Received: 3 December 2022 | Revised: 20 April 2023 | Accepted: 8 May 2023

DOI: 10.1002/vetr.3056

Vet Record

ORIGINAL RESEARCH

Risk factors associated with *Streptococcus suis* cases on pig farms in Spain

Carlos Neila-Ibáñez[1,2,3] | **Sebastián Napp**[1,2,3] | **Lola Pailler-García**[1,2,3] | **Lorena Franco-Martínez**[4] | **José Joaquín Cerón**[4] | **Virginia Aragon**[1,2,3] | **Jordi Casal**[5]

[1]Unitat mixta d'Investigació IRTA-UAB en Sanitat Animal, Centre de Recerca en Sanitat Animal, Universitat Autònoma de Barcelona, Bellaterra, Spain

[2]Institut de Recerca i Tecnologia Agroalimentàries, Centre de Recerca en Sanitat Animal, Universitat Autònoma de Barcelona, Bellaterra, Spain

[3]OIE Collaborating Centre for the Research and Control of Emerging and Re-Emerging Swine Diseases in Europe, Bellaterra, Spain

[4]Interdisciplinary Laboratory of Clinical Analysis, University of Murcia, Espinardo, Spain

[5]Department of Animal Health and Anatomy, Faculty of Veterinary Medicine, Universitat Autònoma de Barcelona, Bellaterra, Spain

Correspondence
Jordi Casal, Department of Animal Health and Anatomy, Faculty of Veterinary Medicine, Universitat Autònoma de Barcelona, Bellaterra, Spain.
Email: Jordi.casal@uab.cat

Funding information
Program for Innovative Global Prevention of *Streptococcus suis* (PIGSs), from program Horizon 2020 of the European Commission. Carlos Neila-Ibáñez was funded by the same project, Grant/Award Number: 727966

Abstract

Background: *Streptococcus suis* can cause meningitis, polyarthritis and acute death in piglets. However, the risk factors associated with *S. suis* infection remain incompletely understood. Therefore, a longitudinal study was carried out, in which six batches from two Spanish pig farms with *S. suis* problems were repeatedly examined to determine possible risk factors.

Methods: A prospective case–control study was conducted, and potential risk factors were evaluated using mixed-effects logistic regression models. The explanatory variables included: (a) concomitant pathogens; (b) biomarkers associated with stress, inflammation and oxidative status; (c) farm environmental factors; 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 risk factors involved in the subsequent development of disease.

Results: Risk factors for *S. suis*-associated disease included porcine reproductive and respiratory syndrome virus co-infection at weaning (odds ratio [OR] = 6.69), sow parity (OR = 0.71), haptoglobin level before weaning (OR = 1.01), relative humidity (OR = 1.11) and temperature (OR = 0.13).

Limitations: Laboratory diagnosis was done at the batch level, with individual diagnosis based on clinical signs only.

Conclusions: This study confirms the multifactorial nature of *S. suis*-associated disease, with both environmental factors and factors related to the host involved in disease development. Controlling these factors may, therefore, help prevent the appearance of disease.

KEYWORDS
animal stress, co-infection, environmental factors, PRRSV, risk factors, *Streptococcus suis*

INTRODUCTION

Streptococcus suis is one of the main bacterial pathogens affecting the pig industry globally, causing economic losses due to substantial postweaning morbidity and mortality and the costs associated with disease control.[1,2] Although *S. suis* is a normal coloniser of the porcine upper respiratory tract,[3] it can cause disease, characterised mainly by meningitis, polyarthritis and acute death, in pigs, especially piglets from 5 to 10 weeks old.[1] Even though globally the proportion of pigs colonised by *S. suis* is close to 100%,[4] the level of associated disease is much lower, with incidence rates below 5% and mortality below 1% reported in nursery units in Spain, Germany and the Netherlands.[2] However, the circumstances that allow strains from the microbiota of healthy animals to produce clinical disease are not completely known.[5] In addition, *S. suis* is considered an emerging zoonotic pathogen, with some important outbreaks in Southeastern Asian countries in the present century.[6]

S. suis is classified into 29 different serotypes depending on the capsular polysaccharide, with serotypes 1, 2, 7 and 9 being the most frequently isolated from clinical cases.[7] Serotypes are distributed worldwide, and the lesions they produce are not serotype dependent.[8] Although many virulence factors have been described for *S. suis*, they are not always present in clinical isolates.[4] Furthermore, there are other factors (unrelated to the pathogen) that may influence the development of the disease, such as the number of piglets weaned per sow, which seemed

to influence mortality during a *S. suis* outbreak in suckling piglets.[9] *S. suis* outbreaks have also been associated with some concomitant viral infections, such as with porcine reproductive and respiratory syndrome virus (PRRSV), porcine circovirus 2 (PCV-2) or swine influenza virus (SIV). These associations are often observed under field conditions, where mixed infections are frequent. However, experimental infections to confirm these hypotheses are complex, so only a few in vivo studies have been published.[10] Piglets born to sows infected with PRRSV during gestation and challenged with *S. suis* at 5 days of age were more susceptible to infection and disease than those born to non-infected sows or those infected only with *S. suis*.[11] In two other experimental studies, piglets inoculated with PRRSV 7 days before being challenged with *S. suis* had higher mortality and more severe lesions than piglets challenged with only one of the pathogens.[12,13] When *S. suis* challenge was performed in piglets 5 days after PCV-2 infection, co-infected piglets exhibited more severe clinical signs and lesions than those inoculated with only one of the pathogens.[14] Similar observations were reported for the SIV co-infection, with co-infected piglets showing more severe clinical signs and increased gene expression of pro-inflammatory mediators than those inoculated with only one of the pathogens.[15] In addition, mixed infections with other bacteria can increase the severity of the lesions caused by *S. suis*, as was reported in a co-infection study with *Bordetella bronchiseptica*, which was used to predispose the nasal mucosa for the *S. suis* inoculation.[16] In that co-infection, *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 in determining the presence and abundance of *S. suis* deserves to be further studied, as the composition of the nasal microbiota may predispose to disease development by other early colonisers.[17,18] Recently, Niazy et al.[19] found a different composition of the tonsillar microbiota in *S. suis*-affected piglets compared with the uninfected group. One of the species found in different abundance was *Glaesserella* (*Haemophilus*) *parasuis*, another pig pathogen with similar clinical manifestations that are often misidentified.[20]

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.[21–23] Although animal stress can be evaluated using different biomarkers, cortisol is probably the most commonly used in pigs.[24] The intensity of inflammatory processes can be measured by acute phase proteins[25] such as haptoglobin, as shown in piglets co-infected with *Mycoplasma hyopneumoniae* and SIV H1N1, which had higher levels of haptoglobin than in non-infected animals.[26] In contrast, transcription of the haptoglobin gene was not altered in blood after an *S. suis* challenge in caesarean-derived colostrum-deprived piglets when compared with non-inoculated piglets.[27] In addition, 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.[28] The levels of these biomarkers may be altered as a result of infectious processes, as demonstrated in SIV and *M. hyopneumoniae* infections.[26]

With the aim of evaluating possible risk factors for *S. suis*-associated disease, *G. parasuis*, SIV, PRRSV and PCV-2 presence, environmental parameters, parity of the dams and biomarkers of stress, inflammation and oxidative status in piglets were analysed in a longitudinal study carried out on two Spanish commercial pig farms.

MATERIALS AND METHODS

Selection of the farms

The study was carried out on two pig farms, 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 the cerebrospinal fluid of suckling piglets (farm A) and weaners (farm B) with neurological clinical signs.

Farm A was a family farm with 500 sows (White Large × Landrace). The nursery was located 2.4 km from the maternity unit. Sows received a metaphylactic treatment with oxytetracycline in their feed from 7 days pre-farrowing until piglets were weaned. Piglets were treated intramuscularly with amoxicillin and gentamicin 2 days before weaning, and vaccinated against *M. hyopneumoniae* and PCV-2 1 day before weaning. In the nursery, piglets with clinical signs compatible with *S. suis*-associated disease were treated intramuscularly twice with amoxicillin, enrofloxacin and dexamethasone, and when *S. suis* clinical disease appeared, all the animals in the batch were treated with amoxicillin in their drinking water for 6 days. The status of the farm was 'positive stable' for PRRSV (i.e., PRRSV was not circulating either in the maternity unit or in the nursery[29]), and neither PCV-2 nor influenza outbreaks were detected during the study. However, a porcine epidemic diarrhoea (PED) outbreak took place in the nursery at the time of the second batch in the study.

Farm B, with 3500 sows (Duroc × White Large × Landrace), belonged to a large commercial production company. The breeding herd and the nursery were located on 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 was 'positive stable' for PRRSV, and neither PCV-2 nor influenza outbreaks were detected during sampling.

On both farms, before the start of the study, swabs were collected from lesions on seven animals found dead or euthanased due to welfare concerns, and these were analysed to confirm an *S. suis* outbreak. *S. suis* presence was determined after swab plating and molecular identification of pure culture bacterial

growth. *S. suis* identification and serotyping was performed by PCR,[30,31] confirming *S. suis* as the cause of disease and showing that the clinical isolates belonged to serotype 7 on both farms. In addition, *S. suis* isolates obtained from lesions were analysed by enterobacterial repetitive intergenic consensus (ERIC)-PCR to determine the number of different strains involved in the outbreak, following the protocol described by Versalovic et al.,[32] but lowering the annealing temperature to 43°C.

Sampling and data collection

Animal sampling was done under institutional authorisation (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.

On each farm, three different batches were sampled. In each batch, 30 piglets were selected from 10 different sows. All piglets with clinical signs compatible with *S. suis* were chosen, and the group was completed by randomly selecting healthy piglets until a group size of 30 was reached. Selected animals were ear-tagged the week before weaning. Animal ages ranged between 17 and 22 days. Clinical signs were checked daily by the veterinarian in charge of the farm according to the protocols of the company. Animals were classified as diseased if they presented clinical signs compatible with *S. suis*, such as neurological signs or lameness, before the sample collection by the veterinarian in charge of the study. Nasal swabs and blood samples were taken from the selected piglets, and nasal and vaginal swabs from their dams. Piglets were sampled again approximately 2 weeks after weaning, when they were between 31 and 36 days of age. If any of the animals not sampled initially subsequently developed lameness or neurological 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. As new individuals were included in the cases group during the follow-ups, the study can be classified as a prospective case–control study.

In total, 117 piglets were sampled between October and December 2019 on farm A, and 90 piglets were sampled between March and May 2021 on farm B. The number of piglets sampled at each visit varied between 30 and 40, whereas the number of sows sampled was 10 for each batch. Information about the ages and sex distribution of the sampled animals is included in Table S1.

During the first two visits, an environmental data logger was placed in the rooms where the piglets were located, at a height of approximately 30 cm. The data logger located at farm A (MHD21ABE17, DeltaOHM, Italy) recorded temperature (°C), relative humidity (%), temperature-humidity index (THI) and CO_2 (ppm), while the data logger placed at farm B recorded only temperature and relative humidity (HD208.1NTCI—HP3517TC1.2, DeltaOHM, Italy). Data were measured every 5 minutes for 60–90 hours after each of the three sampling visits.

Pathogen detection

Nasal and vaginal swabs were resuspended in 500 µL of PBS, and blood was centrifuged at 1048 *g* and 4°C for 10 minutes to obtain serum. Both types of samples were stored at −80°C 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 −80°C until molecular analysis was performed.

The presence of *S. suis*, specifically serotypes 2 (or 1/2, which is indistinguishable from serotype 2 by PCR), 7 and 9 (the most common virulent serotypes in European farms), in the nasal and vaginal samples was evaluated. Meanwhile, the presence of *G. parasuis*, both virulent and non-virulent strains, was evaluated only in the nasal samples. Screening for these pathogens was performed using conventional PCR assays, with the primers and conditions described by Ishida et al.[30] for the presence of *S. suis*, Okura et al.[31] for serotypes 1/2–2, 7 and 9, and Galofré-Milà et al.[33] for *G. parasuis*. Nasal samples were also tested for influenza viral RNA using a quantitative reverse transcription-PCR (RT-qPCR) assay based on the amplification of the conserved segment of the matrix gene, as described by López-Valiñas et al.[34] The presence of PRRSV and PCV-2 in the serum was determined by real-time qPCR assay with commercial kits (VetMAX PRRSV EU and 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 37°C 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 et al.[30]

Analyses of cortisol, haptoglobin and hydrogen peroxide

Cortisol concentration was measured with 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.[35] Haptoglobin concentrations were measured using a commercial

quantitative turbidimetric test (Spinreact, S.A.U, Spain) in an automated analyser (Olympus AU600), which was previously validated by Kaiser et al.[36] H_2O_2 was assessed based on the method of Rhee et al.[37] in an automated analyser (Olympus AU600), as previously validated.[38]

Statistical analysis

To evaluate the effect of the different variables on *S. suis*-associated disease, three different mixed-effects logistic regression models were chosen, depending on the age of the animals and the use of retrospective data:

- Model 1: General risk factor model, considering the presentation of *S. suis*-associated disease at any of the visits, and using all explanatory variables;
- Model 2: Weaning risk factor model, considering the presentation of the disease at the second and third visits (i.e., during the post-weaning period), and using the data collected previously in the farrowing unit; and
- Model 3: Nursery risk factor model, considering the presentation of the disease late in the nursery, and using the data collected in the first nursery visit.

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

First, a bivariate analysis to test the associations between the dependent variables (*S. suis*-associated clinical signs) and the explanatory variables was carried out. Considering the hierarchical structure of the data, due to the potential effects of sow, batch and farm, a mixed-effect logistic regression was used to model the binary independent outcome, including sow, batch and farm as random effects and the remaining dependent variables as fixed effects. Specifically, only those variables with a $p \leq 0.25$ in the bivariate analysis were further evaluated in the multivariate analysis.[39] The final model selection was performed via manual backward selection to better avoid multicollinearity, based on the Akaike information criterion, including only those variables with a $p \leq 0.05$ and excluding those with a variance inflation factor greater than 5.

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

Statistical analyses were conducted with R (v. 4.0.2),[40] using the packages *lme4* (v. 1.1-23)[41] and *rsq* (v. 2.2).[42]

RESULTS

S. suis isolation and disease prevalence

During the study, *S. suis* was isolated from lesions of animals with clinical signs, confirming *S. suis* as the most likely cause of the disease. In total, five different *S. suis* isolates were recovered, one from a tarsal joint and three from cerebrospinal fluid on farm A (serotypes 7, 9 and other serotypes than 1/2, 2, 7 or 9), while one *S. suis* isolate was recovered from fibrin located in the thoracic cavity on farm B (a serotype different to 1/2, 2, 7 and 9). On both farms, strains were recovered from weaned animals. *S. suis* identification was confirmed by *recN* PCR. Isolates from farm A showed different fingerprinting by ERIC-PCR, and they were also different to the isolate from farm B, indicating that several *S. suis* strains were causing disease on farm A.

The prevalence of *S. suis*-associated disease differed between farms and batches (Table 1). Overall, farm A had more diseased animals (from 5.5% to 17.6% in early nursery) than farm B (less than 0.3% for

TABLE 1 Prevalence of *Streptococcus suis*-associated disease and mortality in the six batches at each sampling point.

		Farm A			Farm B		
		Batch 1	Batch 2	Batch 3	Batch 1	Batch 2	Batch 3
***S. suis*-associated disease**	**Suckling piglets**	0% (0/300)	0.3% (1/335)	0% (0/363)	0.2% (3/1786)	0.1% (2/1906)	0.1% (2/1805)
	Early nursery	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 nursery	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 nursery**	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 nursery	7.4% (22/298)	14.2% (47/330)	8.3% (30/360)	0.7% (13/1785)	0.8% (15/1901)	0.5% (9/1795)
	Nursery	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 2 Prevalence of the pathogens obtained in sampled piglets and sows.

			Farm A			Farm B		
			Batch 1	Batch 2	Batch 3	Batch 1	Batch 2	Batch 3
Piglets	**Maternity unit**	Number of samples	30	30	30	30	30	30
		S. suis-associated disease	0%	3%	0%	3%	7%	7%
		Nasal *S. suis*	93%	83%	97%	100%	100%	80%
		Nasal *S. suis* serotype 2 (1/2)	3%	0%	0%	13%	100%	3%
		Nasal *S. suis* serotype 9	20%	77%	57%	0%	10%	0%
		Nasal *G. parasuis* virulent strains	93%	93%	60%	50%	10%	93%
		Nasal *G. parasuis* non-virulent strains	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 nursery	Number of samples	40	40	37	30	30	30
		S. suis-associated disease	38%	30%	24%	0%	3%	0%
		Nasal *S. suis*	100%	100%	100%	100%	100%	57%
		Nasal *S. suis* serotype 2 (1/2)	13%	0%	0%	3%	90%	37%
		Nasal *S. suis* serotype 9	90%	100%	100%	10%	93%	0%
		Nasal *G. parasuis* virulent strains	87%	90%	92%	100%	100%	100%
		Nasal *G. parasuis* non-virulent strains	87%	93%	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%	0%	143%	0%
	Late nursery	Number of samples	40	40	37	30	30	30
		S. suis-associated disease	55%	30%	16%	3%	0%	0%
Sows	**Nasal**	Number of samples	10	10	10	10	10	10
		S. suis	90%	70%	50%	90%	100%	60%
		S. suis serotype 2 (1/2)	30%	0%	0%	60%	40%	80%
		S. suis serotype 9	20%	40%	70%	20%	20%	0%
		G. parasuis virulent strains	50%	50%	30%	50%	50%	80%
		G. parasuis non-virulent strains	80%	60%	30%	100%	100%	50%
	Vaginal	*S. suis*	90%	10%	50%	20%	0%	0%
		S. suis serotype 2 (1/2)	30%	0%	0%	0%	0%	0%
		S. suis serotype 9	10%	10%	30%	0%	0%	0%

Abbreviations: *G. parasuis, Glaesserella parasuis*; PCV-2, porcine circovirus type 2; PRRSV, porcine reproductive and respiratory syndrome virus; *S. suis, Streptococcus suis*; SIV, swine influenza virus; Vir, virulent.

the different phases and batches). Weaners were usually more affected than suckling piglets, especially on farm A.

Prevalence of concomitant pathogens

The prevalence of the pathogens in both piglets and sows is shown in Table 2. *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 the vagina of sows (56.7%). *S. suis* was not detected (i.e., absent in both farrowing and nursery units) only in five animals, all from the third batch of farm B. Data on serotypes 2 (or 1/2) and 9 were included in the analyses because they are the most common in Europe. Serotype 2 (or 1/2) was more prevalent on farm B, contrary to farm A where the most prevalent was serotype 9. As 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 detected in 23 out of 117 animals.

Although *G. parasuis* was detected in all piglets throughout the study, the prevalence of virulent strains varied according to age, being especially high in early nursery pigs (90% and 100% on farms A and B, respectively).

In general, the prevalence of PCV-2 and SIV was low. In contrast, the prevalence of PRRSV on farms A and B was relatively high, particularly in nursery pigs (Table 2).

Biomarker determination

The levels of cortisol, haptoglobin and H_2O_2 varied significantly between the various ages and batches (Table S2). In general, piglets with *S. suis*-associated

disease had higher levels of cortisol, H_2O_2 and haptoglobin than healthy piglets of the same age and batch. For example, in the nursery of farm A, healthy animals had a median of 77.80 mg/dL of haptoglobin compared to 243.37 mg/dL in animals with clinical signs (Figure S1).

Environmental data

Mean room temperatures ranged between 25.8°C and 28.4°C in the farrowing unit and between 26.3°C and 28.4°C in the nursery, while the mean relative humidity ranged between 37.3% and 58.6% in the farrowing unit and between 24.0% and 49.3% in the nursery (Table S3). Relative humidity could not be recorded on one of the visits to farm A due to a device malfunction. CO_2 concentration in the nursery of farm A was almost twice that in the farrowing unit (mean of 2857 vs. 1484 ppm, respectively), while THI values were similar in both units (63.9% in farrowing rooms and 61.0% in the nursery) (Table S3).

Bivariate analysis

The results of the bivariate analysis in the two farms, including the *p*-values and odds ratios of the variables with $p \leq 0.25$, are shown in Figure 1. While 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. *p*-Values and odds ratios for all variables and models are shown in Table S4.

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. The results indicate that animals that were sampled 1 day older (corresponding to the age at which piglets were weaned) present an almost five-fold increase in the odds of developing *S. suis* clinical disease. The animals with higher levels of cortisol and haptoglobin at the first sampling were also more prone to develop disease. The presence of PRRSV in nursery pigs was also statistically significant in this model, becoming the most influential factor (OR = 6.40). Regarding environmental factors, higher mean relative humidity in farrowing rooms increased the odds of *S. suis*-associated disease (OR = 1.10). The only sow factor that was retained in the model was the sow parity, with younger sows being more likely 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 development of *S. suis*-associated disease at weaning (Table 3), namely, the level of haptoglobin in the blood (which indicates inflammation) (OR = 1.01), the average relative humidity in farrowing units (OR = 1.11) and the sow parity (OR = 0.71).

Model 3: Nursery risk factor model

The effect of the variables was also studied for the nursery period with Model 3. The influence of PRRSV co-infection (OR = 6.69) and the average temperature (OR = 0.13) at the beginning of the nursery period were significant, in addition to the parity of the sow (OR = 0.55). Sow parity had the same protective effect as observed in Models 1 and 2.

Models for CO_2 and THI

CO_2 and THI were measured only on farm A. On this farm, only CO_2 range was statistically significant in Models 1 and 2, whereas in Model 3 mean CO_2 was also significant (Table S5). Compared with the models built for the two farms, co-infection with PRRSV and sow parity remained as significant factors with similar values, while haptoglobin was significant in nursery pigs instead of in suckling piglets.

DISCUSSION

S. suis-associated disease is one of the most significant diseases in the pig industry, particularly in intensive pig production systems.[6] Despite the fact that the bacterium is highly prevalent on pig farms due to its role as a natural inhabitant of the microbiota of the porcine upper respiratory tract,[4,43,44] the proportion of animals that are clinically affected is relatively low.[2] This is in accordance with the result of this study, in which *S. suis* was detected in the nasal cavity of all but five of the sampled piglets (97.6% for both farms), but only a small percentage of the animals developed associated disease (0.12%, 1.52%, and 1.47% of the total piglets for each of the sampling points, considering both farms).

S. suis-associated disease has been associated with co-infection with other agents, especially with viruses. Rieckmann et al.[45] reported that even low virulence *S. suis* strains resulted in the development of *S. suis* disease in PRRSV-positive herds. In the present study, PRRSV-infected animals presented a higher risk of developing *S. suis* clinical signs following weaning. The absence of statistical significance for the other two porcine viruses studied, PCV-2 and SIV, does not imply a lack of effect, as their prevalence was low. An important question that arises in co-infection scenarios is which is the primary pathogen, as the simultaneous detection of several pathogens in diseased animals does not allow us to establish the order of infection. However, by looking at the risk factors in the previous stages (Models 2 and 3), we were able to relate the

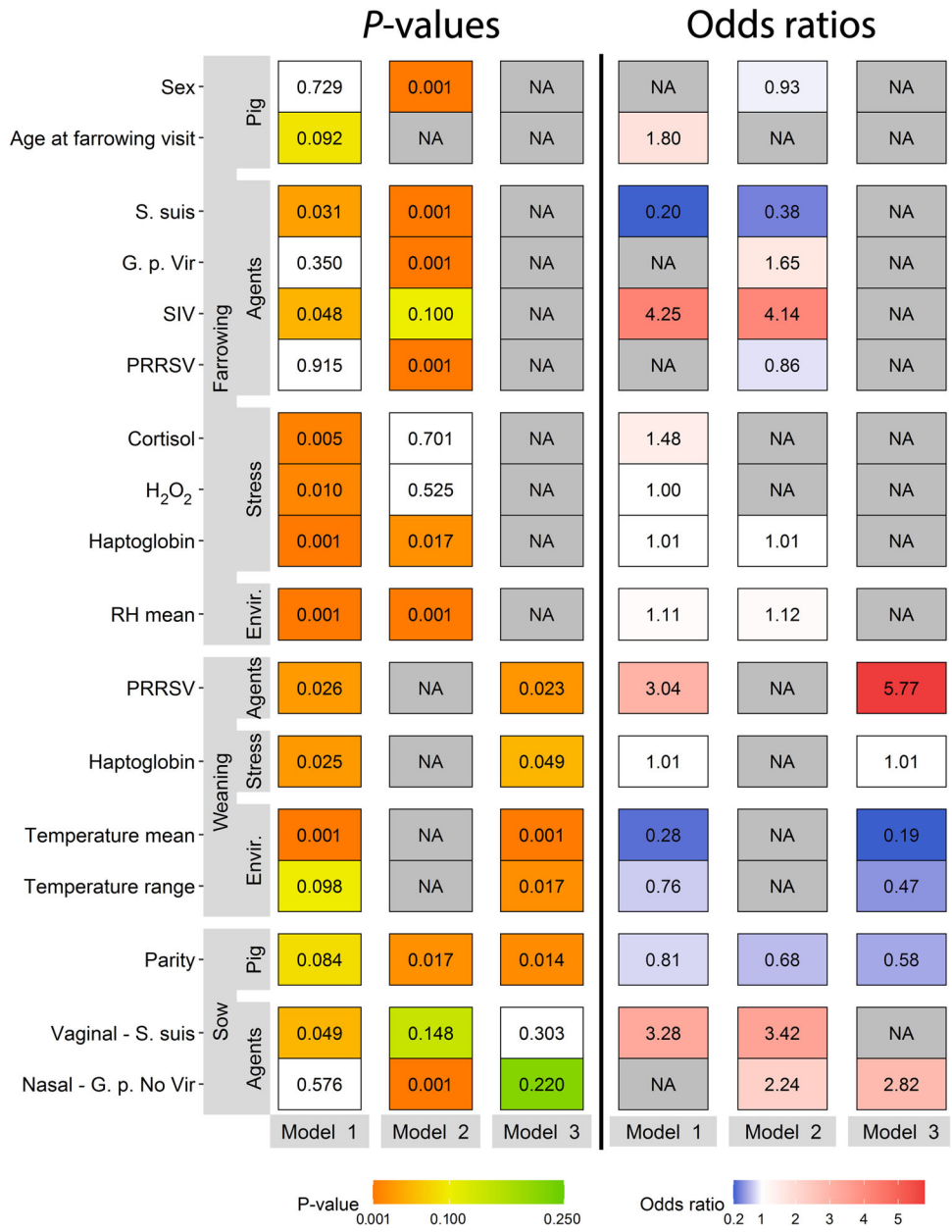

FIGURE 1 Results of the bivariate analysis for all factors with $p \leq 0.25$ on both farms. Model 1: General risk factor model; Model 2: Weaning risk factor model; Model 3: Nursery risk factor model. G. p. No Vir: *G. parasuis* non-virulent strain; G. p. Vir: *Glasserella parasuis* virulent strain; H_2O_2: hydrogen peroxide; PRRSV: porcine reproductive and respiratory syndrome virus; RH: relative humidity; SIV: swine influenza virus; *S. suis*: *Streptococcus suis*

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 colonisers of the porcine upper respiratory tract and affecting young pigs, a direct relationship was not identified in the studied farms. The use of different host cell receptors may explain the absence of interaction, as was observed in previous in vitro studies.[46]

Regarding the environmental conditions, relative humidity and CO_2 can be taken as indirect measures of ventilation and air renewal. Our results showed that piglets located in farrowing units with a higher relative humidity or in nursery units with a higher CO_2 concentration were more prone to develop *S. suis*-associated disease, which reflects the importance of keeping animals in well-ventilated spaces.

Piglets were intentionally sampled before and after weaning, which represents a period of high stress 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, the frequency of disease was expected to be higher.[47] 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 at which this stress occurs.[48] It is also important to point out that stress evaluated in the context of an infectious disease can be considered either a consequence of the disease or a possible cause.[49] The longitudinal study design allowed us to evaluate whether changes in the assessed biomarkers preceded the *S. suis*-associated disease. Prolonged stress stimuli have been

TABLE 3 Variables included in the three mixed-effect logistic regression models built for both farms.

Variable	OR	95% CI	Beta coefficient	*p*-Value
Model 1: General risk factor				
Age of suckling piglets at sampling	4.95	1.87–13.12	1.59	0.001
Cortisol in suckling piglets	1.88	1.32–2.69	0.63	<0.001
Haptoglobin in suckling piglets	1.01	1.01–1.02	0.01	<0.001
Mean of relative humidity in the farrowing unit	1.10	1.02–1.18	0.09	0.013
PRRSV presence in nursery pigs	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 in suckling piglets	1.01	1.00–1.02	0.01	0.028
Mean of relative humidity in the farrowing unit	1.11	1.05–1.17	0.10	<0.001
Sow parity	0.71	0.52–0.97	−0.34	0.031
Model 3: Nursery risk factor				
PRRSV presence in early nursery pigs	6.69	1.55–28.85	1.90	0.011
Mean of temperature in early nursery	0.13	0.05–0.37	−2.04	<0.001
Sow parity	0.55	0.37–0.83	−0.59	0.004

Abbreviations: CI, confident interval; OR, odds ratio; PRRSV, porcine reproductive and respiratory syndrome virus.

associated with elevated levels of cortisol[50]; however, in our study, the association of high cortisol levels and *S. suis*-related disease was observed only in the general model. Haptoglobin has been shown to increase in feed-deprived piglets,[51] 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, in Model 2 we also found that high values of haptoglobin in suckling piglets were correlated with the appearance of the disease 2 weeks later, at the beginning of postweaning. Despite being a statistically significant variable in our models, the magnitude of this influence turned out to be low, increasing the possibility 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).

As sampling was done just before weaning, animals that would be weaned a few days older were more likely to develop *S. suis*-associated disease. Although the explanation for this relationship seems unclear, it might be associated with differences between the two farms, and also with the presence of different strains that would disperse and colonise at different rates. According to Gebhardt et al.,[43] the sex of the animal does not influence postweaning mortality, nor did it influence the appearance of *S. suis*-associated disease in our study.

Hopkins et al.[9] observed that piglets from sows whose previous litters presented with *S. suis* problems were less likely to develop the disease. In our study, we showed 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 influence the development of the piglet microbiota, as has been observed in sows vaccinated against *G. parasuis*.[52]

Our findings suggest that control of infections with other pathogens, improvement of the environmental conditions and reduction of stress may help reduce the incidence of *S. suis* disease, with the consequent decrement in the cost for the producers and the positive effect on public health due to both a reduction in the use of antimicrobials and a reduction of the risk for human infections.[53]

However, one of the limitations of the study was that it was not possible to obtain a laboratory confirmation of the diagnosis for each case of *S. suis*-associated disease, as that would have implied the sacrifice of the animals. Because of that, laboratory diagnosis was carried out only at the farm level, while diagnosis of individuals was based on the presence of clinical signs compatible with *S. suis*-associated disease. Furthermore, given the logistical difficulties associated with a longitudinal study, the evaluation of risk factors could only be carried out on 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 other potential factors may influence the risk of *S. suis*.

CONCLUSION

Our study highlights the multifactorial nature of *S. suis*-associated disease, for which both environmental factors and factors related to the host seem to be involved in the development of the disease. The information presented in this study can help farmers in preventing *S. suis* outbreaks, enabling them to identify and potentially control some of the risk factors involved in the appearance of disease.

AUTHOR CONTRIBUTIONS

Conceptualisation: Carlos Neila-Ibáñez, Sebastián Napp, Virginia Aragon and Jordi Casal. Methodology: Carlos Neila-Ibáñez. Biomarker determination: Lorena Franco-Martínez and José Joaquín Cerón. Data analysis: Carlos Neila-Ibáñez and Lola Pailler-García. Writing the original draft: Carlos Neila-Ibáñez. Writing the review and editing: Carlos Neila-Ibáñez, Sebastián Napp, Lola Pailler-García, Virginia Aragon and Jordi Casal. Funding acquisition: Sebastián Napp, Virginia Aragon and Jordi Casal. All authors read and approved the final manuscript.

ACKNOWLEDGEMENTS

The authors would like to thank the farm animal caretakers and veterinarians for their willingness to collaborate in the study. Diego Pérez and Patricia Pleguezuelos from IRTA-CReSA are thanked for their help in sampling. Núria Galofré-Milà from IRTA-CReSA is thanked for her assistance in the analysis of samples. Carlos Neila-Ibáñez, Sebastián Napp, Lola Pailler-García, Virginia Aragon and Jordi Casal are also grateful to the CERCA programme (Centres de Recerca de Catalunya Programme) from the Generalitat de Catalunya.

CONFLICT OF INTEREST STATEMENT

The authors declare they have no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data are available from the corresponding author upon request.

ETHICS STATEMENT

Sampling of piglets was done under institutional authorisation from IRTA-CReSA (Protocol number 11199) and followed good veterinary practices in accordance with European (Directive 2010/63/EU) and Spanish (Real Decreto 53/2013) regulations and with the approval of the Ethics Commission.

REFERENCES

1. Gottschalk M, Segura M. Streptococci. In: Zimmerman JJ, Karriker LA, Ramirez A, Schwartz KJ, Stevenson GW, Zhang J, editors. Diseases of swine. 11th ed. Hoboken, USA: John Wiley & Sons, Inc.; 2019. p. 934–50.
2. Neila-Ibáñez C, Casal J, Hennig-Pauka I, Stockhofe-Zurwieden N, Gottschalk M, Migura-García L, et al. Stochastic assessment of the economic impact of *Streptococcus suis*-associated disease in German, Dutch and Spanish swine farms. Front Vet Sci. 2021;8:676002.
3. Baele M, Chiers K, Devriese LA, Smith HE, Wisselink HJ, Vaneechoutte M, et al. The Gram-positive tonsillar and nasal flora of piglets before and after weaning. J Appl Microbiol. 2001;91:997–1003.
4. Segura M, Fittipaldi N, Calzas C, Gottschalk M. Critical *Streptococcus suis* virulence factors: are they all really critical? Trends Microbiol. 2017;25:585–99.
5. Segura M, Calzas C, Grenier D, Gottschalk M. Initial steps of the pathogenesis of the infection caused by *Streptococcus suis*: fighting against nonspecific defenses. FEBS Lett. 2016;590:3772–99.
6. Gottschalk M, Xu J, Calzas C, Segura M. *Streptococcus suis*: a new emerging or an old neglected zoonotic pathogen? Future Microbiol. 2010;5:371–91.
7. Segura M, Aragon V, Brockmeier SL, Gebhart C, Greeff A, Kerdsin A, et al. Update on *Streptococcus suis* research and prevention in the era of antimicrobial restriction: 4th International Workshop on *S. suis*. Pathogens. 2020;9:374.
8. Reams RY, Glickman LT, Harrington DD, Thacker HL, Bowersock TL. *Streptococcus suis* infection in swine: a retrospective study of 256 cases. Part II. Clinical signs, gross and microscopic lesions, and coexisting microorganisms. J Vet Diagnostic Investig. 1994;6:326–34.
9. Hopkins D, Poljak Z, Farzan A, Friendship R. Factors contributing to mortality during a *Streptoccocus suis* outbreak in nursery pigs. Can Vet J. 2018;59:623–30.
10. Obradovic MR, Segura M, Segalés J, Gottschalk M. Review of the speculative role of co-infections in *Streptococcus suis*-associated diseases in pigs. Vet Res. 2021;52:49.
11. Feng W, Laster SM, Tompkins M, Brown T, Xu J-S, Altier C, et al. In utero infection by porcine reproductive and respiratory syndrome virus is sufficient to increase susceptibility of piglets to challenge by *Streptococcus suis* type II. J Virol. 2001;75:4889–95.
12. Thanawongnuwech R, Brown GB, Halbur PG, Roth JA, Royer RL, Thacker BJ. Pathogenesis of porcine reproductive and respiratory syndrome virus-induced increase in susceptibility to *Streptococcus suis* infection. Vet Pathol. 2000;37:143–52.
13. Xu M, Wang S, Li L, Lei L, Liu Y, Shi W, et al. Secondary infection with *Streptococcus suis* serotype 7 increases the virulence of highly pathogenic porcine reproductive and respiratory syndrome virus in pigs. Virol J. 2010;7:184.
14. Wang Q, Zhou H, Hao Q, Li M, Liu J, Fan H. Co-infection with porcine circovirus type 2 and *Streptococcus suis* serotype 2 enhances pathogenicity by dysregulation of the immune responses in piglets. Vet Microbiol. 2020;243:108653.
15. Lin X, Huang C, Shi J, Wang R, Sun X, Liu X, et al. Investigation of pathogenesis of H1N1 influenza virus and swine *Streptococcus suis* serotype 2 co-infection in pigs by microarray analysis. PLoS One. 2015;10:e0124086.
16. Vecht U, Wisselink HJ, Van Dijk JE, Smith HE. Virulence of *Streptococcus suis* type 2 strains in newborn germ-free pigs depends on phenotype. Infect Immun. 1992;60:550–6.
17. Correa-Fiz F, Fraile L, Aragon V. Piglet nasal microbiota at weaning may influence the development of Glässer's disease during the rearing period. BMC Genomics. 2016;17:404.
18. Blanco-Fuertes M, Correa-Fiz F, Fraile L, Sibila M, Aragon V. Altered nasal microbiota composition associated with development of polyserositis by *Mycoplasma hyorhinis*. Pathogens. 2021;10:603.
19. Niazy M, Hill S, Nadeem K, Ricker N, Farzan A. Compositional analysis of the tonsil microbiota in relationship to *Streptococcus suis* disease in nursery pigs in Ontario. Anim Microbiome. 2022;4:10.
20. Aragon V, Segales J, Tucker AW. Glässer's disease. In: Zimmerman JJ, Karriker LA, Ramirez A, Schwartz KJ, Stevenson GW, Zhang J, editors. Diseases of swine. 11th ed. Hoboken, USA: John Wiley & Sons, Inc.; 2019. p. 844–53.
21. Vötsch D, Willenborg M, Weldearegay YB, Valentin-Weigand P. *Streptococcus suis* - the "two faces" of a pathobiont in the porcine respiratory tract. Front Microbiol. 2018;9:480.
22. Dee SA, Carlson AR, Winkelman NL, Corey MM. Effect of management practices on the *Streptococcus suis* carrier rate in nursery swine. J Am Vet Med Assoc. 1993;203:295–9.
23. Staats JJ, Feder I, Okwumabua O, Chengappa MM. *Streptococcus suis*: past and present. Vet Res Commun. 1997;21:381–407.
24. Cerón JJ, Contreras-Aguilar MD, Escribano D, Martínez-Miró S, López-Martínez MJ, Ortín-Bustillo A, et al. Basics for the potential use of saliva to evaluate stress, inflammation, immune system, and redox homeostasis in pigs. BMC Vet Res. 2022;18:81.
25. Murata H. Stress and acute phase protein response: an inconspicuous but essential linkage. Vet J. 2007;173:473–4.
26. Deblanc C, Robert F, Pinard T, Gorin S, Quéguiner S, Gautier-Bouchardon AV, et al. Pre-infection of pigs with *Mycoplasma hyopneumoniae* induces oxidative stress that influences outcomes of a subsequent infection with a swine influenza virus of H1N1 subtype. Vet Microbiol. 2013;162:643–51.

27. Neila-Ibáñez C, Brogaard L, Pailler-García L, Martínez J, Segalés J, Segura M, et al. Piglet innate immune response to *Streptococcus suis* colonization is modulated by the virulence of the strain. Vet Res. 2021;52:145.
28. Contreras-Aguilar MD, Escribano D, Martínez-Miró S, López-Arjona M, Rubio CP, Martínez-Subiela S, et al. Application of a score for evaluation of pain, distress and discomfort in pigs with lameness and prolapses: correlation with saliva biomarkers and severity of the disease. Res Vet Sci. 2019;126:155–63.
29. Holtkamp D, Torremorell M, Corzo CA, Linhares D, Nunes M, Yeske P, et al. Proposed modifications to porcine reproductive and respiratory syndrome virus herd classification. J Swine Health Prod. 2021;29:261–70.
30. Ishida S, Tien LHT, Osawa R, Tohya M, Nomoto R, Kawamura Y, et al. Development of an appropriate PCR system for the reclassification of *Streptococcus suis*. J Microbiol Methods. 2014;107:66–70.
31. Okura M, Lachance C, Osaki M, Sekizaki T, Maruyama F, Nozawa T, et al. Development of a two-step multiplex PCR assay for typing of capsular polysaccharide synthesis gene clusters of *Streptococcus suis*. J Clin Microbiol. 2014;52:1714–9.
32. Versalovic J, Koeuth T, Lupski R. Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. Nucleic Acids Res. 1991;19:6823–31.
33. Galofré-Milà N, Correa-Fiz F, Lacouture S, Gottschalk M, Strutzberg-Minder K, Bensaid A, et al. A robust PCR for the differentiation of potential virulent strains of *Haemophilus parasuis*. BMC Vet Res. 2017;13:124.
34. López-Valiñas Á, Sisteré-Oró M, López-Serrano S, Baioni L, Darji A, Chiapponi C, et al. Identification and characterization of swine influenza virus H1N1 variants generated in vaccinated and nonvaccinated, challenged pigs. Viruses. 2021;13:2087.
35. Escribano D, Fuentes-Rubio M, Cerón JJ. Validation of an automated chemiluminescent immunoassay for salivary cortisol measurements in pigs. J Vet Diagnostic Investig. 2012;24:918–23.
36. Kaiser M, Jacobson M, Andersen PH, Bækbo P, Cerón JJ, Dahl J, et al. Inflammatory markers before and after farrowing in healthy sows and in sows affected with postpartum dysgalactia syndrome. BMC Vet Res. 2018;14:83.
37. Rhee SG, Chang TS, Jeong W, Kang D. Methods for detection and measurement of hydrogen peroxide inside and outside of cells. Mol Cells. 2010;29:539–49.
38. Rubio CP, Mainau E, Cerón JJ, Contreras-Aguilar MD, Martínez-Subiela S, Navarro E, et al. Biomarkers of oxidative stress in saliva in pigs: analytical validation and changes in lactation. BMC Vet Res. 2019;15:144.
39. Dohoo IR, Martin SW, Stryhn H. Veterinary epidemiologic research. 1st ed. Charlottetown, Canada: AVC Inc; 2003.
40. R Core Team R: a language and environment for statistical computing. Vienna: R Foundation for Statistical Computing; 2020.
41. Bates D, Mächler M, Bolker BM, Walker SC. Fitting linear mixed-effects models using lme4. J Stat Softw. 2015;67:1–48.
42. Zhang D. CRAN - Package rsq. 2021.
43. Gebhardt JT, Tokach MD, Dritz SS, DeRouchey JM, Woodworth JC, Goodband RD, et al. Postweaning mortality in commercial swine production. I: review of non-infectious contributing factors. Transl Anim Sci. 2020;4:462–84.
44. Goyette-Desjardins G, Auger JP, Xu J, Segura M, Gottschalk M. *Streptococcus suis*, an important pig pathogen and emerging zoonotic agent-an update on the worldwide distribution based on serotyping and sequence typing. Emerg Microbes Infect. 2014;3:e45.
45. Rieckmann K, Pendzialek SM, Vahlenkamp T, Baums CG. A critical review speculating on the protective efficacies of autogenous *Streptococcus suis* bacterins as used in Europe. Porc Heal Manag. 2020;6:12.
46. Mathieu-Denoncourt A, Letendre C, Auger JP, Segura M, Aragon V, Lacouture S, et al. Limited interactions between *Streptococcus suis* and *Haemophilus parasuis* in in vitro co-infection studies. Pathogens. 2018;7:7.
47. Campbell JM, Crenshaw JD, Polo J. The biological stress of early weaned piglets. J Anim Sci Biotechnol. 2013;4:19.
48. Proudfoot KL, Weary DM, von Keyserlingk MAG. Linking the social environment to illness in farm animals. Appl Anim Behav Sci. 2012;138:203–15.
49. Martínez-Miró S, Tecles F, Ramón M, Escribano D, Hernández F, Madrid J, et al. Causes, consequences and biomarkers of stress in swine: an update. BMC Vet Res. 2016;12:171.
50. Dhabhar FS. Enhancing versus suppressive effects of stress on immune function: implications for immunoprotection and immunopathology. Neuroimmunomodulation. 2009;16:300–17.
51. Ott S, Soler L, Moons CPH, Kashiha MA, Bahr C, Vandermeulen J, et al. Different stressors elicit different responses in the salivary biomarkers cortisol, haptoglobin, and chromogranin A in pigs. Res Vet Sci. 2014;97:124–8.
52. Blanco-Fuertes M, Correa-Fiz F, López-Serrano S, Sibila M, Aragon V. Sow vaccination against virulent *Glaesserella parasuis* shapes the nasal microbiota of their offspring. Sci Rep. 2022;12:3357.
53. Rayanakorn A, Goh BH, Lee L-H, Khan TM, Saokaew S. Risk factors for *Streptococcus suis* infection: a systematic review and meta-analysis. Sci Rep. 2018;8(1):13358.

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Neila-Ibáñez C, Napp S, Pailler-García L, Franco-Martínez L, Cerón JJ, Aragon V, et al. Risk factors associated with *Streptococcus suis* cases on pig farms in Spain. Vet Rec. 2023;e3056. https://doi.org/10.1002/vetr.3056