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

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Research Article

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Abstract

Background: *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 mainly around weaning age. However, little is known about the factors responsible for triggering the disease. Therefore, a longitudinal study was carried out, in which a total of six batches from two Spanish swine farms with *S. suis* problems were repeatedly examined, once in the farrowing unit and twice in the weaning unit.

Results: Potential risk factors evaluated included: a) concomitant pathogens such as porcine reproductive respiratory syndrome virus (PRRSV), porcine circovirus type 2, swine influenza virus, and *Glaesserella parasuis*; b) biomarkers associated to stress (cortisol), inflammation (haptoglobin), and oxidative status (hydrogen peroxide); c) farm environmental factors such as temperature, relative humidity, CO2, 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 risk factors involved 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. Risk factors for *S. suis* disease included PRRSV coinfection at weaning (Odds ratio (OR) = 6.69), sow parity (OR = 0.71), haptoglobin before weaning (OR = 1.01), and relative humidity in the farrowing unit as well as temperature at weaning (OR = 1.11 and 0.13, respectively).

Conclusions: This study confirms 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.

Background

*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 and the costs associated to disease control [1, 2]. Although *S. suis* is a normal colonizer of the upper respiratory tract [3], pigs, especially piglets from 5 to 10 week-old, can develop a disease characterized mainly by meningitis, polyarthritis, and acute death [1]. However, the circumstances that allow strains from the microbiota of healthy animals to produce clinical disease are not completely known [4].

*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 [5]. Serotypes are distributed worldwide and the lesions they produce are not serotype dependent [6]. 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 [7]. Although many virulent factors have been described for *S. suis*, they are not always present in clinical isolates [8]. 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 play a role in the mortality during a *S. suis* outbreak in sucking piglets [9]. *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 [10]. 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* [11]. 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 [12, 13]. When *S. suis* challenge was performed in piglets five days after PCV-2 infection, co-infected piglets exhibited more severe clinical signs and lesions than those inoculated only with one of the pathogens [14]. Similar observations were reported for the SIV coinfection, with co-infected piglets showing more severe clinical signs and increased gene expression of pro-inflammatory mediators than those inoculated only with one of the pathogens [15]. 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 [16]. 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 [17, 18]. Recently, Niazy and collaborators [19] 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 [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, with higher levels than in non-infected animals [26]. 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 [27]. Ott and collaborators [28] indicated that cortisol can be used in psychosocial stress situations, such as when animals are mixed, while haptoglobin is not effective in these situations [29]. In addition, biomarkers of oxidative status such as hydrogen peroxide (H$_2$O$_2$) or advanced oxidation protein products can be used as pain indicators and to assess oxidative stress [30],
and can show changes in infectious processes, as demonstrated in SIV and Mycoplasma hyopneumoniae infections [26].

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.

**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 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 they were also different to the isolate from farm B.

Prevalence of *S. suis*-associated disease differed between farms and batches (Table 1). In global, farm A had more diseased animals than farm B. Sucking piglets were more affected in farm B and weaners were more affected in farm A.
Table 1
Prevalence of *S. suis*-associated disease and mortality in different farms and batches.

<table>
<thead>
<tr>
<th></th>
<th>Farm A</th>
<th></th>
<th>Farm B</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Batch 1</td>
<td>Batch 2</td>
<td>Batch 3</td>
<td>Batch 1</td>
</tr>
<tr>
<td><strong>S. suis-associated disease</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Farrowing</td>
<td>0.0%</td>
<td>0.3%</td>
<td>0.0%</td>
<td>0.2%</td>
</tr>
<tr>
<td></td>
<td>(0/300)</td>
<td>(1/335)</td>
<td>(0/363)</td>
<td>(3/1786)</td>
</tr>
<tr>
<td>Early weaning</td>
<td>7.0%</td>
<td>17.6%</td>
<td>5.0%</td>
<td>0.0%</td>
</tr>
<tr>
<td></td>
<td>(21/300)</td>
<td>(59/335)</td>
<td>(18/363)</td>
<td>(0/1786)</td>
</tr>
<tr>
<td>Late weaning</td>
<td>9.1%</td>
<td>13.0%</td>
<td>4.7%</td>
<td>0.1%</td>
</tr>
<tr>
<td><strong>Mortality</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Early weaning</td>
<td>0.7%</td>
<td>1.5%</td>
<td>0.8%</td>
<td>0.1%</td>
</tr>
<tr>
<td>Late weaning</td>
<td>7.4%</td>
<td>14.2%</td>
<td>8.3%</td>
<td>0.7%</td>
</tr>
<tr>
<td></td>
<td>(22/298)</td>
<td>(47/330)</td>
<td>(30/360)</td>
<td>(13/1785)</td>
</tr>
<tr>
<td>Weaning</td>
<td>8.0%</td>
<td>15.5%</td>
<td>9.1%</td>
<td>0.8%</td>
</tr>
<tr>
<td></td>
<td>(24/300)</td>
<td>(52/335)</td>
<td>(33/363)</td>
<td>(14/1786)</td>
</tr>
</tbody>
</table>

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

**Prevalence of infectious agents**

The prevalence of the pathogens in both piglets and sows is shown in Table 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. Data on 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 strains at farrowing was less common on farm B compared to farm A, although this detection evolved on farm B from 50.0, 10.0, and 93.3% for the three batches sampled in farrowing to 100% in the post-weaning unit. In addition, in the case of farm A, six suckers and one weaner were negative to *G. parasuis*, for both virulent and non-virulent 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 1).

**Biomarkers determination**

Three different biomarkers, cortisol, haptoglobin, and H$_2$O$_2$ were used to evaluate the stress, inflammation and oxidative status, respectively. Important differences were observed between the various ages and batches (Additional file 2). In general, piglets with *S. suis*-associated disease had higher levels of cortisol, H$_2$O$_2$, 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 clinical signs (Additional file 3).

**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 (Additional file 4). Relative humidity could not be recorded in one of the visits to the farm A due to a device malfunction. 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) (Additional file 4).

**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 Fig. 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 (Fig. 1).

**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 2. 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 (Fig. 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 sow parity, with younger sows being more prone to have piglets with *S. suis* problems (OR = 0.69).
### Table 2
Variables included in the three models built for both farms.

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

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

### Model 2: Weaning Risk Factor model

Some farrowing variables had a significant impact on the *S. suis*-disease status at weaning (Table 2), namely, the inflammation of piglets indicated by the haptoglobin marker (OR = 1.01), the average relative humidity at farrowing (OR = 1.11), and the sow parity (OR = 0.71). The higher values of haptoglobin, the higher mean relative humidity, the lower the parity of the sow, the higher the odds of developing *S. suis*-disease at weaning.

### 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 parity had the same protective effect than in Model 1 and 2. The presence of PRRSV and lower mean temperature also facilitated the development of *S. suis*-disease.

### Models for CO₂ and THI
CO₂ and THI were measured only in farm A. In this farm, only CO₂ range was statistically significant in Model 1 and 2, whereas in Model 3 mean CO₂ was also significant (Additional file 5). Compared with the models built for the two farms, coinfection with PRRSV and sow parity remained as significant factors with similar values, haptoglobin was significant in weaning instead of in farrowing, while age, cortisol, temperature, and relative humidity were statistically significant (Additional file 5).

**Discussion**

*S. suis*-associated disease is one of the main diseases in the swine industry, in particular in intensive pig production systems [31]. Despite the fact that the bacterium is highly prevalent in swine farms due to its role as natural inhabitant of the microbiota of the porcine upper respiratory tract [7, 8, 32], 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 sampled piglets except five, but only a percentage of the animals developed the disease.

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 development of endemic diseases and their severity is often a challenge [33]. In the present study, various factors that have historically been associated with *S. suis* outbreaks, such as coinfections or temperature and humidity [10, 22], have been studied.

*S suis* disease has been associated with co-infection with other agents, especially with viruses. Rieckmann and collaborators [34] reported that even low virulent *S. suis* strains resulted in the development of *S. suis* disease in PRRSV-positive herds. Disease presentation was 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 while the increase of *S. suis* infections may be linked to epidemics of those viruses.

An important question that arises in co-infection 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 as 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 identified in the studied farms. The use of different host cell receptors may explain the absence of interaction, as was observed in *in vitro* studies [35].
According to Wathes and Whittemore [36], 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 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 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 [37]. All these non-infectious factors may have an impact on the incidence and severity of infectious processes [32], 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 [38]. 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 [39]. In the present study, in addition to cortisol as biomarker of stress, we used the haptoglobin as a biomarker of inflammation and the H$_2$O$_2$, 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 the *S. suis*-associated disease. Prolonged stress stimuli have been associated with elevated levels of cortisol [40], however, in our scenario the association of high cortisol levels and *S. suis*-associated disease was observed only in the general model. Haptoglobin has been shown to increase in feed deprived piglets [28], 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 show in Model 1. However, we also found in Model 2 that high values of haptoglobin in suckling 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 protein, 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 [24]. Despite being a significant variable in our models, the magnitude of this influence turned out to be low, increasing the possibility to develop *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 [24]. 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 the explanation for this relationship seems us unclear, it might be associated to different strains dispersing and colonizing at different rates. According
to Gebhardt and collaborators [32], 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 [9] 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* [41].

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*.

**Conclusion**

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.

**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 clinical signs.

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 clinical signs 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 the study, but a porcine epidemic diarrhoea (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 company. 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 [42] but lowering the annealing temperature to 43°C.

**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. Animal 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. Supplementary information about the sampled animals is included in Additional file 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 2, whereas the number of sows sampled were 10 for each batch.

In the first two visits, an environmental data logger was placed in the area where the piglets were located, at approximately 30 centimetres of height. The data logger located at farm A recorded temperature (°C), relative humidity (%), temperature-humidity index (THI), and CO₂ (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.
### Table 2
Presence of the pathogens studied in sampled piglets and sows.

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

*10 sows were sampled per batch. SIV: swine influenza virus; PRRSV: porcine reproductive and respiratory syndrome virus; PCV-2: porcine circovirus type 2. Vir: virulent.
**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 -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.

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 [43] for the presence of *S. suis*, Okura and collaborators [44] for the serotypes 1/2–2, 7 and 9, and Galofré-Milà and collaborators [45] 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 [46]. 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 37°C and 5% CO₂ 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 [43].

**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 [47].

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 [48].

Hydrogen peroxide (H$_2$O$_2$) was assessed based on the method of Rhee and collaborators [49] in an automated analyser (Olympus AU600) previously validated [50].

**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:

Model 1: General Risk Factor model, considering the presentation of the 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. at weaning), and using the data collected previously in farrowing; and Model 3: Late Weaning Risk Factor model, considering the presentation of the disease at the third visit, and using the data collected in the second visit (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 [51]. The final model selection was 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 [52]), using the packages *lme4* (v. 1.1–23 [53] and *rsq* (v. 2.2 [54]).

**Declarations**

**Ethics approval and consent to participate**
Sampling of piglets was done under institutional authorization from IRTA-CReSA and followed 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 11199).

Consent for publication

Not applicable

Availability of data and materials

The data that support the findings of this study may be available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Author’s contributions

Conceptualization: CN-I, SN, VA, and JC. Methodology: CN-I. Biomarker determination: LF-M, JJC. Data analysis; CN-I and LP-G. Writing the original draft: CN-I. Writing the review and editing: CN-I, SN, LP-G, VA, and JC. Funding acquisition: SN, VA, and JC. All authors read and approved the final manuscript.

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54. Zhang D (2021) CRAN - Package rsq

**Figures**
**Figure 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 Inuenza 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.

**Supplementary Files**

This is a list of supplementary files associated with this preprint. Click to download.

- Additionalfile1.xlsx
- Additionalfile2.xlsx
- Additionalfile3.tif
- Additionalfile4.xlsx
- Additionalfile5.xlsx