis* per affected units evidenced that the disease represents an important burden for pig production, although with substantial differences not only between countries, but also within countries. The mean annual costs for farmers

ranged between 8.7 and 14.1 thousand euros per affected farrowing or nursery unit (with the exception of farrowing units in Spain), while costs in affected fattening units were much lower.

Considering the mean costs per animal summed across all production phases and that millions of pigs are annually produced in Germany, the Netherlands and Spain, *S. suis* causes millions of euros of annual losses to the swine sectors in those countries. However, even though the regions included in the study represent an important proportion of the pig production in the three countries, and that in the remaining regions the majority of pig sector is similarly composed (mainly of highly specialized large farms) (EU, 2020b), the extrapolation of results to the whole of each country may be questionable.

Previous attempts to estimate the losses associated with *S. suis*-disease have highlighted the difficulties due to data limitations. Because of the lack of incidence data, the estimate for the annual losses of *S. suis* type II in Great Britain in 1996, as calculated by Bennett and collaborators (1999), was extremely wide, between 25 thousand and 2 million pounds. In later work, the cost ranged between 100 thousand and 1.3 million pounds (Bennett and IJpelaar, 2005). A high burden of *S. suis* is in agreement with a study by (VanderWaal and Deen, 2018) that reported *S. suis* as one of the most important pathogens for the swine industry nowadays on the basis of the number of publications. Considering the Rushton (2009) framework, only visible losses caused by mortality and expenditures on antimicrobial treatments, autogenous vaccines and laboratory analyses were considered. Yet, *S. suis* has other major negative consequences, such as the impact of *S. suis* as an emerging zoonotic agent, which has increased in the last 15-20 years (Gottschalk and Segura, 2019). However, because of the lack of incidence data, the costs of human infections could not be included in our study. The only study in which the human cost of *S. suis* was calculated was carried out in Vietnam by Huong and collaborators (2019), who estimated that the annual cost was between 2.64 and 3.38 million US$.

Another unaccounted effect of *S. suis* is the potential for AMU to control the disease increasing the risk for development of antimicrobial resistance (Segura et al., 2020). In fact, there are growing evidences of the occurrence of antimicrobial resistance in *S. suis* (Palmieri et al., 2011; Varela et al., 2013; Huang et al., 2016) In order to reduce AMU, new EU regulations to be implemented in 2022 include restrictions on the prophylactic and metaphylactic antimicrobial treatment of animals (EU, 2019a, 2019b). In this context of a progressive reduction of AMU, development of effective alternative tools (e.g., vaccines), is essential to control *S. suis* (Segura, 2020). In the absence of such tools, good biosecurity, plus management practices (e.g., all-in/all-out, groups with similar ages, improvement of ventilation, or avoiding overcrowding) are key for the control of *S. suis* (Varela et al., 2013). Also, Correa-Fiz and collaborators (2020) suggest that some feed additives could be useful to help reduce the impact of *S. suis*-associated disease.

While Lekagul and collaborators (2019) found significant differences between countries in the patterns of the antimicrobial families used, that was not observed for the control *S. suis* in Germany, the Netherlands and Spain, which relied almost exclusively on the use beta-lactams in all the phases. Still, use of cephalosporins was reported in Germany and Spain, an antimicrobial family classified as critically important for human health. Differences in the families of antimicrobials used could be related to differences in market prices, driven by veterinarians' own experiences or country regulations, as in the case of cephalosporins, forbidden in the Netherlands for food producing animals (Jensen et al., 2011; De Briyne et al., 2013).

Our study has several limitations that need to be taken into account. Given the complexity of the questionnaire, and to avoid non-response bias, the veterinarians had to be selected by convenience among known clinical veterinarians. Even though the sample was meant to be representative of the different types of pig production present in the areas of study, some sort of selection bias cannot be ruled out. Also, our results rely on the accuracy with which all the data requested in the

questionnaire was remembered and reported by the veterinarians. While some recall bias is likely, we consider that the parameters estimated were a good approximation of the real values. To improve the precision of the data, the questionnaires were sent a few days in advance to allow the veterinarians to collect the data requested, and then the questionnaire was filled out by phone interview to facilitate the clarification of any possible doubt. Telephone interviews share many of the advantages of face-to-face interviews (e.g., high response rate, opportunity to explain the study) but are less time consuming and less expensive (Dohoo et al., 2003). Furthermore, there are some extra costs associated with *S. suis* in animals that die throughout the production cycle, as for example those animals may have received early metaphylactic treatment or autovaccines for *S. suis* before they died. However, considering that they apply only to some of the costs calculated before and that mortalities in the countries of study are generally low, its impact is likely to be limited. A similar extra costs is incurred by the pigs that do not complete the production cycle because they are slaughtered at earlier stages (e.g. suckling piglets), although its economic impact is also likely to be restricted. For simplification purposes, those extra costs were not considered in the calculations of the cost.

The economic assessment of animal diseases is often hampered by the lack of reliable data, and that is particularly true for swine production diseases. The evaluation of the cost of a disease relies on the availability of three main types of information: the incidence of the disease, how the disease is distributed among the population, and the treatment and control measures (Bennett et al., 1999). That kind of data is essential for detecting changes in the incidence or prevalence of the disease, deciding whether control measures are needed, or evaluating the implementation of those measures. While technological progress has contributed to the development of tools that allow monitoring the occurrence of endemic diseases in almost real-time, e.g., Alba-Casals and collaborators (2020), their application is still restricted to a limited number of farms/companies, which are not necessarily representative of

the whole swine sector. Therefore, alternative methods need to be used for the assessment of endemic diseases and their impact at the country level. We combined questionnaire-based surveys of clinical swine veterinarians with mathematical models. Questionnaires allowed us to collect data on many parameters related to *S. suis*-disease, from a very large number of farms, with a minimum cost; a strategy that may be easily adapted to other production diseases. Bennett and IJpelaar (2005) also relied on surveys, in that case of experts, to obtain the input data needed for the economic evaluation of several livestock diseases. The use of a stochastic model allows both the variability as well as the uncertainty associated with the data on *S. suis* to be incorporated into the calculations. In veterinary medicine, stochastic models have been commonly applied to quantify the risk of introducing a disease into a country through the importation of animals or their products (Vose, 2008; Napp et al., 2010), but are increasingly being used for the calculation of the cost of diseases (e.g., (Onono et al., 2014; Wang et al., 2018; Lyons et al., 2019)).

STUDY II

Risk factors associated to *Streptococcus suis* cases in swine farms in Spain

Submitted

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ABSTRACT

Streptococcus suis, an early colonizer of the upper respiratory tract, can cause the *S. suis*-associated disease, a major infection characterized by meningitis, polyarthritis, and acute death, in piglets around weaning age. Despite being a natural inhabitant of nose and tonsils, animals sometimes develop the disease, knowing little about the factors that can trigger it.

A total of six batches from two Spanish swine farms with confirmed *S. suis* problems were sampled, at the end of the farrowing period and two and four weeks later in weaning unit. It was studied a) concomitant pathogens such as porcine reproductive and respiratory syndrome virus (PRRSV), porcine circovirus type 2, swine influenza virus, and *Glaesserella* (*Haemophilus*) *parasuis*; b) biomarkers associated to stress (cortisol), inflammation (haptoglobin), and oxidative status (hydrogen peroxide); c) farm environmental factors as temperature, relative humidity, CO_2, and temperature-humidity index; and d) parity and *S. suis* presence in sows. Three models were built to study the effect of these variables, including two to assess the protective or risk factor in the subsequent development of the disease.

Presence of *S. suis* problems during the study was confirmed by its isolation in lesions in both farms. They were relevant as predictive in our models the PRRSV coinfection at weaning (Odds ratio (OR) = 6.69), gilts offspring (OR = 0.71), haptoglobin just before weaning (OR = 1.01), and mean of relative humidity in the farrowing unit as well as temperature at weaning (OR = 1.11 and 0.13, respectively). These results reveal the importance for the development *S. suis*-associated disease in the field, of different factors that have been previously studied using experimental models.

Keywords: *Streptococcus suis*, risk factors, coinfection.

INTRODUCTION

Streptococcus suis is one of the main bacterial pathogens causing global economic losses to the swine industry due to substantial post-weaning morbidity and mortality (Gottschalk and Segura, 2019; Neila-Ibáñez et al., 2021a). Although *S. suis* is a normal colonizer of the upper respiratory tract (Baele et al., 2001), pigs, especially piglets from 5 to 10 week-old, can develop a disease characterized mainly by meningitis, polyarthritis, and acute death (Gottschalk and Segura, 2019). However, the circumstances that allow strains from the microbiota of healthy animals to produce clinical disease are not completely known (Segura et al., 2016).

S. suis is classified in different serotypes depending on the capsular polysaccharide, with serotypes 1 to 7, 1/2, 9, and 14, being the most frequently isolated from clinical cases of the 29 originally described (Segura et al., 2020). Serotypes are distributed worldwide and the lesions they produce are not serotype dependent (Reams et al., 1994). Serotype 2 is the most frequently linked to infection in both pigs and humans, but serotype 9 is also highly prevalent in clinical swine isolates from Europe (Goyette-Desjardins et al., 2014). Although many virulent factors have been described for *S. suis*, they are not always present in clinical isolates (Segura et al., 2017). Furthermore, there are other factors (unrelated to the pathogen) that may influence the development of the infection, such as the number of piglets weaned per sow or the litter mortality, which seemed to play a role in the mortality during a *S. suis* outbreak in sucking piglets (Hopkins et al., 2018).

S. suis outbreaks have also been associated with some concomitant viral infections, such as porcine reproductive and respiratory syndrome virus (PRRSV), porcine circovirus 2 (PCV-2), or swine influenza virus (SIV). These associations are mainly observed in field conditions, where mixed infections are frequent; nevertheless, experimental infections to confirm these hypotheses are complex and only a few *in vivo* studies using pigs have been published (Obradovic et

al., 2021a). Piglets born to sows infected with PRRSV during gestation and challenged at 5 days of age with *S. suis* were more susceptible to infection and disease than those born to non-infected sows or those infected only with *S. suis* (Feng et al., 2001). In two other experimental studies, piglets inoculated with PRRSV seven days before being challenged with *S. suis* had a higher mortality rate and more severe lesions than piglets challenged only with one of the pathogens (Thanawongnuwech et al., 2000; Xu et al., 2010). When *S. suis* challenge was performed in piglets five days after PCV-2 infection, coinfected piglets exhibited more severe clinical symptoms and lesions than those inoculated only with one of the pathogens (Wang et al., 2020). Similar observations were reported for the SIV coinfection, with coinfected piglets showing more severe clinical signs and increased gene expression of pro-inflammatory mediators than those inoculated only with one of the pathogens (Lin et al., 2015). In addition, mixed infections with other bacterial agents can increase the severity of the lesions caused by *S. suis*, as was reported in a coinfection study with *Bordetella bronchiseptica,* which was used to predispose the nasal mucosa for the *S. suis* inoculation (Vecht et al., 1992). In that coinfection, *S. suis* was found in lungs with bronchopneumonia only if *B. bronchiseptica* was also present, suggesting that *S. suis* should be considered a secondary pathogen.

Moreover, the role of the respiratory microbiota on the presence and abundance of *S. suis* deserves to be further studied, since the composition of the nasal microbiota may predispose to disease development by other early colonizers (Correa-Fiz et al., 2016; Blanco-Fuertes et al., 2021). Recently, Niazy and collaborators (2022) found a different composition of the tonsillar microbiota in *S. suis*-affected piglets compared with the healthy group. One of the species found in different abundance was *Glaesserella* (*Haemophilus*) *parasuis*, another swine pathogen whose clinical manifestations are often misidentified as *S. suis* infection (Aragon et al., 2019).

Environmental and management factors that irritate the respiratory tract (e.g. high air pollution load) or induce stress in piglets (e.g. excessive temperature fluctuations or overcrowding) have been previously correlated with *S. suis* clinical disease in pigs (Dee et al., 1993; Staats et al., 1997; Vötsch et al., 2018).

Although animal stress can be evaluated using different biomarkers, cortisol is probably the most commonly used in pigs (Cerón et al., 2022). The intensity of the inflammatory process can be measured by acute phase proteins (Murata, 2007) such as haptoglobin, as showed in piglets coinfected with *Mycoplasma hyopneumoniae* and SIV H1N1, with higher levels than in non-infected animals (Deblanc et al., 2013). In contrast, transcription of the haptoglobin gene was not altered in blood after a *S. suis* challenge in cesarean-derived colostrum-deprived piglets when compared with non-inoculated piglets (Neila-Ibáñez et al., 2021b). Ott and collaborators (2014) demonstrated that biomarkers are differently expressed depending on the stressors, highlighting the value of including multiple biomarkers in stress evaluation. For example, cortisol can be used in psychosocial stress situations, such as when animals are mixed, while haptoglobin is not effective in such situations (Escribano et al., 2015). Similarly, biomarkers of oxidative status such as hydrogen peroxide (H_2O_2) or advanced oxidation protein products can be used as pain indicators and to assess oxidative stress (Contreras-Aguilar et al., 2019), becoming suitable markers for infectious processes, as demonstrated in SIV and *Mycoplasma hyopneumoniae* infections (Deblanc et al., 2013).

With the aim of evaluating possible risk factors for *S. suis*-associated disease, some viral and bacterial concomitant pathogens, environmental parameters, parity of the dams and biomarkers of stress, inflammation, and oxidative status in piglets were analysed in a longitudinal study carried out in two Spanish commercial swine farms.

MATERIAL AND METHODS

Selection of the farms

The study was carried out in two swine farms, A and B, located in Catalonia (north-eastern Spain). Both farms had a history of *S. suis*-associated disease, which was confirmed by the isolation of the agent from cerebrospinal fluid of sucking piglets (farm A) and weaners (farm B) with nervous symptoms.

Farm A was a family farm with 500 sows. Weaning units are located at 2.4 km from the maternity. Sows received a metaphylactic treatment with oxytetracycline in feed. Piglets were vaccinated against *Mycoplasma hyopneumoniae* and PCV-2, and treated intramuscularly before weaning with amoxicillin and gentamicin. In the weaning unit, piglets with symptoms compatible with *S. suis* disease were treated intramuscularly twice with amoxicillin, enrofloxacin and dexamethasone, and all the animals in the batch, with amoxicillin in drinking water for 6 days if *S. suis* clinical disease appeared. The status of the farm in relation to PRRSV and PCV-2 was stable, no influenza outbreaks were detected during sampling, but a porcine epidemic diarrhea (PED) outbreak took place in the weaning unit at the time of the second batch in the study.

Farm B, with 3500 sows, belonged to a big producer. Farrowing and weaning units were located in the same farm. Animals with clinical signs compatible with *S. suis* were treated with amoxicillin and dexamethasone intramuscularly, suckling piglets were treated once and weaners twice. The farm had a stable PRRSV and PCV-2 status, and no influenza outbreaks were detected during sampling.

In both farms, swabs collected from lesions of animals found dead or euthanized due to animal welfare, were analysed to confirm a *S. suis* outbreak. *S. suis* presence was determined after swab plating and molecular identification. *S. suis* isolates obtained from lesions were analysed by Enterobacterial Repetitive Intergenic Consensus (ERIC)-

PCR in order to determine the number of different strains involved in the outbreak, following the protocol described by Versalovic and collaborators (1991) but lowering the annealing temperature to 43ºC.

Sampling and data collection

Animal sampling was done under institutional authorization (Ethics Commission in Animal Experimentation of the *Generalitat de Catalunya*, protocol number 11199) and followed good veterinary practices, in accordance with European (Directive 2010/63/EU) and Spanish (*Real Decreto* 53/2013) regulations.

In both farms, three different batches were sampled A total of 30 piglets from 10 different sows were selected per batch and ear-tagged the week before weaning. Animals had ages ranged between 17 and 22 days. All piglets with clinical signs compatible with *S. suis* were chosen, and the group was completed by randomly selecting healthy piglets until reaching 30 animals. Animals were classified as diseased if they presented clinical signs compatible with *S. suis*, such as nervous signs or lameness. Nasal swabs and blood samples were taken from the selected piglets, and nasal and vaginal swabs from their dams. Piglets were sampled again approximately two weeks after weaning, when they were between 31 and 36 days of age. If any of the animals not sampled initially presented lameness or nervous signs, they were also ear-tagged and sampled (up to 10 more). Two weeks later, when piglets were between 45 and 54 days of age, the clinical status of the animals in relation to *S. suis*-associated disease was also recorded. More information about the sampled animals is included in Supplementary Table S3.1.

One hundred and seventeen piglets were sampled between October and December 2019 in farm A, and 90 piglets were sampled between March and May 2021 in farm B. The number of piglets sampled at each visit is shown in Table 3.1, whereas the number of sows sampled were 10 for each batch.

In the first two visits, an environmental data logger was placed, at approximately 30 centimetres of height. The data logger located at farm A recorded temperature (ºC), relative humidity (%), temperature-humidity index (THI), and CO_2 (ppm) (MHD21ABE17, DeltaOHM, Italy), whilst the data logger placed at farm B recorded only temperature and relative humidity (HD208.1NTCI - HP3517TC1.2, DeltaOHM, Italy). Data was measured every 5 minutes for 60 to 90 hours.

Pathogen detection

Nasal and vaginal swabs were resuspended in 500 µL PBS and blood was centrifuged to obtain serum. Both types of samples were stored at -80ºC until they were processed. DNA and RNA were extracted using MagMAX Pathogen RNA/DNA kit (Applied Biosystem™) following the manufacturer´s recommendations, and then stored at -80ºC until molecular analysis.

Presence of *S. suis*, and then detection of the serotypes 1/2 - 2 and 9, were carried out in nasal and vaginal samples, whilst the presence of *G. parasuis*, both virulent and non-virulent strains, was evaluated only in nasal samples. In farm A, serotype 7 was also tested since it had been detected in one of the clinical isolates. Those pathogens were tested using conventional PCR assays, with the primers and conditions described by Ishida and collaborators (2014) for the presence of *S. suis*, Okura and collaborators (2014) for the serotypes 1/2 - 2, 7 and 9, and Galofré-Milà and collaborators (2017) for *G. parasuis*. Nasal samples were also tested for influenza viral RNA by quantitative reverse transcription-PCR (RT-qPCR) assay based on the amplification of the conserved segment of the matrix gene, as described by López-Valiñas and collaborators (2021). PRRSV and PCV-2 presence were determined in serum by real-time qPCR assay with commercial kits (VetMAX™ PRRSV EU & NA 2.0 Kit, Life Technologies, and VetMAX™ Porcine PCV2 Quant Kit, Life Technologies, respectively).

For *S. suis* detection in lesions, a sterile cotton swab was moistened in the lesion or with the fluid in the case of the cerebrospinal

fluid, plated into a chocolate agar plate (Biomerieux), and incubated at 37ºC and 5% CO_2 overnight. The pure culture compatible with *S. suis* was recovered and saved in PBS. DNA was extracted using a Chelex based Instagene Matrix (Bio-Rad Laboratories, Hercules, CA, USA) following the manufacturer's instructions. *S. suis* was confirmed by PCR, using the protocol described by Ishida and collaborators (2014).

Analyses of cortisol, haptoglobin and hydrogen peroxide

Cortisol concentration was measured by a solid-phase, competitive chemiluminescent enzyme immunoassay that uses a polyclonal rabbit anti-cortisol antibody (Immulite/Immulite 1000 cortisol, Siemens Medical Solutions Diagnostics), previously validated for porcine saliva samples (Escribano et al., 2012).

Haptoglobin concentrations were measured by commercial quantitative turbidimetric test (Spinreact, S.A.U, Spain) in an automated analyser (Olympus AU600), previously validated by Kaiser and collaborators (2018).

Hydrogen peroxide (H_2O_2) was assessed based on the method of Rhee and collaborators (2010) in an automated analyser (Olympus AU600) previously validated (Rubio et al., 2019).

Statistical analysis

To evaluate the effect of the different variables on *S. suis*-associated disease, three different mixed-effect logistic regression models were chosen, depending on the age of the animals and the use of retrospective data: 1) Model 1: General Risk Factor model, considering the presentation of the disease at any of the visits, and using all explanatory variables; 2) Model 2: Weaning Risk Factor model, considering the presentation of the disease at the first and second visits (i.e. at weaning), and using all the data collected previously in farrowing; 3) Model 3: Late Weaning Risk Factor model, considering the presentation of the disease at the second visit at weaning, and using the data collected in the first weaning visit.

Mean and range values of each environmental parameter and stress markers were treated as continuous variables. Age (in days) was classified as discrete value, while animal status against the different bacterial and viral pathogens was dichotomised as negative or positive.

First, a bivariate analysis to test associations between the dependent variables (*S. suis*-associated clinical signs) and the explanatory variables, was carried out. Then, a mixed-effect logistic regression model was built with sow, batch and farm as random effects. Only those variables with a $P \leq 0.25$ in the bivariate analysis were further evaluated in the multivariate analysis (Dohoo et al., 2003). The final models selection were performed via manual backward selection, based on the Akaike information criterion (AIC), including only those variables with a $P \leq 0.05$ and excluding those with a variance inflation factor (VIF) > 5.

When building the regression model, the serotypes of *S. suis* analysed were not considered. As CO_2 and THI were only recorded in farm A, the statistical analysis was repeated only for this farm in order to include these two variables associated to the ventilation.

Statistical analyses were conducted with R (v. 4.0.2, [R Core Team, 2020]), using the packages *lme4* (v. 1.1-23, [Bates et al., 2015]) and *rsq* (v. 2.2, [Zhang, 2021]).

RESULTS

S. suis isolation and disease prevalence

S. suis was isolated from lesions of animals with clinical signs, confirming *S. suis* as the most likely cause of the outbreak disease. Five different *S. suis* isolates were recovered, one from a tarsal joint and three from cerebrospinal fluid in farm A, while one *S. suis* isolate was recovered from fibrin located in the thoracic cavity in farm B. *S. suis* identification was confirmed by *recN* PCR. Isolates from farm A showed different fingerprinting by ERIC-PCR, and different to the isolate from farm B.

Prevalence of *S. suis*-associated disease differed between farms and batches (Table 3.2). In global, farm A had more diseased animals than farm B, which is consistent with the higher number of *S. suis* isolates recovered in farm A. Farrowing units were more affected in farm B and weaners were more affected in farm A.

Prevalence of infectious agents

The prevalence of the pathogens in both piglets and sows are shown in Table 3.1. *S. suis* was detected in all batches and in a high proportion of nasal samples (93.0% for piglets and 76.7% for sows), but it was less common in vagina of sows (56.7%). *S. suis* was not detected (i.e. absent in both farrowing and weaning) only in five animals, all from the third batch of farm B. Serotypes 2 and 9 were included in the analysis because they are the most prevalent in Europe. Serotype 2 was more prevalent in farm B, contrary to farm A where the most prevalent was serotype 9. Since one clinical isolate from farm A belonged to serotype 7, the presence of this serotype was analysed in samples from this farm, and it was only detected in 23 out of 117 animals.

Even though *G. parasuis* was detected in all piglets throughout the study, the presence of virulent strain was lower on farm B at farrowing compared to the same unit on farm A, although this detection evolved on farm B from 50.0, 10.0, and 93.3% for the three batches sampled to 100%. In addition, in the case of farm A, six suckers and one weaner were negative for both type of strains.

In general, the prevalence of PCV-2 and SIV were low. In contrast, the prevalence of PRRSV in both farms were relatively high, especially in weaning (Table 3.1).

Biomarkers determination

Three different biomarkers, cortisol, haptoglobin, and H_2O_2 were used to evaluate the stress, inflammation and oxidative status. Important differences were observed between the various ages and batches (Supplementary Table S3.2). In general, piglets with *S. suis*-associated

disease had higher levels of cortisol, H_2O_2, and particularly of haptoglobin than healthy piglets of the same age and batch. For example, in the weaning unit of farm A, healthy animals had a median of 77.80 mg/dL of haptoglobin compared to 243.37 mg/dL in animals with symptoms (Supplementary Figure S3.1).

Table 3.1 | Presence of the pathogens studied in sampled piglets and sows. SIV: swine influenza virus; PRRSV: porcine reproductive and respiratory syndrome virus; PCV-2: porcine circovirus type 2. Vir: virulent.

			Farm A			Farm B		
			Batch 1	**Batch 2**	**Batch 3**	**Batch 1**	**Batch 2**	**Batch 3**
Piglet	**Farrowing**	*S. suis*-associated disease	0/30	1/30	0/30	3/30	2/30	2/30
		Nasal *S. suis*	93%	83%	97%	100%	100%	80%
		Nasal *S. suis* serotype 2	3%	0%	0%	13%	100%	3%
		Nasal *S. suis* serotype 9	20%	77%	57%	0%	10%	0%
		Nasal *G. parasuis* (vir)	93%	93%	60%	50%	10%	93%
		Nasal *G. parasuis* (non vir)	100%	97%	70%	100%	100%	100%
		Nasal SIV	20%	0%	0%	7%	10%	0%
		Blood PRRSV	0%	10%	17%	7%	0%	37%
		Blood PCV-2	0%	0%	10%	0%	10%	3%
	Early weaning	*S. suis*-associated disease	15/40	12/40	9/37	0/30	1/30	0/30
		Nasal *S. suis*	100%	100%	100%	100%	100%	57%
		Nasal *S. suis* serotype 2	13%	0%	0%	3%	90%	37%
		Nasal *S. suis* serotype 9	90%	100%	100%	10%	93%	0%
		Nasal *G. parasuis* (vir)	87%	90%	92%	100%	100%	100%
		Nasal *G. parasuis* (non vir)	87%	92%	84%	97%	100%	77%
		Nasal SIV	5%	0%	3%	0%	0%	0%
		Blood PRRSV	36%	75%	41%	17%	70%	47%
		Blood PCV-2	0%	10%	5%	10%	0%	10%
	Late weaning	*S. suis*-associated disease	22/40	12/40	6/37	1/30	0/30	0/30
Sow*	**Nasal**	*S. suis*	90%	70%	50%	90%	100%	60%
		S. suis serotype 2	30%	0%	0%	60%	40%	80%
		S. suis serotype 9	20%	40%	70%	20%	20%	0%
		G. parasuis virulent	50%	50%	30%	50%	50%	80%
		G. parasuis non-virulent	80%	60%	30%	100%	100%	50%
	Vaginal	*S. suis*	90%	10%	50%	20%	0%	0%
		S. suis serotype 2	30%	0%	0%	0%	0%	0%
		S. suis serotype 9	10%	10%	30%	0%	0%	0%

*10 sows were sampled per batch.

Table 3.2 | Prevalence of *S. suis*-associated disease and mortality in different farms and batches.

		Farm A			Farm B		
		Batch 1	**Batch 2**	**Batch 3**	**Batch 1**	**Batch 2**	**Batch 3**
***S. suis*-associated disease**	**Farrowing**	0.0% (0/300)	0.3% (1/335)	0.0% (0/363)	0.2% (3/1786)	0.1% (2/1906)	0.1% (2/1805)
	Early weaning	7.0% (21/300)	17.6% (59/335)	5.0% (18/363)	0.0% (0/1786)	0.1% (1/1906)	0.0% (0/1805)
	Late weaning	9.1% (27/298)	13.0% (43/330)	4.7% (17/360)	0.1% (1/1785)	0.3% (5/1901)	0.1% (2/1795)
Mortality	**Early weaning**	0.7% (2/300)	1.5% (5/335)	0.8% (3/363)	0.1% (1/1786)	0.3% (5/1906)	0.5% (10/1805)
	Late weaning	7.4% (22/298)	14.2% (47/330)	8.3% (30/360)	0.7% (13/1785)	0.8% (15/1901)	0.5% (9/1795)
	Weaning	8.0% (24/300)	15.5% (52/335)	9.1% (33/363)	0.8% (14/1786)	1.0% (20/1906)	1.1% (19/1805)

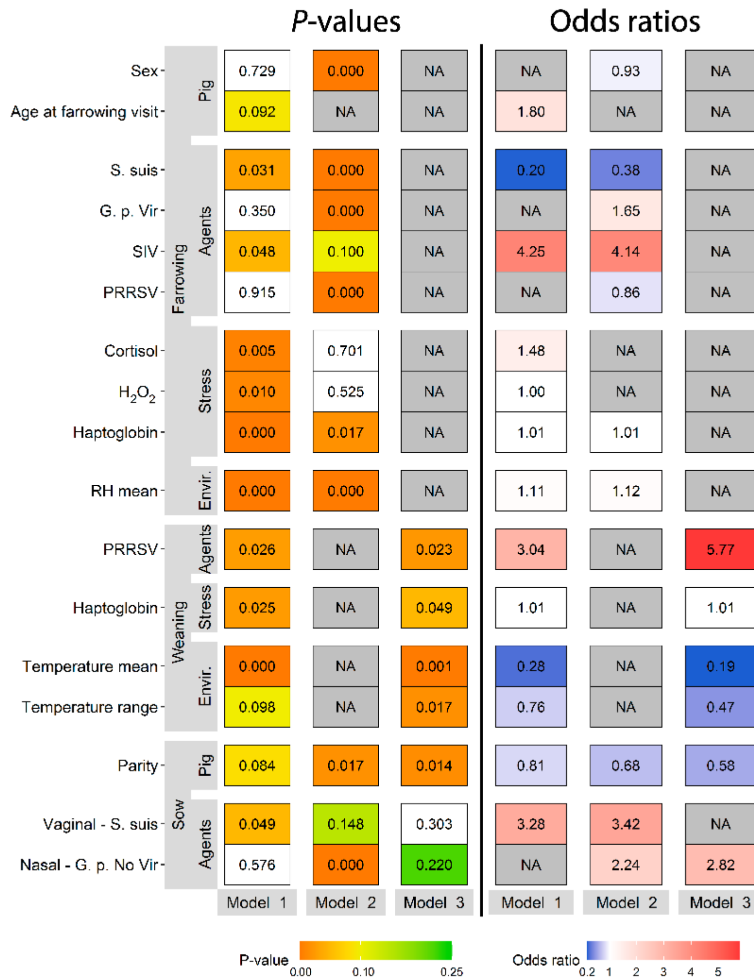
Table shows the number of animals present in the batch and how many of them presented clinical signs compatible with *S. suis* infection.

Environmental data

Mean room temperatures ranged between 25.8 and 28.4ºC in farrowing and between 26.3 and 28.4ºC in weaning, and mean relative humidity ranged between 37.3 and 58.6% in farrowing and between 24.0 and 49.3% in weaning (Supplementary Table S3.3). Relative humidity could not be recorded due to a device malfunction in one of the visits to the farm A. CO_2 concentration in farm A was almost twice at weaning than at farrowing (mean of 2,857 ppm vs 1,484 ppm, respectively), whilst THI values were similar in both units (63.9% at farrowing and 61.0% at weaning) (Supplementary Table S3.3).

Bivariate analysis

The results of the bivariate analysis of the two farms, including the *P*-values and odds ratios of the variables with $p \leq 0.25$, are shown in Figure 1. Whilst the odds ratios fluctuated between models, the effects of the different factors (identified as either a risk or a protective factor) were consistent throughout all of them (Figure 1).



	P-values Model 1	P-values Model 2	P-values Model 3	Odds ratios Model 1	Odds ratios Model 2	Odds ratios Model 3
Sex	0.729	0.000	NA	NA	0.93	NA
Age at farrowing visit	0.092	NA	NA	1.80	NA	NA
S. suis	0.031	0.000	NA	0.20	0.38	NA
G. p. Vir	0.350	0.000	NA	NA	1.65	NA
SIV	0.048	0.100	NA	4.25	4.14	NA
PRRSV	0.915	0.000	NA	NA	0.86	NA
Cortisol	0.005	0.701	NA	1.48	NA	NA
H_2O_2	0.010	0.525	NA	1.00	NA	NA
Haptoglobin	0.000	0.017	NA	1.01	1.01	NA
RH mean	0.000	0.000	NA	1.11	1.12	NA
PRRSV	0.026	NA	0.023	3.04	NA	5.77
Haptoglobin	0.025	NA	0.049	1.01	NA	1.01
Temperature mean	0.000	NA	0.001	0.28	NA	0.19
Temperature range	0.098	NA	0.017	0.76	NA	0.47
Parity	0.084	0.017	0.014	0.81	0.68	0.58
Vaginal - S. suis	0.049	0.148	0.303	3.28	3.42	NA
Nasal - G. p. No Vir	0.576	0.000	0.220	NA	2.24	2.82

Figure 3.1 | Bivariate analysis. Results of the bivariate analysis for all factors with $P \leq 0.25$ in both farms. Model 1: General Risk Factor model; Model 2: Weaning Risk Factor model; Model 3: Late Weaning Risk Factor model. G. p. Vir: *Glasserella parasuis* virulent strain; SIV: swine influenza virus; PRRSV: porcine reproductive and respiratory syndrome virus; RH: relative humidity; G. p. No Vir: *Glasserella parasuis* non-virulent strain. Piglets were sampled few days before and after weaning, taking nasal swabs and blood. Sows were sampled at the same time that first piglet sampling, taking nasal and vaginal swabs. A data logger was placed in the farms after sampling, recording different variables during 3 days every 5 minutes.

Multivariable model

Model 1: General Risk Factor model

The results from Model 1 (i.e. *S. suis*-associated disease at any time), are presented in Table 3.3. The results indicate that an increase of one day of age at the moment in which suckers were sampled (i.e., the age at which animals were weaned) is linked to an increase of almost 5-fold in the odds of developing *S. suis* clinical disease. Those animals with higher levels of cortisol and haptoglobin at the first sampling, were also more prone to develop disease. Even though concomitant infections with SIV at farrowing and PRRSV at weaning were statistically significant in the bivariate analysis (Figure 1), only the presence of PRRSV at weaning was statistically significant in this model, becoming the most influential factor (OR = 6.40). Regarding environmental factors, higher mean relative humidity at farrowing increased the odds of *S. suis*-disease (OR = 1.10). The only sow factor that was retained in the model was the parity, being younger sows more prone to have piglets with *S. suis* problems (OR = 0.69).

Model 2: Weaning Risk Factor model

Some farrowing variables had a significant impact on the *S. suis*-disease status at weaning (Table 3.3), namely, the stress of piglets indicated by the haptoglobin marker (OR = 1.01), the average relative humidity at farrowing period (OR = 1.11), and the sow parity (OR = 0.71). The lower the parity of the sow was, the greater the possibility of developing symptoms in offspring, as well as on stressed piglets that were kept at a higher mean relative humidity.

Model 3: Late Weaning Risk Factor model

The effect of the variables was also studied for the weaning unit with Model 3. The influence of PRRSV coinfection (OR = 6.69) and the average temperature (OR = 0.13) at the beginning of the weaning unit were significant, in addition to the parity of the sow (OR = 0.55). Sow's parity had the same protective effect than in Model 1 and 2, and the

presence of PRRSV and batches with low mean of temperature were more prone of developing *S. suis* compatible disease.

Models including only Farm A

The models performed in Farm A with CO_2 and THI variables showed that CO_2 range was significant in Model 1 and 2, whereas in Model 3 was CO_2 mean (Supplementary Table S3.4). Compared with the models built for the two farms, remained coinfection with PRRSV and sow parity as significant factors with similar values, haptoglobin was significant in weaning instead of in farrowing, and age, cortisol, temperature, and relative humidity were not included in any of them (Supplementary Table S3.4).

Table 3.3 | Variables included in the three models built for both farms.

Variable	OR	CI	Beta coefficient	*P*-value
Model 1: General Risk Factor				
Age at farrowing sampling	4.95	1.87 - 13.12	1.59	0.001
Cortisol at farrowing	1.88	1.32 - 2.69	0.63	<0.001
Haptoglobin at farrowing	1.01	1.01 - 1.02	0.01	<0.001
Mean of relative humidity at farrowing	1.10	1.02 - 1.18	0.09	0.013
PRRSV presence at weaning	6.40	1.74 - 23.53	1.85	0.005
Sow parity	0.69	0.52 - 0.93	-0.36	0.016
Model 2: Weaning Risk Factor				
Haptoglobin at farrowing	1.01	1.00 - 1.02	0.01	0.028
Mean of relative humidity at farrowing	1.11	1.05 - 1.17	0.10	<0.001
Sow parity	0.71	0.52 - 0.97	-0.34	0.031
Model 3: Late Weaning Risk Factor				
PRRSV presence at early weaning	6.69	1.55 - 28.85	1.90	0.011
Mean of temperature at early weaning	0.13	0.05 - 0.37	-2.04	<0.001
Sow parity	0.55	0.37 - 0.83	-0.59	0.004

OR: Odds ratio. CI: Confident interval 95%.

DISCUSSION

S. suis-associated disease is one of the main diseases in the swine industry, in particular in intensive pig production systems (Gottschalk et al., 2010). Despite the fact that the bacterium is highly prevalent in swine farms, the proportion of animals that are clinically affected is relatively low.

Knowledge of why disease outbreaks occur is important to establish control measures to reduce their impact, not only for animal well-being but also for animal production. The identification of the possible causes of incidence and severity of these disease outbreaks is often a challenge (Gebhardt et al., 2020). In the present study, various factors that have historically been associated with *S. suis* outbreaks, such as coinfections or temperature and humidity (Dee et al., 1993; Obradovic et al., 2021a), have been studied.

S suis disease has been linked with coinfection with other agents, especially with viruses. Rieckmann and collaborators (2020) reported that even low virulent *S. suis* strains resulted in the development of *S. suis* disease in PRRSV positive herds. Those observations have been confirmed in the present study, since PRRSV infected animals presented a higher risk of developing *S. suis* clinical signs during post-weaning. The absence of statistical significance for the other two porcine viruses studied, PCV-2 and SIV, does not imply a lack of effect, since their prevalence were low, and their influence on *S. suis* infections may be linked to epidemics of those viruses.

An important question that arises in coinfection scenarios is which is the primary pathogen, since the simultaneous detection of several pathogens in diseased animals does not allow to establish the order of infections. Sampling before the disease outbreak and subsequent animal tracking is a complex task and not always successful (selected animals may not develop the infection). However, by looking at the risk factors in the previous stages (models 2 and 3), we were able to relate the PRRSV infection at 5 weeks of age with the subsequent development of clinical signs consistent with *S. suis* at 7 weeks of age.

Despite both *G. parasuis* and *S. suis* being early colonizers of the porcine upper respiratory tract and affecting young pigs, a direct relationship has not been observed in the studied farms. The use of different host cell receptors may explain the absence of interaction, as was observed in *in vitro* studies (Mathieu-Denoncourt et al., 2018).

S. suis was detected in the nasal cavity of all sampled piglets except five, which is in accordance with its role as natural inhabitant of the microbiota of the porcine upper respiratory tract and with what has been reported by other authors (Goyette-Desjardins et al., 2014; Segura et al., 2017; Werinder et al., 2020).

According to Wathes and Whittemore (2006), keeping animals out of the comfort temperature (28ºC for suckers at the end of farrowing and 22ºC for weaners weighing between 10 and 15 kg) result in thermal stress. In our study higher temperatures just after weaning seemed beneficial for reducing the risk of *S. suis* disease despite being higher than the comfort values reported.

Relative humidity and CO_2 can be taken as indirect measures of the ventilation and air renewal. Our results showed that piglets located in farrowing units with a higher relative humidity and in weaning units with higher CO_2 concentration were prone to develop *S. suis*-associated disease, which reflects the importance of keeping animals in well-ventilated spaces. The use of data loggers in the present experiment has made possible to see the importance of ventilation.

Piglets were intentionally sampled before and after weaning, which represents a period of high stress level due to various factors such as an abrupt separation from the sow, change from milk to solid feed, movement to weaner pens, or the creation of new hierarchical groups by commingling litters; and thus, frequency of the disease, was expected to be higher (Campbell et al., 2013). All these non-infectious factors may have an impact on the incidence and severity of infectious processes (Gebhardt et al., 2020), but the risk of developing clinical disease also depends on the duration of the stressful situation, which may result in acute or chronic stress, and is also influenced by the age in which this stress occurs (Proudfoot et al., 2012). It is also important to point out that the stress evaluated in the context of an infectious disease can be considered either a consequence (of the disease) or a possible cause (Martínez-Miró et al., 2016). In the present study, in addition to cortisol

as biomarker of stress, we used the haptoglobin as a biomarker of inflammation and the H_2O_2, a biomarker of redox status, in order to get information on different aspects of the animal's condition. Moreover, the longitudinal study design allowed evaluating whether changes in these biomarkers preceded *S. suis*-associated disease. Prolonged stress stimuli have been associated with elevated levels of cortisol (Dhabhar, 2009), however, in our scenario the association of *S. suis*-associated disease and high cortisol levels was observed only in the general model (i.e. Model 1). Haptoglobin has been shown to increase in feed deprived piglets (Ott et al., 2014), which can occur when an animal does not have access to feed due to mobility problems caused by the arthritis typical of *S. suis* infection, as shown in Model 1. However, we also found in Model 2 that high values of haptoglobin in sucking piglets were correlated with the appearance of the disease two weeks later, at the beginning of post-weaning. This is a characteristic feature of acute phase proteins, and in particular of haptoglobin, which can increase before the appearance of clinical signs, being one of the most earliest and sensitive biomarkers of inflammation (Cerón et al., 2022). Despite being a significant variable in our models, the magnitude of this influence turned out to be low, increasing the odds of developing *S. suis*-associated disease by 1.01 for each mg/dL of haptoglobin in serum (observed values of haptoglobin ranged from 8 to 322.9 mg/dL). Currently, there is a trend to replace serum stress analysis with saliva samples, which has advantages such as it is a non-invasive technique that is easy to collect, and therefore is less stressful for the animals (Cerón et al., 2022). In our case, we used the blood samples already collected to study the presence of viral pathogens.

As sampling was done just before weaning, animals that would be weaned few days older were more likely to develop *S. suis*-associated disease, although explanation about this relationship seems us unclear. According to Gebhardt and collaborators (2020), animal gender does not influence post-weaning mortality, nor did it influence the appearance of *S. suis*-associated disease in our study.

Hopkins and collaborators (2018) observed that piglets from sows whose previous litters presented *S. suis* problems were less prone to developing the disease. In our study, we evaluated the influence of the dam on the occurrence of *S. suis* infection showing that piglets born from older sows were less likely to present problems, and that result was very consistent across all the models. The reasons for this finding could be the higher immunological protection conferred by the colostrum intake, or changes in the sow vaginal or nasal microbiota which consequently may have an effect on the development of the piglet microbiota, as it has been studied with sows vaccinated against *G. parasuis* (Blanco-Fuertes et al., 2022).

Due to its complexity, the study could only be carried out in two farms. Therefore, the consistency of the risk factors identified would need to be corroborated with further studies using a larger number of farms, as well as studies in other countries, where potentially other factors may influence the risk of *S. suis*. Our study highlights the multifactorial nature of a pathobiont such as *S. suis*, for which both environmental factors and factors related to the host seem to be involved in the development of the disease. The light shed in this study can help preventing *S. suis* outbreaks by controlling some of the variables involved in its appearance.

STUDY III

Piglet innate immune response to *Streptococcus suis* colonization is modulated by the virulence of the strain

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ABSTRACT

Streptococcus suis is a zoonotic pathogen of swine involved in arthritis, polyserositis, and meningitis. Colonization of piglets by *S. suis* is very common and occurs early in life. The clinical outcome of infection is influenced by the virulence of the *S. suis* strains and the immunity of the animals. Here, the role of innate immunity was studied in cesarean-derived colostrum-deprived piglets inoculated intranasally with either virulent *S. suis* strain 10 (S10) or non-virulent *S. suis* strain T15. Colonization of the inoculated piglets was confirmed at the end of the study by PCR and immunohistochemistry. Fever (≥ 40.5 °C) was more prevalent in piglets inoculated with S10 compared to T15 at 4 h after inoculation. During the 3 days of monitoring, no other major clinical signs were detected. Accordingly, only small changes in transcription of genes associated with the antibacterial innate immune response were observed at systemic sites, with S10 inducing an earlier response than T15 in blood. Local inflammatory response to the inoculation, evaluated by transcriptional analysis of selected genes in nasal swabs, was more sustained in piglets inoculated with the virulent S10, as demonstrated by transcription of inflammation-related genes, such as IL1B, IL1A, and IRF7. In contrast, most of the gene expression changes in trachea, lungs, and associated lymph nodes were observed in response to the non-virulent T15 strain. Thus, *S. suis* colonization in the absence of systemic infection induces an innate immune response in piglets that appears to be related to the virulence potential of the colonizing strain.

Keywords: *Streptococcus suis*, colonization, innate immunity, pig immunity, gene expression, bacterial virulence.

INTRODUCTION

Streptococcus suis is a major bacterial pathogen of swine, involved in meningitis, arthritis, septicemia, and acute death, among other clinical syndromes. Disease caused by *S. suis* is more prevalent in nursery pigs, but sucklers and young fatteners can also be affected (Gottschalk and Segura, 2019). Additionally, *S. suis* is a zoonotic agent that is receiving increased scientific interest due to Chinese outbreaks in humans in 1998 and 2005 (Gottschalk et al., 2007).

S. suis is an early colonizer of the swine upper respiratory tract, mainly found in tonsil and nasal cavity (O'Dea et al., 2018). Newborn piglets experience the first contact with the bacterium in the birth canal during parturition, as *S. suis* colonizes the sow's vaginal tract (Amass et al., 1997). In addition, animals housed in the same pen are exposed to horizontal colonization by direct contact or aerosol, especially during outbreaks when animals may shed bacteria in high numbers (Cloutier et al., 2003).

S. suis strains are heterogeneous both with respect to antigenicity and virulence (Segura et al., 2020). Presently, 29 confirmed serotypes have been described based on the antigenicity of capsular polysaccharides (Kerdsin et al., 2014; Okura et al., 2016). Pigs are usually colonized by more than one serotype, but only a few strains can induce disease. Serotype 2 is the most frequently isolated serotype from affected organs in diseased individuals (swine and humans) in most parts of the world (Goyette-Desjardins et al., 2014). However, different virulence results have been reported for the same *S. suis* serotype, or even the same *S. suis* strain (Berthelot-Hérault et al., 2005; Fittipaldi et al., 2011).

Different animal models have been used to study *S. suis* pathogenesis, including pigs, mice, rabbits, and zebrafish (Segura et al., 2017). Clinical disease has been reproduced by respiratory, intraperitoneal, and intravenous routes in pigs, but reproduction of the disease is difficult (Segura et al., 2017). After colonization, the

development of disease depends on the virulence potential of the strain and the interplay between the host response and the bacteria (Li et al., 2010). Severe disease is caused by excessive inflammation (Ye et al., 2009), and *in vitro* studies have demonstrated strong induction of pro-inflammatory cytokines and chemokines by *S. suis* serotype 2 (Segura et al., 1999, 2002, 2006; Vadeboncoeur et al., 2003). A major obstacle to the investigation of *S. suis* disease *in vivo* is the fact that systemic disease is not easily induced by challenge via the natural oronasal route of infection. In fact, systemic disease by intranasal challenge is not induced or is strongly reduced in the absence of acetic acid pretreatment or prior viral infection (Feng et al., 2001; Pallarés et al., 2003). Thus, to reproduce systemic disease, it seems necessary to inoculate *S. suis* either by injection (e.g. intravenous or intraperitoneal) or intranasally after irritation of the mucosa by pre-treatment.

Only few *in vivo S. suis* challenge studies in pigs have focused on characterizing the host immune responses. Genes related to bacterial recognition (*TLR4*, *MYD88*) and inflammatory responses (*IL6*, *CXCL8*, *CCL2*) have been shown to be expressed in lungs of pigs after intranasal *S. suis* challenge (serotype 2, strain 05ZY), and these responses were enhanced by coinfection with influenza A virus (H1N1) (Lin et al., 2015). Intravenous challenge of pigs with *S. suis* serotype 2 (strain SC19) induced expression of bacterial pattern recognition receptors (*TLR2*, *CD14*) in the lung, as well as components of the inflammatory response (*IL1B*, *IL6*, *TNF*, *CXCL8*) (Liu et al., 2011). Nasal challenge (after acetic acid pre-treatment) with *S. suis* serotype 2 (strain 05ZY) induced a primarily TLR2-dependent cytokine response in the spleen (Li et al., 2010). The hepatic response has been investigated *in vivo*, showing that clinical and subclinical disease after *S. suis* serotype 2 (strain SS02-0119) challenge by subcutaneous inoculation was accompanied by an acute phase response consisting of the acute phase proteins (APPs) serum amyloid A, C-reactive protein, haptoglobin, pig-MAP, and apolipoprotein A-I (Sorensen et al., 2006). These studies have thus

shown that inflammatory responses can be induced *in vivo* by *S. suis* serotype 2 challenge.

The present study, performed in cesarean-derived colostrum-deprived (CDCD) piglets, describes the host early immune response in blood, nasal mucosa, and various tissues to intranasal inoculation with *S. suis* T15 and S10. These strains belong to serotype 2 and have shown different virulence in pigs, based on the frequency of clinical signs, leukocytosis, and mortality reported in previous animal experiments (Vecht et al., 1997). Inoculation of the strains was performed without pre-treatment of the mucosa in order to examine the natural response of the host when encountering *S. suis* strains of different virulence. How this early response might affect disease development is also discussed.

MATERIALS AND METHODS

Animal study

Animal experimentation was performed in the BSL3 facilities of IRTA-CReSA (Bellaterra, Spain) following good veterinary practices, in accordance with European (Directive 2010/63/EU) and Spanish (*Real Decreto* 53/2013) regulation. The experimental study was approved by the Ethics Commission in Animal Experimentation of the *Generalitat de Catalunya* (Protocol number 10201). Four pregnant sows were transported to IRTA facilities and housed for two weeks before delivery, which was performed by cesarean section. The genetic background of these piglets was (Duroc × White Large) ♀ × Landrace ♂, a commercial breed. Piglets were fed the milk substitutive Patavie Porc (Oriane-Celtilait) ad libitum during the first 2 days. Afterwards, animals received Neopigg (Provimi Cargill) mixed with the milk or dry after 10-15 days of age. Piglets were treated with colistin (Colimicina SP, SP Veterinaria S.A., Spain) and enrofloxacin (Baytril 0.5%, Bayer Hispania S.L., Spain), both orally, during the first nine days of life. Twenty piglets, housed in the same box, were included in the study. At 25 days of age (3 days before inoculation), blood samples and nasal swabs were taken from all piglets. Piglets were randomly assigned to 5 groups of 4 piglets

each for inoculation and euthanasia, and housed in 3 separated boxes, depending on the inoculum assigned to them. One group was inoculated with strain T15 and euthanized 1 day post-inoculation (dpi), while a second group that was also inoculated with strain T15 was euthanized at 3 dpi. Similarly, two groups were inoculated with strain S10 and were euthanized at 1 and 3 dpi, respectively. A fifth group was inoculated with PBS (Phosphate Buffered Saline) and euthanized at 1 dpi, as negative control. On day 28 of life, inoculation was performed intranasally with a nasal atomizer (MAD Nasal™, Teleflex, Athlone, Ireland) with 2 mL of 1.1×10^9 colony forming unit (CFU)/mL of *S. suis* T15 (non-virulent serotype 2 strain) or with 2 mL of 1.8×10^9 CFU/mL of *S. suis* S10 (virulent serotype 2 strain), while the control group was inoculated with 2 mL of PBS. For the three groups, the inoculated volume was split between the two nostrils. Strains were provided by Dr Astrid de Greeff and Dr Norbert Stockhofe from Wageningen Bioveterinary Research (Wageningen University & Research, the Netherlands). After inoculation, piglets were supervised for clinical signs, including rectal temperature.

To study the innate immune response to the inoculated strains, early time points were chosen for sampling. Nasal swabs and blood were collected 4 h after inoculation, 1, 2 and 3 dpi. After euthanasia, piglets were examined by necropsy and lesion scores were calculated as a combination of the severity of the lesions and the number of body sites affected. In addition, samples from tissues (trachea, cranial and caudal lobes of the lung, submandibular and tracheobronchial lymph nodes, spleen, and liver) were collected. To ensure RNA integrity, blood samples were obtained in PAXgene Blood RNA tubes (Becton Dickinson, Spain) which were kept at room temperature 4 h and subsequently stored at 4 °C for 72 h and ultimately transferred to -20 °C. Nasal swabs and tissues were immediately submerged in RNAlater (Invitrogen, Spain) and stored at 4 °C overnight to allow thorough penetration of the stabilizing solution into the tissue and subsequently stored at -20 °C until RNA extraction was performed.

Detection of *S. suis* serotype 2 by PCR and immunohistochemistry

Additional nasal swabs were taken at necropsy for detection of *S. suis* by PCR. Swabs were resuspended in PBS and DNA was extracted using the Nucleospin Blood kit (Macherey-Nagel, Germany). Four µL of DNA (between 42.0 and 867.2 ng) were used in the PCR to detect the serotype of the challenge strains, serotype 2, as previously described (Okura et al., 2014).

For immunohistochemistry (IHC), tissue samples from respiratory tract, including nasal turbinates, cribriform plate of ethmoid, trachea, and caudal lung lobe, as well as submandibular and tracheobronchial lymph nodes, were fixed by immersion in 10% buffered formalin and embedded in paraffin. Bacterial antigen detection in tissues was performed by IHC using a rabbit monoclonal anti-*S. suis* serotype 2 antibody (SSI Diagnostica, DK), followed by BrightVision Alkaline Phosphatase (AP)-conjugated anti-rabbit immunoglobulin G (IgG; Immunologic) and Vector Red (Vector Labs). Slides were counter stained with hematoxylin (Ferrando et al., 2015). Additionally, another consecutive slide from each tissue was stained with hematoxylin-eosin to study the lesions.

RNA extraction and quality control

Extraction of total RNA from lymph nodes, lungs, trachea, spleen, and liver was performed using the miRNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Briefly, approximately 30 mg of RNAlater stabilized tissue was homogenized in 1 mL QIAzol Lysis Reagent (in kit) using M-tubes (Miltenyi Biotec) and a gentleMACS Dissociator (Miltenyi Biotec). Total RNA was isolated from the homogenate by column-based extraction, including on-column DNase digestion of contaminating genomic DNA using the RNase-Free DNase Set (Qiagen) according to the manufacturer's instructions. Total RNA was eluted in 50 µL RNase-free water and stored at -80 °C.

Extraction of total RNA from whole blood collected in PAXgene Blood RNA Tubes was performed using the PAXgene Blood miRNA

Kit (Qiagen) according to the manufacturer's instructions, including on-column DNase digestion as above. RNA was eluted in 40 µL BR5 buffer (in kit) and stored at -80 °C.

Total RNA from RNAlater stabilized nasal swabs was extracted using an in-house optimized protocol. First, the RNAlater containing the swab was mixed with one volume (1 mL) RNA Lysis Buffer from the Quick-RNA Microprep Kit (Zymo Research) and vortexed followed by 5 min incubation at room temperature. Then the swab was removed, and the sample transferred to a 15 mL tube and mixed with 2.5 volumes (5 mL) cold (<0 °C) 100% ethanol, followed by vortexing and 30 min incubation at -20 °C. The supernatant was carefully removed with a pipette and the precipitate was washed twice with 70% ethanol at room temperature. The precipitate was dissolved in 1 mL RNase-free water and 700 µL was transferred to a Zymo-Spin IC Column (from the Quick-RNA Microprep Kit) and centrifuged at 10000 × g for 30 s and flow-through was discarded. This was repeated until the entire sampled had been passed through the column. From this point the Quick-RNA Microprep Kit protocol for RNA purification was followed according to the manufacturer's instructions, including on-column DNase digestion of contaminating genomic DNA. Total RNA was eluted in 15 µL RNase-free water and stored at -80 °C.

RNA concentration (ng/µL) and purity (A260/A280 and A260/A230 ratios) were assessed using a NanoDrop 1000 spectrophotometer (Thermo Scientific). RNA integrity number (RIN) was measured using an Agilent 2100 Bioanalyzer (Agilent Technologies) and the RNA 6000 Nano Kit (Agilent Technologies) (Supplementary Table S4.1).

Transcriptional analysis

Two replicates of cDNA were synthesized from each RNA sample using the QuantiTect Reverse Transcription Kit (Qiagen) according to the manufacturer's instructions employing 500 ng RNA for each synthesis for all tissue and blood RNA samples. For the nasal swabs, due to limited

amounts of sample and low RNA yields, cDNA synthesis was performed using as much RNA as was possible for the individual samples, varying from 33 to 323 ng per cDNA synthesis. Two no-reverse transcriptase controls (reaction not containing reverse transcriptase, negative controls) were made for each tissue/sample type.

All cDNA samples (diluted 1:10 in low-EDTA TE buffer) were pre-amplified using the TaqMan PreAmp Master Mix Kit (Applied Biosystems) in combination with a primer mix (each primer at 200 nM) containing all primer pairs to be used in the subsequent qPCR analysis (see below for details on primer design). All cDNA samples from tissues and whole blood were pre-amplified using 18 cycles of amplification, while cDNA samples from nasal swabs were pre-amplified using 22 cycles of pre-amplification. Following pre-amplification, residual primers were digested using Exonuclease I (New England BioLabs). Pre-amplified, exonuclease treated cDNA was diluted 1:10 in low-EDTA TE buffer for use in qPCR, and pools of pre-amplified, exonuclease treated cDNA were prepared from each of the tissue/sample types to produce dilution series in order to experimentally determine qPCR efficiency of all assays (primer pairs) for all investigated tissue/sample types. In addition, a non-template control was prepared to check for background fluorescence build-up of all primer pairs in the absence of cDNA template.

qPCR analysis was carried out using the high-throughput platform BioMark (Fluidigm) using 192.24 Dynamic Array IFC chips (Fluidigm) (192 samples in combination with 24 assays, used for lung tissues, trachea, nasal swabs) or 96.96 Dynamic Array IFC chips (Fluidigm) (96 samples in combination with 96 assays, used for lymph nodes, liver, spleen, blood). All assays (primer pairs) employed in the present study were designed in-house and purchased from Sigma-Aldrich. All qPCR primer sequences and amplification efficiencies can be found in Supplementary Table S4.2. Whenever possible, primers pairs were designed to span intron/exon borders in order to prevent amplification of potentially contaminating genomic DNA. qPCR was

carried out using a sample mix comprising TaqMan Gene Expression Master Mix (Applied Biosystems), DNA Binding Dye (Fluidigm), EvaGreen Dye (Biotium), and pre-amplified, exonuclease treated cDNA (diluted 1:10 in low-EDTA TE buffer). The individual assay mixes consisted of Assay Loading Reagent (Fluidigm) and primer pairs (20 µM for each primer). After loading all samples and reagents onto the chips using appropriate controllers (RX IFC Controller [Fluidigm] for 192.24 Dynamic Array IFC chips and HX IFC Controller [Fluidigm] for 96.96 Dynamic Array IFC chips), chips were transferred to the BioMark instrument for 35 cycles of amplification followed by melting curve analysis to ensure specific amplification.

Amplification curves, melting curves, and standard curves (dilution series) were evaluated using the Fluidigm Real-Time PCR Analysis software (v. 4.1.3). The GenEx software (v. 6) was used to correct C_q values with the obtained qPCR efficiencies, to evaluate potential reference genes for data normalization with the geNorm (Vandesompele et al., 2002) and NormFinder (Andersen et al., 2004) algorithms and subsequently perform normalization, to average technical repeats, and to convert C_q values to linear scale by computing relative quantities. Different subsets of reference genes were found appropriate for data normalization in different tissues based on the abovementioned reference gene evaluation: whole blood: *YWHAZ*, *RPL13A*, *HPRT1*; nasal swabs: *B2M*, *RPL13A*, *PPIA*; trachea: *RPL13A*, *GAPDH*, *HPRT1*; lung: *GAPDH*, *HPRT1*, *B2M*, *RPL13A*; submandibular lymph node: *PPIA*, *YWHAZ*, *RPL13A*; tracheobronchial lymph node: *B2M*, *HPRT1*, *PPIA*, *YWHAZ*, *RPL13A*, *ACTB*; liver: *PPIA*, *YWHAZ*, *RPL13A*; spleen: *HPRT1*, *PPIA*, *YWHAZ.*

Statistical analysis

Data analyses were performed with R (v. 4.0.2, (R Core Team, 2020)). Rectal temperature after inoculation was analyzed using ANOVA with Tukey's Honest Significant Difference (HSD) post-hoc test with interaction between inoculated groups and time points. In order to

compare changes in gene expression between groups, relative transcript quantities were calculated; for longitudinal samples (nasal swabs and blood) gene expression levels at 4 h post-inoculation and 1, 2, and 3 dpi were normalized against 3 days before inoculation. Statistical significance of the gene expression changes in whole blood and nasal swab samples was assessed by linear mixed effects regression with interaction between the different time points and the inoculum groups, taking into account animal ID as random-effect. For necropsy samples (all other tissue samples, taken at 1 and 3 dpi), normalization was done against the values of the PBS inoculated group. Statistical significance of the gene expression changes in necropsy tissues was analyzed using ANOVA with Tukey's HSD post-hoc test with interaction between inoculated groups and time points. A confidence level of 95% was considered as statistically significant ($P < 0.05$).

RESULTS

Clinical signs and lesions after inoculation

Few clinical signs were observed, comprising mild tremors at 2 and 3 dpi in two piglets inoculated with S10. Differences among the groups were observed in rectal temperature after the inoculation of the two strains. Although both groups of *S. suis* inoculated piglets had higher temperature than the control group (PBS inoculated) at 4 h after the challenge, this difference was statistically significant only in the piglets inoculated with S10 (ANOVA Tukey's HSD, $P = 0.044$; Figure 4.1). Furthermore, the number of piglets with rectal temperature higher than 40.5 °C at 4 h post-inoculation was greater in the group inoculated with S10 (5 out of 8) than in the T15 group (1 out of 8); however, no statistical difference was found between the two *S. suis*-inoculated groups. Temperatures at later time points were also recorded, and although S10 gave rise to higher temperature than T15 at 2 dpi, no statistical differences were found (Figure 4.1).

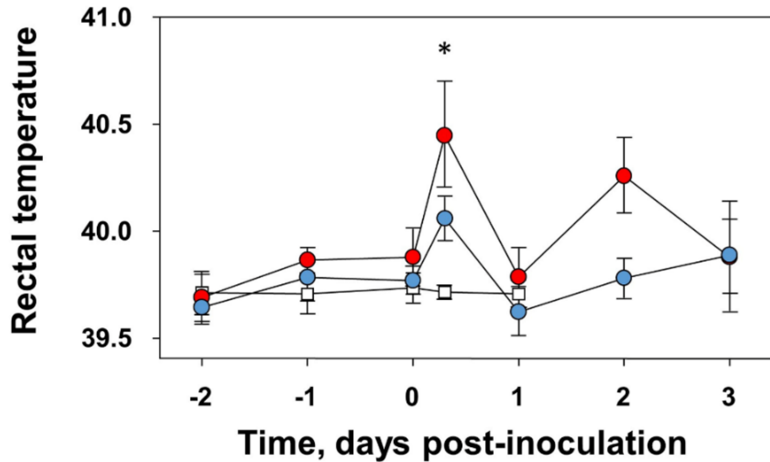

Figure 4.1 | Rectal temperature before and after *S. suis* intranasal inoculation. Mean and standard deviation of rectal temperatures of the piglets before and after intranasal inoculation with *S. suis* strains T15 (non-virulent, blue circles) or S10 (virulent, red circles) (n = 8 from -2 to 1 day post-inoculation [dpi]; n = 4 at 2 and 3 dpi, for both strains). A group of piglets was inoculated with PBS as control (white squares; n = 4 for all time points). * Statistically significant ($P < 0.05$) difference between S10 and PBS groups.

Gross lesions identified at necropsy were in general mild, affecting animals in all groups, including the PBS challenged group. None of these lesions could be ascribed to the *S. suis* challenge as *S. suis* was not re-isolated or detected by PCR from any of the lesions.

In the histological evaluation, no apparent lesions were found in most of the tissues (98/120) and were not consistent with characteristic *S. suis* pathology, with no differences among the three groups.

S. suis serotype 2 detection in the respiratory tract after inoculation

S. suis serotype 2 was detected by PCR in nasal swabs taken postmortem in piglets inoculated with T15 (7/8) or S10 (7/8). Amplification was more intense in nasal swabs from piglets inoculated with S10 than with T15, especially at 3 dpi (three samples from T15 inoculated piglets yielded a weak amplification and one was negative in the PCR, while two samples from S10 inoculated piglets yielded a moderate amplification, one a strong amplification and one was negative in the PCR).

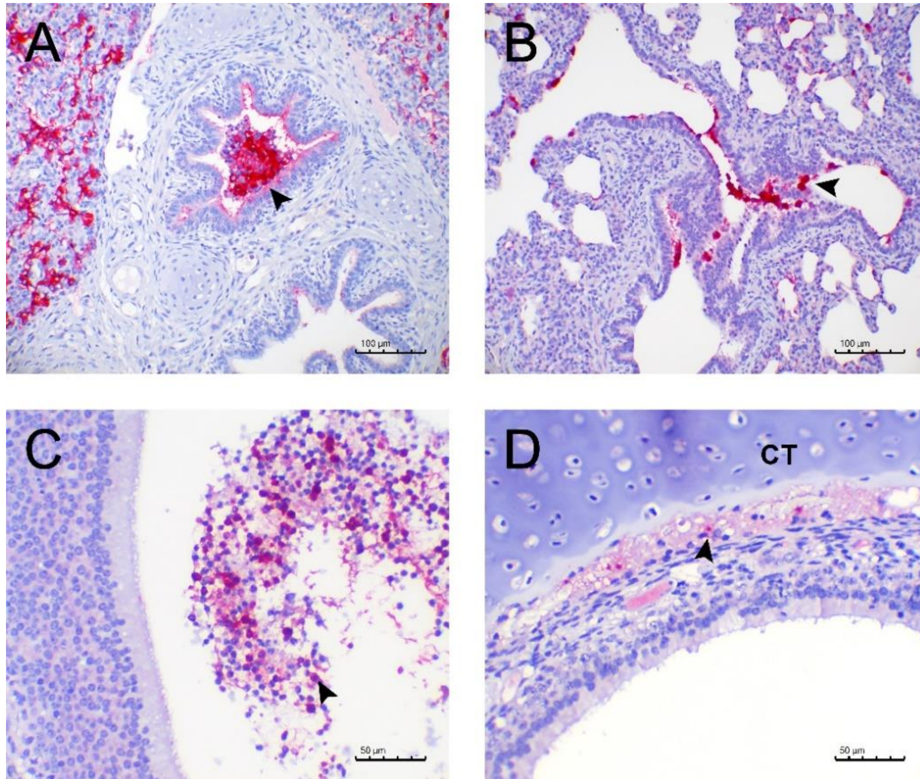

Figure 4.2 | Detection of *S. suis* serotype 2 by immunohistochemistry. Piglets were intranasally inoculated with *S. suis* T15 (non‑virulent) or S10 (virulent) strains, and tissues were collected at 1 and 3 days post‑inoculation (dpi). A: Bacteria present at 1 dpi in the bronchiole (arrowhead) and alveolar lumen of a piglet inoculated with T15. B: Bacteria present at 1 dpi in the epithelial surface of bronchioles (arrowhead) and alveoli of a piglet inoculated with S10. C: Presence of bacteria at 3 dpi in neutrophils in the mucus of the cribriform plate of ethmoid of a piglet inoculated with T15 (arrowhead). D: Bacteria next to the cartilage of the cribriform plate of ethmoid (CT) at 3 dpi, from a piglet inoculated with S10 (arrowhead).

Using IHC, *S. suis* serotype 2 was detected in the upper respiratory tract for both strains, mostly in the mucus but also in the epithelium of the nasal cavity (2/8 for T15 and 4/8 for S10), cribriform plates of ethmoid (7/8 for T15 and 8/8 for S10), and tracheas (3/8 for T15 and 1/8 for S10). Immunolabelling was also found in the alveolar lumen of the lungs (4/8 for T15 and 5/8 for S10) (Figures 4.2A and B), but there was no detection in any of the lymph nodes analyzed. In the cribriform plate of ethmoid, T15 bacteria were found only in the mucus (Figure 4.2C), while some S10 bacteria were detected deep in the tissue, close to

the cartilage (Figure 4.2D). This latter location was not observed in any of the animals infected with the T15 strain. Thus, *S. suis* serotype 2 was detected in all inoculated animals by either PCR (14/16) or IHC (15/16), but not in the piglets from the non-infected control group.

Local and systemic transcriptional responses to virulent and non-virulent *S. suis* inoculation

High quality RNA was obtained from lymph nodes, trachea, lungs, spleen, liver, and whole blood. RNA obtained from nasal swabs was of sub-optimal quality, and care was therefore taken when choosing a strategy for transcriptional analysis of these samples. This included limiting the focus to relatively few genes that could be expected to be strongly induced during an inflammatory antibacterial response, as well as assaying the transcription of several of the investigated genes with two independent assays (two different primer pairs targeting the same mRNA transcript at non-overlapping sites). Mean and range of RNA quality for the different tissues are summarized in Supplementary Table S4.1. Gene expression in longitudinal samples (whole blood and nasal swabs) and in necropsy samples (all other tissues) were compared to the expression at 3 days before challenge and the PBS group, respectively, as indicated above (see section “Materials and methods”).

Generally, only small changes in gene expression were observed in *S. suis*-challenged animals, with the majority of the transcriptional regulation being <2-fold either up- or down-regulated, for both *S. suis* strains. In addition, only some of these changes were statistically significant, probably due to the considerable individual variation in gene expression levels observed within the groups of animals.

However, a group of genes showed quite pronounced transcriptional responses in nasal swab samples and clearly demonstrated differential host responses after virulent and non-virulent challenge, with more genes consistently up-regulated by S10 at 4 h post-inoculation than by T15 (Figures 4.3 and 4.4). These genes included pro- and anti-inflammatory genes *IL1A*, *IL1B*, *IL1RN*, and *IRF1*, as well as

the chemokine *CXCL10*, and were induced early in the nasal mucosa after challenge with both strains (Figure 4.3, Supplementary Table S4.3). This response seemed to continue unabated throughout the experiment in the piglets inoculated with the virulent S10 strain whereas the response to the T15 challenge was shorter and had a tendency to return to baseline levels by day 3 (Figures 4.3 and 4.4). Despite the changes observed between strains (Figures 4.3 and 4.4, Supplementary Table S4.3), specially at 3 dpi, none of these were statistically significant, probably due to the low number of piglets.

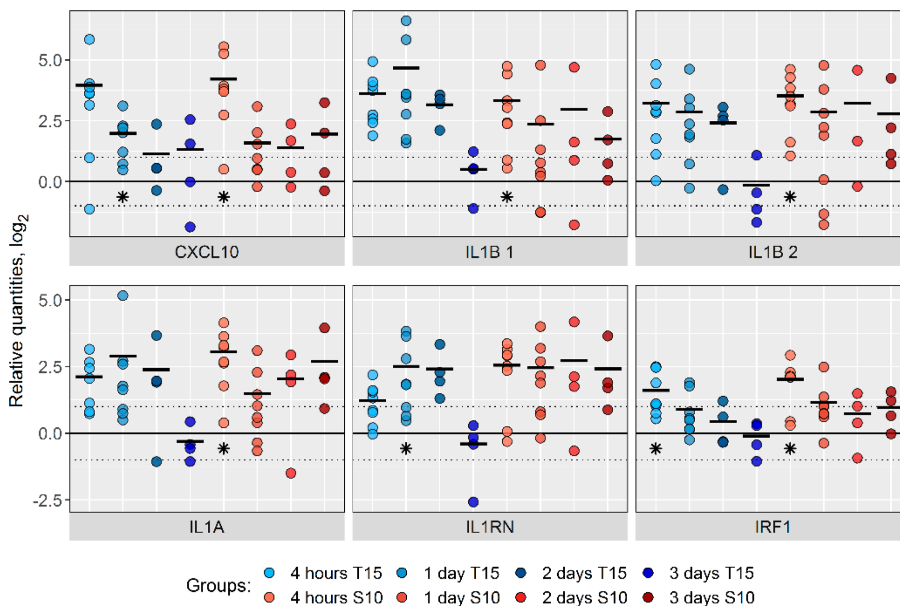

Figure 4.3 | Relative gene expression in nasal samples after *S. suis* intranasal inoculation. Log_2 of the individual values and mean (black bars) of the relative gene expression in nasal samples in *S. suis* inoculated piglets (gene expression was normalized relative to the 3 days before challenge mean for each inoculated group). Piglets were intranasally inoculated with *S. suis* T15 (non-virulent, blue bars) or S10 (virulent, red bars) strains, and nasal swabs were taken at 4 h and 1, 2 and 3 days after inoculation. Genes showing significant difference ($P < 0.05$) in pairwise analysis when comparing different time points in the *S. suis* inoculated groups or between strains at the same time point, and with a mean greater than 2-fold change ($log_2 = 1$) are shown. * $P < 0.05$ when comparing versus their respective 3 days before challenge time point. n = 8 for both strains at 4 h post-infection and 1 dpi, n = 4 for both strains at 2 and 3 dpi. *IL1B 1* and *IL1B 2* are both *IL1B* assays consisting of two different primer pairs targeting non-overlapping sites in the *IL1B* transcript. All expression values and significant differences can be found in Supplementary Table S4.3.

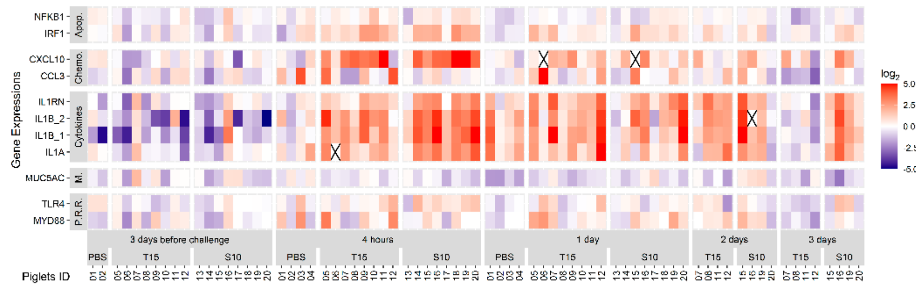

Figure 4.4 | Gene expression in nasal samples after *S. suis* inoculation. Samples were taken at 3 days before, and 4 h, 1, 2, and 3 days after the intranasal inoculation of *S. suis* strains T15 (non-virulent) and S10 (virulent). A group of piglets inoculated with PBS are also shown and served as control. All the genes found to be quantifiable are shown irrespectively of their statistical significance. Gene expression was normalized relative to the 3 days before challenge mean for each inoculated group and $\log_2$ transformed. Values are presented as a heat map. Numbers in abscissa axis represent animal ID. Color scale was limited to ± 5 and out of bounds values displayed with the maximum intensity color. Gene functional groups: Apop.: Apoptosis; Chemo.: Chemokines; M.: Miscellaneous; P.R.R.: Pattern Recognition Receptors. *IL1B_1* and *IL1B_2* are both *IL1B* assays consisting of two different primer pairs targeting non-overlapping sites in the *IL1B* transcript. Samples marked with a black cross: expression level not quantifiable.

In contrast to the observations in the nasal samples, gene expression in the submandibular lymph node was generally less affected with fewer and smaller changes and only by the T15 strain (at 3 dpi), with no significant modulation of gene expression observed in animals inoculated with S10 strain at 1 or 3 dpi (Figure 4.5). At day 3 after the challenge with the non-virulent T15 strain, genes *IL1B* and *PTGS2* (indicative of inflammation) and *CCL2* and *SELP* (involved in recruitment of immune cells) were significantly >2-fold up-regulated *vs*. PBS control (Figure 4.5). In the case of *PTGS2* and *CCL2*, significant differences were also found between T15 and S10 inoculated animals, with up-regulation only by the non-virulent T15 strain. In the tracheobronchial lymph node, significant up-regulation was only observed for *CASP1* at 1 dpi in S10 inoculated pigs (Figure 4.5). Genes with significant changes lower than 2-fold when compared *vs*. the PBS group for both lymph nodes are included in Supplementary Figure S4.1. In addition, individual expression changes in submandibular and tracheobronchial lymph nodes are presented in Figure 4.6. Although

some changes were observed in individual piglets, the response showed high variation within the groups and no statistical differences were found in our model (Figure 4.6 and Supplementary Table S4.4). As an example, *IL1RN* in tracheobronchial lymph node: mean ± standard deviation of 3.92 ± 2.78 and 3.79 ± 1.18 for T15 strain at 1 and 3 dpi respectively; and 5.88 ± 3.49 and 4.06 ± 2.44 for S10 strain at 1 and 3 dpi respectively (all tissues values are available in Supplementary Table S4.4).

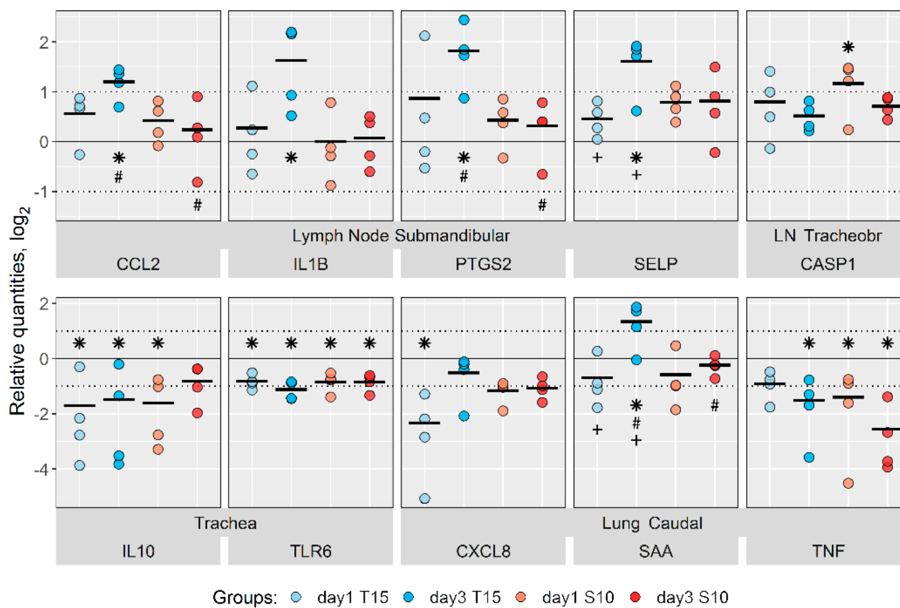

Figure 4.5 | Relative gene expression in different tissues after *S. suis* intranasal inoculation. Log_2 of the individual values and mean (black bars) of the relative gene expression in different tissues in *S. suis* inoculated piglets (gene expression was normalized relative to the PBS group). Piglets were intranasally inoculated with *S. suis* T15 (non-virulent, blue bars) or S10 (virulent, red bars), and necropsies were performed at 1 and 3 days post-infection. The values and means are shown for the indicated groups (challenge strain and time point) having at least one significant difference when compared to the PBS group and with a mean higher than 2-fold change (log_2 = 1). LN Tracheobr: Tracheobronchial lymph node. * indicates significant differences (P < 0.05) versus the PBS group. Differences between strains at the same time point are labelled with # and differences between time points for the same strain are labelled with +, P < 0.05, in both cases. n = 4 for each group. All expression values and significant differences can be found in Supplementary Table S4.4.

In trachea, *IL10* and *TLR6* were significantly down-regulated in response to both strains, which coincided with a general trend towards down-regulation of the majority of genes examined in this tissue (Figure 4.5, Supplementary Figure S4.2). In lungs, more changes were detected in the caudal than in the cranial lobe, which only showed minor changes in *TLR6* in the S10 inoculated group (Supplementary Figure S4.1). In the caudal lobe, significant regulation with a >2-fold change was observed for *CXCL8* (IL8) (down-regulated by T15 at 1 dpi), *SAA* (up-regulated by T15 at 3 dpi), and *TNF* (down-regulated by T15 at 3 dpi, and by S10 at 1 and 3 dpi) compared to the PBS group (Figure 4.5). When comparing the responses to the two strains, *SAA* (>2-fold, Figure 4.5) and *TLR2* (<2-fold, Supplementary Figure S4.1) were significantly higher for the non-virulent T15 than the virulent S10 at 3 dpi. A few other significantly different ($P < 0.05$) <2-fold changes compared to the PBS group, between time points for the same strains, or between strains at the same time point, are shown in Supplementary Figure S4.1.

Analysis of whole blood indicated changes in gene expression patterns in response to the challenge with both *S. suis* strains, although the majority of changes were below 2-fold, with subtle differences in the temporal dynamics depending on the challenge strain (Figures 4.7 and 4.8, Supplementary Table S4.5). The blood response to strain S10 was rapid with six genes showing >2-fold up-regulation at 4 h after challenge (*CASP1*, *CD14*, *IRF7*, *STAT1*, *STAT2*, and *TLR4*), and maintaining this difference at 2 dpi for *STAT1* and *STAT2* (Figure 4.7, Supplementary Table S4.5). The response to the non-virulent strain T15 was more delayed, with a peak in the number of significantly up-regulated genes with a >2-fold change on 1 dpi (five genes, *CASP1*, *CCL4*, *IRF7*, *STAT1*, and *STAT2*), and only one gene (*TLR4*) significantly up-regulated at 4 h after the challenge (Figure 4.7, Supplementary Table S4.5). The different response observed to both strains was statistically different only at 1 dpi for the following genes: *CASP1*, *CCL4*, *IRF7*, *STAT1*, and *STAT2*, with higher values in animals challenged with the non-virulent strain T15 (Figure 4.7, Supplementary Table S4.5). Significant differences lower

than 2-fold between strains were observed only at 1 dpi for the genes *IL1B*, *JAK2*, *TICAM1* (*TRIF*), and *TNF*, with higher values for T15 than for the S10, and also for *NFKBIA*, but in the opposite direction (Supplementary Table S4.5).

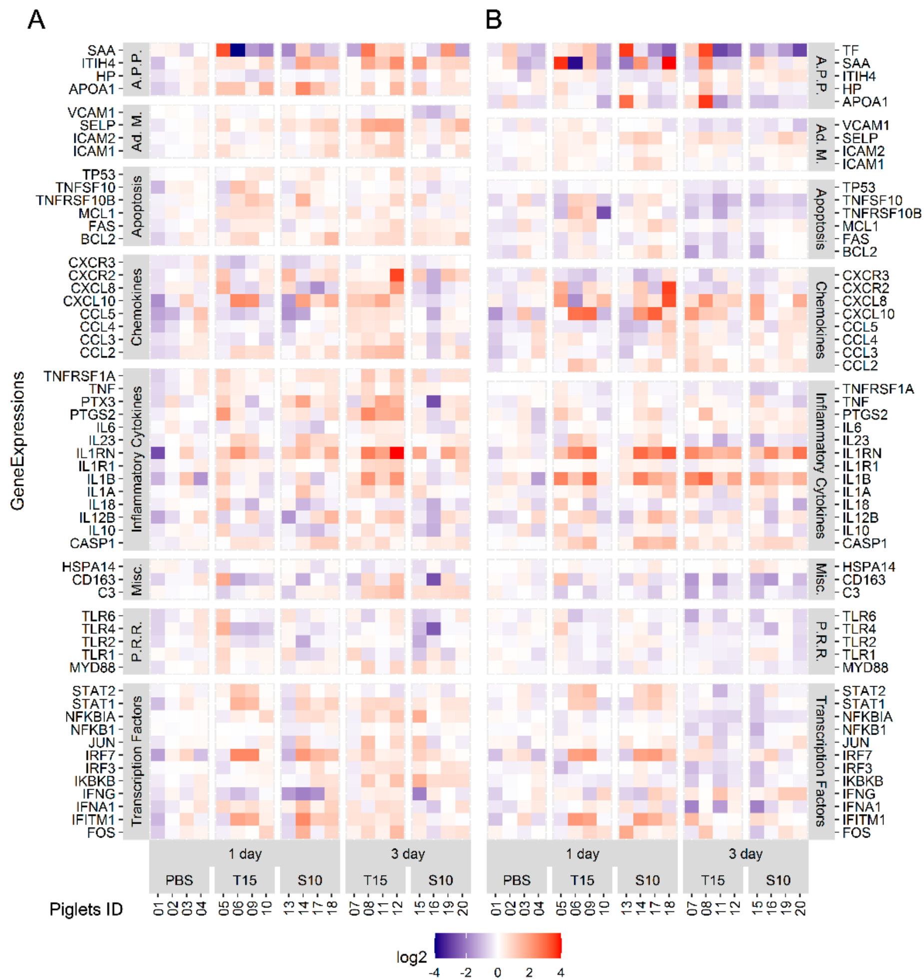

Figure 4.6 | Gene expression in submandibular and tracheobronchial lymph nodes after *S. suis* intranasal inoculation. Samples from the submandibular (A) and tracheobronchial (B) lymph nodes were collected at 1 and 3 days after the intranasal inoculation of *S. suis* T15 (non-virulent) and S10 (virulent). Results at 1 day post-inoculation from piglets inoculated with PBS are also included as control. All the genes found to be quantifiable are shown irrespectively of their statistical significance. Gene expression was normalized relative to the PBS group and $\log_2$ transformed. Values are presented as a heat map. Numbers in abscissa axis represent animal ID. Color scale was limited to ± 4 and out of bounds values displayed with the maximum intensity color. Gene functional groups: A.P.P.: Acute Phase Proteins; Ad. M.: Adhesion Molecules; Misc.: Miscellaneous; P.R.R.: Pattern Recognition Receptors.

Consistent with the absence of systemic disease and/or overt systemic reactions to the intranasal *S. suis* inoculation, very few genes were significantly affected in liver and spleen (Supplementary Figure S4.3). Despite the small magnitude of these changes (all with <2-fold changes), some statistical differences between strains were observed in liver at 1 dpi, with lower values in piglets inoculated with the virulent strain S10 for *BCL2*, *TNFRSF1A*, and *TP53*. Individual values for all the genes analyzed in these tissues are presented as heat maps in Supplementary Figure S4.4.

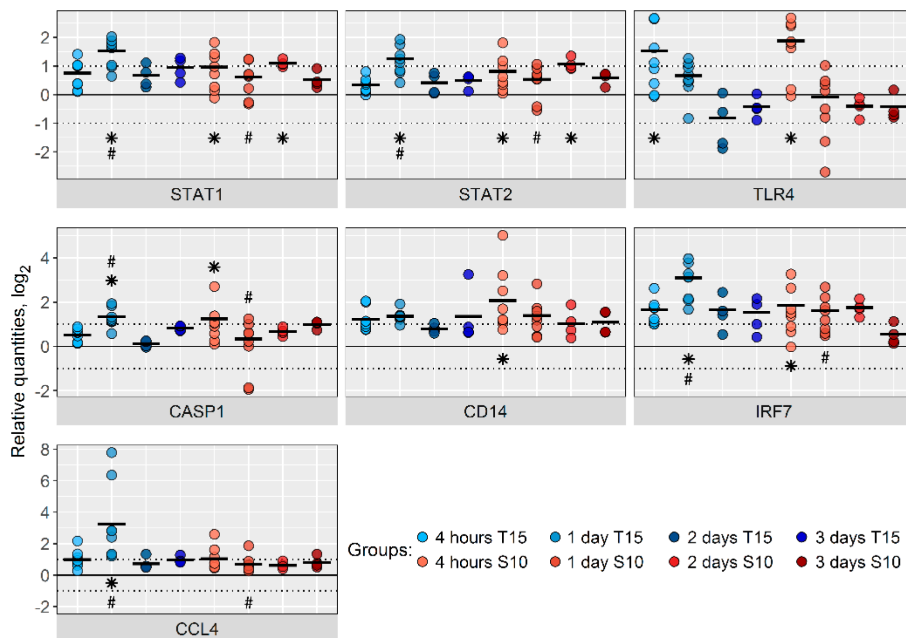

Figure 4.7 | Relative gene expression in blood after *S. suis* intranasal inoculation. Log_2 of the individual values and mean (black bars) of the relative gene expression in blood in *S. suis* inoculated piglets (gene expression was normalized relative to the 3 days before challenge mean for each inoculated group). Piglets were intranasally inoculated with *S. suis* T15 (non-virulent, blue bars) or S10 (virulent, red bars), and blood samples were taken at 4 h and 1, 2, and 3 days after inoculation. Genes with at least 1 significant difference in pairwise analysis ($P < 0.05$) when comparing different time points in the *S. suis* inoculated groups or between strains at the same time point, and with a mean greater than 2-fold change ($log_2 = 1$) are shown. * indicates significant differences ($P < 0.05$) versus their respective 3 days before challenge time point. Differences between strains at the same time point are labelled with # ($P < 0.05$). n = 8 for both strains at 4 h post-infection and 1 dpi, n = 4 for both strains at 2 and 3 dpi. All expression values and significant differences can be found in Supplementary Table S4.5.

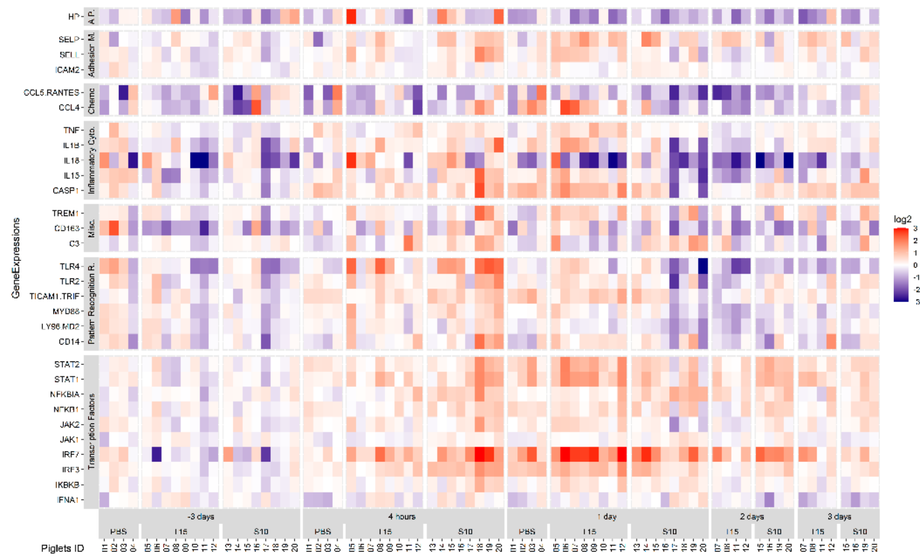

Figure 4.8 | Gene expression in blood after *S. suis* intranasal inoculation. Samples were taken at 3 days before, and 4 h, 1, 2, and 3 days after the intranasal inoculation of *S. suis* T15 (non-virulent) and S10 (virulent). A group of piglets inoculated with PBS are also shown and served as control. All genes found to be quantifiable are shown irrespectively of their statistical significance. Gene expression was normalized relative to the 3 days before challenge mean for each inoculated group and $\log_2$ transformed. Values are presented as a heat map. Numbers in abscissa axis represent animal ID. Color scale was limited to ± 3 and out of bounds values displayed with the maximum intensity color. Gene functional groups: A.P.: Acute Phase Proteins; Adhesion M.: Adhesion Molecules; Chemo.: Chemokines; Misc.: Miscellaneous; Pattern Recognition R.: Pattern Recognition Receptors.

DISCUSSION

Pathogens use different mechanisms to evade the innate immune system, the first line of defense against them, and to colonize the host. In *S. suis* infection, the host's immune response combined with the virulence of the infecting strain play important roles in achieving colonization and, subsequently, in the possible development of the disease (Segura et al., 2016).

Although it is difficult to reproduce disease with this bacterium using the intranasal route of inoculation, it has been used on numerous occasions to study host-pathogen interactions (Segura et al., 2016). In the present study, despite the fact that systemic disease did not develop

in the inoculated animals in the short course of the study, we did observe various host responses (including fever) induced by strains S10 and T15, with different known virulence potential, during the first steps of infection. For most of the sample types examined in this work, the limited number of animals and high individual variations made it difficult to correlate a clear gene expression pattern or inflammatory marker consistently with the virulence of the strain. However, for the nasal mucosa the transcriptional response did in fact reflect the virulence potential of the inoculated *S. suis* strain. Despite these limitations, this study used an experimental model that reproduces *S. suis* natural infection of pigs, providing for the first time a comprehensive overview of the host innate immune response induced by *S. suis* during upper respiratory tract colonization. In addition, the present study paves the way for more extensive mechanistic studies on modulation of host immunity by this important swine pathogen.

Interestingly, both strains induced an early pro-inflammatory response locally in the nasal mucosa; however, the return to baseline gene expression levels was faster for the non-virulent strain (T15). Among genes up-regulated by both strains at the nasal mucosa, *IL1B* is a cytokine that acts as a master regulator of inflammation by controlling a variety of innate immune processes (Kaneko et al., 2019). Several studies have reported the capacity of *S. suis* to induce IL-1 cytokine family members by a variety of cell types (Auger et al., 2017; Lavagna et al., 2019). In addition to *IL1B*, up-regulation of the interferon-regulatory factor 1 (*IRF1*) suggests activation of the interferon (IFN) pathway during *S. suis* colonization, including expression of *CXCL10*, a chemokine gene that can be up-regulated in response to IFN-γ/IRF1 signaling pathway (Shultz et al., 2009). The IFN pathways can play either a regulatory or a pathological role depending on the virulence of the *S. suis* strain or the specific clinical manifestation of the disease, as previously suggested (Lachance et al., 2013; Lavagna et al., 2019).

In the submandibular lymph node, gene expression related to recruitment of immune cells (such as expression of the chemokine *CCL2*

and the adhesion molecule P selectin encoded by *SELP*) and to inflammation (*IL1B*) was mainly observed after colonization with the non-virulent strain. These seemingly contradictory results observed between nasal mucosa and the submandibular lymph node may reflect intrinsic properties of the strains, with different molecular composition, including the presence of virulence-associated proteins in the S10 strain (e.g., the Muramidase-Released Protein [MRP] or the extracellular factor [EF] protein) that are absent in T15 (Vecht et al., 1997).

In the absence of clinical manifestations and histopathological lesions, the observed modulation of the innate immune response by *S. suis* colonization could be considered a homeostasis-restoring state of inflammation (Chovatiya and Medzhitov, 2014), which is considered different from pathological inflammation. It has been suggested that such state may be maintained by pattern recognition receptors (PRRs) expressed in stromal and/or immune cells, detecting endogenous ligands and/or pathogens (Chovatiya and Medzhitov, 2014). In agreement with this concept, expression of interleukin-1 receptor antagonist (IL-1RA; encoded by *IL1RN*) was observed in nasal samples and that of the enzyme cyclooxygenase-2 (COX-2), encoded by *PTGS2*, was found in submandibular lymph node (and mainly induced by the non-virulent strain). COX-2-derived metabolites are important regulators of inflammation (Szymanski et al., 2012) and IL-1RA competitively inhibits IL-1 binding to cell-surface receptors. Maintenance of a balance between IL-1 and IL-1RA is important in preventing the development or progression of inflammatory disease (Arend and Guthridge, 2000). It has been suggested in other models that selective induction of IL-1RA might facilitate mucosal colonization by bacteria. IL-1RA also plays a critical role in maintaining a homeostatic and balanced microbiota (Barton et al., 2003; Rogier et al., 2017). Further studies are required to delineate the link between *S. suis* colonization and the induction of a homeostasis-restoring state of inflammation, including a potential regulatory role of IL-1RA and/or COX-2.

The systemic response was limited as no clinical invasive disease was observed and *S. suis* was not found in blood. The observed minor changes in gene expression in systemic samples could be a consequence of the ongoing local response at the upper respiratory track. Indeed, up-regulated genes were associated to the IL-1 or the IFN pathways (such as *CASP1*, *IRFs*, and *STATs*), which seem to predominate during the innate immune response induced by *S. suis* colonization. However, massive activation of these and other pro-inflammatory pathways (cytokine storm) are known to be involved in pathological inflammation during *S. suis* systemic disease leading to septic shock (Bi et al., 2014). Nevertheless, the transcriptional patterns in blood showed that the host response to the virulent challenge was rapid, peaking within hours after challenge, which coincided with elevated body temperature. In contrast, the circulating response to the non-virulent challenge was more delayed and did not coincide with the fever response. This correlation between up-regulation of pro-inflammatory cytokines and fever agrees with the initial course of disease in other pig infection models (Senthilkumar et al., 2019). Internal organs such as liver, spleen, kidney, or heart, are invaded after *S. suis* reaches systemic circulation (Fittipaldi et al., 2012). However, in the present study, the piglets did not develop systemic disease and, accordingly, the splenic and hepatic response to both *S. suis* strains showed a low number of genes significantly affected and with low magnitude. This lack of systemic disease may be explained by the route of inoculation, intranasal, unaided by acetic acid or viral coinfection (Feng et al., 2001; Pallarés et al., 2003), the short time of the study, or the ability of the host to control the infection before bacteria could reach the bloodstream.

Transcriptional results from trachea and lungs indicate that the host response or bacterial spread beyond the nasal cavity and further down the respiratory tract for the duration of the experiment was limited. Specific serotype detection by IHC was achieved in the tissues in which more mucus remained after the paraffin treatment, like the alveolar sac in lungs or the characteristic sinuous tissue of the cribriform plate of the

ethmoid bone. In other respiratory tissues, bacteria were only detected in mucus or a few of them attached to the epithelium, which is consistent with the sub-clinical infection and the low response observed in the trachea. Colonization thus appears to primarily affect the host response locally at the site of colonization, with little or no widely disseminated response beyond the nasal cavity. Regarding the localization of *S. suis* S10 detected in the cribriform plate of the ethmoid, it cannot be ruled out that this site may serve as a non-hematogenous route to the central nervous system. This route has previously been suggested for *Streptococcus pneumoniae* (Marra and Brigham, 2001) and demonstrated for others bacteria such as *Neisseria meningitidis* and *Burkholderia pseudomallei* (Sjölinder and Jonsson, 2010; St. John et al., 2014), as well as for ameboflagellates (*Naegleria fowleri*; (Jarolim et al., 2000)) or viruses (SARS-CoV-2; (Meinhardt et al., 2021)). This hypothesis deserves further analysis.

This study provides information for understanding the colonization of *S. suis* (first step of infection) and the potential mechanisms involved in the early local innate immune response, which might either favor colonization without disease development or rather colonization followed by systemic invasion. Our results seem to reflect a host response to this non-virulent *S. suis*, which is characterized by rapid control at the site of inoculation, probably mediated by a sustained immune response at the associated lymph node. In contrast, the virulent strain used seem to prevent a robust lymph node response, and, in consequence, they are maintained at the site of inoculation, where they continue to elicit inflammatory mediators. Several factors might dictate these outcomes, including host and environment factors, as well as the virulence potential of the strain.

STUDY IV

Protection of selected commensals against a challenge with virulent *Streptococcus suis*

Manuscript

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ABSTRACT

Due to the current situation with the use of antimicrobials in livestock, both to control and to prevent diseases, it is necessary to develop alternative control tools that avert the onset of bacterial diseases and the appearance of antimicrobial resistance. *Streptococcus suis*, an early colonizer of the porcine upper respiratory tract, is responsible for one of the most important bacterial diseases in piglets at weaning age.

In the present study, the suitability of the use of putative nasal and tonsillar probiotics isolated from healthy animals against the development of *S. suis*-associated disease was examined. Different combination of isolates from the genera *Rothia* (isolates R1 and R2), *Moraxella* (M) and a non-virulent *S. suis* strain (Ss) were tested. Piglets were nasal and orally inoculated with the putative probiotics on days 1 and 6 of life, with one group as control. Later, the piglets were intranasally challenged at 31 days of life with the virulent *S. suis* strain P1/7. Of the 25 challenged piglets, 14 died between days 3 and 6 after P1/7 inoculation. Although the group inoculated with the R1+R2+M combination showed the best results in the different parameters studied (clinical signs after infection, survival, and lesions at necropsy), the double inoculation with the selected probiotics was not enough to completely protect the piglets against a lethal challenge with a virulent strain of *S. suis*. The experimental model of the present study lays the foundations for future approaches to study the performance of nasal probiotics in endemic pig diseases.

Keywords: probiotics; *Streptococcus suis*; disease prevention, colonization.

INTRODUCTION

The effect of antimicrobials has been extensively studied in pig production because of their impact on animal health and growth (Cromwell, 2002; Thacker, 2013). However, due to the problems associated with antimicrobial resistances (AMR), the development of new tools for reducing the use of antimicrobials is essential, especially in livestock (Ghosh et al., 2019). The use of antimicrobial as growth promoters in animals was totally banned in the European Union in 2006 (EU, 2003), nevertheless, other countries such as U.S.A. and China have only applied partial restrictions recently, in 2017 and 2020 respectively (Centner, 2016; Hu and Cowling, 2020). On the other hand, the use of antimicrobials to prevent or control diseases is also restricted by the new regulations published by the EU for their application at the beginning of 2022 (EU, 2019b, 2019a).

S. suis, an encapsulated Gram-positive coccus, is considered one of the most important pathogen that affects pigs worldwide and an important cause antimicrobial use (Gottschalk and Segura, 2019; Neila-Ibáñez et al., 2021a). Since birth, *S. suis* colonizes the upper respiratory tract of piglets, constituting part of the natural microbiota of tonsils and nasal cavity (Devriese et al., 1994; Amass et al., 1997). The respiratory tract is also the main entry route for *S. suis* infection (Gottschalk and Segura, 2019). Despite pigs of all ages can develop disease, piglets from 5 to 10 weeks of age are the most affected group (Gottschalk and Segura, 2019). The most common clinical signs in animals that develop infection are incoordination, paddling, lameness, swollen joints, and acute death (Gottschalk and Segura, 2019). When an outbreak occurs, herd mortality can reach 20% if piglets do not receive antimicrobial treatment (Cloutier et al., 2003). *S. suis* is also an emerging zoonotic agent, generally producing individual cases in Western countries but it has also caused large outbreaks in Southeast Asian countries (Gottschalk and Segura, 2019). Multiples *S. suis* serotypes have been described based on the capsular polysaccharide, which, among other factors, makes vaccination

a difficult task (Segura, 2015). Therefore, disease prevention is generally based on an alarming use of metaphylactic antimicrobials, leading to a risk of generating antimicrobial resistance (Seitz et al., 2016; Yongkiettrakul et al., 2019; Segura et al., 2020).

The development of new tools to overcome the restrictions on preventive antimicrobials, such as prebiotics or probiotics, has increased in recent years. Among other functions, intestinal probiotics improve the gut health due their role in maintaining the function of the intestinal barrier, working as promoters of the anti-inflammatory response, or inhibiting the growth of potentially pathogens (Reid and Friendship, 2002; Kenny et al., 2011; Liao and Nyachoti, 2017). Referring to the swine respiratory tract, the set of bacteria that inhabit the nasal cavity or the tonsil, known as nasal or tonsil microbiota, seem to play a role in the predisposition to swine diseases, such as *Streptococcus suis*, Glässer's disease, or *Mycoplasma hyorhinis* (Correa-Fiz et al., 2016; Blanco-Fuertes et al., 2021; Niazy et al., 2022). Modulation of this nasal microbiota with the use of upper respiratory tract probiotics could prevent the onset of these diseases by modulating the immune system and occupying the ecological niche of these potentially pathogens (Costa-Hurtado et al., 2020). Although the performance of nasal probiotics has been studied in humans, their use to fight against respiratory diseases in livestock has not been extensively explored (Man et al., 2017; Shokryazdan et al., 2017; Dimitri-Pinheiro et al., 2020).

Different tonsillar microbiota composition was observed in piglets with *S. suis* disease and healthy piglets (Niazy et al., 2022), suggesting that a timely stabilization of the microbiota may prevent the development of the disease caused by *S. suis*. Despite this fact, to the best of our knowledge there are no previous attempt to test the effect of nasal/oral probiotic candidates against infection by virulent *S. suis*. Su and collaborators (2008) observed a possible reduction of *S. suis* in the hindgut of post-weaned piglets after the oral administration of a *Lactobacillus sobrius* strain. Other *in vitro* studies have identified strains with potential capacity to inhibit *S. suis*, such as *Lactobacillus*

plantarum, *Pediococcus acidilactici*, and *Bacillus coagulans*, which were recovered from feces (Gu et al., 2015; Sirichokchatchawan et al., 2018), or *Streptococcus pluranimalium*, recovered from a piglet nose (Vaillancourt et al., 2022).

The goal of the present study was to examine the suitability of different candidate probiotic cocktails against a lethal challenge with *S. suis*.

MATERIALS AND METHODS

Commensal selection and inoculum preparation

The commensals used in the present study were recovered from the nose or tonsils of healthy piglets and selected based on different criteria:

-Isolate R1 is a strain of *Rothia* characterized by expressing a natural antimicrobial via large non-ribosomal peptide synthase (NRPS), with activity in vitro against *S. suis*, and having a relatively good interaction with tonsil organoids. Pena Cortes and collaborators (2018) reported *Rothia* as a core tonsil microorganism in piglets after birth, however, this specific species variant was relatively rare in tonsil samples (0.006% of total microbiota composition in previously sampled farms).

-R2 is another *Rothia* strain. The Amplicon Sequence Variant (ASV) corresponding to R2 was negatively correlated with *S. suis* and was more abundant than R1, accounting for 0.5% of the microbiota composition. Biosynthetic genes seems not to appear in its genome sequence.

-Isolate M is a strain of *Moraxella*, which was among the most abundant ASVs in tonsil microbiota (5.3%) and was negatively correlated with the abundance of *S. suis*. Moraxella was also reported as an abundant genus in the tonsillar microbiota by Pena Cortes and collaborators (2018). This specific strain had adherence ability, was sensitive to serum and phagocytosis, and showed lower antimicrobial resistance than other *Moraxella* isolates (resistant to tetracycline and trimethroprim-sulfonamide, whilst sensitive to colistin, enrofloxacin, amoxicillin with

clavulanate, ceftiofur, gentamicin, lincomycin-spectinomycin, erythromycin, tulathromycin, and marbofloxacin) (López-Serrano et al., 2020).

-Isolate Ss is a *S. suis* serotype 31 that was selected because it was an abundant ASV, showed lower antimicrobial resistance compared with other *S. suis* isolates recovered from healthy piglets and was classified in a clade associated with carriage isolates; i.e., non-virulent due to the absence of classical virulence factors.

All strains were plated on chocolate agar (Biomerieux, Spain) and incubated overnight at 37ºC with 5% CO_2. Bacterial suspensions were prepared in 20% glycerol in PBS and inocula were prepared by mixing the corresponding strains at the concentrations indicated in Table 5.1. Four combinations of commensals were produced (Table 5.1) and were tested in newborn piglets (see below). Pigs in each study group were inoculated with 2 mL of the corresponding combination of commensals. A control group was inoculated with 20% glycerol in PBS.

Table 5.1 | Quantities of commensal combinations inoculated, expressed in colony forming unit (CFU)/mL.

	1st inoculum - Day 1	**2nd inoculum - Day 6**
	R1+M+Ss group	
R1	1.5×10^5	2.1×10^7
M	2.6×10^5	2.8×10^7
Ss	2.6×10^5	4.6×10^7
	R1+R2+M+Ss group	
R1	3.0×10^5	2.8×10^7
R2	2.4×10^5	1.9×10^7
M	2.6×10^5	2.4×10^7
Ss	2.9×10^5	1.9×10^7
	R1+R2+M group	
R1	2.3×10^5	2.0×10^7
R2	2.3×10^5	1.4×10^7
M	3.6×10^5	2.0×10^7
	Ss group	
Ss	2.2×10^5	2.4×10^7

Animal study

Animal experimentation was performed in the BSL3 facilities of IRTA-CReSA (Bellaterra, Spain) following good veterinary practices, in accordance with European (Directive 2010/63/EU) and Spanish (*Real Decreto* 53/2013) regulation and with the approval of the Ethics Commission in Animal Experimentation of the *Generalitat de Catalunya* (Protocol number 10201).

Four pregnant sows of sixth parity were selected from a farm with low incidence of *S. suis*-disease. These animals were tested negative to *S. suis* serotypes 1/2 - 2 in the PCR described by Ishida and collaborators (2014) in swabs collected from nose and vagina 1 month before delivery. Sows were transported to the BSL3 facilities and were housed for two weeks before delivery. Sows were treated with ceftiofur (Naxcel) intramuscularly 7 and 4 day before farrowing, and with phenoxymethylpenicillin (Penilevel) intravaginally on days 4, 3, and 2 before farrowing. Farrowing was induced at day 113 of gestation with prostaglandin (Veteglan). The offspring had the genetics of a commercial breed (Duroc ♀ × Landrace ♂).

Newborn piglets were allowed to nurse with their biological mother during approximately 1 day. After this time, sows were removed from the study and the 44 viable piglets were identified, randomized in the 5 groups described above (Table 5.1), and housed in two clean units. Groups R1+M+Ss, group R1+R2+M+Ss, and group Ss were housed in one unit, whilst groups R1+R2+M, and control group were housed in a separate unit. All the actions performed during the experiment are summarized in Table 5.2.

Piglets were hand fed during the first 2 days with Patavie Porc (Oriane-Celtilait), a milk substitutive. Afterwards, animals received Neopigg (Provimi Cargill) mixed with the milk substitutive for one week, and dry Neopigg for the rest of the study.

In order to mimic the natural route of colonization, inoculation of bacterial commensals was carried out intranasally and orally with a nasal atomizer (MAD Nasal™, Teleflex, Athlone, Ireland). The inoculation of the commensals was performed twice, at day 1 and 6 of life, with 1 mL intranasally (0.5 mL per nostril) and 1 mL orally per piglet of the concentration of bacteria described in Table 5.1.

Table 5.2 | Summary of actions performed during the experiment.

Inoculum group	Inoculation of commensals	Colistin treatment	P1/7 Challenge	Clinical signs and RT[2]	Necropsy	Number of animals
R1+M+Ss	Day 1 and 6 of life	Day 3 to 5 of life	Day 31 of life	Daily -1 to 9 DPI	10 DPI	6
R1+R2+M+Ss						6
R1+R2+M						6
Control	NA[1]					5

[1] NA: No applicable, control group was inoculated only with PBS. [2] RT: Rectal temperature.

Piglets were treated orally with colistin (Colimicina SP, SP Veterinaria S.A., Spain) from day 3 to 5 to stop a diarrheal episode produced by *E. coli*. From the 44 piglets that were included in the study, 25 remained alive at day 10 of life due to this episode. The surviving piglets had the following distribution: 6 piglets in the groups inoculated with commensal mixtures (R1+M+Ss, R1+R2+M+Ss, and R1+R2+M groups), 5 piglets in the control group, and 2 piglets in the group inoculated only with *S. suis*. Due to the low number of piglets in the Ss group, this group was not taken into account in the analysis of the results.

At day 31 of life, all piglets were challenged intranasally with 1 mL (10^{10} CFU/mL) of the virulent P1/7 *S. suis* serotype 2 strain. The inoculum was split between the two nostrils using the MAD nasal atomizer. Rectal temperature and clinical signs were recorded from 1 days before challenge until the day before the end of the study, set at day 41 of life (10 days post infection [dpi]).

Clinical signs were scored and classified as follow: 0 if no sign was present, 1 if the clinical sign was mild, 2 moderate, and 3 severe. Piglets were euthanized for animal welfare if a score 3, or two consecutive days with score 2, were observed.

Piglets were examined at necropsy and lesion scores, from 0 to 3 (as for clinical signs), were established for each lesion. A global pathological score was calculated as a mean of the scores of the individual lesions observed in pericardial, thoracic and abdominal cavity, brain and joints (joint score was the mean of the scores observed in the carpal and tarsal joints). Swabs for bacterial culture were collected from different locations: pericardium, thoracic, and abdominal cavities, carpal and tarsal joints, bronchoalveolar lavage fluid (BALF) and cerebrospinal fluid. Nasal swabs were also collected at necropsy and stored in 500 µL of PBS.

Microscopic lesions were also evaluated in tissues from the respiratory tract (nasal turbinate, cribriform plate of ethmoid, trachea, and lung) and brain. These tissues were fixed by immersion in 10% buffered formalin, embedded in paraffin and stained with hematoxylin–eosin.

P1/7 isolation and *S. suis* serotype 2 detection

To assess if the disease observed was due to the challenge, swabs collected at necropsy were plated on chocolate agar (Biomerieux, Spain) and were incubated overnight at 37ºC with 5% CO_2. Cultures were scored depending on the number of colonies presented (in the case of joints, final score was the mean of the four joints sampled): 1 from 1 to 20 colonies, 2 from 21 to 200 colonies, and 3 with more of 200 colonies. Colonies compatible with *S. suis* were collected, resuspended in 500 µL of PBS, and DNA was extracted using a Chelex based Instagene Matrix (Bio-Rad Laboratories, Hercules, CA, USA) following the manufacturer's instructions. An Enterobacterial Repetitive Intergenic Consensus (ERIC)-PCR, a fingerprinting method, was performed with 10 µL of DNA from these isolates recovered at 10 ng/µL. The same amount of DNA from P1/7 strain was used as control, as previously described by Versalovic and collaborators (1991).

To assess the P1/7 colonization, DNA from nasal swabs collected at necropsy and saved in PBS, was extracted using the Nucleospin Blood

kit (Macherey–Nagel, Germany). In this case, 4 µL of DNA with a concentration from 22.2 to 42.5 ng/µL was used to detected serotype 2, the serotype of the challenge strain, following the steps described by Okura and collaborators (2014).

Statistical analysis

Survival log rank test was performed to study mortality differences between groups after the challenge. Rectal temperature, clinical signs observed after challenge, lesions at necropsy, and P1/7 recovered were studied by one-way analysis of variance (ANOVA). All statistical analyses were conducted with R (v. 4.0.2, (R Core Team, 2020)).

RESULTS

Clinical signs and survival after challenge

The most common clinical signs after challenge were mild apathy, which was detected in 17 out of 23 animals, and in some of them evolved to moderate apathy, specifically in two animals from groups R1+M+Ss and R1+R2+M+Ss. Fever (> 40.0ºC) was detected at least one day in 16 out of 23 piglets, but no differences were observed among the different groups after challenge (Figure 5.1). The distribution of the clinical signs registered by group is shown in Table 5.3.

Table 5.3 | Distribution of the different clinical signs registered after challenge.

Clinical sign	R1+M+Ss	R1+R2+M+Ss	R1+R2+M	Control
Apathy	6/6	5/6	3/6	3/5
Fever	6/6	4/6	3/6	3/5
Dyspnea	3/6	5/6	3/6	3/5
Semi-prostration	2/6	3/6	2/6	2/5
Tremors	3/6	2/6	2/6	2/5
No clinical signs	0/6	1/6	3/6	1/5

Clinical signs after *S. suis* infection were observed in lower frequency in the R1+R2+M group (3 out of 6, Table 5.3), although lesion severity was not different between groups (P = 0. 241, Figure 5.2).

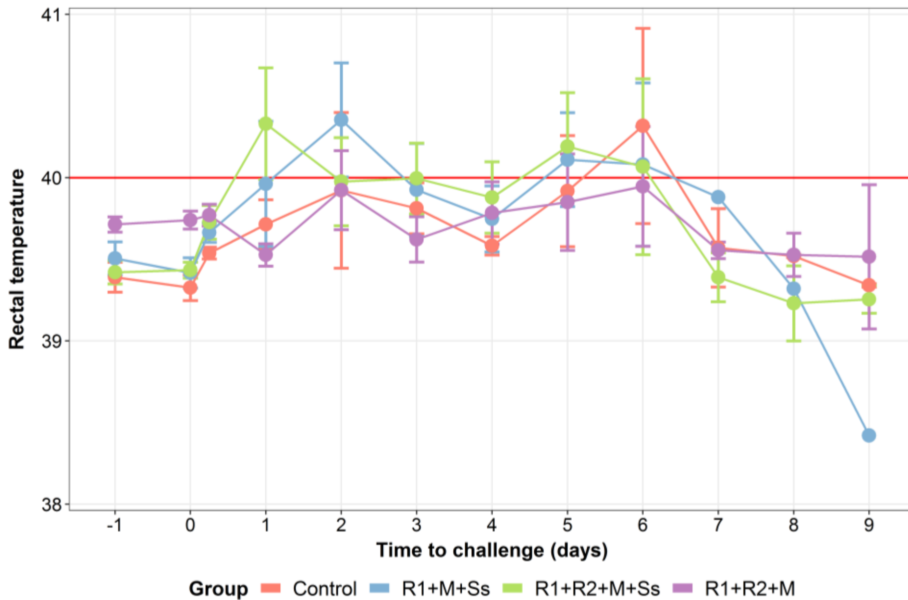

Figure 5.1 | Mean temperatures and standard deviation for the different groups after challenge with *S. suis* P1/7. Animals were inoculated oral and nasally with commensal candidates *Rothia* R1 and R2, *Moraxella* M, and *Streptococcus suis* Ss in combinations as indicated in the legend below the graph. Inoculation of commensals was performed at day 1 and 6 of life, and one group was inoculated with PBS as control. Piglets were challenged at day 31 of life with *S. suis* virulent reference strain P1/7. Temperature was recorded from 1 day before the challenge till the day before the end of experiment, which was set at 10 days post-infection.

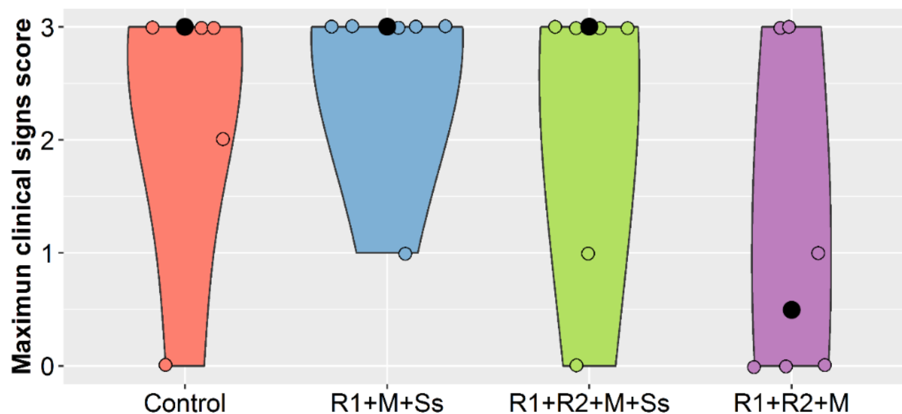

Figure 5.2 | Individual maximum clinical sign score registered after challenge with *S. suis* P1/7. Animals were inoculated oral and nasally with commensal candidates *Rothia* R1 and R2, *Moraxella* M, and *Streptococcus suis* Ss in combinations as indicated in the abscissa axis. Inoculation of commensals was performed at day 1 and 6 of life, and one group was inoculated with PBS as control. Piglets were challenged at day 31 of life with *S. suis* virulent reference strain P1/7. The end of experiment was set at 10 days post-infection. Black dots represent the median for each group.

In total, 14 piglets died or were euthanatized due to the severity of the clinical signs between 3 to 6 dpi (Table 5.4). No significant differences in survival were found among the 4 groups (*P* = 0.319, Figure 5.3), but twice as many animals survived in R1+R2+M compared to R1+R2+M+Ss and control group, and three more animals when compared with R1+M+Ss (Table 5.4). The two animals from the Ss group that survived the diarrheal episode remained alive after the *S. suis* P1/7 challenge until the end of the experiment.

Table 5.4 | Chronology of deaths due to the challenge and animals that survived. dpi: days post-infection.

Groups	Piglets per group at challenge	Mortality			Survivors at 10 dpi (%)
		Total	N	dpi	
R1+M+Ss	6	5	2	3	1 (16.7%)
			1	4	
			1	5	
			1	6	
R1+R2+M+Ss	6	4	2	4	2 (33.3%)
			1	5	
			1	6	
R1+R2+M	6	2	1	3	4 (66.7%)
			1	6	
Control	5	3	1	3	2 (40.0%)
			1	5	
			1	6	
Ss	2	2	0	0	2 (100%)

Necropsy findings

At necropsy, the lesions found were those described for *S. suis*, such as pericarditis, pleurisy, peritonitis, meningitis, and arthritis. Noteworthy the lesions found in the necropsies performed at 10 dpi were only mild (6 piglets with peritonitis, 1 with pericarditis, and 1 with peritonitis and arthritis), suggesting that the animals that survived the challenge were clearing the infection. Only three piglets, belonging to R1+M+Ss, R1+R2+M+Ss, and control group did not show any lesion at 10 dpi. The group with the lower score, based on the number and the severity of the lesions, was the R1+R2+M group, followed by the R1+M+Ss group, control group, and R1+R2+M+Ss group (Figure 5.4, Table 5.5), however, differences were not found between groups (*P* = 0.853).

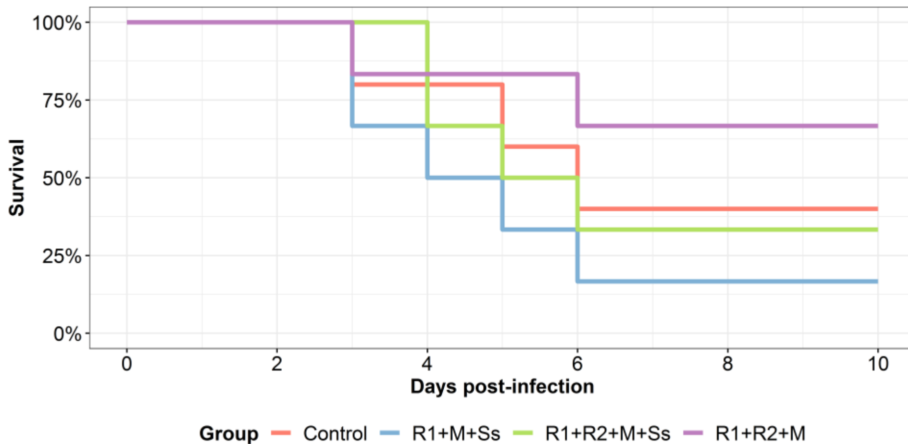

Figure 5.3 | Piglet survival after challenge with *S. suis* P1/7. Animals were inoculated oral and nasally with commensal candidates *Rothia* R1 and R2, *Moraxella* M, and *Streptococcus suis* Ss in combinations as indicated in the legend below the graph. Inoculation of commensals was performed at day 1 and 6 of life, and one group was inoculated with PBS as control. Piglets were challenged at day 31 of life with *S. suis* virulent reference strain P1/7. The end of experiment was set at 10 days post-infection.

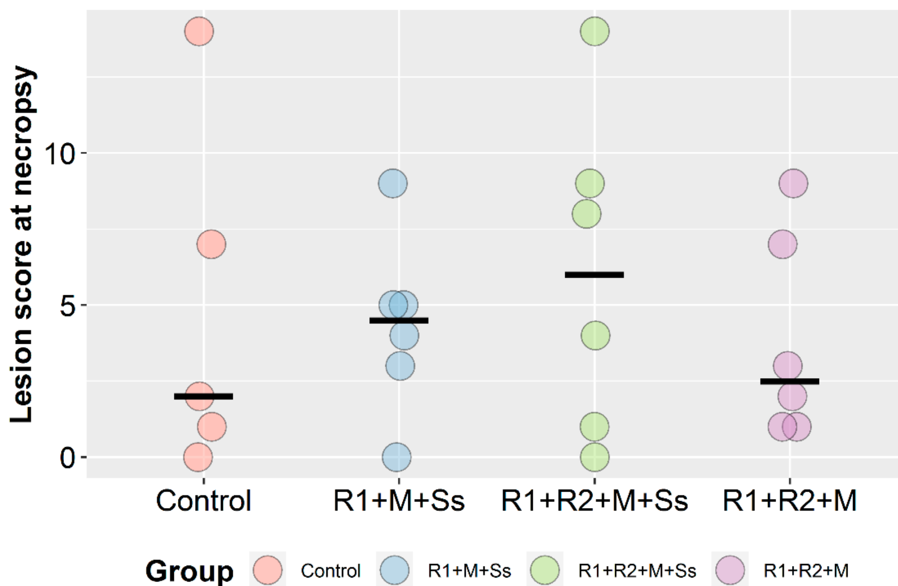

Figure 5.4 | Global lesion score of individual pigs after challenge with *S. suis* P1/7. Animals were inoculated oral and nasally with commensal bacteria *Rothia* R1 and R2, *Moraxella* M, and *Streptococcus suis* Ss in combinations as indicated in the legend below the graph. Inoculation of commensals was performed at day 1 and 6 of life, and one group was inoculated with PBS as control. Piglets were challenged at day 31 of life with *S. suis* virulent reference strain P1/7. The end of experiment was set at 10 days post-infection. Black lines represent the median for each group.

Table 5.5 | Number of piglets with macroscopic lesions and mean global scores recorded at necropsy for the different groups. Animals were inoculated oral and nasally with commensal bacteria *Rothia* R1 and R2, *Moraxella* M, and *Streptococcus suis* Ss in combinations as indicated in the table.

Lesion	R1+M+Ss	R1+R2+M+Ss	R1+R2+M	Control
Pericarditis	2/6	2/6	0/6	2/5
Pleurisy	4/6	4/6	2/6	0/5
Peritonitis	5/6	5/6	6/6	3/5
Arthritis	1/6	2/6	3/6	2/5
Meningitis*	0/6	0/6	0/6	3/5
Mean global lesion score	3.8	4.8	2.7	4.2

*Meningitis lesions were observed microscopically.

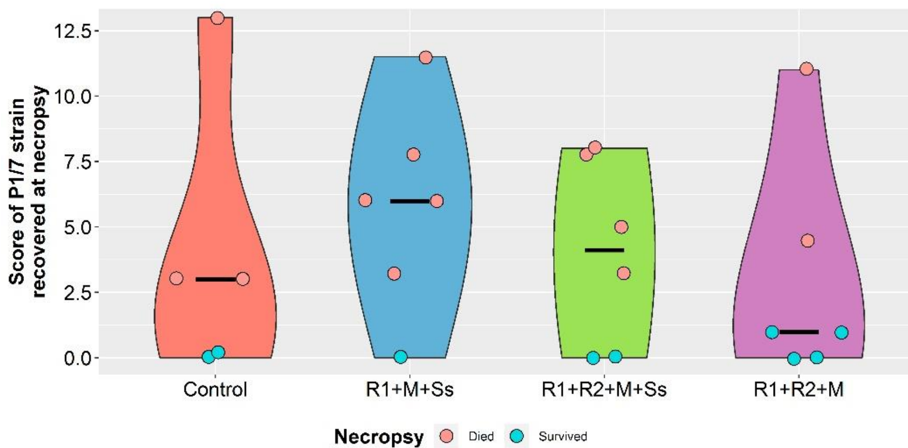

Figure 5.5 | Score from *S. suis* P1/7 strain recovered at necropsy. Animals were inoculated oral and nasally with commensal candidates *Rothia* R1 and R2, *Moraxella* M, and *Streptococcus suis* Ss in combinations as indicated in the legend below the graph. Inoculation of commensals was performed at day 1 and 6 of life, and one group was inoculated with PBS as control. Piglets were challenged at day 31 of life with *S. suis* virulent reference strain P1/7. The end of experiment was set at 10 days post-infection. Black lines represent the median for each group.

S. suis colonization and infection

Colonization was confirmed in all piglets by the detection of *S. suis* serotype 2 in nasal swabs at necropsy. The challenge strain, confirmed by ERIC-PCR, was recovered from at least one tissue of all piglets that died due to the challenge and only from 3 of the animals euthanized at 10 dpi: from the joint of one animal of the control group (2 colonies) and from the thoracic cavities (1 colony), and BALF (2 colonies) of two

animals of the R1+R2+M group. Bacterial scores from the different groups are represented in Figure 5.5; the differences observed were not significant (P = 0.714). This lack of isolation of *S. suis* or isolation of very limited number of bacteria at necropsies performed at 10 dpi, supports the previously proposed hypothesis that surviving animals were clearing the infection at this time point.

DISCUSSION

The effect of the microbiota on health has been widely studied, both in humans and swine (Man et al., 2017; Niederwerder, 2017). In pigs, different nasal microbiota composition has been described in animals that developed bacterial infectious diseases, such as *M. hyorhinis* or *G. parasuis* (Correa-Fiz et al., 2016; Blanco-Fuertes et al., 2021). For *S. suis*, only the microbiota in tonsils has been studied, showing different composition in piglets with *S. suis* disease and healthy piglets (Niazy et al., 2022). A common point in all these studies is that healthy animals had a greater microbiota diversity, which is correlated with a decreased risk of developing respiratory disease in swine (Pirolo et al., 2021).

In the present study we tested the protection capacity of selected commensals against a challenge with virulent *S. suis* inoculated intranasally. Clinical signs after challenge, such as fever, dyspnea, or semi-prostration, were compatible with *S. suis* infection (Sanford and Tilker, 1982; Clifton-Hadley et al., 1986; Gottschalk and Segura, 2019). Moreover, the macroscopic lesions at necropsy were also compatible with this infection (Reams et al., 1994; Staats et al., 1997; Gottschalk and Segura, 2019), and it was confirmed by isolation of the *S. suis* challenge strain from the lesions. The differences found between groups were not statistically significant, probably due to the low number of piglets in each group or other factors related to the infection model used. In the case of the group inoculated with non-virulent *S. suis*, we cannot ensure that the survival observed was due to this inoculation, since only two animals were alive when challenged with the virulent strain. Nevertheless, this possible protective effect was not observed when the

non-virulent *S. suis* strain was administered together with *Rothia* and *Moraxella*.

The selected commensals were inoculated in piglets that had contact, although limited, with their mothers. These putative protective commensals are species that are naturally found in the upper respiratory microbiota (Pena Cortes et al., 2018), and we cannot rule out the presence of the commensal species in non-inoculated groups. In fact, we know that all the piglets were colonized by *S. suis* before the challenge (not shown). The detection of the specific strains used for colonization could be planned for future studies by the development of specific PCRs or by shot gun sequencing.

The study of the immune response to the commensal inoculation and to the *S. suis* challenge in the different groups would be interesting to complement our results and shed some light on the putative mechanisms of protection. In this aspect, the Interleukin-1 (IL-1) cytokine family has been reported to be induced by *S. suis* infection in different cell types using a mouse model (Auger et al., 2017; Lavagna et al., 2019), and it was further confirmed in the swine model (Neila-Ibáñez et al., 2021b), which makes it an interesting group of cytokines to be studied and to examine the effect of the commensal candidates.

Despite the possible suitability of the potential commensals of the group R1+R2+M (*Rothia* and *Moraxella*), more studies have to be carried out to examine the effect of different factors that affect the development of the microbiota in piglets. This model presents the limitation that an appropriate microbiota cannot be developed as in a normal situation on the farm, since to reduce the interference of the natural colonization from their mothers, piglets were separated from the sows shortly after delivery and antimicrobial treatments were performed, both to the piglets and the sows (Correa-Fiz et al., 2019; Obregon-Gutierrez et al., 2021). In consequence, the immune maturation of the piglets may be affected and have a reflection in the results (Niederwerder, 2017).

In conclusion, this study sheds some light on the possibility of using selected candidates as probiotics in order to establish a beneficial respiratory microbiota in early stages for prevention of the infection caused by *S. suis* in pigs. However, the dual inoculation with the selected low-to medium prevalent strains was not sufficient to protect the piglets against a virulent *S. suis* challenge. In particular, it is important to point out that the use of a *S. suis* non-virulent strains did not offer any improvement and its use may be difficult to regulate due to the zoonotic nature of the bacteria.

GENERAL DISCUSSION

Currently, tight production margins in the pig production sector have entailed a modernization of the production, producing more in less time, and with higher quality. However, these modifications have consequences on animal health, increasing the probability of disease outbreaks (Saladrigas-García et al., 2021).

Streptococcus suis causes severe disease in young pigs both in traditional and modern intensive swine farms worldwide (Gottschalk and Segura, 2019). It can produce systemic disease in piglets, resulting in meningitis, polyarthritis, polyserositis, valvular endocarditis, septicemia, and acute death. The disease affects mostly post-weaning piglets, but suckers and fatteners can also be affected (Gottschalk and Segura, 2019).

Besides the impact on pigs, *S. suis* is a pathogen of concern for the public health authorities due to its zoonotic potential. This latter aspect is especially important in areas where proper hygienic-sanitary measures are not followed and there is a human consumption of raw pork products, as it is the case in some Southeast Asian countries (Rao et al., 2021). However, despite being considered endemic, the real impact of *S. suis*-associated disease in pig farms worldwide is unknown, mainly because reporting is not mandatory.

Due to its colonizing role, *S. suis* infection is highly prevalent. In contrast, the prevalence of the disease is not high and depends on the presence of several factors such as the age of the animal and the virulence of the strains. While both virulent and non-virulent strains are part of the upper respiratory tract microbiota, and participate in the maintenance of this complex bacterial network, under certain unknown circumstances virulent strains are able to evade the host defenses and develop infection (Gottschalk and Segura, 2019). Thus, the host may show a differential immune response to the colonization by a virulent or a non-virulent *S. suis* strain, as it has been observed in cesarean-derived colostrum-deprived piglets, which had a more sustained inflammatory response in the nasal cavity to the virulent strain.

Within animal diseases, it is critical the economic assessment for decision-making, including the allocation of resources for their control. Therefore, the establishment of a baseline level for *S. suis* disease prevalence is essential to allow the detection of changes over time. That way, when different control and prevention strategies are applied, their real impact on the prevalence of the disease can be estimated. For the swine production countries studied, clinical veterinarians reported different strategies to fight against *S. suis*, highlighting especially the difference between the Netherlands and Spain. Whilst Dutch veterinarians reported frequent use of autogenous vaccines, Spanish veterinarians used more antimicrobials. This fact coincides with the historical interest shown in the Netherlands with an early restriction in the use of antimicrobials in livestock compared to Spain (EMA, 2021). Despite Germany, Spain, and the Netherlands having the same regulatory prospect for the reduction of antimicrobial treatments, it is a reality that producers in those countries are using different strategies to control *S. suis* disease, although it is foreseeable that these measures will tend to converge in the future. These different prevention strategies also influenced the cost associated with *S. suis*-disease, with the highest costs in Germany, followed by the Netherlands and lastly Spain.

The use of autogenous vaccines represented a significant cost for pig producers if they are applied. However, they have some limitations because of the genetic heterogeneity of *S. suis* isolates, including the presence of clinical and non-clinical isolates, even within the same herd (Vela et al., 2003). Although hundreds of putative virulence factors have been pointed out for strains causing disease, none of them is decisive for the pathogenicity of *S. suis* (Segura et al., 2017; Tram et al., 2021). Therefore, studies that focus on the identification and characterization of true and essential virulence factors will help in the development of novel vaccines to prevent infection and will increase the understanding of *S. suis* pathogenesis.

Eliminating *S. suis* from a herd may be a very difficult task, and, if it is achieved, keeping a *S. suis*-free herd is not feasible (Gottschalk

and Segura, 2019). Therefore, considering the progressive reduction on the use of antimicrobials and the current absence of an effective vaccine, complementary prevention approaches, as the use of probiotics, are required. Orally administered probiotics have been used for the control of different pathogens such as enterotoxigenic *Escherichia coli* in post-weaning diarrhea in pigs or *Mannheimia haemolytica* in bovine respiratory disease (Kayser et al., 2019; Xin et al., 2020). In general, the effect of probiotics seems to be modest and strain-dependent (Cameron and McAllister, 2019). However, there are no studies about the performance of probiotics specifically administered intranasally in pigs. The results presented in this thesis, using nasal and oral administration of probiotics along with a subsequent intranasal challenge, represents the first step for the use of colonizers of the upper respiratory tract to prevent the development of diseases caused by pathogenic bacteria naturally present in the respiratory microbiota. Influence of these selected probiotics on the local immune response, especially in the Interleukin-1 cytokine family whose expression is increased in the presence of *S. suis*, and their performance in modifying the early microbiota and its posterior stabilization deserve further study.

In the present thesis, the effect of different variables traditionally associated with *S. suis* disease were studied for the first time. The reduction of the predisposing factors observed in this study may help to avert the development of *S. suis*-associated disease. The effect of some of these factors, such as PRRSV coinfection after weaning, can be mitigated with vaccination and the improvement of biosecurity, to prevent the entry of new viral strains into the farms. Also a correct control of the environmental parameters, and reducing the stress of the animals, e.g. including environmental enrichment materials could help reduce the onset of the disease. In addition, another measure could be increased surveillance of piglets born to sows of lower parity, especially from gilts, in order to begin an early antimicrobial therapeutic treatment as soon as clinical signs compatible with the disease are observed, as well as a possible vaccination against the pathogen in younger sows. All

these actions can reduce the spread of the disease with the consequent reduction of its cost and increase in animal welfare.

However, it would be also important to perform future studies using more farms and more countries to confirm the results observed. That will also allow to assess some factors which could not be evaluated in our study, as other porcine immunosuppressant virus, e.g. porcine circovirus type 2 or swine influenza virus, to better understand the effect of these coinfection in the field.

CONCLUSIONS

1. *S. suis*-disease is highly prevalent in farms of the studied areas of Spain, the Netherlands, and Germany. Weaners pigs are the age group most severely affected with up to 4% of animals with clinical disease and 0.9% mortality.

2. *S. suis* represents an important burden for pig production in Spain, the Netherlands, and Germany, as the mean estimated cost per animal ranged between 0.60 and 1.30 €. The cost was essentially influenced by the control measures, which in Spain was mainly based on the use of antimicrobials, in the Netherlands on the use of autovaccines, and in Germany on a combination of both.

3. Factors including weaning at higher age, higher stress at farrowing, higher mean relative humidity at farrowing, presence of PRRSV at weaning, or being born to younger sows, were associated with a higher risk of *S. suis*-disease. Therefore, strategies to control those factors will likely reduce the burden caused by *S. suis*.

4. The innate immune response against *S. suis* in cesarean-derived colostrum-deprived piglets depends on the virulence of the strain, reflecting the ability of the host to control and maintain a low inflammation after a challenge with a non-virulent strain, probably mediated by a sustained immune response at the associated lymph node.

5. In nasal mucosa, the inflammatory response observed after the inoculation of a *S. suis* virulent strain in cesarean-derived colostrum-deprived piglets was faster and stronger than for the non-virulent strain. The associated lymph node response did not seem to be able to avoid invasion after colonization.

6. The inoculation of two *Rothia* and one *Moraxella* in newborn piglets provided partial protection against a lethal *S. suis* challenge, as indicated by the higher survival and milder clinical signs and lesions against a lethal *S. suis* challenge.

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SUPPLEMENTARY MATERIAL

STUDY I

Supplementary Table S2.1 | Values used for the parameters in the study in the different countries.

Parameter	Abbreviation	German values	Dutch values	Spanish values	Unit	Source of data
Proportion of weight loss in suckling piglets	w_p	≈0	≈0	≈0	-	Questionnaire / E.o.
Average value of a suckling piglet *	v_p	13.6	9.5	11.7	Euros	EU (2020a) / E.o.
Proportion of weight loss in nursery pigs	w_n	≈0	≈0	≈0	-	Questionnaire / E.o.
Average value of a nursery pig ⁺	v_n	45.6	28.1	27.0	Euros	EU (2020a) / E.o.
Proportion of weight loss in fatteners	w_f	≈0	≈0	≈0	-	Questionnaire / E.o.
Average value of a fattener ±	v_f	79.1	46.3	45.1	Euros	EU (2020a) / E.o.
Average number of sows in a year in farrowing units	S	2689	2886	4768	Sows	Questionnaire
Average number of piglets weaned per sow per year	y_p	30.2	30.6	27.5	Piglets/year	Hoste (2020)
Average total mortality during nursery	m_n	2.9%	2.8%	4.6%	-	Hoste (2020)
Average number of cycles per year in nursery	c_n	6.3	6.8	6.8	Cycles/year	E.o.
Average number of fatteners produced in a year in fattening units	N_f	19225	15957	19704	Fatteners	Questionnaire
Average cost of autogenous vaccines per animal	k	0.9	0.9	0.9	Euros	E.o.
Proportion of clinical cases confirmed by the laboratory in suckling piglets	p_p	75.0%	81.0%	86.0%	-	Questionnaire
Proportion of clinical cases confirmed by the laboratory in nursery pigs	p_n	77.2%	91.0%	76.0%	-	Questionnaire
Proportion of clinical cases confirmed by the laboratory in fattening	p_f	45.8%	80.6%	50.0%	-	Questionnaire
Average cost of laboratory analysis per farm	s	64.0	79.2	33.2	Euros	E.o.
Average animal weight in suckling piglets *	-	5.5	5.7	5.4	Kg	E.o.
Average animal weight in nursery pigs ⁺	-	18.4	16.9	12.5	Kg	E.o.
Average animal weight in fattening ±	-	31.9	27.9	20.9	Kg	E.o.
Average animal price in 2019	-	2.48	1.66	2.16	€/kg	EU (2020a)

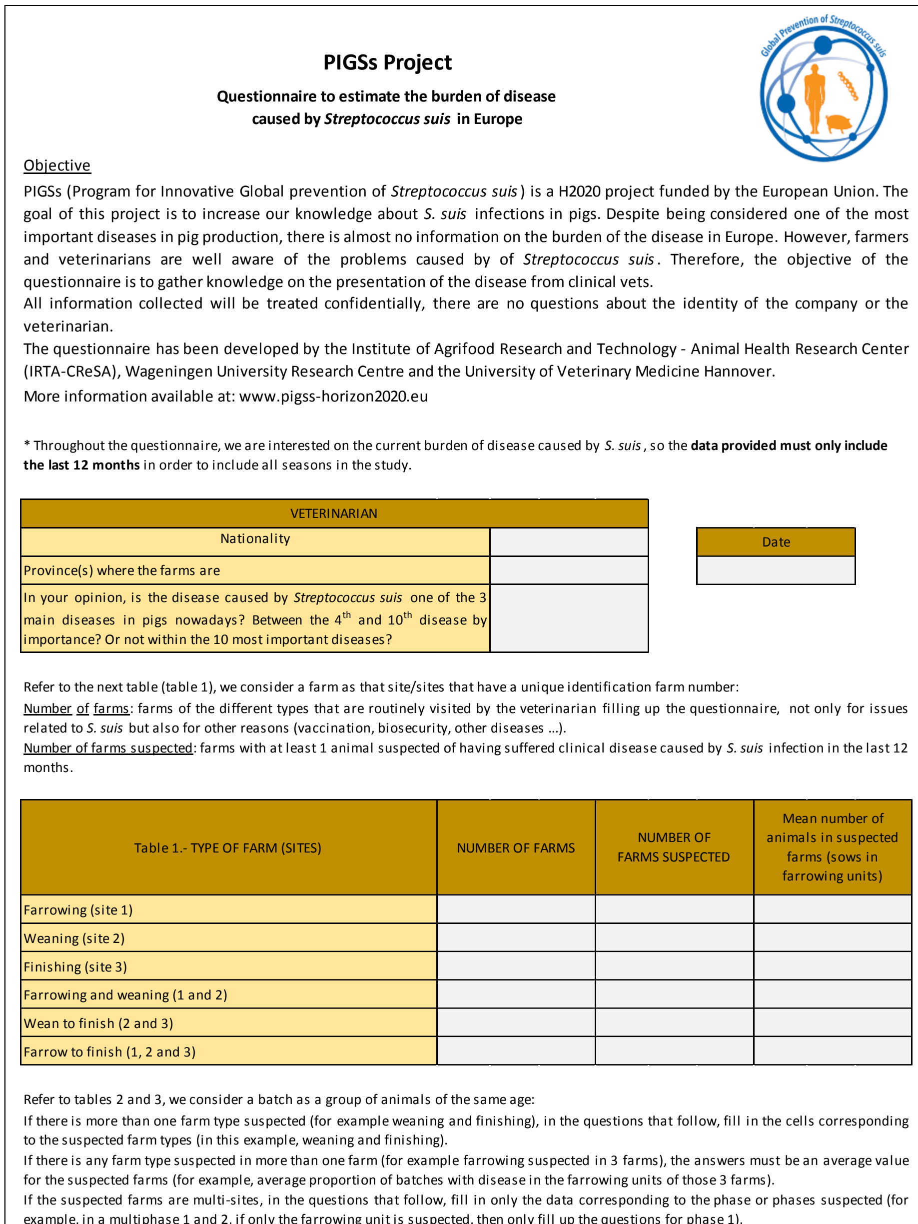
Pigs produced in 2019	-	55.1	16.6	53.0	Millions of heads	EU (2020a)
Amoxicillin – Parenteral	-	1.6	1.4	0.5	Cent €/ kg of live weight	E.o.
Amoxicillin – Premix	-	0.2	0.3	0.2	Cent €/ kg of live weight	E.o.
Amoxicillin – Oral powder	-	0.3	0.3	0.2	Cent €/ kg of live weight	E.o.
Ceftiofur – Parenteral	-	1.9	-	0.9	Cent €/ kg of live weight	E.o.
Penicillin – Parenteral	-	1.1	0.7	0.5	Cent €/ kg of live weight	E.o.
Trimethoprim sulfamethoxazole – Parenteral	-	0.7	0.6	-	Cent €/ kg of live weight	E.o.
Trimethoprim sulfamethoxazole – Premix	-	0.2	0.2	-	Cent €/ kg of live weight	E.o.
Trimethoprim sulfamethoxazole – Oral powder	-	0.3	0.2	-	Cent €/ kg of live weight	E.o.

*Values were calculated 3 days before movement to a nursery unit. + Values were calculated in the middle of the nursery period. ± Values were calculated 5 days after movement to fattening unit. E.o.: Expert opinion.

Supplementary Table S2.2 | Mean total annual cost per affected production unit in the countries of study (in thousands of euros). In brackets, 90% confidence interval (CI) of the corresponding cost.

Country	Mean total cost (90% CI) in thousands of euros
Germany	8.7 (3.0-16.7)
The Netherlands	10.2 (0.5-22.9)
Spain	2.7 (0.1-8.3)
Germany	9.9 (1.8-25.2)
The Netherlands	11.2 (2.4-23.2)
Spain	14.1 (4.0-27.1)
Germany	1.2 (0.1-3.4)
The Netherlands	0.8 (0.0-2.4)
Spain	0.4 (0.0-1.4)

Supplementary Data S2.1 | Questionnaire to estimate the burden of disease caused by *Streptococcus suis* in Europe.



PIGSs Project

Questionnaire to estimate the burden of disease caused by *Streptococcus suis* in Europe

Global Prevention of Streptococcus suis

Objective

PIGSs (Program for Innovative Global prevention of *Streptococcus suis*) is a H2020 project funded by the European Union. The goal of this project is to increase our knowledge about *S. suis* infections in pigs. Despite being considered one of the most important diseases in pig production, there is almost no information on the burden of the disease in Europe. However, farmers and veterinarians are well aware of the problems caused by of *Streptococcus suis*. Therefore, the objective of the questionnaire is to gather knowledge on the presentation of the disease from clinical vets.
All information collected will be treated confidentially, there are no questions about the identity of the company or the veterinarian.
The questionnaire has been developed by the Institute of Agrifood Research and Technology - Animal Health Research Center (IRTA-CReSA), Wageningen University Research Centre and the University of Veterinary Medicine Hannover.
More information available at: www.pigss-horizon2020.eu

* Throughout the questionnaire, we are interested on the current burden of disease caused by *S. suis*, so the **data provided must only include the last 12 months** in order to include all seasons in the study.

VETERINARIAN	
Nationality	
Province(s) where the farms are	
In your opinion, is the disease caused by *Streptococcus suis* one of the 3 main diseases in pigs nowadays? Between the 4th and 10th disease by importance? Or not within the 10 most important diseases?	

Date

Refer to the next table (table 1), we consider a farm as that site/sites that have a unique identification farm number:
Number of farms: farms of the different types that are routinely visited by the veterinarian filling up the questionnaire, not only for issues related to *S. suis* but also for other reasons (vaccination, biosecurity, other diseases ...).
Number of farms suspected: farms with at least 1 animal suspected of having suffered clinical disease caused by *S. suis* infection in the last 12 months.

Table 1.- TYPE OF FARM (SITES)	NUMBER OF FARMS	NUMBER OF FARMS SUSPECTED	Mean number of animals in suspected farms (sows in farrowing units)
Farrowing (site 1)			
Weaning (site 2)			
Finishing (site 3)			
Farrowing and weaning (1 and 2)			
Wean to finish (2 and 3)			
Farrow to finish (1, 2 and 3)			

Refer to tables 2 and 3, we consider a batch as a group of animals of the same age:
If there is more than one farm type suspected (for example weaning and finishing), in the questions that follow, fill in the cells corresponding to the suspected farm types (in this example, weaning and finishing).
If there is any farm type suspected in more than one farm (for example farrowing suspected in 3 farms), the answers must be an average value for the suspected farms (for example, average proportion of batches with disease in the farrowing units of those 3 farms).
If the suspected farms are multi-sites, in the questions that follow, fill in only the data corresponding to the phase or phases suspected (for example, in a multiphase 1 and 2, if only the farrowing unit is suspected, then only fill up the questions for phase 1).

Table 2.-In farms suspected (with at least one piglet with *S. suis* problems)	IN FARROWING (1)	IN WEANING (2)	IN FINISHING (3)
Proportion of **batches with disease** associated to *S. suis* (in suspected farms)			
Proportion of **animals with disease** associated to *S. suis* (in suspected batches)			
Proportion of mortality, **animals dead** due to *S. suis* (over total population in suspected batches)			
Have suspected batches reduced weight gain, were wasted or had lack of uniformity? If **yes**, indicate proportion of those who did.			

Refer to table 3, the total percentage of batches suspected of *S. suis* clinical disease by season has to be 100 % (for example, for phase 1, 40% of the suspected batches occurred in spring, 10% in summer, 30% in autumn and 20% in winter).

Table 3.- Seasonality of the batches suspected of *S. suis* clinical disease (% by season)	IN FARROWING (1)	IN WEANING (2)	IN FINISHING (3)
Spring			
Summer			
Autumn			
Winter			

Refer to table 4, choose (for each phase) the three most important causes: assign the number 1 for the most important cause, 2 for the second and 3 for the third.

Table 4.- According to your experience, what are the main risk factors associated with the disease in your farms?	IN FARROWING (1)	IN WEANING (2)	IN FINISHING (3)
Animal density			
Other concomitant diseases (PRRS, circovirus, influenza...)			
Temperature fluctuation			
Poor ventilation			
Recent movement to post-weaning / fattening units			
Failure in the antimicrobial treatment			
Others (indicate)			

Refer to table 5, the total percentage of clinical signs has to be 100% for each phase that presents the disease.

Table 5.- Among the animals with disease, proportion with the following clinical signs:	IN FARROWING (1)	IN WEANING (2)	IN FINISHING (3)
Nervous signs			
Arthritis (lameness)			
Acute death			
Other (indicate which)			

Table 6.- Related with laboratory diagnosis, **in the last year**:	IN FARROWING (1)	IN WEANING (2)	IN FINISHING (3)
Proportion of **farms** with suspected *S. suis* disease from which **samples** are sent to the laboratory for confirmation			
Proportion of those **farms** that sent samples to the laboratory in which ***S. suis*** has been **isolated**			

Refer to the next tables (tables 7a, 7b, 7c and 7d):

The route could be water, feed or parenteral. Fill up all the sites where the disease was present. Indicate if there is no treatment in the correspondig cell (for example, the full batch is not treated when a case of *S. suis* occurs in transition).

Table 7a.- **Routine** treatment (for **all** farms)	IN FARROWING (1)			IN WEANING (2)			IN FINISHING (3)		
	% farms	Route	Duration	% farms	Route	Duration	% farms	Route	Duration
Antibiotic 1 (Name:________________)	%			%			%		
Antibiotic 2 (Name:________________)	%			%			%		
Antibiotic 3 (Name:________________)	%			%			%		
Antibiotic 4 (Name:________________)	%			%			%		
Antibiotic 5 (Name:________________)	%			%			%		

Table 7b.- In case of disease, therapeutic treatment of the **animals affected**	IN FARROWING (1)			IN WEANING (2)			IN FINISHING (3)		
	% farms	Route	Duration	% farms	Route	Duration	% farms	Route	Duration
Antibiotic 1 (Name:________________)	%			%			%		
Antibiotic 2 (Name:________________)	%			%			%		
Antibiotic 3 (Name:________________)	%			%			%		
Antibiotic 4 (Name:________________)	%			%			%		
Antibiotic 5 (Name:________________)	%			%			%		

Table 7c.- In case of disease, therapeutic treatment of the **rest of the pen**	IN FARROWING (1)			IN WEANING (2)			IN FINISHING (3)		
	% farms	Route	Duration	% farms	Route	Duration	% farms	Route	Duration
Antibiotic 1 (Name:________________)	%			%			%		
Antibiotic 2 (Name:________________)	%			%			%		
Antibiotic 3 (Name:________________)	%			%			%		
Antibiotic 4 (Name:________________)	%			%			%		
Antibiotic 5 (Name:________________)	%			%			%		

Table 7d.- In case of disease, therapeutic treatment of the **rest of the batch**	IN FARROWING (1)			IN WEANING (2)			IN FINISHING (3)		
	% farms	Route	Duration	% farms	Route	Duration	% farms	Route	Duration
Amoxicilin	%			%			%		
Antibiotic 2 (Name:________________)	%			%			%		
Antibiotic 3 (Name:________________)	%			%			%		
Antibiotic 4 (Name:________________)	%			%			%		
Antibiotic 5 (Name:________________)	%			%			%		

Refer to table 8, answer with Yes or No in the different phases in which the disease is observed. If the answer is Yes **,** indicate the percentage of farms in which autovaccines is used.

Table 8.- Use of autovaccines in the last 12 months	IN FARROWING (1)	IN WEANING (2)	IN FINISHING (3)
Yes / **No** (if **Yes**, % of farms)	%	%	%
In what proportion of the farms where the autovaccine was applied do you consider it was successful in preventing clinical disease compatible with *S. suis* ?	%	%	%

Thank you very much for your collaboration.

Supplementary Data S2.2 | Calculation of weights

Within each country, we collected the data from a series of questionnaires/veterinarians, each of which provided information of a number of farms that contained a number of farrowing units with an average number of sows, a number of nursery units with an average number of nursery pigs, and a number of fattening units with an average number of fatteners. We wanted to account for the fact that those parameters from the veterinarians providing information on more units and animals should have more weight, but at the same time, we wanted to avoid the parameters being essentially determined just by a few veterinarians. Therefore, we restricted the weights of the veterinarians to up to 20% depending on the number of farms they provided information for. Those averaged weighted values were the inputs fed to the mathematical model.

First, the unadjusted weights of the questionnaires/veterinarians for suckling piglets were calculated as:

$$\boldsymbol{w_{p,i}(unadjusted)} = \frac{\mathbf{1}}{\mathbf{2}}\left(\frac{\boldsymbol{u_{p,i}}}{\sum \boldsymbol{u_{p,i}}} + \frac{\boldsymbol{n_{p,i}}}{\sum \boldsymbol{n_{p,i}}}\right)$$

Where $\boldsymbol{u_{p,i}}$ was the number of farrowing units for which questionnaire/veterinarian $\boldsymbol{i}$ had data, and $\boldsymbol{n_{p,i}}$ was the average number of sows in those farrowing units from questionnaire/veterinarian $\boldsymbol{i}$.

Then, those values were limited to between 1% and 20%:

If $\boldsymbol{w_{p,i} < 0.01}$ then $\boldsymbol{w_{p,i}(limited) = 0.01}$; if $\boldsymbol{w_{p,i} > 0.2}$ then $\boldsymbol{w_{p,i}(limited) = 0.2}$; otherwise $\boldsymbol{w_{p,i}(limited) = w_{p,i}}$.

Finally, values were readjusted so that the sum of final weights equals 1:

$$\boldsymbol{w_{p,i}} = \frac{\boldsymbol{w_{p,i}(limited)}}{\sum \boldsymbol{w_{p,i}(limited)}}$$

Supplementary Data S2.3 | Estimation of the number of animals produced by unit per year.

In a farrowing unit, the number of animals (suckling piglets) produced per year ($\boldsymbol{N_{p,i}}$) was estimated as:

$$\boldsymbol{N_{p,i} = S_{p,i} \times y}$$

Where $\boldsymbol{S_{p,i}}$ was the mean number of sows in farrowing units in questionnaire $\boldsymbol{i}$, and $\boldsymbol{y}$ was the average number of piglets weaned per sow per year. We assumed that the mortality associated to *S. suis* occurred at the end of farrowing.

As disease in nursery units may occur at any time, the mean number of nursery pigs produced per year according to questionnaire/veterinarian $\boldsymbol{i}$ ($\boldsymbol{N_{n,i}}$) was calculated as:

$$\boldsymbol{N_{n,i} = \frac{N_{p,i} + \left[N_{p,i} \times (1 - m_{tn})\right]}{2}}$$

Where $\boldsymbol{m_{tn}}$ was the average total mortality during nursery due to any cause.

In fattening units, since *S. suis* affects fatteners at the beginning of the fattening period, the mean number of fatteners produced per year ($\boldsymbol{N_f}$) was considered equal to the number of nursery pigs at the end of the nursery period.

In farms without a farrowing unit, the number of nursery pigs produced per year for questionnaire/veterinarian $\boldsymbol{i}$ ($\boldsymbol{N_{n,i}}$) was estimated:

$$\boldsymbol{N_{n,i} = n_{n,i} \times c_n}$$

Where $\boldsymbol{n_{n,i}}$ was the average number of nursery pigs in the nursery units in questionnaire $\boldsymbol{i}$, and $\boldsymbol{c_n}$ was the average number of cycles in nursery per year. And, in a farm without a farrowing unit, the number of fattening pigs produced per year ($\boldsymbol{N_{f,i}}$) was estimated as:

$$\boldsymbol{N_{f,i} = n_{f,i} \times c_f}$$

Where $\boldsymbol{n_f}$ was the average number of fatteners in the fattening units in questionnaire $\boldsymbol{i}$, and $\boldsymbol{c_f}$ was the average number of fattening cycles per year.

Supplementary Data S2.4. | Numerical example of the calculation of the average cost due to *S. suis* for each pig at the end of the production cycle produced in country X.

In country X, the parameters related to *S. suis*-associated disease were:

a) the average costs of *S. suis* per suckling piglet in affected farrowing units ($c_{p_{total}}$) was 0.3 €.

b) the average costs of *S. suis* per nursery pig in affected nursery units ($c_{n_{total}}$) was 0.8 €.

c) the average costs of *S. suis* per fattener in affected fattening units ($c_{f_{total}}$) was 0.2 €.

d) the proportion of farrowing units affected by *S. suis*-disease (f_p) was 0.5 (i.e., 50%).

e) the proportion of nursery units affected by *S. suis*-disease (f_n) was 0.6.

f) the proportion of fattening units affected by *S. suis*-disease (f_f) was 0.4.

Therefore, in country X, the average cost per suckling piglet ($a_{p_{total}}$), the average cost per nursery pig ($a_{n_{total}}$) and the average cost per fattener ($a_{f_{total}}$) can be calculated as:

$$\boldsymbol{a_{p_{total}} = c_{p_{total}} \times f_p = 0.3 \times 0.5 = 0.15} \text{ €}$$

$$\boldsymbol{a_{n_{total}} = c_{n_{total}} \times f_n = 0.8 \times 0.6 = 0.48} \text{ €}$$

$$\boldsymbol{a_{f_{total}} = c_{f_{total}} \times f_f = 0.2 \times 0.4 = 0.08} \text{ €}$$

Then, the average cost due to *S. suis* for each pig at the end of the production cycle produced in country X can be calculated as:

$$\boldsymbol{c_{Spain} = a_{p_{total}} + a_{n_{total}} + a_{f_{total}} = 0.15 + 0.48 + 0.08 = 0.71} \text{ €}$$

STUDY II

Supplementary Table S3.1 | Information of the sampled animals.

Mean and range piglet age and sex, as well as mean and range sow parity for the different batches sampled.

		Farm A			Farm B		
		Batch 1	**Batch 2**	**Batch 3**	**Batch 1**	**Batch 2**	**Batch 3**
Piglet	**Mean and range piglet age at farrowing**	22	21	21	19.5 (17 - 21)	20.1 (20 - 21)	19.2 (18 - 21)
	Mean and range piglet age at early weaning	34	36	34	33.5 (31 - 35)	34.1 (34 - 35)	33.2 (32 - 35)
	Mean and range piglet age at late weaning	49	54	47	47.5 (45 - 49)	48.1 (48 - 49)	47.2 (46 - 49)
	Piglets sex (F-M)	17 - 23	18 - 22	19 - 18	16 - 14	15 - 15	13 - 17
Sow	**Mean parity (range)**	4.0 (1 - 6)	3.9 (1 - 7)	5.9 (3 - 8)	3.1 (1 - 8)	5.4 (4 - 8)	1.6 (1 - 3)

Supplementary Table S3.2 | Values of the biomarkers analysed in serum.

Minimum, maximum, and mean of the cortisol, H_2O_2, and haptoglobin for the different batches sampled.

			Farrowing			Weaning		
			Cortisol (ug/dL)	**H_2O_2 (µmol/L)**	**Haptoglobin (mg/dL)**	**Cortisol (ug/dL)**	**H_2O_2 (µmol/L)**	**Haptoglobin (mg/dL)**
Min - Max	**Farm A**	**Batch 1**	0.080 - 3.240	343.0 - 1,103.0	21.54 - 322.91	0.641 - 8.750	236.0 - 957.0	8.00 - 275.04
Mean			0.679	772.3	194.88	2.841	598.8	90.77
Min - Max		**Batch 2**	0.240 - 4.990	186.0 - 1,029.0	8.0 - 296.6	0.532 - 23.300	210.0 - 957.0	8.00 - 300.94
Mean			1.447	555.4	167.20	3.734	639.4	144.44
Min - Max		**Batch 3**	0.332 - 5.150	174.0 - 934.0	90.99 - 292.56	0.939 - 50.000	180.0 - 1,323.0	8.0 - 312.1
Mean			1.596	578.5	183.50	3.548	744.6	132.0
Min - Max	**Farm B**	**Batch 1**	0.661 - 16.700	67.0 - 1,261.0	8.00 - 312.24	0.499 - 4.580	202.0 - 1,237.0	8.00 - 277.61
Mean			2.585	498.8	84.71	1.734	607.9	108.28
Min - Max		**Batch 2**	0.404 - 23.000	55.0 - 1,111.0	8.00 - 277.26	0.739 - 4.440	283.0 - 1,183.0	8.00 - 255.73
Mean			3.160	311.1	46.70	2.208	797.1	121.55
Min - Max		**Batch 3**	0.887 - 12.600	28.0 - 1,212.0	8.00 - 297.03	0.450 - 4.450	101.0 - 1,170.0	8.00 - 209.78
Mean			2.888	438.6	78.93	2.155	718.4	98.08

Supplementary Table S3.3 | Environmental parameters.

Range and mean of temperature (ºC), relative humidity (%), CO_2 (ppm), and temperature-humidity index (THI) at farrowing and weaning units for the different batches. CO_2 and temperature was only recorded for farm A. Values of relative humidity and THI could not be retrieved for farm A batch 1.

			Farrowing				Weaning			
			Temperature (ºC)	**Relative humidity (%)**	**CO_2 (ppm)**	**Temperature-humidity index**	**Temperature (ºC)**	**Relative humidity (%)**	**CO_2 (ppm)**	**Temperature-humidity index**
Range	**Farm A**	**Batch 1**	6.9	40.8	2,259	13.0	3.1	-	3,221	-
Mean			27.1	58.6	1,236	67.5	26.7	-	2,237	-
Range		**Batch 2**	8.2	42.0	1,951	14.3	4.6	33.0	2,969	7.4
Mean			28.4	37.9	1,852	63.3	28.4	34.3	3,666	62.2
Range		**Batch 3**	5.7	34.4	1,837	7.8	3.9	36.2	3,405	7.9
Mean			25.8	37.3	1,364	60.71	28.3	24.0	2,668	59.7
Range	**Farm B**	**Batch 1**	7.3	51.2	-	-	8.7	38.4	-	-
Mean			26.1	52.2	-	-	26.3	40.8	-	-
Range		**Batch 2**	5.8	51.8	-	-	6.7	28.0	-	-
Mean			26.2	47.8	-	-	26.5	49.3	-	-
Range		**Batch 3**	6.8	37.1	-	-	8.8	37.5	-	-
Mean			26.6	52.6	-	-	27.4	43.1	-	-

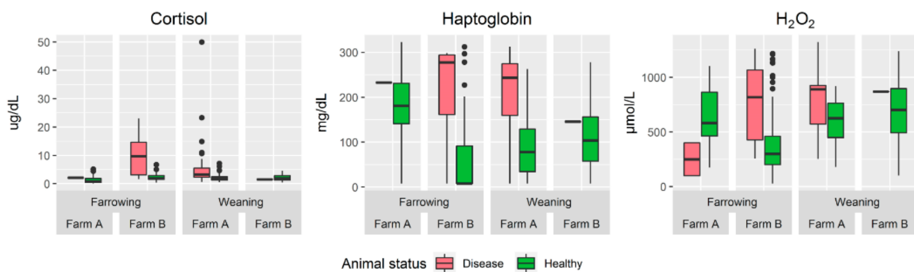
Supplementary Table S3.4 | Variables included in the models for farm A.

These models include CO_2 and THI variables, only studied in farm A.

Variable	Odds ratio	CI	Beta coefficient	*P*-value
Model 1: General Risk Factor				
CO_2 range at farrowing	1.01	1.01 - 1.02	0.01	<0.001
PRRSV presence at weaning	5.50	1.18 - 25.63	1.70	0.030
Haptoglobin at weaning	1.02	1.01 - 1.03	0.02	0.006
Sow parity	0.67	0.45 - 0.99	-0.40	0.045
Model 2: Weaning Risk Factor				
CO_2 range at farrowing	1.01	1.00 - 1.01	0.01	<0.001
Sow parity	0.71	0.51 - 0.97	-0.35	0.033
Model 3: Late Weaning Risk Factor				
PRRSV presence at early weaning	9.19	1.97 - 42.82	2.22	0.004
CO_2 mean at early weaning	1.00	1.00 - 1.00	0.00	<0.001
Sow parity	0.47	0.30 - 0.75	-0.75	0.001

Supplementary Figure S3.1 | Boxplot of the biomarkers studied for healthy animals and those with clinical symptoms compatible with *S. suis* at sampling.

Representation of the three biomarkers studied in serum samples (cortisol, haptoglobin, and H_2O_2), differentiating between healthy piglets and piglets with clinical signs consistent with *S. suis*-associated disease, i.e. arthritis and meningitis, at the moment of sampling.



STUDY III

Supplementary Table S4.1 | RNA Quality.

Mean values with the minimum and maximum range for RNA concentration and qualities for each sample type.

Tissue/sample type	RNA concentration (ng/µl)	A_{260}/A_{280} ratio	A_{260}/A_{230} ratio	RIN	Total samples
Whole blood	2039 (1043-3107)	2.1 (2.1-2.2)	2.1 (1.6-2.2)	7.8 (6.9-9.1)	76
Nasal swabs	30 (6.8-87.3)	1.9 (1.5-2.1)	1.4 (0.6-2.4)	1.7 (1.0-4.7)	74
Trachea	400 (124-719)	2.1 (2.0-2.2)	1.6 (1.0-2.0)	7.9 (5.4-9.2)	20
Lung (cranial lobe)	847 (182-1369)	2.2 (2.1-2.2)	1.7 (0.6-2.3)	7.8 (5.7-9.0)	20
Lung (caudal lobe)	1047 (413-1691)	2.2 (2.1-2.2)	1.9 (1.3-2.2)	7.7 (5.9-8.9)	20
Submandibular lymph node	2036 (1038-3101)	2.1 (2.0-2.2)	2.1 (1.8-2.2)	7.5 (4.3-9.5)	20
Tracheobronchial lymph node	1431 (648-2382)	2.1 (2.1-2.2)	2.2 (2.0-2.2)	7.2 (4.8-9.1)	20
Liver	1519 (600-2422)	2.1 (2.0-2.1)	1.7 (1.1-2.1)	9.2 (8.4-9.8)	20
Spleen	1914 (1206-2991)	2.1 (2.1-2.2)	2.1 (1.7-2.3)	9.0 (7.1-9.9)	20

Supplementary Table S4.2 | Primer sequences and amplification efficiency.

List of primers used, including primer sequences, amplicon length and amplification efficiency by tissue.

- Pro- and anti-inflammatory cytokines, cytokine receptors, and other inflammation-related factors:

Gene	Gene name	Forward primer sequence	Reverse primer sequence
IL1A	Interleukin 1, Alpha	TGTGCTAAATAACCTGGATGAGG	GGTTCGTCTTCGTTTTGAGC
IL1B (assay 1)	Interleukin 1, Beta	TCTCTCACCCCTTCTCCTCA	GACCCTAGTGTGCCATGGTT
IL1B (assay 2)	Interleukin 1, Beta	CCAAAGAGGGACATGGAGAA	GGGCTTTTGTTCTGCTTGAG
IL6 (assay 1)	Interleukin 6	CCTCTCCGGACAAAACTGAA	TCTGCCAGTACCTCCTTGCT
IL6 (assay 2)	Interleukin 6	TGGGTTCAATCAGGAGACCT	CAGCCTCGACATTTCCCTTA
IL10	Interleukin 10	TACAACAGGGGCTTGCTCTT	GCCAGGAAGATCAGGCAATA
IL12A	Interleukin 12, Subunit Alpha	CCACCTGGACCATCTCAGTT	CAGCAGATTTTGGGAGTGGT
IL12B	Interleukin 12, p40	GACCAGAAAGAGCCCAAAAAC	AGGTGAAACGTCCGGAGTAA
IL15	Interleukin 15	CGTCATTTTGCAAGAGTCCA	TGGACGATAAACTGCTGTTTGC
IL18	Interleukin 18	CTGCTGAACCGGAAGACAAT	TCCGATTCCAGGTCTTCATC
IL23	Interleukin 23	CAACAGTCAGTCCTGCTTGC	GCTCCCCTGTGAAAATGTCT
IL1R1	Interleukin 1, Receptor Type 1	CCGGGTGAGTGACTTTGTCT	TTCCTTCAGCACTGGGTCTT
IL1RN	Interleukin 1, Receptor Antagonist	TGCCTGTCCTGTGTCAAGTC	GTCCTGCTCGCTGTTCTTTC
TNF (assay 1)	Tumor Necrosis Factor	CACGTTGTAGCCAATGTCAAAG	GAGGTACAGCCCATCTGTCG
TNF (assay 2)	Tumor Necrosis Factor	CCCCCAGAAGGAAGAGTTTC	CGGGCTTATCTGAGGTTTGA
TNFRSF1A	Tumor Necrosis Factor, Receptor 1	AGTGAAATGTCCCAGGTGGA	TTCTTTCTGCAGCCACACAC
CASP1	Caspase 1	GAAGGACAAACCCAAGGTGA	TGGGCTTTCTTAATGGCATC
NLRP3	NLR Family Pyrin Domain Containing 3	GACTTTCCAGGAGTTCTTTGCTG	CCTGGTTTACAAGGCCAAAG
PTGS2	Prostaglandin-Endoperoxide Synthase 2, COX2	AGGCTGATACTGATAGGAGAAACG	GCAGCTCTGGGTCAAACTTC
PTX3	Pentraxin 3	TGCCAGCAGGTTGTGAAAC	GGCACTGAAAGCCTCAAGTT

Amplification efficiency (%), marked with * and in bold those with at least one significant difference ($P < 0.05$) except for Blood and Nasal swab										
Gene	**Amplicon length (nt)**	**Blood**	**Nasal swab**	**Trachea**	**Lung (cranial)**	**Lung (caudal)**	**Submandibular lymph node**	**Tracheobronchial lymph node**	**Liver**	**Spleen**
IL1A	135		107	92	88	90	99	100		101
IL1B (assay 1)	60		84				**106***		95	91
IL1B (assay 2)	123	91	101	90	92	92		94		
IL6 (assay 1)	118						99	99	105	106
IL6 (assay 2)	116			96	93	95				
IL10	110			**90***	90	90	100	106	101	97
IL12A	94									98
IL12B	70						90	98		99
IL15	86	104								
IL18	100	94					98	94	88	98
IL23	86						94	101		99
IL1R1	73						102	102		101
IL1RN	90		93				96	96	94	99
TNF (assay 1)	129						107	109	98	98
TNF (assay 2)	92	87		92	92	**93***				
TNFRSF1A	71						89	95	**90***	96
CASP1	147	98					92	**98***	92	104
NLRP3	194								110	
PTGS2	100						**97***	100		99
PTX3	104						101			95

- Chemokines and chemokine receptors:

Gene	**Gene name**	**Forward primer sequence**	**Reverse primer sequence**
CCL2	C-C Motif Chemokine, Ligand 2	CTTCTGCACCCAGGTCCTT	CGCTGCATCGAGATCTTCTT
CCL3 (assay 1)	C-C Motif Chemokine, Ligand 3	CTCTGCAGCCAGGTCTTCTC	CTACGAATTTGCGAGGAAGC
CCL3 (assay 2)	C-C Motif Chemokine, Ligand 4	CCAGGTCTTCTCTGCACCAC	GCTACGAATTTGCGAGGAAG
CCL4	C-C Motif Chemokine, Ligand 5	CCGTGGTATTCCAGACCAAA	ACTCCTGGACCCAGTCATCA

CCL5 (RANTES)	C-C Motif Chemokine, Ligand 6	CTCCATGGCAGCAGTCGT	AAGGCTTCCTCCATCCTAGC
IL8 (assay 1)	Interleukin 8, CXCL8	TTGCCAGAGAAATCACAGGA	TGCATGGGACACTGGAAATA
IL8 (assay 2)	Interleukin 8, CXCL8	GAAGAGAACTGAGAAGCAACAACA	TTGTGTTGGCATCTTTACTGAGA
CXCL10	C-X-C Motif Chemokine, Ligand 10	CCCACATGTTGAGATCATTGC	GCTTCTCTCTGTGTTCGAGGA
CXCR2	C-X-C Motif Chemokine, Receptor 2	ACAGCTGCCTCAATCCTCTC	ATGGCCATGATCTTGAGGAG
CXCR3 (assay 1)	C-X-C Motif Chemokine, Receptor 3	GTAGGGTGGACGTAGCCAAG	GGAACTTGACACCCACGAAG
CXCR3 (assay 2)	C-X-C Motif Chemokine, Receptor 3	CTGGTGGACACCCTCATGTA	TGGCTACGTCCACCCTACTT

Amplification efficiency (%), marked with * and in bold those with at least one significant difference (*P*<0.05) except for Blood and Nasal swab										
Gene	**Amplicon length (nt)**	**Blood**	**Nasal swab**	**Trachea**	**Lung (cranial)**	**Lung (caudal)**	**Submandibular lymph node**	**Tracheobronchial lymph node**	**Liver**	**Spleen**
CCL2	93			90	90	92	**100***	101	90	101
CCL3 (assay 1)	97		90				**101***	92		99
CCL3 (assay 2)	90			90	95	89				
CCL4	69	104					95	93	96	97
CCL5 (RANTES)	121	99		93	90	93	106	96	93	103
IL8 (assay 1)	78						107	105		101
IL8 (assay 2)	99			94	92	**90***			97	
CXCL10	141		90				99	98		106
CXCR2	76						99	108		101
CXCR3 (assay 1)	96						94	97		
CXCR3 (assay 2)	73									103

- Pattern recognition receptors and associated signalling:

Gene	**Gene name**	**Forward primer sequence**	**Reverse primer sequence**
TLR1	Toll Like Receptor 1	CCTTCAAGACCTTAACACACAGAG	CAGATTTACTGCGGTGCTGA
TLR2 (assay 1)	Toll Like Receptor 2	GTTTTACGGAAATTGTGAAACTG	TCCACATTACCGAGGGATTT
TLR2 (assay 2)	Toll Like Receptor 2	CGGAGGTTGCATATTCCACAG	TGTGAAAGGGAACAGGGAAC
TLR4 (assay 1)	Toll Like Receptor 4	TTTCCACAAAAGTCGGAAGG	CAACTTCTGCAGGACGATGA
TLR4 (assay 2)	Toll Like Receptor 4	TGGTGTCCCAGCACTTCATA	CAACTTCTGCAGGACGATGA

TLR6	Toll Like Receptor 6	TGGATGTTAGCTCGAATTCTTTG	GAACCTTGATCCTGGGAGGT
CD14	CD14	AAGCTCACCGTGCTTGATCT	CCTTCCAGGGTCAGGTCAT
MYD88	Myeloid Differentiation Primary Response Protein 88	CCAGACTAAGTTTGCACTCAGC	AGGATGCTGGGGAACTCTTT
LY96 (MD2)	Lymphocyte Antigen 96 (MD2)	CAGTAAAGGTTGAGCCCTGTG	TTTGCGCATTGGTAAAGTCA
TICAM1 (TRIF)	Toll Like Receptor, Adaptor Molecule 1	CTGCCTTCCCACAGCCTC	AGCCCCAGTTGTACCATTTGA

Amplification efficiency (%), marked with * and in bold those with at least one significant difference (*P*<0.05) except for Blood and Nasal swab										
Gene	**Amplicon length (nt)**	**Blood**	**Nasal swab**	**Trachea**	**Lung (cranial)**	**Lung (caudal)**	**Submandibular lymph node**	**Tracheobronchial lymph node**	**Liver**	**Spleen**
TLR1	100						90	90	94	99
TLR2 (assay 1)	136			89	95	**95***				
TLR2 (assay 2)	128	101					101	101	94	102
TLR4 (assay 1)	145								97	
TLR4 (assay 2)	116	104	103				102	93		105
TLR6	141			**92***	**97***	90	91	107	101	96
CD14	92	95								
MYD88	99	90	99				91	94		**96***
LY96 (MD2)	140	90								
TICAM1 (TRIF)	109	97								

- Transcription factors, interferons, and cytokine-related signalling

Gene	Gene name	Forward primer sequence	Reverse primer sequence
NFKB1	Nuclear Factor Kappa B Subunit 1	TCCACAAGGCAGCAAATAGA	AAGCTGAGTTTGCGAAAGGA
NFKBIA	Nuclear Factor of Kappa Light Polypeptide Gene Enhancer in B-Cells Inhibitor, Alpha	GAGGATGAGCTGCCCTATGAC	CCATGGTCTTTTAGACACTTTCC
IKBKB	Inhibitor of Nuclear Factor Kappa-B Kinase, Subunit Beta, IKK-β	TGGGATCACATCGGACAAACTG	CTTCACCTCGTTCTCCCGTC
FOS	Proto-oncogene c-Fos	CTCCAAGCGGAGACAGACC	CTTCTCCTTCAGCAGGTTGG
JUN	c-Jun	AGTGAAAACCTTGAAAGCGCAG	TGGCACCCACTGTTAACGTG
IRF1	Interferon Regulatory, Factor 1	TGAAGCTGCAACAGATGAGG	CTTCCCATCCACGTTTGTCT

IRF3	Interferon Regulatory, Factor 3	GCTACACCCTCTGGTTCTGC	GAGACACATGGGGACAACCT
IRF7 (assay 1)	Interferon Regulatory, Factor 7	GTGTGCTCCTGTACGGGTCT	CTGCAGCAGCTTCTCTGTGT
IRF7 (assay 2)	Interferon Regulatory, Factor 7	GCTCCCCACACTACACCATC	TCCAACTTCACCAGGACGA
STAT1	Signal Transducer and Activator of Transcription 1	CCTTGCAGAATAGAGAACATGATAC	CCTTTCTCTTGTTGTCAAGCATT
STAT2	Signal Transducer and Activator of Transcription 2	TTTGCCCCATGATCTGAGACAC	ACGTTGGTGTTCTGGCTAGC
IFNA1	Interferon Alpha 1	TTCCAGCTCTTCAGCACAGA	AGCTGCTGATCCAGTCCAGT
IFNG	Interferon Gamma	CCATTCAAAGGAGCATGGAT	TTCAGTTTCCCAGAGCTACCA
JAK1	Janus Kinase 1	TGGGCATGGCTGTGTTGG	CTTGTAGCTGATGTCCTTGGGA
JAK2	Janus Kinase 2	CTCAGATATGCAAGGGTATGGAGT	CCACCAATATATTCCTTGTTGCCA
IFITM1	Interferon-Induced Transmembrane Protein1	GCTTTCGCCTACTCCGTGA	CCAGGATCAGAGCCCAGATG

Amplification efficiency (%), marked with * and in bold those with at least one significant difference (*P*<0.05) except for Blood and Nasal swab										
Gene	**Amplicon length (nt)**	**Blood**	**Nasal swab**	**Trachea**	**Lung (cranial)**	**Lung (caudal)**	**Submandibular lymph node**	**Tracheobronchial lymph node**	**Liver**	**Spleen**
NFKB1	83	98	95				98	100		98
NFKBIA	85	96					98	**95***		98
IKBKB	85	100					96	103		**88***
FOS	78						93	93		90
JUN	114						104	102		106
IRF1	100		99							
IRF3	95	94					117	105	101	95
IRF7 (assay 1)	125	97					100		99	100
IRF7 (assay 2)	91							98		
STAT1	108	91					95	96		95
STAT2	120	88					98	98		97
IFNA1	86	100					101	101	100	102
IFNG	76						93	96	95	95
JAK1	86	95								
JAK2	81	100								
IFITM1	112						102	100		104

- Apoptosis

Gene	Gene name	Forward primer sequence	Reverse primer sequence
CASP3	Caspase 3	CTGGCAAACCCAAACTTTTC	GTCCCACTGTCCGTCTCAAT
CASP8	Caspase 8	CCGAAACTTGGACCATAATGA	GATGATGCCCTTGTCTCCAT
FAS	Fas Receptor	GGTGAAAAGACGGTGCAGAAG	ACGTCTTTTATCATTGGCACCTC
FASLG	Fas Ligand	CACCCCAATCTACCCTCTGAG	GTGTCTTCCCATTCCAGAGGG
BCL2	Bcl-2 (B-cell Lymphoma 2)	CCCTGTGGATGACTGAGTACC	AACCACACATGCACCTACCC
MCL1	MCL1, BCL2 Family Apoptosis Regulator	GAGGCTGGGATGGGTTTGTG	TGCCAAACCAGCTCCTACTC
TNFRSF10B (assay 1)	TRAIL Receptor 2 (TRAIL-R)	CACACAGACATGCCAATTCC	GAAAGGACAGAACCCCAACA
TNFRSF10B (assay 2)	TRAIL Receptor 2 (TRAIL-R)	GTCAGTGCACGGGAAGTTTT	CTTCCCATGGAGAGGAAACA
TNFSF10	TRAIL short (TRAIL)	GTCCACAGAGAGTGGCTGCT	TGGCCCAAAGCTTTTTCATA
TP53	Tumor Protein p53	TGAATGACGCCTTGGAGCTG	TTTATGGCGGGAGGGAGACT

Amplification efficiency (%), marked with * and in bold those with at least one significant difference (*P*<0.05) except for Blood and Nasal swab										
Gene	**Amplicon length (nt)**	**Blood**	**Nasal swab**	**Trachea**	**Lung (cranial)**	**Lung (caudal)**	**Submandibular lymph node**	**Tracheobronchial lymph node**	**Liver**	**Spleen**
CASP3	79								99	
CASP8	73								100	
FAS	87						95	95	98	96
FASLG	98								94	
BCL2	83						99	102	**96***	97
MCL1	110						**98***	103	97	102
TNFRSF10B (assay 1)	75								94	
TNFRSF10B (assay 2)	76						96	88		97
TNFSF10	94						99	96	92	102
TP53	113						104	97	**101***	101

- Acute phase proteins

Gene	Gene name	Forward primer sequence	Reverse primer sequence
APOA1	Apolipoprotein A1	GTTCTGGGACAACCTGGAAA	GCTGCACCTTCTTCTTCACC
CRP (assay 1)	C-Reactive Protein	GGTGGGAGACATTGGAGATG	GAAGGTCCCACCAGCATAGA
CRP (assay 2)	C-Reactive Protein	CTTTTGCCCAGACAGACATGAT	GAGTGGTTTGGTGAGCCTTG
HP (assay 1)	Haptoglobin	ACAGATGCCACAGATGACAGC	CGTGCGCAGTTTGTAGTAGG
HP (assay 2)	Haptoglobin	CAGGAGATGGAGTGTACACCTTG	TGCTTCACATTCAGGCAGTT
ITIH4 (assay 1)	Inter-Alpha-Trypsin Inhibitor Heavy Chain Family Member 4	ATGACAGCAAGCGAACAGTG	GGGGATCCCTCTTGGTAATC
ITIH4 (assay 2)	Inter-Alpha-Trypsin Inhibitor Heavy Chain Family Member 4	AGGCCCTCACCATATCACAG	GTTGCCATCCAGGACTGTTT
LTF	Lactoferrin	GGAAAAGACTGCCCAGACAA	ACACTCCGTGTTGTCGTTGA
ORM1	Orosomucoid 1	ACCCCCAGTACAATGAGTCG	TTAACAGCAGGTCAGCAACG
SAA (isoform 2)	Serum Amyloid A, isoform 2	TGGAGAGCCTACTCGGACAT	CCTTTGGGCAGCATCATAGT
SAA (isoform 3)	Serum Amyloid A, isoform 3	CTCAAGGAAGCTGGTCAAGG	GGACATTCTCTCTGGCATCG
SAA (isoform 2/3)	Serum Amyloid A, isoform 2/3	CAGAGATGGGCATCATTCCT	TGGCATCGCTGATCACTTTA
TF	Transferrin	TAAACAGCAGGCTCAATTTGG	ATTGGGTGTCATCCCTGAAG

Amplification efficiency (%), marked with * and in bold those with at least one significant difference (*P*<0.05) except for Blood and Nasal swab										
Gene	**Amplicon length (nt)**	**Blood**	**Nasal swab**	**Trachea**	**Lung (cranial)**	**Lung (caudal)**	**Submandibular lymph node**	**Tracheobronchial lymph node**	**Liver**	**Spleen**
APOA1	86						93	96	98	98
CRP (assay 1)	85			90	91	92				
CRP (assay 2)	98								96	
HP (assay 1)	105	108		96	91	91				
HP (assay 2)	83						105	106	101	108
ITIH4 (assay 1)	85			93	91	96	100	104		
ITIH4 (assay 2)	110								99	
LTF	78									99
ORM1	210								**99***	

SAA (isoform 2)	90			96	89	**90***	103	101		99
SAA (isoform 3)	178						97	98		
SAA (isoform 2/3)	184						98	105	97	103
TF	104							106	97	101

- Adhesion molecules

Gene	Gene name	Forward primer sequence	Reverse primer sequence
ICAM1 (assay 1)	Intercellular Adhesion Molecule 1	AAGCTTCTCCTGCTCTGCTG	GGGGTCCATACAGGACACTG
ICAM1 (assay 2)	Intercellular Adhesion Molecule 1	GCCCAATTGAAGCTGAATGT	CACCTGGGTCTGGTTCTTGT
ICAM2 (assay 1)	Intercellular Adhesion Molecule 2	CGTGTTCTCCCCTCCAAAG	GATGGTGAGGGTTTCAAAGG
ICAM2 (assay 2)	Intercellular Adhesion Molecule 2	CGGACACCTCATTCACAGAG	TGCCACAAACAAGAAGAGCA
SELL	Selectin L	CCAAGAGAGCCCTCTGTTACAC	CCCGTAGTACCCTGCATCAC
SELP (assay 1)	Selectin P	CCTAGCAGGGCCATTGAC	CCCACCCATCACTAAACCTG
SELP (assay 2)	Selectin P	AGTATGCAGAGCTGTCAAATGC	GAAGCTGCAGGTTGATCCAT
VCAM1 (assay 1)	Vascular Cell Adhesion Molecule 1	CTTGACGTGAAAGGAAGAGAAAG	GGATGCACAATAGAGCACGA
VCAM1 (assay 2)	Vascular Cell Adhesion Molecule 1	CTCCTTAATAATACCTGCCATCG	TTTTGATTTTTGAGCGTCTACAAG

Amplification efficiency (%), marked with * and in bold those with at least one significant difference (*P*<0.05) except for Blood and Nasal swab										
Gene	**Amplicon length (nt)**	**Blood**	**Nasal swab**	**Trachea**	**Lung (cranial)**	**Lung (caudal)**	**Submandibular lymph node**	**Tracheobronchial lymph node**	**Liver**	**Spleen**
ICAM1 (assay 1)	89						101			
ICAM1 (assay 2)	108							100	101	95
ICAM2 (assay 1)	121								101	99
ICAM2 (assay 2)	121	100					98	101		
SELL	116	93								
SELP (assay 1)	85						**99***	103		99
SELP (assay 2)	106	101							98	
VCAM1 (assay 1)	72						92			99
VCAM1 (assay 2)	97							95	84	

- Miscellaneous

Gene	Gene name	Forward primer sequence	Reverse primer sequence
C3	Complement Component 3	ATCAAATCAGGCTCCGATGA	GGGCTTCTCTGCATTTGATG
C5	Complement Component 5	AAGCTGGAGAAGCCGTTGC	TTTTCGAGGTTAGCGTTCGT
CFH	Complement Factor H	AGTGTGTGGGTCGTCCTTG	GGTAGCTGTCTTTCTCCTGAGC
CD163	CD163	CACATGTGCCAACAAAATAAGAC	CACCACCTGAGCATCTTCAA
HSPA14	Heat Shock 70 kDa Protein 14	CACTGGAAAAAGCAATATTCTGG	AAATGTGTGCCTCCGATGTT
MUC5AC	Mucin 5AC	CCCAGATCTGCAGCACCTAC	GTAACACAGGCCACCTGCTT
TREM1	Triggering Receptor Expressed on Myeloid Cells 1	CACAAATGTGACGGATATCACC	TGACATCCTGGTGACAACAAA

Amplification efficiency (%), marked with * and in bold those with at least one significant difference (*P*<0.05) except for Blood and Nasal swab										
Gene	**Amplicon length (nt)**	**Blood**	**Nasal swab**	**Trachea**	**Lung (cranial)**	**Lung (caudal)**	**Submandibular lymph node**	**Tracheobronchial lymph node**	**Liver**	**Spleen**
C3	76	81					**97***	96	92	96
C5	82								89	
CFH	75								95	
CD163	130	100					100	103	96	101
HSPA14	132						96	102		99
MUC5AC	94		98							
TREM1	118	92								

- Reference genes

Gene	Gene name	Forward primer sequence	Reverse primer sequence
ACTB	Beta-Actin	CTACGTCGCCCTGGACTTC	GCAGCTCGTAGCTCTTCTCC
B2M	Beta-2 Microglobulin	TGAAGCACGTGACTCTCGAT	CTCTGTGATGCCGGTTAGTG
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase	ACCCAGAAGACTGTGGATGG	AAGCAGGGATGATGTTCTGG
HPRT1	Hypoxanthine-Guanine Phosphoribosyltransferase	ACACTGGCAAAACAATGCAA	TGCAACCTTGACCATCTTTG
PPIA	Peptidylprolyl, Isomerase A	CAAGACTGAGTGGTTGGATGG	TGTCCACAGTCAGCAATGGT
RPL13A	60S Ribosomal Protein L13a	ATTGTGGCCAAGCAGGTACT	AATTGCCAGAAATGTTGATGC
YWHAZ	14-3-3 Protein Zeta/Delta	GCTGCTGGTGATGATAAGAAGG	AGTTAAGGGCCAGACCCAAT

Amplification efficiency (%), marked with * and in bold those with at least one significant difference (*P*<0.05) except for Blood and Nasal swab										
Gene	**Amplicon length (nt)**	**Blood**	**Nasal swab**	**Trachea**	**Lung (cranial)**	**Lung (caudal)**	**Submandibular lymph node**	**Tracheobronchial lymph node**	**Liver**	**Spleen**
ACTB	77							96		
B2M	65		92		101	95		107		
GAPDH	79			90	98	98				
HPRT1	71	93		91	95	98		91		98
PPIA	138		96				108	105	97	105
RPL13A	76	95	97	93	98	92	105	99	98	
YWHAZ	124	92					103	100	96	102

Supplementary Table S4.3 | Gene expression in nasal samples.

Gene expression from nasal swabs samples, including means, standard deviations, and *P*-values.

- Mean ± Standard Deviation

Gene	**PBS**			**T15**					**S10**				
	-3Days	**4Hours**	**1dpi**	**-3Days**	**4Hours**	**1dpi**	**2dpi**	**3dpi**	**-3Days**	**4Hours**	**1dpi**	**2dpi**	**3dpi**
CCL3	1 ± 0.17	9.55 ± 13.40	1.64 ± 0.86	1 ± 0.63	3.90 ± 5.59	5.01 ± 9.62	1.31 ± 1.10	0.24 ± 0.11	1 ± 0.73	1.36 ± 0.70	2.62 ± 3.56	2.17 ± 2.10	1.18 ± 0.74
CXCL10	1 ± 0.06	0.69 ± 0.52	0.64 ± 0.32	1 ± 0.64	15.53 ± 17.78	3.94 ± 2.51	2.21 ± 1.98	2.52 ± 2.50	1 ± 0.54	18.56 ± 15.55	3.01 ± 2.64	2.63 ± 1.97	3.88 ± 3.99
IL1A	1 ± 0.32	1.71 ± 1.49	3.74 ± 3.44	1 ± 1.02	4.34 ± 2.71	7.48 ± 11.62	5.23 ± 5.26	0.81 ± 0.38	1 ± 0.54	8.31 ± 5.19	2.81 ± 2.69	4.10 ± 3.00	6.44 ± 6.10
IL1B_1	1 ± 1.06	5.87 ± 3.11	37.43 ± 47.11	1 ± 1.13	12.27 ± 8.96	25.41 ± 34.17	8.95 ± 3.30	1.42 ± 0.77	1 ± 2.11	10.02 ± 9.22	5.11 ± 9.26	7.79 ± 12.15	3.34 ± 2.84

IL1B_2	1 ± 0.58	1.55 ± 1.33	4.52 ± 4.02	1 ± 1.59	9.28 ± 9.01	7.29 ± 7.69	5.34 ± 3.21	0.90 ± 0.83	1 ± 1.92	11.54 ± 7.67	7.30 ± 9.27	9.31 ± 12.66	6.87 ± 8.18
IL1RN	1 ± 0.82	1.76 ± 0.92	3.50 ± 2.38	1 ± 0.96	2.34 ± 1.18	5.71 ± 5.05	5.34 ± 3.33	0.76 ± 0.44	1 ± 0.79	5.89 ± 3.46	5.50 ± 5.06	6.61 ± 7.81	5.35 ± 4.87
IRF1	1 ± 0.11	1.30 ± 0.96	0.70 ± 0.14	1 ± 0.54	3.05 ± 1.70	1.86 ± 1.10	1.36 ± 0.72	0.93 ± 0.39	1 ± 0.71	4.07 ± 2.03	2.23 ± 1.45	1.67 ± 0.98	1.96 ± 0.85
MUC5AC	1 ± 0.38	1.73 ± 0.75	0.53 ± 0.23	1 ± 0.86	0.64 ± 0.20	0.55 ± 0.26	0.57 ± 0.33	0.57 ± 0.33	1 ± 0.72	0.84 ± 0.33	1.06 ± 0.66	1.73 ± 0.80	0.54 ± 0.30
MYD88	1 ± 0.10	5.36 ± 6.05	1.52 ± 0.51	1 ± 0.64	2.56 ± 3.07	1.99 ± 1.75	1.36 ± 0.62	0.44 ± 0.21	1 ± 0.63	1.37 ± 0.71	2.11 ± 1.62	2.29 ± 1.59	1.16 ± 0.46
NFKB1	1 ± 0.08	0.88 ± 0.34	0.48 ± 0.14	1 ± 0.43	1.66 ± 0.85	1.12 ± 0.31	1.26 ± 0.23	0.59 ± 0.28	1 ± 0.80	1.31 ± 0.51	1.00 ± 0.41	0.98 ± 0.29	0.80 ± 0.31
TLR4	1 ± 0.01	0.88 ± 0.76	0.44 ± 0.14	1 ± 0.42	1.98 ± 1.28	1.90 ± 1.41	1.53 ± 0.85	0.69 ± 0.15	1 ± 0.39	2.10 ± 1.08	1.08 ± 0.34	1.08 ± 0.56	1.76 ± 1.47

- *P*-values, part I

Gene	***P*-value PBS Group**			***P*-value T15 Group**									
	-3Days *vs.*		**4Hours *vs.***	**-3Days *vs.***				**4Hours *vs.***			**1dpi *vs.***		**2dpi *vs.***
	4Hours	**1dpi**	**1dpi**	**4Hours**	**1dpi**	**2dpi**	**3dpi**	**1dpi**	**2dpi**	**3dpi**	**2dpi**	**3dpi**	**3dpi**
CCL3	0.755	1.000	0.655	0.641	0.327	1.000	0.998	0.984	0.856	0.624	0.617	0.365	0.996
CXCL10	1.000	1.000	1.000	**0.017***	0.968	0.999	0.999	0.106	0.128	0.144	0.998	0.999	1.000
IL1A	0.999	0.898	0.923	0.700	0.082	0.678	1.000	0.724	0.999	0.772	0.933	0.187	0.712
IL1B_1	1.000	0.975	0.969	0.852	0.207	0.979	1.000	0.768	0.999	0.934	0.759	0.425	0.989
IL1B_2	1.000	0.951	0.945	0.137	0.379	0.844	1.000	0.978	0.899	0.324	0.993	0.597	0.894
IL1RN	1.000	0.987	0.993	0.912	**0.033***	0.181	1.000	0.216	0.536	0.925	1.000	0.094	0.251
IRF1	0.999	0.997	0.948	**0.004***	0.516	0.979	1.000	0.201	0.116	**0.025***	0.956	0.674	0.981
MUC5AC	0.848	0.957	0.220	0.430	0.210	0.381	0.381	0.992	0.992	0.992	1.000	1.000	1.000
MYD88	0.626	1.000	0.472	0.166	0.591	0.977	0.986	0.920	0.725	0.152	0.977	0.460	0.869
NFKB1	0.987	0.369	0.454	0.092	0.991	0.922	0.731	0.225	0.740	**0.016***	0.990	0.510	0.365
TLR4	0.998	0.674	0.705	0.309	0.394	0.915	0.987	1.000	0.949	0.246	0.975	0.307	0.762

- *P*-values, part II

Gene	*P*-value S10 Group									
	-3Days *vs.*				4Hours *vs.*			1dpi vs.		2dpi *vs.*
	4Hours	1dpi	2dpi	3dpi	1dpi	2dpi	3dpi	2dpi	3dpi	3dpi
CCL3	1.000	0.979	0.997	1.000	0.992	0.999	1.000	1.000	0.994	0.999
CXCL10	**<0.001***	0.987	0.997	0.974	**0.003***	**0.013***	**0.027***	1.000	1.000	0.999
IL1A	**0.040***	0.949	0.878	0.444	0.194	0.623	0.964	0.996	0.797	0.963
IL1B_1	**0.048***	0.693	0.428	0.976	0.533	0.978	0.434	0.961	0.991	0.856
IL1B_2	**0.011***	0.262	0.344	0.654	0.647	0.977	0.647	0.994	0.999	0.976
IL1RN	0.087	0.135	0.127	0.346	1.000	0.998	0.999	0.989	1.000	0.989
IRF1	**<0.001***	0.299	0.932	0.773	**0.041***	**0.022***	0.057	0.933	0.993	0.997
MUC5AC	0.982	1.000	0.283	0.761	0.948	0.124	0.943	0.359	0.672	0.052
MYD88	0.995	0.776	0.874	1.000	0.939	0.967	0.998	1.000	0.894	0.919
NFKB1	0.599	1.000	0.998	0.873	0.590	0.604	0.235	0.999	0.878	0.976
TLR4	0.081	1.000	1.000	0.583	0.126	0.290	0.966	1.000	0.684	0.771

- *P*-values, part III

Gene	*P*-value -3Days			*P*-value 4Hours			*P*-value 1dpi			*P*-value 2dpi	*P*-value 3dpi
	PBS *vs.*		T15 *vs.*	PBS *vs.*		T15 *vs.*	PBS *vs.*		T15 *vs.*	T15 *vs.*	T15 *vs.*
	T15	S10	S10	T15	S10	S10	T15	S10	S10	S10	S10
CCL3	0.988	0.998	0.991	0.924	0.293	0.354	0.244	0.907	0.311	0.995	0.977
CXCL10	0.992	0.994	0.999	**0.030***	**0.001***	0.390	0.876	0.913	0.995	0.991	0.950
IL1A	0.995	0.995	1.000	0.823	0.153	0.297	0.755	0.703	0.159	0.944	0.282
IL1B_1	0.999	0.982	0.972	0.731	**0.045***	0.104	0.592	0.894	0.794	0.486	0.798
IL1B_2	1.000	1.000	0.999	0.220	**0.038***	0.561	0.916	0.815	0.964	0.672	0.472
IL1RN	0.982	0.993	0.994	0.754	0.107	0.236	0.126	0.353	0.752	0.997	0.235
IRF1	0.999	0.992	0.967	**0.024***	**0.003***	0.672	0.187	0.153	0.991	1.000	0.663
MUC5AC	0.376	0.887	0.350	0.607	0.463	0.956	0.564	0.217	0.694	0.084	0.888
MYD88	0.908	0.985	0.913	0.921	0.345	0.081	0.278	0.595	0.767	1.000	0.988
NFKB1	0.406	0.671	0.772	0.645	0.806	0.941	0.530	0.401	0.961	0.909	0.754
TLR4	0.366	0.512	0.913	0.972	0.505	0.522	0.264	0.813	0.471	0.945	0.124

Supplementary Table S4.4 | Gene expression from tissues samples, including means, standard deviations, and *P*-values.

- Trachea

Gene	Mean ± SD				
	PBS	T15		S10	
	Day1	Day1	Day3	Day1	Day3
CCL2	1 ± 1.06	0.70 ± 0.22	0.47 ± 0.09	0.60 ± 0.35	0.54 ± 0.23
CCL3	1 ± 0.37	0.88 ± 0.46	0.93 ± 0.63	0.70 ± 0.49	0.65 ± 0.23
CCL5	1 ± 0.29	0.72 ± 0.16	1.13 ± 0.21	0.90 ± 0.34	1.10 ± 0.30
CRP	1 ± 0.27	0.96 ± 0.39	0.92 ± 0.21	0.58 ± 0.22	0.65 ± 0.17
HP	1 ± 0.57	0.63 ± 0.21	0.57 ± 0.21	0.82 ± 0.31	0.63 ± 0.31
IL10	1 ± 0.11	0.31 ± 0.34	0.36 ± 0.37	0.33 ± 0.25	0.57 ± 0.25
IL1A	1 ± 0.52	1.50 ± 0.80	0.97 ± 0.23	0.80 ± 0.33	0.62 ± 0.29
IL1B	1 ± 0.29	0.60 ± 0.27	0.86 ± 0.87	0.50 ± 0.21	0.78 ± 0.37
IL6	1 ± 0.15	0.75 ± 0.36	0.69 ± 0.26	1.00 ± 0.25	0.79 ± 0.31
IL8	1 ± 0.29	0.54 ± 0.40	0.60 ± 0.27	0.65 ± 0.36	0.63 ± 0.15
ITIH4	1 ± 1.13	0.91 ± 0.21	0.75 ± 0.33	0.74 ± 0.14	0.90 ± 0.18
SAA	1 ± 0.26	1.00 ± 0.48	1.72 ± 1.16	2.29 ± 2.51	0.87 ± 0.34
TLR2	1 ± 0.30	0.89 ± 0.21	0.92 ± 0.15	1.04 ± 0.28	0.87 ± 0.34
TLR6	1 ± 0.35	0.57 ± 0.10	0.46 ± 0.11	0.56 ± 0.13	0.56 ± 0.11
TNF	1 ± 0.39	0.78 ± 0.94	0.31 ± 0.23	0.28 ± 0.20	0.27 ± 0.25

Gene	*P*-value								
	ANOVA	PBS group *vs*. T15		PBS group *vs*. S10		T15 *vs*. S10		Day1 *vs*. Day 3	
		Day1	Day3	Day1	Day3	Day1	Day3	T15	S10
CCL2	0.649	0.925	0.619	0.803	0.725	0.998	1.000	0.969	1.000
CCL3	0.776	0.995	0.999	0.878	0.804	0.979	0.904	1.000	1.000
CCL5	0.253	0.605	0.958	0.982	0.986	0.885	1.000	0.256	0.833
CRP	0.125	0.999	0.993	0.211	0.374	0.292	0.608	1.000	0.994
HP	0.420	0.575	0.445	0.943	0.567	0.937	0.999	0.999	0.934
IL10	**0.016***	**0.024***	**0.036***	**0.029***	0.246	1.000	0.804	0.999	0.747
IL1A	0.166	0.596	1.000	0.973	0.791	0.284	0.831	0.548	0.983
IL1B	0.575	0.751	0.993	0.562	0.964	0.997	0.999	0.930	0.902
IL6	0.393	0.710	0.511	1.000	0.823	0.723	0.980	0.997	0.834
IL8	0.284	0.261	0.400	0.505	0.459	0.986	1.000	0.998	1.000
ITIH4	0.953	0.999	0.965	0.960	0.999	0.991	0.995	0.993	0.994
SAA	0.475	1.000	0.927	0.616	1.000	0.615	0.875	0.927	0.529
TLR2	0.868	0.970	0.993	1.000	0.955	0.919	0.999	1.000	0.892
TLR6	**0.008***	**0.035***	**0.007***	**0.034***	**0.032***	1.000	0.939	0.924	1.000
TNF	0.156	0.964	0.313	0.273	0.260	0.611	1.000	0.667	1.000

- Cranial lobe - Lungs

Gene	Mean ± SD				
	PBS	T15		S10	
	Day1	Day1	Day3	Day1	Day3
CCL2	1 ± 0.12	1.74 ± 0.69	1.44 ± 0.57	3.04 ± 4.03	1.27 ± 0.45
CCL3	1 ± 0.25	1.75 ± 1.33	1.24 ± 0.41	1.55 ± 0.89	0.71 ± 0.21

CCL5	1 ± 0.43	0.94 ± 0.29	1.25 ± 0.16	0.92 ± 0.42	1.10 ± 0.22
CRP	1 ± 0.30	1.24 ± 0.24	1.26 ± 0.29	1.25 ± 0.24	1.32 ± 0.26
HP	1 ± 0.48	2.12 ± 0.83	1.90 ± 0.67	1.65 ± 0.80	1.82 ± 0.17
IL10	1 ± 0.24	2.24 ± 1.24	1.49 ± 0.37	1.15 ± 0.53	0.96 ± 0.41
IL1A	1 ± 0.49	0.47 ± 0.32	0.69 ± 0.15	0.65 ± 0.22	0.71 ± 0.17
IL1B	1 ± 0.30	2.15 ± 1.85	1.88 ± 0.63	1.94 ± 1.90	1.18 ± 0.38
IL6	1 ± 0.37	0.81 ± 0.25	0.78 ± 0.35	1.34 ± 0.67	0.87 ± 0.19
IL8	1 ± 0.54	0.91 ± 0.50	0.98 ± 0.49	0.95 ± 0.40	0.94 ± 0.08
ITIH4	1 ± 0.29	1.11 ± 0.37	0.83 ± 0.24	1.34 ± 0.56	0.79 ± 0.20
SAA	1 ± 0.25	0.72 ± 0.28	1.76 ± 1.23	4.18 ± 6.14	2.43 ± 1.40
TLR2	1 ± 0.19	1.04 ± 0.24	1.45 ± 0.33	1.39 ± 0.74	1.63 ± 0.31
TLR6	1 ± 0.12	1.07 ± 0.22	1.05 ± 0.33	1.00 ± 0.23	1.71 ± 0.50
TNF	1 ± 0.34	1.16 ± 0.57	1.32 ± 1.10	1.45 ± 0.90	0.62 ± 0.15

Gene	***P*-value**								
	ANOVA	**PBS group *vs*. T15**		**PBS group *vs*. S10**		**T15 *vs*. S10**		**Day1 *vs*. Day 3**	
		Day1	**Day3**	**Day1**	**Day3**	**Day1**	**Day3**	**T15**	**S10**
CCL2	0.581	0.979	0.997	0.545	1.000	0.854	1.000	0.999	0.668
CCL3	0.339	0.625	0.991	0.834	0.982	0.995	0.857	0.864	0.534
CCL5	0.598	0.999	0.797	0.997	0.992	1.000	0.958	0.654	0.937
CRP	0.503	0.709	0.663	0.672	0.449	1.000	0.996	1.000	0.995
HP	0.184	0.144	0.317	0.612	0.403	0.827	1.000	0.986	0.996
IL10	0.079	0.109	0.832	0.998	1.000	0.184	0.786	0.511	0.994
IL1A	0.221	0.142	0.597	0.494	0.636	0.907	1.000	0.835	0.999
IL1B	0.625	0.691	0.852	0.818	1.000	0.999	0.927	0.998	0.904
IL6	0.304	0.957	0.931	0.744	0.991	0.361	0.997	1.000	0.482
IL8	0.998	0.998	1.000	1.000	1.000	1.000	1.000	0.999	1.000
ITIH4	0.221	0.990	0.955	0.653	0.917	0.886	1.000	0.780	0.231
SAA	0.474	1.000	0.995	0.539	0.953	0.459	0.997	0.985	0.906
TLR2	0.195	1.000	0.558	0.665	0.246	0.750	0.968	0.647	0.924
TLR6	**0.022***	0.998	0.999	1.000	**0.037***	0.998	0.055	1.000	**0.037***
TNF	0.523	0.997	0.964	0.891	0.938	0.976	0.630	0.997	0.483

- Caudal lobe – Lungs

Gene	**Mean ± SD**				
	PBS	**T15**		**S10**	
	Day1	**Day1**	**Day3**	**Day1**	**Day3**
CCL2	1 ± 0.36	0.97 ± 0.51	1.01 ± 0.34	1.90 ± 2.01	0.70 ± 0.29
CCL3	1 ± 0.53	1.02 ± 0.17	0.62 ± 0.13	0.74 ± 0.39	0.35 ± 0.16
CCL5	1 ± 0.48	0.56 ± 0.21	0.63 ± 0.11	0.52 ± 0.20	0.72 ± 0.16
CRP	1 ± 0.94	1.16 ± 0.83	0.98 ± 0.17	1.10 ± 0.73	0.72 ± 0.36
HP	1 ± 0.41	1.78 ± 0.59	1.49 ± 0.72	1.41 ± 1.04	0.70 ± 0.34
IL10	1 ± 0.92	0.20 ± 0.13	0.26 ± 0.20	0.27 ± 0.23	0.26 ± 0.11
IL1A	1 ± 1.16	1.03 ± 0.61	1.18 ± 0.34	0.87 ± 0.47	0.75 ± 0.43
IL1B	1 ± 0.56	0.60 ± 0.22	0.84 ± 0.50	0.77 ± 0.31	0.76 ± 0.36
IL6	1 ± 0.31	1.51 ± 0.13	1.09 ± 0.39	1.90 ± 1.75	1.02 ± 0.45
IL8	1 ± 0.57	0.20 ± 0.16	0.70 ± 0.32	0.45 ± 0.12	0.48 ± 0.12
ITIH4	1 ± 0.86	0.77 ± 0.17	0.68 ± 0.34	0.52 ± 0.34	0.43 ± 0.20
SAA	1 ± 0.29	0.62 ± 0.40	2.54 ± 1.21	0.67 ± 0.49	0.85 ± 0.19
TLR2	1 ± 0.11	1.31 ± 0.12	1.91 ± 0.48	1.22 ± 0.57	1.15 ± 0.11
TLR6	1 ± 0.51	1.24 ± 0.58	1.81 ± 0.76	1.27 ± 0.44	1.98 ± 0.46
TNF	1 ± 0.47	0.53 ± 0.18	0.35 ± 0.21	0.38 ± 0.25	0.17 ± 0.15

<table>
<tr><th rowspan="3">Gene</th><th colspan="9">P-value</th></tr>
<tr><th rowspan="2">ANOVA</th><th colspan="2">PBS group vs. T15</th><th colspan="2">PBS group vs. S10</th><th colspan="2">T15 vs. S10</th><th colspan="2">Day1 vs. Day 3</th></tr>
<tr><th>Day1</th><th>Day3</th><th>Day1</th><th>Day3</th><th>Day1</th><th>Day3</th><th>T15</th><th>S10</th></tr>
<tr><td>CCL2</td><td>0.491</td><td>1.000</td><td>1.000</td><td>0.687</td><td>0.991</td><td>0.657</td><td>0.990</td><td>1.000</td><td>0.431</td></tr>
<tr><td>CCL3</td><td>0.048*</td><td>1.000</td><td>0.464</td><td>0.781</td><td>0.070</td><td>0.743</td><td>0.748</td><td>0.425</td><td>0.429</td></tr>
<tr><td>CCL5</td><td>0.131</td><td>0.177</td><td>0.318</td><td>0.128</td><td>0.570</td><td>1.000</td><td>0.989</td><td>0.995</td><td>0.828</td></tr>
<tr><td>CRP</td><td>0.904</td><td>0.997</td><td>1.000</td><td>1.000</td><td>0.974</td><td>1.000</td><td>0.980</td><td>0.995</td><td>0.929</td></tr>
<tr><td>HP</td><td>0.214</td><td>0.483</td><td>0.833</td><td>0.900</td><td>0.966</td><td>0.932</td><td>0.475</td><td>0.969</td><td>0.567</td></tr>
<tr><td>IL10</td><td>0.100</td><td>0.126</td><td>0.176</td><td>0.181</td><td>0.170</td><td>0.999</td><td>1.000</td><td>1.000</td><td>1.000</td></tr>
<tr><td>IL1A</td><td>0.909</td><td>1.000</td><td>0.995</td><td>0.998</td><td>0.982</td><td>0.996</td><td>0.887</td><td>0.998</td><td>0.999</td></tr>
<tr><td>IL1B</td><td>0.729</td><td>0.640</td><td>0.981</td><td>0.927</td><td>0.921</td><td>0.973</td><td>0.999</td><td>0.909</td><td>1.000</td></tr>
<tr><td>IL6</td><td>0.506</td><td>0.908</td><td>1.000</td><td>0.572</td><td>1.000</td><td>0.963</td><td>1.000</td><td>0.951</td><td>0.595</td></tr>
<tr><td>IL8</td><td>0.025*</td><td>0.017*</td><td>0.649</td><td>0.136</td><td>0.177</td><td>0.784</td><td>0.855</td><td>0.202</td><td>1.000</td></tr>
<tr><td>ITIH4</td><td>0.453</td><td>0.949</td><td>0.854</td><td>0.578</td><td>0.420</td><td>0.932</td><td>0.931</td><td>0.999</td><td>0.999</td></tr>
<tr><td>SAA</td><td>0.003*</td><td>0.913</td><td>0.025*</td><td>0.944</td><td>0.997</td><td>1.000</td><td>0.013*</td><td>0.005*</td><td>0.994</td></tr>
<tr><td>TLR2</td><td>0.019*</td><td>0.716</td><td>0.014*</td><td>0.887</td><td>0.967</td><td>0.996</td><td>0.049*</td><td>0.149</td><td>0.999</td></tr>
<tr><td>TLR6</td><td>0.123</td><td>0.972</td><td>0.302</td><td>0.957</td><td>0.152</td><td>1.000</td><td>0.991</td><td>0.626</td><td>0.419</td></tr>
<tr><td>TNF</td><td>0.007*</td><td>0.169</td><td>0.030*</td><td>0.040*</td><td>0.005*</td><td>0.922</td><td>0.889</td><td>0.867</td><td>0.824</td></tr>
</table>

- Submandibular lymph node

<table>
<tr><th rowspan="3">Gene</th><th colspan="5">Mean ± SD</th></tr>
<tr><th>PBS</th><th colspan="2">T15</th><th colspan="2">S10</th></tr>
<tr><th>Day1</th><th>Day1</th><th>Day3</th><th>Day1</th><th>Day3</th></tr>
<tr><td>APOA1</td><td>1 ± 0.45</td><td>2.24 ± 0.78</td><td>1.74 ± 0.85</td><td>2.72 ± 1.87</td><td>1.52 ± 0.59</td></tr>
<tr><td>BCL2</td><td>1 ± 0.37</td><td>1.42 ± 0.40</td><td>1.52 ± 0.23</td><td>1.62 ± 0.76</td><td>1.59 ± 0.34</td></tr>
<tr><td>C3</td><td>1 ± 0.09</td><td>0.95 ± 0.30</td><td>1.90 ± 0.81</td><td>1.08 ± 0.34</td><td>1.68 ± 0.10</td></tr>
<tr><td>CASP1</td><td>1 ± 0.19</td><td>1.35 ± 0.26</td><td>1.45 ± 0.28</td><td>1.58 ± 0.55</td><td>1.35 ± 0.19</td></tr>
<tr><td>CCL2</td><td>1 ± 0.39</td><td>1.48 ± 0.44</td><td>2.30 ± 0.49</td><td>1.34 ± 0.37</td><td>1.18 ± 0.54</td></tr>
<tr><td>CCL3</td><td>1 ± 0.31</td><td>0.79 ± 0.15</td><td>1.53 ± 0.33</td><td>1.01 ± 0.24</td><td>1.02 ± 0.44</td></tr>
<tr><td>CCL4</td><td>1 ± 0.57</td><td>0.92 ± 0.13</td><td>1.39 ± 0.20</td><td>0.93 ± 0.32</td><td>1.00 ± 0.21</td></tr>
<tr><td>CCL5</td><td>1 ± 0.94</td><td>0.68 ± 0.14</td><td>1.52 ± 0.09</td><td>0.73 ± 0.44</td><td>0.94 ± 0.37</td></tr>
<tr><td>CD163</td><td>1 ± 0.39</td><td>1.26 ± 1.47</td><td>1.37 ± 0.86</td><td>0.82 ± 0.31</td><td>0.83 ± 0.62</td></tr>
<tr><td>CXCL10</td><td>1 ± 0.74</td><td>2.92 ± 2.58</td><td>2.39 ± 0.74</td><td>2.28 ± 1.59</td><td>0.86 ± 0.23</td></tr>
<tr><td>CXCR2</td><td>1 ± 0.30</td><td>1.39 ± 0.67</td><td>3.69 ± 5.04</td><td>1.70 ± 0.83</td><td>1.70 ± 0.89</td></tr>
<tr><td>CXCR3</td><td>1 ± 0.42</td><td>0.77 ± 0.30</td><td>1.08 ± 0.23</td><td>0.88 ± 0.38</td><td>0.95 ± 0.20</td></tr>
<tr><td>FAS</td><td>1 ± 0.23</td><td>1.17 ± 0.31</td><td>1.34 ± 0.31</td><td>0.98 ± 0.22</td><td>1.28 ± 0.11</td></tr>
<tr><td>FOS</td><td>1 ± 0.32</td><td>1.25 ± 0.46</td><td>1.74 ± 0.72</td><td>1.61 ± 1.12</td><td>1.24 ± 0.39</td></tr>
<tr><td>HP</td><td>1 ± 0.26</td><td>0.78 ± 0.12</td><td>1.15 ± 0.30</td><td>0.89 ± 0.07</td><td>1.11 ± 0.33</td></tr>
<tr><td>HSPA14</td><td>1 ± 0.24</td><td>0.95 ± 0.15</td><td>0.87 ± 0.12</td><td>1.00 ± 0.20</td><td>0.88 ± 0.21</td></tr>
<tr><td>ICAM1</td><td>1 ± 0.21</td><td>1.27 ± 0.26</td><td>1.66 ± 0.39</td><td>1.35 ± 0.42</td><td>1.11 ± 0.39</td></tr>
<tr><td>ICAM2</td><td>1 ± 0.11</td><td>1.01 ± 0.19</td><td>1.54 ± 0.47</td><td>1.15 ± 0.28</td><td>1.24 ± 0.28</td></tr>
<tr><td>IFITM1</td><td>1 ± 0.52</td><td>2.23 ± 1.49</td><td>1.54 ± 0.17</td><td>2.58 ± 1.95</td><td>1.09 ± 0.23</td></tr>
<tr><td>IFNA1</td><td>1 ± 0.41</td><td>1.51 ± 0.25</td><td>1.40 ± 0.39</td><td>1.74 ± 0.64</td><td>0.99 ± 0.46</td></tr>
<tr><td>IFNG</td><td>1 ± 0.31</td><td>0.68 ± 0.19</td><td>1.18 ± 0.24</td><td>0.65 ± 0.69</td><td>0.84 ± 0.45</td></tr>
<tr><td>IKBKB</td><td>1 ± 0.25</td><td>1.06 ± 0.10</td><td>1.68 ± 0.76</td><td>1.24 ± 0.38</td><td>2.02 ± 0.71</td></tr>
<tr><td>IL10</td><td>1 ± 0.19</td><td>1.03 ± 0.59</td><td>1.22 ± 0.35</td><td>0.68 ± 0.17</td><td>0.74 ± 0.35</td></tr>
<tr><td>IL12B</td><td>1 ± 0.64</td><td>1.05 ± 0.55</td><td>1.81 ± 0.53</td><td>1.41 ± 1.10</td><td>1.08 ± 0.56</td></tr>
<tr><td>IL18</td><td>1 ± 0.13</td><td>1.22 ± 0.94</td><td>0.87 ± 0.09</td><td>0.79 ± 0.31</td><td>0.67 ± 0.19</td></tr>
<tr><td>IL1A</td><td>1 ± 0.23</td><td>1.10 ± 0.32</td><td>1.68 ± 0.33</td><td>1.41 ± 0.71</td><td>1.23 ± 0.35</td></tr>
<tr><td>IL1B</td><td>1 ± 0.93</td><td>1.21 ± 0.68</td><td>3.10 ± 1.66</td><td>1.00 ± 0.51</td><td>1.05 ± 0.37</td></tr>
<tr><td>IL1R1</td><td>1 ± 0.25</td><td>1.30 ± 0.19</td><td>1.69 ± 0.44</td><td>1.20 ± 0.27</td><td>1.17 ± 0.53</td></tr>
</table>

IL1RN	1 ± 0.79	2.33 ± 1.49	6.88 ± 6.49	2.42 ± 0.82	2.62 ± 1.33
IL23	1 ± 0.21	1.48 ± 0.48	1.23 ± 0.23	1.66 ± 0.68	1.20 ± 0.22
IL6	1 ± 0.28	0.92 ± 0.19	1.24 ± 0.26	0.77 ± 0.19	0.96 ± 0.31
IL8	1 ± 0.27	1.46 ± 1.16	2.23 ± 1.43	0.81 ± 0.63	0.68 ± 0.20
IRF3	1 ± 0.06	1.02 ± 0.25	1.33 ± 0.36	1.15 ± 0.37	1.36 ± 0.23
IRF7	1 ± 0.71	3.23 ± 2.64	1.52 ± 0.42	2.50 ± 1.61	1.46 ± 0.43
ITIH4	1 ± 0.39	1.51 ± 0.34	2.98 ± 1.69	2.30 ± 1.27	2.35 ± 1.28
JUN	1 ± 0.17	1.05 ± 0.21	1.52 ± 0.77	1.14 ± 0.73	1.19 ± 0.72
MCL1	1 ± 0.18	1.58 ± 0.05	1.27 ± 0.32	1.20 ± 0.27	1.02 ± 0.22
MYD88	1 ± 0.23	1.15 ± 0.34	1.43 ± 0.57	1.08 ± 0.20	1.35 ± 0.38
NFKB1	1 ± 0.05	1.00 ± 0.04	1.11 ± 0.24	0.94 ± 0.19	1.04 ± 0.12
NFKBIA	1 ± 0.05	1.25 ± 0.42	1.60 ± 0.63	1.34 ± 0.42	1.73 ± 1.01
PTGS2	1 ± 0.15	1.83 ± 1.71	3.55 ± 1.48	1.35 ± 0.42	1.25 ± 0.45
PTX3	1 ± 0.47	1.21 ± 0.64	2.24 ± 1.29	2.10 ± 1.11	0.99 ± 0.62
SAA	1 ± 0.28	2.70 ± 4.96	2.67 ± 2.74	0.83 ± 0.76	1.61 ± 1.99
SELP	1 ± 0.20	1.37 ± 0.32	3.05 ± 1.03	1.73 ± 0.37	1.76 ± 0.82
STAT1	1 ± 0.40	1.96 ± 1.02	1.62 ± 0.27	1.53 ± 0.66	1.37 ± 0.19
STAT2	1 ± 0.19	1.64 ± 0.60	0.89 ± 0.16	1.17 ± 0.39	0.95 ± 0.19
TLR1	1 ± 0.16	1.31 ± 0.33	1.03 ± 0.51	1.05 ± 0.51	0.94 ± 0.44
TLR2	1 ± 0.30	0.92 ± 0.39	1.07 ± 0.07	0.84 ± 0.30	0.80 ± 0.29
TLR4	1 ± 0.38	1.18 ± 1.31	1.04 ± 0.27	0.88 ± 0.19	0.63 ± 0.36
TLR6	1 ± 0.40	1.17 ± 0.59	1.11 ± 0.38	1.11 ± 0.35	0.91 ± 0.39
TNF	1 ± 0.37	1.24 ± 0.26	1.47 ± 0.36	1.15 ± 0.23	1.12 ± 0.15
TNFRSF1A	1 ± 0.40	1.45 ± 0.40	1.71 ± 0.70	1.39 ± 0.24	1.43 ± 0.49
TP53	1 ± 0.10	1.19 ± 0.27	1.31 ± 0.40	1.22 ± 0.22	1.19 ± 0.25
TRAIL	1 ± 0.37	1.46 ± 0.81	1.03 ± 0.18	1.09 ± 0.32	0.82 ± 0.10
TRAIL-R	1 ± 0.16	1.62 ± 0.68	1.25 ± 0.41	1.53 ± 1.00	1.22 ± 0.46
VCAM1	1 ± 0.09	1.01 ± 0.15	1.01 ± 0.15	1.05 ± 0.14	0.72 ± 0.33

Gene	***P*-value**								
	ANOVA	**PBS group *vs.* T15**		**PBS group *vs.* S10**		**T15 *vs.* S10**		**Day1 *vs.* Day 3**	
		Day1	**Day3**	**Day1**	**Day3**	**Day1**	**Day3**	**T15**	**S10**
APOA1	0.217	0.470	0.849	0.182	0.950	0.961	0.998	0.957	0.496
BCL2	0.345	0.686	0.519	0.352	0.393	0.973	0.999	0.998	1.000
C3	**0.016***	1.000	0.055	0.999	0.206	0.993	0.937	**0.041***	0.306
CASP1	0.187	0.556	0.329	0.134	0.547	0.851	0.993	0.992	0.858
CCL2	**0.009***	0.580	**0.007***	0.815	0.978	0.993	**0.022***	0.123	0.985
CCL3	**0.045***	0.863	0.164	1.000	1.000	0.851	0.185	**0.028***	1.000
CCL4	0.282	0.997	0.471	0.998	1.000	1.000	0.482	0.305	0.998
CCL5	0.180	0.894	0.586	0.934	1.000	1.000	0.490	0.174	0.972
CD163	0.836	0.991	0.968	0.998	0.998	0.943	0.889	1.000	1.000
CXCL10	0.225	0.367	0.657	0.718	1.000	0.969	0.573	0.984	0.636
CXCR2	0.546	0.999	0.505	0.993	0.993	1.000	0.750	0.641	1.000
CXCR3	0.680	0.833	0.995	0.980	0.999	0.987	0.972	0.626	0.997
FAS	0.198	0.868	0.329	1.000	0.533	0.814	0.995	0.851	0.465
FOS	0.543	0.984	0.545	0.703	0.984	0.937	0.833	0.835	0.935
HP	0.217	0.691	0.903	0.964	0.965	0.962	0.999	0.240	0.698
HSPA14	0.752	0.996	0.855	1.000	0.879	0.995	1.000	0.968	0.873
ICAM1	0.121	0.810	0.099	0.616	0.992	0.996	0.206	0.505	0.852
ICAM2	0.106	1.000	0.114	0.952	0.781	0.966	0.584	0.128	0.992
IFITM1	0.249	0.556	0.960	0.325	1.000	0.992	0.980	0.905	0.382
IFNA1	0.122	0.509	0.713	0.184	1.000	0.946	0.688	0.996	0.172
IFNG	0.387	0.819	0.973	0.764	0.979	1.000	0.774	0.480	0.970

IKBKB	0.056	1.000	0.363	0.961	0.080	0.987	0.878	0.454	0.245
IL10	0.252	1.000	0.912	0.728	0.839	0.650	0.372	0.950	0.999
IL12B	0.467	1.000	0.508	0.923	1.000	0.949	0.596	0.564	0.961
IL18	0.515	0.958	0.993	0.962	0.843	0.675	0.973	0.807	0.996
IL1A	0.211	0.997	0.198	0.652	0.932	0.832	0.566	0.325	0.974
IL1B	**0.027***	0.998	**0.046***	1.000	1.000	0.998	0.053	0.081	1.000
IL1R1	0.142	0.763	0.101	0.931	0.959	0.994	0.301	0.565	1.000
IL1RN	0.130	0.971	0.100	0.963	0.943	1.000	0.330	0.273	1.000
IL23	0.231	0.482	0.926	0.208	0.957	0.972	1.000	0.906	0.527
IL6	0.167	0.991	0.658	0.689	0.999	0.906	0.530	0.403	0.807
IL8	0.142	0.946	0.328	0.998	0.985	0.832	0.148	0.733	1.000
IRF3	0.252	1.000	0.471	0.937	0.389	0.957	1.000	0.516	0.815
IRF7	0.235	0.238	0.985	0.595	0.990	0.950	1.000	0.479	0.844
ITIH4	0.165	0.966	0.147	0.508	0.470	0.859	0.928	0.387	1.000
JUN	0.746	1.000	0.722	0.997	0.989	0.999	0.930	0.783	1.000
MCL1	**0.015***	**0.018***	0.467	0.717	1.000	0.177	0.524	0.338	0.772
MYD88	0.443	0.979	0.486	0.998	0.662	0.999	0.998	0.805	0.831
NFKB1	0.628	1.000	0.840	0.982	0.996	0.982	0.961	0.839	0.894
NFKBIA	0.462	0.974	0.617	0.922	0.440	0.999	0.998	0.913	0.883
PTGS2	**0.025***	0.798	**0.026***	0.989	0.997	0.966	**0.049***	0.193	1.000
PTX3	0.159	0.997	0.320	0.428	1.000	0.623	0.313	0.495	0.420
SAA	0.782	0.897	0.903	1.000	0.997	0.860	0.980	1.000	0.993
SELP	**0.005***	0.916	**0.003***	0.504	0.463	0.929	0.075	**0.015***	1.000
STAT1	0.272	0.196	0.585	0.714	0.902	0.832	0.971	0.920	0.994
STAT2	0.054	0.124	0.992	0.956	1.000	0.360	0.999	0.057	0.902
TLR1	0.752	0.818	1.000	1.000	1.000	0.894	0.998	0.871	0.996
TLR2	0.691	0.993	0.997	0.932	0.868	0.995	0.706	0.946	1.000
TLR4	0.800	0.995	1.000	0.999	0.922	0.967	0.888	0.998	0.978
TLR6	0.908	0.977	0.996	0.996	0.998	1.000	0.960	1.000	0.960
TNF	0.240	0.751	0.182	0.940	0.971	0.991	0.439	0.776	1.000
TNFRSF1A	0.363	0.655	0.258	0.766	0.704	1.000	0.912	0.938	1.000
TP53	0.584	0.838	0.489	0.752	0.839	1.000	0.969	0.969	1.000
TRAIL	0.361	0.580	1.000	0.998	0.975	0.753	0.960	0.629	0.897
TRAIL-R	0.617	0.614	0.977	0.737	0.986	0.999	1.000	0.905	0.947
VCAM1	0.156	1.000	1.000	0.995	0.288	0.997	0.271	1.000	0.159

- Tracheobronchial lymph node

Gene	Mean ± SD				
	PBS	T15		S10	
	Day1	Day1	Day3	Day1	Day3
APOA1	1 ± 0.57	0.95 ± 0.38	3.78 ± 5.84	2.52 ± 3.38	0.63 ± 0.13
BCL2	1 ± 0.45	1.04 ± 0.34	0.66 ± 0.24	1.43 ± 0.43	0.92 ± 0.35
C3	1 ± 0.18	0.75 ± 0.21	0.71 ± 0.26	0.86 ± 0.23	0.70 ± 0.18
CASP1	1 ± 0.10	1.74 ± 0.75	1.43 ± 0.28	2.25 ± 0.74	1.64 ± 0.23
CCL2	1 ± 0.22	1.54 ± 0.68	1.64 ± 0.47	1.25 ± 0.40	1.41 ± 0.25
CCL3	1 ± 0.54	0.94 ± 0.19	1.27 ± 0.33	1.03 ± 0.48	1.12 ± 0.24
CCL4	1 ± 0.44	1.15 ± 0.28	1.26 ± 0.41	1.15 ± 0.58	1.29 ± 0.26
CCL5	1 ± 0.55	0.78 ± 0.08	1.08 ± 0.36	1.06 ± 0.93	1.21 ± 0.40
CD163	1 ± 0.21	1.29 ± 0.79	0.64 ± 0.37	0.90 ± 0.40	0.56 ± 0.28
CXCL10	1 ± 0.98	4.11 ± 3.93	2.34 ± 1.45	3.80 ± 3.59	1.82 ± 1.06
CXCR2	1 ± 0.26	1.67 ± 1.14	0.83 ± 0.34	3.87 ± 5.11	0.92 ± 0.27
CXCR3	1 ± 0.45	0.67 ± 0.14	0.91 ± 0.33	0.87 ± 0.45	0.92 ± 0.29

FAS	1 ± 0.17	0.93 ± 0.28	0.72 ± 0.12	0.94 ± 0.19	0.92 ± 0.24
FOS	1 ± 0.15	1.39 ± 0.42	1.31 ± 0.69	1.85 ± 1.11	1.07 ± 0.45
HP	1 ± 0.23	1.06 ± 0.44	1.46 ± 0.68	0.94 ± 0.23	0.90 ± 0.17
HSPA14	1 ± 0.15	1.00 ± 0.14	1.03 ± 0.10	1.10 ± 0.21	1.26 ± 0.26
ICAM1	1 ± 0.24	1.12 ± 0.21	1.00 ± 0.13	1.42 ± 0.40	0.98 ± 0.20
ICAM2	1 ± 0.13	1.18 ± 0.15	1.11 ± 0.21	1.15 ± 0.20	1.19 ± 0.07
IFITM1	1 ± 0.56	2.45 ± 1.90	1.52 ± 0.84	2.43 ± 1.39	1.30 ± 0.33
IFNA1	1 ± 0.32	1.10 ± 0.22	0.73 ± 0.50	1.15 ± 0.47	0.71 ± 0.37
IFNG	1 ± 0.35	1.22 ± 0.53	1.54 ± 0.59	1.19 ± 0.73	1.72 ± 0.51
IKBKB	1 ± 0.16	0.83 ± 0.06	0.72 ± 0.18	0.96 ± 0.23	0.80 ± 0.19
IL10	1 ± 0.42	1.20 ± 0.53	1.04 ± 0.20	1.01 ± 0.24	0.90 ± 0.27
IL12B	1 ± 0.53	1.31 ± 0.64	1.34 ± 0.51	1.68 ± 0.98	1.07 ± 0.42
IL18	1 ± 0.32	1.12 ± 0.66	0.82 ± 0.18	0.87 ± 0.16	0.88 ± 0.49
IL1A	1 ± 0.24	1.22 ± 0.37	1.38 ± 0.55	1.39 ± 0.54	1.31 ± 0.27
IL1B	1 ± 0.55	4.15 ± 2.80	4.59 ± 2.58	3.35 ± 1.86	2.69 ± 0.75
IL1R1	1 ± 0.11	1.05 ± 0.11	1.07 ± 0.18	1.16 ± 0.24	0.93 ± 0.14
IL1RN	1 ± 0.38	3.92 ± 2.78	3.79 ± 1.18	5.88 ± 3.49	4.06 ± 2.44
IL23	1 ± 0.19	1.35 ± 0.76	0.69 ± 0.21	1.23 ± 0.44	0.69 ± 0.17
IL6	1 ± 0.30	0.99 ± 0.18	1.15 ± 0.26	1.11 ± 0.19	1.32 ± 0.17
IL8	1 ± 0.25	2.13 ± 1.58	2.47 ± 1.41	3.65 ± 4.38	2.18 ± 1.46
IRF3	1 ± 0.23	0.80 ± 0.24	0.66 ± 0.13	1.00 ± 0.23	0.87 ± 0.27
IRF7	1 ± 0.58	2.47 ± 2.03	1.17 ± 0.45	2.77 ± 1.72	1.29 ± 0.56
ITIH4	1 ± 0.32	1.15 ± 0.26	1.27 ± 0.51	0.99 ± 0.44	1.38 ± 0.18
JUN	1 ± 0.25	1.12 ± 0.14	1.09 ± 0.57	1.28 ± 0.22	1.08 ± 0.45
MCL1	1 ± 0.46	1.39 ± 0.37	1.04 ± 0.23	1.45 ± 0.40	1.07 ± 0.19
MYD88	1 ± 0.15	0.84 ± 0.16	0.91 ± 0.21	0.98 ± 0.27	0.83 ± 0.08
NFKB1	1 ± 0.10	0.96 ± 0.17	0.70 ± 0.13	1.02 ± 0.07	0.85 ± 0.28
NFKBIA	1 ± 0.11	0.89 ± 0.16	0.68 ± 0.07	1.01 ± 0.19	0.69 ± 0.08
PTGS2	1 ± 0.36	1.34 ± 0.68	1.46 ± 0.77	1.32 ± 0.51	1.29 ± 0.12
SAA	1 ± 0.70	3.97 ± 6.17	1.90 ± 2.64	6.62 ± 9.87	1.41 ± 0.59
SELP	1 ± 0.08	1.27 ± 0.17	1.53 ± 0.20	1.64 ± 0.48	1.44 ± 0.53
STAT1	1 ± 0.26	1.47 ± 0.75	0.91 ± 0.15	1.46 ± 0.62	0.87 ± 0.22
STAT2	1 ± 0.26	1.66 ± 0.90	0.79 ± 0.24	1.64 ± 0.58	0.95 ± 0.37
TF	1 ± 0.79	1.47 ± 0.85	3.41 ± 5.29	3.41 ± 6.00	0.35 ± 0.18
TLR1	1 ± 0.22	0.95 ± 0.20	0.85 ± 0.04	0.99 ± 0.26	1.12 ± 0.37
TLR2	1 ± 0.09	0.95 ± 0.27	0.84 ± 0.05	1.15 ± 0.18	0.89 ± 0.17
TLR4	1 ± 0.17	1.16 ± 0.59	0.87 ± 0.25	0.97 ± 0.20	0.75 ± 0.25
TLR6	1 ± 0.15	0.87 ± 0.36	0.74 ± 0.30	1.04 ± 0.24	0.85 ± 0.27
TNF	1 ± 0.23	1.07 ± 0.27	1.12 ± 0.25	1.05 ± 0.22	1.02 ± 0.58
TNFRSF1A	1 ± 0.05	0.96 ± 0.27	0.86 ± 0.17	1.05 ± 0.26	0.70 ± 0.19
TP53	1 ± 0.12	0.91 ± 0.12	0.78 ± 0.10	1.08 ± 0.07	1.01 ± 0.20
TRAIL	1 ± 0.53	1.24 ± 0.79	0.55 ± 0.13	1.17 ± 0.41	0.58 ± 0.09
TRAIL-R	1 ± 0.17	1.21 ± 1.09	0.61 ± 0.19	1.08 ± 0.29	0.72 ± 0.04
VCAM1	1 ± 0.32	1.11 ± 0.22	0.72 ± 0.09	0.96 ± 0.19	0.74 ± 0.17

Gene	***P*-values**								
	ANOVA	**PBS group *vs*. T15**		**PBS group *vs*. S10**		**T15 *vs*. S10**		**Day1 *vs*. Day 3**	
		Day1	**Day3**	**Day1**	**Day3**	**Day1**	**Day3**	**T15**	**S10**
APOA1	0.556	1.000	0.698	0.952	1.000	0.946	0.597	0.597	0.683
BCL2	0.108	1.000	0.686	0.490	0.998	0.587	0.845	0.845	0.587
C3	0.280	0.484	0.343	0.873	0.321	0.950	1.000	1.000	0.999
CASP1	**0.039***	0.272	0.750	**0.022***	0.401	0.619	0.971	0.971	0.895
CCL2	0.307	0.447	0.284	0.927	0.680	0.881	0.944	0.944	0.997

CCL3	0.771	0.999	0.851	1.000	0.991	0.998	0.979	0.979	0.743
CCL4	0.860	0.985	0.889	0.986	0.846	1.000	1.000	1.000	0.994
CCL5	0.846	0.976	1.000	1.000	0.981	0.947	0.997	0.997	0.929
CD163	0.217	0.900	0.793	0.998	0.654	0.760	0.999	0.999	0.312
CXCL10	0.406	0.450	0.942	0.546	0.990	1.000	0.998	0.998	0.861
CXCR2	0.358	0.994	1.000	0.450	1.000	0.683	1.000	1.000	0.986
CXCR3	0.736	0.672	0.995	0.983	0.997	0.923	1.000	1.000	0.870
FAS	0.410	0.989	0.353	0.995	0.977	1.000	0.675	0.675	0.612
FOS	0.408	0.912	0.957	0.385	1.000	0.851	0.982	0.982	1.000
HP	0.318	0.999	0.495	1.000	0.996	0.992	0.313	0.313	0.625
HSPA14	0.266	1.000	0.999	0.932	0.300	0.940	0.409	0.409	1.000
ICAM1	0.121	0.958	1.000	0.179	1.000	0.471	1.000	1.000	0.953
ICAM2	0.484	0.537	0.844	0.694	0.472	0.999	0.961	0.961	0.980
IFITM1	0.305	0.421	0.967	0.437	0.996	1.000	0.999	0.999	0.783
IFNA1	0.386	0.996	0.863	0.982	0.820	1.000	1.000	1.000	0.681
IFNG	0.400	0.979	0.651	0.988	0.397	1.000	0.991	0.991	0.922
IKBKB	0.191	0.634	0.212	0.998	0.525	0.807	0.960	0.960	0.909
IL10	0.824	0.927	1.000	1.000	0.994	0.935	0.982	0.982	0.962
IL12B	0.617	0.957	0.944	0.588	1.000	0.927	0.976	0.976	1.000
IL18	0.837	0.993	0.970	0.990	0.993	0.901	1.000	1.000	0.837
IL1A	0.673	0.946	0.704	0.680	0.832	0.973	0.999	0.999	0.980
IL1B	0.130	0.199	0.117	0.457	0.735	0.975	0.645	0.645	0.998
IL1R1	0.388	0.991	0.967	0.652	0.968	0.880	0.716	0.716	1.000
IL1RN	0.115	0.429	0.472	0.065	0.386	0.761	1.000	1.000	1.000
IL23	0.133	0.758	0.830	0.938	0.828	0.992	1.000	1.000	0.220
IL6	0.262	1.000	0.866	0.959	0.301	0.939	0.824	0.824	0.829
IL8	0.608	0.953	0.888	0.492	0.945	0.874	1.000	1.000	0.999
IRF3	0.214	0.729	0.250	1.000	0.923	0.727	0.672	0.672	0.888
IRF7	0.208	0.491	1.000	0.319	0.997	0.997	1.000	1.000	0.606
ITIH4	0.494	0.973	0.829	1.000	0.581	0.964	0.991	0.991	0.991
JUN	0.855	0.989	0.996	0.802	0.997	0.966	1.000	1.000	1.000
MCL1	0.254	0.519	1.000	0.398	0.998	0.999	1.000	1.000	0.616
MYD88	0.607	0.746	0.958	1.000	0.697	0.830	0.970	0.970	0.983
NFKB1	0.078	0.997	0.128	1.000	0.686	0.983	0.724	0.724	0.222
NFKBIA	**0.004***	0.737	**0.021***	1.000	**0.030***	0.698	1.000	1.000	0.196
PTGS2	0.807	0.894	0.750	0.916	0.941	1.000	0.991	0.991	0.998
SAA	0.566	0.931	0.999	0.586	1.000	0.953	1.000	1.000	0.981
SELP	0.127	0.790	0.237	0.110	0.400	0.563	0.996	0.996	0.825
STAT1	0.224	0.627	0.999	0.646	0.995	1.000	1.000	1.000	0.475
STAT2	0.099	0.438	0.978	0.467	1.000	1.000	0.991	0.991	0.195
TF	0.663	1.000	0.875	0.876	0.999	0.938	0.753	0.753	0.938
TLR1	0.639	0.999	0.895	1.000	0.955	1.000	0.529	0.529	0.968
TLR2	0.152	0.991	0.698	0.712	0.892	0.452	0.994	0.994	0.911
TLR4	0.514	0.955	0.979	1.000	0.824	0.914	0.986	0.986	0.722
TLR6	0.553	0.964	0.659	1.000	0.929	0.915	0.977	0.977	0.950
TNF	0.987	0.998	0.984	0.999	1.000	1.000	0.993	0.993	1.000
TNFRSF1A	0.186	0.998	0.870	0.998	0.285	0.967	0.799	0.799	0.966
TP53	**0.047***	0.849	0.183	0.898	1.000	0.363	0.150	0.150	0.668
TRAIL	0.159	0.948	0.657	0.986	0.715	0.999	1.000	1.000	0.274
TRAIL-R	0.474	0.977	0.822	1.000	0.939	0.996	0.998	0.998	0.500
VCAM1	0.079	0.942	0.376	0.999	0.444	0.858	1.000	1.000	0.118

- Liver

Gene	Mean ± SD				
	PBS	T15		S10	
	Day1	Day1	Day3	Day1	Day3
APOA1	1 ± 0.09	0.95 ± 0.22	0.98 ± 0.07	1.00 ± 0.30	1.12 ± 0.25
BCL2	1 ± 0.19	1.39 ± 0.16	1.05 ± 0.07	0.74 ± 0.25	1.01 ± 0.23
C3	1 ± 0.17	0.92 ± 0.19	1.12 ± 0.13	0.98 ± 0.20	1.12 ± 0.13
C5	1 ± 0.12	1.00 ± 0.20	1.16 ± 0.26	1.21 ± 0.29	1.13 ± 0.09
CASP1	1 ± 0.19	1.19 ± 0.51	1.08 ± 0.30	1.45 ± 0.72	1.26 ± 0.29
CASP3	1 ± 0.15	0.95 ± 0.15	0.96 ± 0.15	0.78 ± 0.05	0.77 ± 0.12
CASP8	1 ± 0.15	1.22 ± 0.39	0.94 ± 0.09	0.96 ± 0.25	0.85 ± 0.06
CCL2	1 ± 0.12	1.25 ± 0.38	0.94 ± 0.29	0.74 ± 0.21	0.91 ± 0.23
CCL4	1 ± 0.36	0.92 ± 0.27	0.80 ± 0.25	0.75 ± 0.19	0.79 ± 0.33
CCL5	1 ± 1.28	0.32 ± 0.09	0.38 ± 0.04	0.31 ± 0.13	0.41 ± 0.05
CD163	1 ± 0.16	1.01 ± 0.54	0.87 ± 0.31	0.83 ± 0.15	0.88 ± 0.13
CFH	1 ± 0.12	0.96 ± 0.17	1.17 ± 0.38	0.97 ± 0.21	1.26 ± 0.36
CRP	1 ± 0.69	1.18 ± 0.48	1.94 ± 0.89	1.90 ± 1.19	1.82 ± 0.49
FAS	1 ± 0.43	1.16 ± 0.25	1.03 ± 0.38	0.84 ± 0.38	0.91 ± 0.17
FASLG	1 ± 0.60	0.56 ± 0.12	0.45 ± 0.11	0.42 ± 0.08	0.47 ± 0.12
HP	1 ± 0.35	1.30 ± 0.23	1.37 ± 0.08	1.54 ± 0.39	1.22 ± 0.24
ICAM1	1 ± 0.15	1.39 ± 0.18	1.26 ± 0.21	0.96 ± 0.39	0.99 ± 0.13
ICAM2	1 ± 0.46	0.96 ± 0.38	0.75 ± 0.35	0.51 ± 0.11	0.67 ± 0.21
IFNA1	1 ± 0.21	1.33 ± 0.33	0.96 ± 0.03	0.89 ± 0.18	0.92 ± 0.20
IFNG	1 ± 1.30	0.33 ± 0.19	0.49 ± 0.30	0.28 ± 0.17	0.62 ± 0.21
IL10	1 ± 0.36	0.98 ± 0.34	0.74 ± 0.46	0.55 ± 0.12	0.55 ± 0.13
IL18	1 ± 0.22	0.94 ± 0.71	0.82 ± 0.45	0.75 ± 0.15	0.90 ± 0.47
IL1B	1 ± 0.50	0.73 ± 0.46	1.04 ± 0.78	0.41 ± 0.26	0.75 ± 0.37
IL1RN	1 ± 0.31	1.68 ± 0.62	1.10 ± 0.32	1.14 ± 0.07	0.98 ± 0.20
IL6	1 ± 0.20	1.22 ± 0.73	1.10 ± 0.66	0.58 ± 0.25	1.02 ± 0.18
IL8	1 ± 0.80	0.85 ± 0.44	0.77 ± 0.53	0.58 ± 0.12	0.55 ± 0.25
IRF3	1 ± 0.09	0.91 ± 0.26	0.91 ± 0.03	0.91 ± 0.19	1.06 ± 0.07
IRF7	1 ± 0.71	1.98 ± 1.51	0.74 ± 0.15	1.21 ± 0.72	0.83 ± 0.09
ITIH4	1 ± 0.49	1.20 ± 0.36	1.52 ± 0.84	1.50 ± 1.09	1.32 ± 0.33
MCL1	1 ± 0.17	1.12 ± 0.32	0.94 ± 0.21	0.85 ± 0.16	0.73 ± 0.08
NLRP3	1 ± 0.35	1.00 ± 0.37	1.02 ± 0.31	0.86 ± 0.29	1.07 ± 0.16
ORM1	1 ± 0.14	0.86 ± 0.11	0.83 ± 0.08	1.04 ± 0.21	0.68 ± 0.16
SAA	1 ± 1.23	1.18 ± 0.89	2.12 ± 2.60	3.29 ± 2.55	2.86 ± 1.94
SELP	1 ± 0.34	1.06 ± 0.31	1.14 ± 0.38	0.72 ± 0.13	0.93 ± 0.23
TF	1 ± 0.12	0.89 ± 0.16	0.93 ± 0.11	0.85 ± 0.05	0.95 ± 0.09
TLR1	1 ± 0.33	1.20 ± 0.23	1.36 ± 0.26	1.40 ± 0.43	1.29 ± 0.39
TLR2	1 ± 0.13	1.20 ± 0.30	1.23 ± 0.39	1.23 ± 0.43	1.33 ± 0.10
TLR4	1 ± 0.12	0.92 ± 0.43	0.92 ± 0.22	1.01 ± 0.17	1.04 ± 0.07
TLR6	1 ± 0.17	1.50 ± 0.35	1.59 ± 0.31	1.44 ± 0.52	1.49 ± 0.23
TNF	1 ± 0.40	0.98 ± 0.23	0.95 ± 0.35	0.68 ± 0.29	0.98 ± 0.31
TNFRSF1A	1 ± 0.08	1.29 ± 0.05	1.24 ± 0.15	0.92 ± 0.28	1.11 ± 0.14
TP53	1 ± 0.13	0.95 ± 0.15	0.89 ± 0.08	0.66 ± 0.13	0.98 ± 0.08
TRAIL	1 ± 0.42	1.34 ± 0.65	0.88 ± 0.08	0.92 ± 0.30	0.64 ± 0.09
TRAIL-R	1 ± 0.33	2.02 ± 1.39	0.82 ± 0.07	1.21 ± 0.90	0.92 ± 0.26
VCAM1	1 ± 0.31	1.00 ± 0.48	0.76 ± 0.09	0.84 ± 0.37	0.85 ± 0.20

Gene	*P*-value								
	ANOVA	**PBS group *vs*. T15**		**PBS group *vs*. S10**		**T15 *vs*. S10**		**Day1 *vs*. Day 3**	
		Day1	**Day3**	**Day1**	**Day3**	**Day1**	**Day3**	**T15**	**S10**
APOA1	0.809	0.996	1.000	1.000	0.923	0.995	0.862	1.000	0.929
BCL2	**0.005***	0.071	0.996	0.346	1.000	**0.002***	0.999	0.134	0.305
C3	0.340	0.952	0.836	1.000	0.819	0.985	1.000	0.441	0.717
C5	0.520	1.000	0.801	0.628	0.904	0.628	0.999	0.801	0.981
CASP1	0.659	0.971	0.999	0.616	0.914	0.919	0.976	0.996	0.973
CASP3	0.061	0.983	0.994	0.173	0.136	0.379	0.261	1.000	1.000
CASP8	0.254	0.651	0.996	0.999	0.871	0.500	0.974	0.445	0.955
CCL2	0.146	0.672	0.998	0.626	0.987	0.091	1.000	0.488	0.884
CCL4	0.711	0.993	0.847	0.725	0.831	0.917	1.000	0.974	1.000
CCL5	0.427	0.480	0.562	0.466	0.617	1.000	1.000	1.000	0.999
CD163	0.865	1.000	0.971	0.920	0.975	0.901	1.000	0.960	0.999
CFH	0.436	0.999	0.885	1.000	0.668	1.000	0.992	0.779	0.588
CRP	0.336	0.997	0.478	0.518	0.603	0.708	0.999	0.667	1.000
FAS	0.718	0.961	1.000	0.956	0.996	0.664	0.987	0.982	0.997
FASLG	0.064	0.236	0.099	0.075	0.122	0.958	1.000	0.983	0.999
HP	0.144	0.556	0.380	0.097	0.807	0.757	0.937	0.997	0.501
ICAM1	0.064	0.174	0.536	0.999	1.000	0.111	0.498	0.923	1.000
ICAM2	0.240	1.000	0.818	0.263	0.623	0.345	0.996	0.899	0.955
IFNA1	0.069	0.245	0.999	0.945	0.984	0.071	0.999	0.167	0.999
IFNG	0.505	0.556	0.769	0.491	0.898	1.000	0.998	0.996	0.937
IL10	0.150	1.000	0.777	0.300	0.305	0.333	0.905	0.814	1.000
IL18	0.940	1.000	0.979	0.931	0.998	0.974	0.999	0.995	0.989
IL1B	0.437	0.938	1.000	0.488	0.956	0.895	0.928	0.905	0.867
IL1RN	0.075	0.098	0.995	0.982	1.000	0.238	0.99	0.187	0.971
IL6	0.418	0.963	0.998	0.718	1.000	0.352	0.999	0.996	0.692
IL8	0.669	0.991	0.957	0.731	0.696	0.929	0.97	0.999	1.000
IRF3	0.545	0.906	0.915	0.913	0.984	1.000	0.665	1.000	0.662
IRF7	0.257	0.461	0.991	0.996	0.998	0.669	1.000	0.250	0.965
ITIH4	0.808	0.994	0.825	0.836	0.963	0.968	0.994	0.963	0.995
MCL1	0.140	0.911	0.993	0.848	0.397	0.379	0.636	0.712	0.922
NLRP3	0.904	1.000	1.000	0.968	0.997	0.964	0.999	1.000	0.868
ORM1	**0.023***	0.650	0.508	0.996	**0.047***	0.438	0.576	0.999	**0.023***
SAA	0.415	1.000	0.925	0.492	0.672	0.568	0.982	0.959	0.998
SELP	0.366	0.999	0.960	0.670	0.997	0.511	0.854	0.994	0.842
TF	0.389	0.658	0.902	0.333	0.975	0.975	0.998	0.987	0.658
TLR1	0.502	0.910	0.574	0.486	0.741	0.924	0.998	0.963	0.991
TLR2	0.640	0.888	0.812	0.819	0.551	1.000	0.990	1.000	0.988
TLR4	0.929	0.991	0.987	1.000	0.999	0.983	0.950	1.000	1.000
TLR6	0.168	0.281	0.151	0.398	0.297	0.999	0.992	0.994	0.999
TNF	0.611	1.000	0.999	0.637	1.000	0.683	1.000	1.000	0.697
TNFRSF1A	**0.023***	0.126	0.243	0.950	0.863	**0.034***	0.753	0.994	0.471
TP53	**0.007***	0.981	0.724	**0.009***	0.999	**0.026***	0.851	0.953	**0.014***
TRAIL	0.183	0.708	0.991	0.998	0.673	0.526	0.895	0.450	0.838
TRAIL-R	0.224	0.361	0.997	0.994	1.000	0.579	1.000	0.224	0.981
VCAM1	0.769	1.000	0.822	0.947	0.959	0.945	0.995	0.818	1.000

- Spleen

Gene	Mean ± SD				
	PBS	T15		S10	
	Day1	Day1	Day3	Day1	Day3
APOA1	1 ± 0.45	1.19 ± 0.36	1.35 ± 0.34	1.23 ± 0.70	1.10 ± 0.41
BCL2	1 ± 0.36	0.96 ± 0.38	0.43 ± 0.21	0.80 ± 0.20	0.66 ± 0.26
C3	1 ± 0.12	0.97 ± 0.16	0.77 ± 0.41	1.33 ± 0.92	1.00 ± 0.32
CASP1	1 ± 0.51	2.41 ± 1.78	1.24 ± 0.54	2.46 ± 0.93	1.04 ± 0.21
CCL2	1 ± 0.75	1.26 ± 0.58	0.84 ± 0.35	0.90 ± 0.36	0.62 ± 0.26
CCL3	1 ± 0.54	1.05 ± 0.21	1.02 ± 0.45	0.73 ± 0.16	0.91 ± 0.33
CCL4	1 ± 0.40	0.98 ± 0.27	1.00 ± 0.52	0.97 ± 0.42	0.95 ± 0.44
CCL5	1 ± 1.05	0.76 ± 0.24	0.76 ± 0.25	0.81 ± 0.54	0.86 ± 0.32
CD163	1 ± 0.41	2.09 ± 0.51	0.99 ± 0.31	1.67 ± 1.22	1.37 ± 0.49
CXCL10	1 ± 1.59	2.06 ± 2.24	0.67 ± 0.70	1.11 ± 1.33	0.33 ± 0.20
CXCR2	1 ± 0.42	1.03 ± 0.46	0.91 ± 0.49	1.22 ± 0.63	1.13 ± 0.48
CXCR3	1 ± 0.45	0.44 ± 0.16	0.83 ± 0.31	0.68 ± 0.41	0.92 ± 0.24
FAS	1 ± 0.27	0.80 ± 0.24	0.58 ± 0.19	0.63 ± 0.14	0.80 ± 0.32
FOS	1 ± 0.19	1.26 ± 0.90	0.86 ± 0.29	1.01 ± 0.64	0.87 ± 0.49
HP	1 ± 0.45	0.62 ± 0.25	0.61 ± 0.40	0.87 ± 0.30	0.66 ± 0.11
HSPA14	1 ± 0.25	0.87 ± 0.09	1.00 ± 0.17	1.15 ± 0.11	1.12 ± 0.23
ICAM1	1 ± 0.17	0.94 ± 0.16	0.73 ± 0.19	0.89 ± 0.18	0.72 ± 0.20
ICAM2	1 ± 0.31	0.71 ± 0.13	0.74 ± 0.15	0.64 ± 0.15	0.79 ± 0.09
IFITM1	1 ± 0.74	1.74 ± 1.31	0.67 ± 0.34	1.40 ± 0.69	0.62 ± 0.15
IFNA1	1 ± 0.22	0.99 ± 0.18	0.60 ± 0.43	0.89 ± 0.11	0.65 ± 0.33
IFNG	1 ± 0.51	0.97 ± 0.41	1.73 ± 1.06	0.84 ± 0.24	1.29 ± 0.37
IKBKB	1 ± 0.18	0.83 ± 0.05	0.68 ± 0.13	0.68 ± 0.17	0.79 ± 0.16
IL10	1 ± 0.47	0.86 ± 0.09	0.80 ± 0.52	0.55 ± 0.22	1.01 ± 0.52
IL12A	1 ± 0.72	1.61 ± 1.14	0.55 ± 0.37	1.15 ± 0.22	0.66 ± 0.07
IL12B	1 ± 0.65	2.22 ± 2.36	1.44 ± 0.98	1.34 ± 0.73	0.90 ± 0.54
IL18	1 ± 0.21	1.25 ± 0.38	1.34 ± 0.61	1.43 ± 0.15	1.43 ± 0.64
IL1A	1 ± 0.49	1.39 ± 0.47	1.23 ± 0.42	1.30 ± 0.49	0.98 ± 0.33
IL1B	1 ± 1.02	1.62 ± 1.38	1.00 ± 0.38	1.12 ± 0.68	0.68 ± 0.26
IL1R1	1 ± 0.22	1.50 ± 0.44	1.41 ± 0.30	1.45 ± 0.68	0.94 ± 0.32
IL1RN	1 ± 1.19	1.99 ± 1.68	0.50 ± 0.18	1.10 ± 1.13	0.42 ± 0.22
IL23	1 ± 0.28	1.43 ± 0.83	0.57 ± 0.20	1.11 ± 0.38	0.77 ± 0.23
IL6	1 ± 0.29	1.31 ± 0.24	1.18 ± 0.25	1.13 ± 0.16	1.05 ± 0.47
IL8	1 ± 0.40	0.94 ± 0.29	2.28 ± 1.44	1.73 ± 1.74	1.37 ± 1.04
IRF3	1 ± 0.13	1.01 ± 0.19	0.69 ± 0.18	0.90 ± 0.13	1.00 ± 0.26
IRF7	1 ± 0.91	1.86 ± 1.64	0.50 ± 0.26	1.23 ± 0.52	0.39 ± 0.08
JUN	1 ± 0.15	0.97 ± 0.26	0.62 ± 0.14	0.81 ± 0.23	0.78 ± 0.27
LTF	1 ± 1.03	0.75 ± 0.50	0.43 ± 0.38	0.65 ± 0.31	0.27 ± 0.14
MCL1	1 ± 0.37	0.83 ± 0.22	0.65 ± 0.11	0.88 ± 0.12	0.73 ± 0.11
MYD88	1 ± 0.09	0.96 ± 0.14	0.77 ± 0.14	0.73 ± 0.06	0.77 ± 0.11
NFKB1	1 ± 0.06	1.13 ± 0.16	0.82 ± 0.24	0.94 ± 0.02	0.85 ± 0.18
NFKBIA	1 ± 0.05	1.10 ± 0.33	0.79 ± 0.09	1.11 ± 0.07	0.98 ± 0.17
PTGS2	1 ± 0.57	0.67 ± 0.25	0.67 ± 0.71	0.37 ± 0.23	0.49 ± 0.33
PTX3	1 ± 0.23	0.99 ± 0.22	1.21 ± 0.10	1.19 ± 1.02	0.90 ± 0.67
SAA	1 ± 0.29	0.98 ± 0.40	3.50 ± 3.41	65.00 ± 127.75	1.82 ± 0.89
SELP	1 ± 0.34	1.23 ± 0.46	0.98 ± 0.20	1.21 ± 0.44	1.02 ± 0.25
STAT1	1 ± 0.40	1.47 ± 0.84	0.67 ± 0.23	1.03 ± 0.28	0.67 ± 0.19
STAT2	1 ± 0.42	1.45 ± 0.76	0.62 ± 0.14	1.05 ± 0.30	0.61 ± 0.09
TF	1 ± 0.63	0.82 ± 0.43	0.51 ± 0.38	1.19 ± 0.78	0.85 ± 0.60

TLR1	1 ± 0.27	1.03 ± 0.19	0.96 ± 0.11	1.41 ± 0.41	1.05 ± 0.27
TLR2	1 ± 0.15	0.92 ± 0.17	0.90 ± 0.18	0.83 ± 0.03	0.90 ± 0.18
TLR4	1 ± 0.25	1.14 ± 0.29	0.96 ± 0.28	1.05 ± 0.24	0.98 ± 0.31
TLR6	1 ± 0.18	0.99 ± 0.19	0.80 ± 0.32	1.06 ± 0.27	0.78 ± 0.21
TNF	1 ± 0.22	1.16 ± 0.30	1.06 ± 0.30	0.81 ± 0.09	0.84 ± 0.39
TNFRSF1A	1 ± 0.23	1.12 ± 0.08	0.87 ± 0.19	0.85 ± 0.20	0.90 ± 0.25
TP53	1 ± 0.09	0.86 ± 0.09	0.76 ± 0.09	0.93 ± 0.33	0.80 ± 0.18
TRAIL	1 ± 0.74	0.83 ± 0.63	0.19 ± 0.04	0.40 ± 0.17	0.36 ± 0.21
TRAIL-R	1 ± 0.47	1.34 ± 0.81	0.46 ± 0.18	1.02 ± 0.45	0.65 ± 0.20
VCAM1	1 ± 0.19	1.12 ± 0.07	0.85 ± 0.36	1.30 ± 0.38	0.85 ± 0.12

Gene	***P*-value**								
	ANOVA	**PBS group *vs*. T15**		**PBS group *vs*. S10**		**T15 *vs*. S10**		**Day1 *vs*. Day 3**	
		Day1	**Day3**	**Day1**	**Day3**	**Day1**	**Day3**	**T15**	**S10**
APOA1	0.864	0.976	0.832	0.953	0.998	1.000	0.942	0.990	0.994
BCL2	0.083	1.000	0.092	0.869	0.496	0.936	0.796	0.128	0.957
C3	0.598	1.000	0.957	0.861	1.000	0.825	0.960	0.973	0.855
CASP1	0.097	0.277	0.996	0.249	1.000	1.000	0.998	0.446	0.271
CCL2	0.493	0.944	0.991	0.998	0.806	0.842	0.964	0.761	0.923
CCL3	0.736	1.000	1.000	0.835	0.997	0.730	0.992	1.000	0.955
CCL4	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
CCL5	0.972	0.975	0.972	0.989	0.997	1.000	0.999	1.000	1.000
CD163	0.155	0.198	1.000	0.631	0.933	0.896	0.927	0.192	0.968
CXCL10	0.512	0.821	0.997	1.000	0.958	0.869	0.997	0.638	0.931
CXCR2	0.918	1.000	0.999	0.971	0.996	0.984	0.968	0.996	0.999
CXCR3	0.182	0.168	0.945	0.663	0.997	0.830	0.994	0.482	0.844
FAS	0.151	0.765	0.136	0.238	0.753	0.847	0.676	0.664	0.857
FOS	0.857	0.965	0.996	1.000	0.997	0.970	1.000	0.851	0.996
HP	0.374	0.498	0.461	0.977	0.587	0.822	0.999	1.000	0.889
HSPA14	0.252	0.856	1.000	0.753	0.890	0.237	0.879	0.868	0.998
ICAM1	0.144	0.990	0.243	0.899	0.229	0.993	1.000	0.459	0.680
ICAM2	0.114	0.221	0.303	0.086	0.504	0.978	0.994	1.000	0.769
IFITM1	0.223	0.646	0.970	0.943	0.952	0.966	1.000	0.311	0.608
IFNA1	0.172	1.000	0.295	0.981	0.422	0.988	0.999	0.324	0.732
IFNG	0.262	1.000	0.431	0.995	0.953	0.998	0.825	0.390	0.815
IKBKB	**0.040***	0.501	0.051	**0.045***	0.281	0.573	0.851	0.613	0.820
IL10	0.513	0.987	0.955	0.542	1.000	0.820	0.942	1.000	0.513
IL12A	0.188	0.655	0.854	0.997	0.940	0.833	0.999	0.179	0.814
IL12B	0.600	0.644	0.987	0.995	1.000	0.852	0.971	0.895	0.985
IL18	0.648	0.929	0.811	0.666	0.666	0.979	0.999	0.998	1.000
IL1A	0.627	0.736	0.946	0.866	1.000	0.999	0.932	0.986	0.843
IL1B	0.634	0.836	1.000	1.000	0.982	0.917	0.982	0.835	0.942
IL1R1	0.235	0.489	0.665	0.574	1.000	1.000	0.554	0.998	0.465
IL1RN	0.280	0.684	0.959	1.000	0.934	0.761	1.000	0.315	0.889
IL23	0.127	0.655	0.673	0.996	0.951	0.844	0.968	0.100	0.823
IL6	0.641	0.607	0.908	0.969	0.999	0.918	0.968	0.974	0.994
IL8	0.458	1.000	0.523	0.889	0.990	0.858	0.785	0.479	0.990
IRF3	0.120	1.000	0.173	0.935	1.000	0.912	0.176	0.153	0.939
IRF7	0.178	0.649	0.928	0.996	0.860	0.845	1.000	0.241	0.668
JUN	0.148	0.999	0.152	0.720	0.625	0.832	0.831	0.215	1.000
LTF	0.423	0.967	0.608	0.898	0.383	0.999	0.994	0.922	0.867
MCL1	0.216	0.794	0.187	0.929	0.401	0.997	0.984	0.742	0.841
MYD88	**0.009***	0.983	0.064	**0.027***	0.065	0.072	1.000	0.162	0.988

NFKB1	0.081	0.765	0.485	0.977	0.661	0.437	0.998	0.080	0.931
NFKBIA	0.124	0.926	0.462	0.905	1.000	1.000	0.555	0.142	0.839
PTGS2	0.402	0.846	0.840	0.336	0.540	0.878	0.982	1.000	0.995
PTX3	0.919	1.000	0.984	0.989	0.999	0.985	0.937	0.979	0.949
SAA	0.448	1.000	1.000	0.529	1.000	0.528	1.000	1.000	0.541
SELP	0.761	0.889	1.000	0.913	1.000	1.000	1.000	0.856	0.941
STAT1	0.128	0.597	0.837	1.000	0.836	0.654	1.000	0.143	0.788
STAT2	0.066	0.566	0.697	1.000	0.694	0.665	1.000	0.082	0.595
TF	0.584	0.992	0.762	0.990	0.996	0.895	0.920	0.942	0.921
TLR1	0.175	1.000	1.000	0.245	0.998	0.312	0.988	0.996	0.368
TLR2	0.643	0.937	0.895	0.530	0.875	0.921	1.000	1.000	0.966
TLR4	0.896	0.955	1.000	0.999	1.000	0.992	1.000	0.893	0.994
TLR6	0.360	1.000	0.745	0.996	0.695	0.994	1.000	0.762	0.479
TNF	0.380	0.921	0.998	0.861	0.928	0.410	0.810	0.983	1.000
TNFRSF1A	0.337	0.909	0.897	0.829	0.954	0.356	1.000	0.439	0.996
TP53	0.373	0.826	0.381	0.984	0.533	0.983	0.999	0.926	0.827
TRAIL	0.110	0.982	0.136	0.379	0.319	0.681	0.981	0.314	1.000
TRAIL-R	0.137	0.852	0.519	1.000	0.838	0.875	0.977	0.120	0.812
VCAM1	0.116	0.966	0.919	0.480	0.923	0.837	1.000	0.601	0.147

Supplementary Table S4.5 | Gene expression in blood.

Gene expression from blood samples, including means, standard deviations, and *P*-values.

- Mean ± Standard Deviation

Gene	PBS			T15					S10				
	-3Days	4Hours	1dpi	-3Days	4Hours	1dpi	2dpi	3dpi	-3Days	4Hours	1dpi	2dpi	3dpi
C3	1 ± 0.22	0.93 ± 0.12	1.04 ± 0.30	1 ± 0.26	1.63 ± 1.21	1.13 ± 0.95	1.20 ± 0.71	0.86 ± 0.39	1 ± 0.14	1.43 ± 0.54	1.52 ± 0.65	0.84 ± 0.23	1.19 ± 0.82
CASP1	1 ± 0.31	1.33 ± 0.31	1.61 ± 0.25	1 ± 0.35	1.43 ± 0.29	2.54 ± 0.78	1.09 ± 0.11	1.77 ± 0.14	1 ± 0.31	2.37 ± 1.75	1.27 ± 0.76	1.60 ± 0.20	1.99 ± 0.22
CCL4	1 ± 0.61	1.27 ± 1.09	2.30 ± 1.43	1 ± 0.33	1.00 ± 0.57	3.26 ± 2.48	0.73 ± 0.41	0.97 ± 0.21	1 ± 1.38	1.04 ± 0.74	0.70 ± 0.51	0.62 ± 0.21	0.81 ± 0.36
CCL5.RANTES	1 ± 0.84	1.46 ± 1.86	1.69 ± 1.84	1 ± 0.77	0.75 ± 0.45	0.84 ± 0.38	0.30 ± 0.14	1.09 ± 0.34	1 ± 0.80	0.81 ± 0.48	0.46 ± 0.21	0.47 ± 0.11	0.99 ± 0.48
CD14	1 ± 0.45	0.71 ± 0.21	0.73 ± 0.44	1 ± 0.41	1.22 ± 0.52	1.36 ± 0.27	0.79 ± 0.20	1.36 ± 1.27	1 ± 0.35	2.07 ± 1.45	1.31 ± 0.78	1.03 ± 0.65	1.10 ± 0.52
CD163	1 ± 0.93	0.40 ± 0.22	0.37 ± 0.21	1 ± 0.30	2.52 ± 1.39	2.56 ± 1.15	1.46 ± 1.01	2.35 ± 1.55	1 ± 0.88	1.50 ± 0.78	0.86 ± 0.50	0.61 ± 0.28	0.76 ± 0.52
HP	1 ± 0.30	0.96 ± 0.48	0.72 ± 0.42	1 ± 0.83	1.61 ± 2.21	0.55 ± 0.29	0.53 ± 0.23	0.74 ± 0.77	1 ± 0.84	1.74 ± 1.61	0.59 ± 0.20	0.49 ± 0.14	0.57 ± 0.25
ICAM2	1 ± 0.31	0.92 ± 0.13	0.94 ± 0.15	1 ± 0.23	1.09 ± 0.18	1.11 ± 0.22	0.98 ± 0.09	1.03 ± 0.12	1 ± 0.16	1.13 ± 0.23	1.21 ± 0.10	1.06 ± 0.11	0.93 ± 0.13
IFNA1	1 ± 0.29	0.74 ± 0.27	0.92 ± 0.37	1 ± 0.29	0.96 ± 0.31	0.91 ± 0.20	0.86 ± 0.11	0.68 ± 0.19	1 ± 0.21	1.15 ± 0.32	1.15 ± 0.34	0.78 ± 0.32	0.63 ± 0.12
IKBKB	1 ± 0.28	0.99 ± 0.05	0.98 ± 0.17	1 ± 0.20	1.07 ± 0.20	1.36 ± 0.34	0.96 ± 0.19	1.06 ± 0.24	1 ± 0.17	1.51 ± 0.51	1.29 ± 0.20	1.19 ± 0.17	1.07 ± 0.18
IL15	1 ± 0.10	0.68 ± 0.22	0.76 ± 0.21	1 ± 0.58	1.48 ± 0.55	2.09 ± 0.75	0.62 ± 0.14	1.27 ± 0.49	1 ± 0.30	2.14 ± 1.51	0.83 ± 0.40	1.11 ± 0.69	1.65 ± 1.27

IL18	1 ± 1.00	0.64 ± 0.29	0.48 ± 0.37	1 ± 0.93	1.96 ± 2.12	0.85 ± 1.54	0.27 ± 0.08	0.47 ± 0.25	1 ± 0.74	1.77 ± 1.06	0.83 ± 0.69	0.30 ± 0.26	0.80 ± 0.18
IL1B	1 ± 0.34	0.71 ± 0.26	1.03 ± 0.33	1 ± 0.55	1.52 ± 0.55	1.92 ± 0.96	0.77 ± 0.15	1.50 ± 0.20	1 ± 0.55	1.85 ± 1.48	0.92 ± 0.58	1.08 ± 0.32	1.21 ± 0.38
IRF3	1 ± 0.13	0.96 ± 0.09	1.55 ± 0.15	1 ± 0.16	1.46 ± 0.33	2.24 ± 0.63	1.15 ± 0.21	1.20 ± 0.28	1 ± 0.26	2.45 ± 0.58	1.78 ± 0.52	1.65 ± 0.33	1.41 ± 0.23
IRF7	1 ± 0.25	0.88 ± 0.19	2.05 ± 1.31	1 ± 0.41	3.14 ± 1.41	8.58 ± 4.53	3.15 ± 1.67	2.89 ± 1.47	1 ± 0.74	3.62 ± 2.90	3.06 ± 1.75	3.39 ± 0.81	1.48 ± 0.49
JAK1	1 ± 0.23	1.09 ± 0.23	1.17 ± 0.32	1 ± 0.19	0.88 ± 0.12	0.99 ± 0.12	0.93 ± 0.16	0.89 ± 0.16	1 ± 0.17	1.15 ± 0.14	1.16 ± 0.19	1.12 ± 0.24	0.84 ± 0.08
JAK2	1 ± 0.14	0.89 ± 0.13	0.91 ± 0.13	1 ± 0.29	1.34 ± 0.37	1.58 ± 0.32	1.00 ± 0.25	1.24 ± 0.27	1 ± 0.27	1.53 ± 0.71	0.94 ± 0.29	1.25 ± 0.26	1.32 ± 0.26
LY96.MD2	1 ± 0.29	0.79 ± 0.10	0.73 ± 0.15	1 ± 0.36	1.20 ± 0.44	0.93 ± 0.21	0.66 ± 0.16	0.97 ± 0.31	1 ± 0.25	1.43 ± 0.45	0.75 ± 0.23	0.91 ± 0.28	1.17 ± 0.25
MYD88	1 ± 0.28	0.94 ± 0.04	0.90 ± 0.17	1 ± 0.31	1.32 ± 0.61	1.22 ± 0.38	0.58 ± 0.08	0.90 ± 0.27	1 ± 0.31	1.63 ± 0.47	0.96 ± 0.34	0.88 ± 0.21	0.90 ± 0.24
NFKB1	1 ± 0.22	1.35 ± 0.14	1.48 ± 0.14	1 ± 0.28	1.05 ± 0.25	1.35 ± 0.32	1.00 ± 0.39	1.15 ± 0.32	1 ± 0.16	1.62 ± 0.33	1.43 ± 0.57	1.65 ± 0.24	1.45 ± 0.23
NFKBIA	1 ± 0.25	1.06 ± 0.21	1.21 ± 0.11	1 ± 0.21	1.27 ± 0.43	1.38 ± 0.45	1.48 ± 0.35	1.19 ± 0.63	1 ± 0.22	1.69 ± 0.62	1.68 ± 0.44	1.45 ± 0.36	1.20 ± 0.29
SELL	1 ± 0.20	0.93 ± 0.12	1.00 ± 0.21	1 ± 0.28	1.32 ± 0.56	1.68 ± 0.50	0.91 ± 0.27	1.34 ± 0.31	1 ± 0.28	1.78 ± 0.93	1.07 ± 0.44	1.06 ± 0.35	1.28 ± 0.46
SELP	1 ± 0.39	0.95 ± 0.48	1.11 ± 0.44	1 ± 0.30	1.23 ± 0.45	1.95 ± 0.56	1.60 ± 0.38	1.68 ± 0.33	1 ± 0.27	1.57 ± 0.49	1.62 ± 0.96	1.10 ± 0.52	1.39 ± 0.52
STAT1	1 ± 0.17	1.01 ± 0.09	1.49 ± 0.62	1 ± 0.30	1.69 ± 0.57	2.88 ± 0.91	1.60 ± 0.44	1.94 ± 0.51	1 ± 0.24	1.95 ± 0.91	1.54 ± 0.60	2.14 ± 0.18	1.44 ± 0.31
STAT2	1 ± 0.19	1.29 ± 0.15	1.65 ± 0.65	1 ± 0.34	1.26 ± 0.24	2.40 ± 0.84	1.34 ± 0.34	1.41 ± 0.22	1 ± 0.20	1.76 ± 0.82	1.45 ± 0.50	2.10 ± 0.31	1.51 ± 0.21
TICAM1.TRIF	1 ± 0.27	1.59 ± 0.04	1.93 ± 0.53	1 ± 0.37	1.09 ± 0.22	1.46 ± 0.41	0.80 ± 0.24	1.08 ± 0.23	1 ± 0.25	1.92 ± 0.36	1.39 ± 0.64	1.41 ± 0.31	1.44 ± 0.17
TLR2	1 ± 0.41	0.96 ± 0.15	0.88 ± 0.19	1 ± 0.50	1.44 ± 0.48	1.42 ± 0.25	0.72 ± 0.20	1.20 ± 0.53	1 ± 0.35	1.87 ± 1.02	0.99 ± 0.57	1.23 ± 0.38	1.26 ± 0.39

TLR4	1 ± 0.53	0.48 ± 0.12	0.50 ± 0.10	1 ± 0.49	2.88 ± 2.23	1.58 ± 0.58	0.57 ± 0.36	0.75 ± 0.20	1 ± 0.55	3.69 ± 1.99	0.94 ± 0.62	0.76 ± 0.16	0.74 ± 0.25
TNF	1 ± 0.44	1.05 ± 0.28	0.79 ± 0.27	1 ± 0.17	1.22 ± 0.44	1.77 ± 0.43	0.88 ± 0.10	1.26 ± 0.11	1 ± 0.24	1.41 ± 0.19	1.21 ± 0.15	1.21 ± 0.15	1.04 ± 0.24
TREM1	1 ± 0.34	0.89 ± 0.13	0.83 ± 0.34	1 ± 0.32	1.21 ± 0.43	1.62 ± 0.57	0.98 ± 0.42	1.45 ± 0.62	1 ± 0.29	1.77 ± 1.43	1.32 ± 0.81	1.20 ± 0.66	1.46 ± 0.75

- *P*-values, part I

Gene	***P*-value PBS Group**			***P*-value T15 Group**									
	-3Days *vs.*		**4Hours *vs.***	**-3Days *vs.***				**4Hours *vs.***			**1dpi *vs.***		**2dpi *vs.***
	4Hours	**1dpi**	**1dpi**	**4Hours**	**1dpi**	**2dpi**	**3dpi**	**1dpi**	**2dpi**	**3dpi**	**2dpi**	**3dpi**	**3dpi**
C3	1.000	1.000	0.999	0.312	0.994	0.988	0.996	0.552	0.825	0.327	1.000	0.958	0.944
CASP1	0.872	0.421	0.935	0.710	**<0.001***	0.999	0.347	**0.015***	0.938	0.912	**0.010***	0.389	0.609
CCL4	0.997	0.514	0.721	1.000	**0.014***	0.998	1.000	**0.014***	0.998	1.000	**0.031***	0.063	0.999
CCL5.RANTES	0.769	0.428	0.980	0.899	0.980	0.298	0.999	0.997	0.711	0.868	0.550	0.953	0.270
CD14	0.919	0.938	1.000	0.936	0.706	0.989	0.805	0.987	0.811	0.988	0.576	1.000	0.622
CD163	**0.009***	**0.005***	1.000	0.195	0.177	0.986	0.558	1.000	0.734	0.999	0.710	0.999	0.893
HP	1.000	0.996	0.998	0.715	0.882	0.963	0.998	0.199	0.462	0.679	1.000	0.993	0.998
ICAM2	0.932	0.970	1.000	0.824	0.704	1.000	0.975	0.999	0.951	0.999	0.899	0.993	0.993
IFNA1	0.477	0.987	0.780	0.995	0.863	0.554	**0.038***	0.975	0.738	0.078	0.942	0.207	0.703
IKBKB	1.000	1.000	1.000	0.979	**0.022***	1.000	0.986	0.088	0.968	1.000	0.069	0.284	0.970
IL15	0.641	0.837	0.997	0.711	**0.046***	0.988	0.907	0.504	0.570	1.000	0.058	0.627	0.743
IL18	0.714	0.375	0.981	**0.011***	0.983	0.979	1.000	**0.002***	**0.019***	0.075	1.000	0.994	0.986
IL1B	0.876	1.000	0.839	0.542	0.064	0.959	0.838	0.753	0.328	1.000	**0.047***	0.789	0.544
IRF3	1.000	**0.044***	**0.026***	0.069	**<0.001***	0.943	0.861	**<0.001***	0.637	0.775	**<0.001***	**<0.001***	1.000
IRF7	1.000	0.784	0.713	0.427	**<0.001***	0.632	0.734	**<0.001***	1.000	1.000	**0.007***	**0.004***	1.000
JAK1	0.959	0.722	0.979	0.492	1.000	0.945	0.718	0.555	0.983	1.000	0.963	0.764	0.991
JAK2	0.974	0.989	1.000	0.333	**0.018***	1.000	0.778	0.665	0.603	0.996	0.101	0.602	0.876
LY96.MD2	0.554	0.291	0.991	0.489	0.980	0.496	0.996	0.203	**0.043***	0.895	0.766	0.924	0.391
MYD88	0.996	0.969	0.999	0.179	0.558	0.299	1.000	0.947	**0.004***	0.311	**0.022***	0.655	0.495
NFKB1	0.268	0.058	0.944	0.990	**0.028***	0.998	0.691	0.086	1.000	0.883	0.236	0.801	0.897
NFKBIA	0.999	0.938	0.982	0.578	0.267	0.305	0.950	0.982	0.936	0.992	0.997	0.903	0.807

SELL	0.999	1.000	0.999	0.554	**0.019***	1.000	0.614	0.441	0.635	0.999	0.063	0.798	0.596
SELP	1.000	0.992	0.971	0.673	**<0.001***	0.194	0.094	**<0.001***	0.768	0.554	0.211	0.379	0.998
STAT1	1.000	0.583	0.610	0.174	**<0.001***	0.514	0.108	**0.003***	0.999	0.964	**0.010***	0.105	0.931
STAT2	0.888	0.244	0.772	0.799	**<0.001***	0.752	0.605	**<0.001***	0.998	0.981	**0.008***	**0.016***	1.000
TICAM1.TRIF	0.106	**0.003***	0.622	0.964	**0.011***	0.834	0.972	0.062	0.527	1.000	**0.004***	0.247	0.578
TLR2	1.000	0.991	0.998	0.339	0.395	0.939	0.915	1.000	0.174	0.966	0.203	0.979	0.606
TLR4	0.523	0.571	1.000	**0.009***	0.814	0.975	0.997	0.135	**0.012***	**0.024***	0.591	0.753	0.999
TNF	0.997	0.600	0.392	0.551	**<0.001***	0.970	0.593	**0.004***	0.361	0.999	**<0.001***	0.062	0.359
TREM1	0.998	0.990	1.000	0.929	0.145	1.000	0.571	0.529	0.983	0.907	0.410	0.996	0.714

- *P*-values, part II

Gene	***P*-value S10 Group**									
	-3Days *vs.*				**4Hours *vs.***			**1dpi *vs.***		**2dpi *vs.***
	4Hours	**1dpi**	**2dpi**	**3dpi**	**1dpi**	**2dpi**	**3dpi**	**2dpi**	**3dpi**	**3dpi**
C3	0.535	0.325	0.979	0.990	0.996	0.380	0.937	0.237	0.826	0.889
CASP1	**0.003***	0.942	0.652	0.173	**0.025***	0.431	0.923	0.940	0.466	0.931
CCL4	1.000	0.944	0.943	0.996	0.915	0.919	0.991	1.000	0.999	0.997
CCL5.RANTES	0.936	0.221	0.225	0.986	0.655	0.555	1.000	0.991	0.774	0.591
CD14	**0.017***	0.877	1.000	1.000	0.158	0.090	0.124	0.944	0.975	1.000
CD163	0.590	0.993	0.645	0.842	0.338	0.101	0.205	0.834	0.957	0.998
HP	0.535	0.910	0.860	0.912	0.131	0.188	0.241	0.998	1.000	1.000
ICAM2	0.468	0.083	0.971	0.962	0.871	0.959	0.292	0.603	0.063	0.774
IFNA1	0.598	0.587	0.793	0.195	1.000	0.176	**0.013***	0.171	**0.013***	0.864
IKBKB	**0.001***	0.148	0.791	0.997	0.369	0.205	**0.036***	0.948	0.560	0.956
IL15	**0.007***	0.984	0.995	0.428	**0.001***	0.119	0.803	0.929	0.218	0.752
IL18	0.162	0.985	0.274	0.912	0.052	**0.004***	0.078	0.495	0.991	0.826
IL1B	0.078	0.999	0.999	1.000	**0.044***	0.140	0.258	1.000	0.999	0.998
IRF3	**<0.001***	**<0.001***	**0.039***	0.372	**0.004***	**0.007***	**<0.001***	0.977	0.453	0.862
IRF7	**0.022***	0.110	0.150	0.990	0.961	0.999	0.241	0.998	0.540	0.484
JAK1	0.328	0.246	0.746	0.449	1.000	0.998	**0.019***	0.992	**0.013***	0.089
JAK2	**0.022***	0.995	0.752	0.547	**0.008***	0.672	0.854	0.565	0.365	0.998
LY96.MD2	**0.030***	0.392	0.924	0.970	**<0.001***	**0.019***	0.404	0.976	0.265	0.693
MYD88	**<0.001***	0.999	0.796	0.858	**<0.001***	**<0.001***	**<0.001***	0.891	0.934	1.000

NFKB1	**<0.001***	**0.010***	**0.007***	0.158	0.592	0.998	0.543	0.911	0.995	0.790
NFKBIA	**<0.001***	**<0.001***	0.127	0.768	1.000	0.853	0.179	0.870	0.193	0.797
SELL	**0.002***	0.996	1.000	0.857	**0.007***	**0.034***	0.227	1.000	0.954	0.938
SELP	**0.011***	**0.005***	0.996	0.430	0.999	0.164	0.876	0.106	0.774	0.739
STAT1	**0.010***	0.305	**0.012***	0.702	0.572	0.980	0.567	0.403	0.998	0.384
STAT2	**0.020***	0.341	**0.005***	0.431	0.687	0.787	0.920	0.203	0.999	0.421
TICAM1.TRIF	**<0.001***	0.200	0.485	0.395	**0.034***	0.116	0.159	1.000	1.000	1.000
TLR2	**0.005***	1.000	0.994	0.985	**0.004***	0.098	0.125	0.993	0.982	1.000
TLR4	**<0.001***	1.000	0.986	0.984	**<0.001***	**<0.001***	**<0.001***	0.993	0.992	1.000
TNF	0.065	0.620	0.836	1.000	0.686	0.787	0.272	1.000	0.866	0.934
TREM1	**0.044***	0.756	0.987	0.719	0.447	0.406	0.850	0.992	0.998	0.959

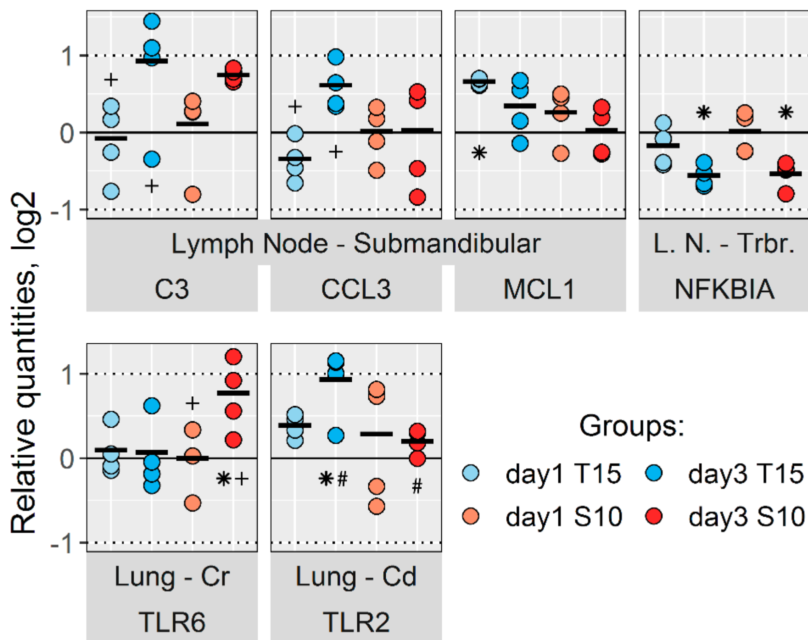
- *P*-values, part III

Gene	***P*-value -3Days**			***P*-value 4Hours**			***P*-value 1dpi**			***P*-value 2dpi**	***P*-value 3dpi**
	PBS *vs.*		**T15 *vs.***	**PBS *vs.***		**T15 *vs.***	**PBS *vs.***		**T15 *vs.***	**T15 *vs.***	**T15 *vs.***
	T15	**S10**	**S10**	**T15**	**S10**	**S10**	**T15**	**S10**	**S10**	**S10**	**S10**
C3	0.947	0.724	0.844	0.128	0.093	0.980	0.848	0.085	0.125	0.876	0.529
CASP1	0.671	0.595	0.988	0.629	0.590	0.059	0.700	0.062	**<0.001***	0.682	0.972
CCL4	0.976	0.823	0.589	0.821	0.954	0.523	0.778	0.164	**0.008***	0.947	0.916
CCL5.RANTES	1.000	0.967	0.962	0.351	0.509	0.937	0.234	0.064	0.693	0.995	0.940
CD14	0.921	0.740	0.901	0.574	**0.047***	0.199	0.398	0.756	0.751	0.997	0.533
CD163	**<0.001***	**0.003***	0.444	0.964	0.727	0.810	0.893	0.943	0.607	0.854	0.554
HP	0.981	0.981	1.000	0.458	0.337	0.961	0.991	0.997	0.997	0.961	0.898
ICAM2	0.139	0.286	0.873	0.906	0.918	0.579	0.902	0.643	0.242	0.730	0.805
IFNA1	0.697	0.929	0.849	0.133	**0.033***	0.710	0.795	0.233	0.436	0.990	0.993
IKBKB	0.896	0.712	0.909	1.000	0.066	**0.019***	0.132	0.562	0.494	0.726	0.904
IL15	**0.031***	0.072	0.897	0.968	0.177	**0.039***	0.836	0.272	**0.031***	0.564	0.573
IL18	0.843	0.717	0.959	0.289	0.664	0.692	0.963	0.999	0.963	0.556	0.762
IL1B	0.456	0.528	0.988	0.643	0.170	0.496	0.760	0.373	**0.038***	0.901	0.768
IRF3	0.457	0.373	0.981	0.575	**<0.001***	**<0.001***	0.511	0.512	**0.023***	0.239	0.847
IRF7	0.824	0.962	0.915	0.605	**0.043***	0.166	**0.009***	0.894	**0.006***	0.525	0.926
JAK1	0.126	0.181	0.972	0.998	**0.041***	**0.012***	0.822	0.115	0.197	0.333	0.864
JAK2	0.193	0.296	0.949	0.908	0.225	0.283	0.378	0.417	**0.006***	0.478	0.853

LY96.MD2	0.336	0.102	0.694	0.514	0.353	0.935	0.997	0.342	0.172	1.000	0.912
MYD88	0.396	0.301	0.972	0.891	0.279	0.384	0.972	0.521	0.247	0.889	0.712
NFKB1	0.917	0.996	0.823	0.514	0.463	**0.019***	0.990	0.922	0.950	0.129	0.951
NFKBIA	0.975	0.690	0.738	0.556	**0.002***	**0.006***	0.652	**0.009***	**0.025***	0.636	0.647
SELL	0.734	0.862	0.956	0.839	**0.044***	0.060	0.311	0.965	0.095	0.887	0.991
SELP	0.960	0.946	0.998	0.508	0.072	0.339	**0.016***	0.138	0.466	0.465	0.863
STAT1	0.705	0.862	0.938	0.578	0.072	0.288	**0.036***	0.804	**<0.001***	0.193	0.703
STAT2	0.973	0.970	1.000	0.927	0.433	0.127	0.103	0.539	**<0.001***	0.103	0.972
TICAM1.TRIF	0.553	0.969	0.267	0.588	0.635	0.061	0.889	0.032	**0.031***	0.595	0.975
TLR2	0.654	0.651	1.000	0.788	0.122	0.237	0.646	0.896	0.236	0.614	0.961
TLR4	0.162	0.193	0.993	**0.034***	**<0.001***	0.158	0.807	0.977	0.569	0.987	0.996
TNF	0.065	0.054	0.994	0.305	0.797	0.560	**0.004***	0.952	**0.001***	0.393	0.484
TREM1	0.994	0.992	1.000	0.774	0.125	0.253	0.175	0.544	0.623	0.955	0.979

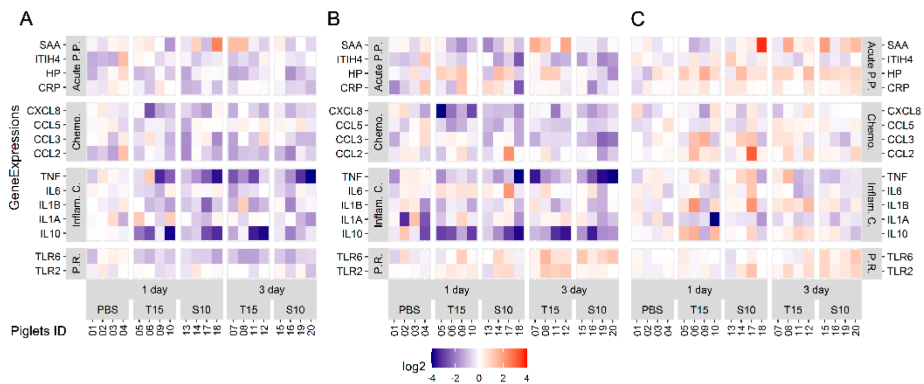
Supplementary Figure S4.1 | Significant gene expression with < 2-fold changes in lymph nodes, trachea and lungs after *S. suis* intranasal inoculation.

Log_2 of the individual values and mean (black bars) of the relative gene expression in different tissues of *S. suis* inoculated piglets. Piglets were intranasally inoculated with *S. suis* T15 (non-virulent, blue bars) or S10 (virulent, red bars), and necropsies were performed at 1 and 3 days post-infection. Gene expression was normalized relative to the PBS group. The values and means are shown for the indicated groups (challenge strain and time point) having at least one significant difference when compared to the PBS group and with a mean lower than 2-fold change (log_2 = 1). LN Trbr: Tracheobronchial lymph node; Lung Cr: Lung Cranial; Lung Cd: Lung Caudal. * indicates significant differences ($P < 0.05$) versus the PBS group. Differences between strains at the same time point are labelled with # and differences between time points for the same strain are labelled with +, $P < 0.05$, in both cases. $n = 4$ for each group. All expression values and significant differences can be found in Additional file 5.



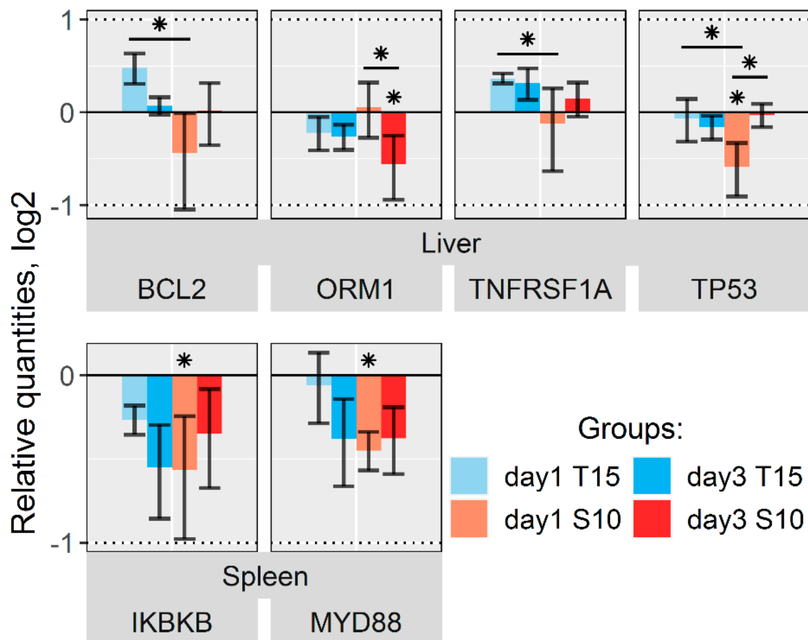
Supplementary Figure S4.2 | Gene expression in different respiratory tissues after *S. suis* intranasal inoculation.

Samples from trachea (A), caudal lung (B), and cranial lung (C) were collected at 1 and 3 days after the intranasal inoculation of *S. suis* T15 (non-virulent) and S10 (virulent). Results at 1 day post-inoculation from piglets inoculated with PBS are also included as control. All the genes found to be quantifiable are shown irrespectively of their statistical significance. Gene expression was normalized relative to the PBS group and $\log_2$ transformed. Values are presented as a heat map. Numbers in abscissa axis represent animal ID. Color scale was limited to ± 4 and out of bounds values displayed with the maximum intensity color. Gene functional groups: Acute P.P.: Acute Phase Proteins; Chemo.: Chemokines; P.R.: Pattern Recognition Receptors.



Supplementary Figure S4.3 | Significant gene expression in spleen and liver after *S. suis* intranasal inoculation.

Log_2 of the individual values and mean (black bars) of the relative gene expression in spleen and liver in *S. suis* inoculated piglets. Piglets were intranasally inoculated with *S. suis* T15 (non-virulent, blue bars) or S10 (virulent, red bars), and necropsies were performed at 1 and 3 days post-infection. Gene expression was normalized relative to the PBS group. The values and means are shown for the indicated groups (challenge strain and time point) having at least one significant difference when compared to the PBS group. * indicates significant differences ($P < 0.05$) versus the PBS group. Differences between strains at the same time point are labelled with # and differences between time points for the same strain are labelled with +, $P < 0.05$, in both cases. n = 4 for each group. All expression values and significant differences can be found in Additional file 5.



Supplementary Figure S4.4 | Gene expression in spleen and liver after *S. suis* intranasal inoculation.

Samples from spleen (A) and liver (B) were collected at 1 and 3 days after the intranasal inoculation of *S. suis* T15 (non-virulent) and S10 (virulent). Results at 1 day post-inoculation from piglets inoculated with PBS are also included as control. All the genes found to be quantifiable are shown irrespectively of their statistical significance. Gene expression was normalized relative to the PBS group and $\log_2$ transformed. Values are presented as a heat map. Numbers in abscissa axis represent animal ID. Color scale was limited to ± 3 and out of bounds values displayed with the maximum intensity color. Gene functional groups: A.P.P. and Acute P.P.: Acute Phase Proteins; Ad. M.: Adhesion Molecules; Chemo.: Chemokines; Misc.: Miscellaneous; P.R.R.: Pattern Recognition Receptors; Trans. F.: Transcription Factors.