Animal Microbiome

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Nasal microbial diversity is associated with survival in piglets infected by a highly virulent PRRSV-1 strain

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Abstract

Background Porcine reproductive and respiratory syndrome virus (PRRSV) is a major threat to swine industry worldwide, especially virulent variants arising during the last years, such as Spanish PRRSV-1 Rosalia strain. The role of the nasal microbiota in respiratory viral infections is still to be unveiled but may be promisingly related with the health status of the animals and thus, their susceptibility. The goal of this project was to study the nasal microbiota composition of piglets during a highly virulent PRRSV-1 outbreak comparing animals that died due to the infection with animals that survived it. The microbiota composition was inferred by V3–V4 regions of the 16S rRNA gene sequencing and bioinformatics analysis. To deepen the analysis, we added samples taken from piglets before the outbreak as well as from the sows giving birth to piglets under study.

Results Piglets that survived the PRRSV-1 outbreak reported a more diverse and different nasal microbiota at three weeks of age compared to piglets dying, which was highly related with the litter of origin and the sow of the piglets. In addition, a high abundance of classical swine nasal colonizers belonging to genera such as *Bergeyella*, *Glaesserella*, *Neisseria* and *Moraxella* (among others), was related with good outcome. On the other hand, a dysbiotic community dominated by *Escherichia* and a different clade of *Moraxella* was found in piglets with bad outcome. Moreover, samples taken before the outbreak showed similar dynamics prior to virulent PRRSV-1 arrival, suggesting that microbiota-related susceptibility was already occurring in the animals and that the increase in mortality seen was related to the new highly virulent strain.

Conclusion Our study suggests that the susceptibility to an infection such as PRRSV could be related to the nasal microbiota composition at the moment of infection and may serve as starting point to explore animal resilience. Since the dysbiosis detected as an initial response to infection may be not specific for this virus, further investigations should explore this phenomenon in the context of other viral infections.

Keywords Pig, Swine, Virus, PRRSV, Nasal microbiota

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Introduction

For the last three decades, porcine reproductive and respiratory syndrome virus (PRRSV) has established as one of the major pathogens of swine worldwide. In sows, PRRSV infection is characterized by outbreaks of reproductive disease, which hallmark feature is abortion of fresh or mummified foetuses after day 90 of gestation. Stillbirths, weak-born piglets and an increase in preweaning mortality are other common consequences of PRRSV infection [1]. In piglets, the infection manifests as a respiratory disease that is usually complicated with other viral or bacterial respiratory agents. Furthermore, the association of secondary bacterial infections leads to increased use of antimicrobials and mortality in piglets [2].

PRRSV belongs to the genus *Betaarterivirus* within the family *Arteriviridae*, Order *Nidovirales*. Two PRRSV species are recognised, PRRSV-1 and PRRSV-2 [3]. One of the key features of both PRRSV species is the high genetic and antigenic diversity and the existence of strains of diverse virulence, among which the so-called highly pathogenic PRRSV that emerged in China in 2006 has been one of the most devastating ones [4]. Once, it was thought that highly virulent PRRSV strains only existed for PRRSV-2, while PRRSV-1 was considered of lower virulence. However, the description of strains such as Lena and Rosalia [5, 6] made clear that highly virulent strains also exist within PRRSV-1. Besides causing severe abortion storms and mortality of sows, highly virulent strains can cause high mortalities in the farrowing units and nurseries of the affected farms. This increased mortality is probably the result of a combination of factors, including the high viral loads and the consequent inflammatory response, the induction of apoptosis in the thymus of the infected piglets and the frequency of secondary infections [7].

The role of the microbiota in health and disease has been extensively studied and nowadays is one of the major research areas in biomedical sciences. However, in the veterinary field the available information is still limited. In previous studies, it was shown that PRRSV infection impacts the composition of the gut microbiome and that this impact depends on the virulence of the PRRSV strain [8–10]. Also, other studies [9, 11, 12] have shown that the diversity and composition of the gut microbiome may influence the outcome of PRRSV or porcine circovirus 2 infections, highlighting the complex interplay of the microbiota and these viral infections. However, little is known about the role and the interaction between the respiratory microbiota and respiratory viral infections in pigs. Among the few available studies, Gierse et al. [13] showed that infection with a swine influenza virus H1N1 resulted in lower richness of the nasal microbial community, while other studies [14] showed little impact of a H3N2 influenza A virus infection on the richness of the nasal microbiota, but associated the increase of certain species with the PRRSV and/or IAV challenge. Similarly, Hau et al. [15] have recently shown that the nasal microbiota seems to be resilient to influenza A virus infection.

In the present study, we examined the nasal microbiota composition of piglets naturally infected in the first three weeks of life by a highly virulent PRRSV-1 strain of a newly emerged clade in Spain, comparing the microbial communities of the animals that ended up dying due to the infection with the microbiota of those that survived it.

Material and methods

Farm, pigs and sampling

The farm where the study was conducted was a 1400-sow operation, working with weekly farrowing batches (approximately 62 sows/batch). Piglets were weaned at 4 weeks of age when they were moved to nursery pens. At 9 weeks of age, they were moved to a fattening unit where they remained until reaching market weight.

Although viral circulation occurred in growers, the farm was considered stable for PRRSV; namely weaned pigs routinely tested negative for PRRSV by RT-qPCR (LSI Vetmax PRRSV EUNA 2.0, Thermofisher), indicating that the virus was not circulating in the farrowing units. In late December 2021, a highly virulent PRRSV-1 strain belonging to a new clade recently reported [16] entered the farm. The strain had a 97.7% nucleotide identity (whole genome sequence) with strain R1, the first isolate described for this clade. The introduction of the new PRRSV strain caused a reproductive outbreak and most piglets already tested positive for the virus by RT-qPCR before weaning. To note, before the outbreak sows were routinely vaccinated against PRRSV three times per year using a modified live commercial vaccine that was administered intramuscularly. Piglets were routinely vaccinated at three weeks of age against PCV2. The farm was also known to be infected by an influenza A virus (IAV, H1N2; 1C.1.2.1 clade).

Sampling of piglets was conducted during a follow-up study initially aimed at determining the dynamics of IAV in the farm. For that purpose, a cohort of 40 piglets from the same batch belonging to ten randomly chosen litters (4 piglets randomly selected per sow) was followed from birth until the end of the nursery period. Blood samples were collected at 1, 3, 4, 5, 6, 7, 8 and 12 weeks of age to assess the evolution of pigs. Nasal samples were also taken at 3 weeks of age in piglets and simultaneously from their sows by introducing the swab until the nostril, and immediately transported to the lab at 4 °C in

transport medium for viral isolation (Virocult®). Animals were followed until 12 weeks of age and mortality was recorded. Sampled animals were not removed from the farm.

Since the mortality in the followed cohort surpassed 50%, we deemed interesting to determine the possible relationship between the outcome of PRRSV infection and the nasal microbiota composition. In the study, we included 31 animals from ten different litters after discarding animals not infected by PRRSV at 3 weeks of age. All animals included in this study developed typical clinical signs of PRRSV infection. Of those 31 animals, 16 survived until the end of the follow-up period (group S) while 15 died before that timepoint (group D). All animals included in the study were infected by PRRSV at the moment of collecting the nasal swabs; of these, 13 were also positive for IAV (8 from D and 5 from S groups). Samples taken from eight of the sows giving birth to these litters were also included (n = 8). Moreover, we disposed of some nasal swabs from 3-week-old piglets and their sows together with the clinical outcome information (survived or dead by 12 weeks of age), taken before the introduction of the highly virulent PRRSV strain. Thus, 8 samples from piglets (4 litters) and their respective sows (n = 4) were also included in the study for comparative purposes (S_0, n = 4; and D_0, n = 4). All of them were negative for PRRSV at the moment of sampling.

Determination of PRRSV and IAV infections

For the detection of PRRSV a commercial RT-qPCR kit was used (LSI Vetmax PRRSV EUNA 2.0, Thermofisher). The kit includes an internal positive control to ensure the adequacy of the RNA extraction process. IAV was detected in nasal swabs using an RT-qPCR, whose primers and probe have been described elsewhere [17].

DNA extraction, 16S rRNA gene library preparation and sequencing

Genomic DNA was extracted from nasal swabs with the NucleoSpin Blood kit (Machinery Nagel, GmbH & Co, Düren; Germany), following manufacturer's instructions. The concentration of the eluted DNA was measured in a BioDrop DUO device (BioDrop Ltdre). Genomic libraries of the variable regions V3-V4 from 16S rRNA gene were prepared by PCR using Illumina recommended primers (fwd 5′TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG3′, rev 5′GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC3′) and sequencing was done with Illumina MiSeq pair-end (2X300 bp, MS-102–2003 MiSeq Reagent Kit v2, 500 cycles) at the Servei de Genòmica, Universitat Autònoma de Barcelona (Spain). The length of the amplicons (460 bp) was verified on a Bioanalyzer DNA 1000 chip (Agilent). After sorting reads into samples (demultiplexing), samples were further processed through bioinformatics analyses.

Microbiota bioinformatics analysis

Raw reads obtained in the sequencing were processed and analyzed using quantitative insights into microbial ecology (QIIME2) software, version 2023.9 [18]. Then, primers were removed with *q2 cutadapt* [19]. Quality filtering, low-quality 3′ positions removal, paired-end merging, elimination of chimeras, and classification of reads into Amplicon Sequence Variants (ASVs) was performed with DADA2 [20] integrated as a QIIME2 plugin. Two additional filtering steps were performed to curate the pool of ASVs. Firstly, to remove any non-prokaryotic sequence, ASVs not matching Greengenes database [21] vs. 13.8. (available at http://greengenes.microbio.me/greengenes_release/gg_13_8_otus/) aligning at 65% identity and 50% query coverage were filtered out using VSEARCH [22] within *q2 quality control* [23]. Secondly, non-bacterial sequences classified as *Archaea*, *Chloroplast* or *Mitochondria* using the same Greengenes 13.8 taxonomy were also discarded.

Since the reads included in the analysis were obtained from two different runs, all data was merged for the downstream analysis. *Q2 greengenes2* [24] was used to infer the taxonomy of the ASVs using the *non-v4-16s* function, where ASVs were mapped to unique 16S rRNA sequences in Greengenes2 (available at https://ftp.microbio.me/greengenes_release/current/), referred as features. The phylogenetic tree was obtained from the same repository.

Diversity analyses were performed using *q2 diversity* at a common depth of 18,090, corresponding to the lowest sample depth. Alpha diversity was estimated with the Chao1 index for richness estimation [25], and the Shannon index [26], which combines richness and evenness measures. The significance of the differences between the study groups were tested with pairwise t-tests (999 random permutations), included in *alpha-group-significance* [27]. Jaccard [28] and Bray–Curtis [29] dissimilarity indexes were used to calculate the qualitative and quantitative beta diversity (diversity between samples), respectively. Distance matrices were computed and principal coordinate analysis (PCoA) and PCoA biplots [30, 31] were performed using *core-metrics* and visualized using Emperor [32]. The effect size of the variables under study was measured using the Adonis function from vegan package (R software) [33], by nesting the variables. To diminish the run effect, it was always included as the first variable in the nested formulas. The significance of the comparisons

was calculated with PERMANOVA pairwise tests (999 random permutations) using *beta-group-significance* [34].

To detect differentially abundant taxa among study groups, three different methods were followed. First, the Songbird [35] differential ranking method was used as a QIIME2 plugin to rank the features with the covariates in this study. Second, two different and complementary methods were used to compare and validate the associations: dsf-dr [36] and ANCOM with bias correction [37], both also used as QIIME2 plugins.

The functional prediction from the inferred metagenome was done with PICRUSt2 [38], using EPA-NG [39] for phylogenetic placement of reads, KEGG orthologs database [40] to infer gene families and functional pathways and modules with MinPath [41]. To do so, all singletons (features present only in one sample and/or one time) were removed. To visualize and compute statistics on PICRUSt2 output, Statistical Analysis of Microbial Profiles (STAMP) [42] was used. Welch's test [43] with Bonferroni [44] correction was used to find differentially abundant modules between the study groups. KEGG modules were also inferred with the reconstruction function of the KEGG-mapper online tool (available in https://www.genome.jp/kegg/mapper/reconstruct.html) and compared qualitatively between the groups under study (presence/absence and degree of completeness of the modules). In all statistical tests, significance was set at $P<0.05$.

Microbiome analysis output data was further processed and used to create figures and tables in RStudio version 2022.07.0 [45] with the packages qiime2r [46], reshape2 [47], ggplot2 [48] and tidyverse [49].

Results

The nasal microbiota composition differed according to the outcome of the piglets

After raw read processing, 4180 different ASVs were obtained with a mean frequency of 157,350 counts per sample. ASVs were mapped to Greengenes2 database and a total number of 1447 features (greengenes2 IDs) was obtained, with a mean frequency of 123,725 counts per sample.

When the nasal microbiota composition was compared between the animals that survived (group S; n = 16) or died (group D; n = 15) after the highly virulent PRRSV-1 outbreak, a higher bacterial richness was observed in group S than in group D (Chao1 index $P=0.001$, Fig. 1A), while no differences were observed according to the Shannon index ($P=0.66$). The composition differed also between groups S and D in the beta diversity analyses, which grouped into separate clusters in both qualitative and quantitative analyses (Jaccard and Bray–Curtis PERMANOVA $P=0.001$; Fig. 1B), with an estimated effect size of 8.3% and 23% for Jaccard and Bray–Curtis matrices, respectively.

In this study, the piglets came from three types of litters: three litters where all piglets died, three where

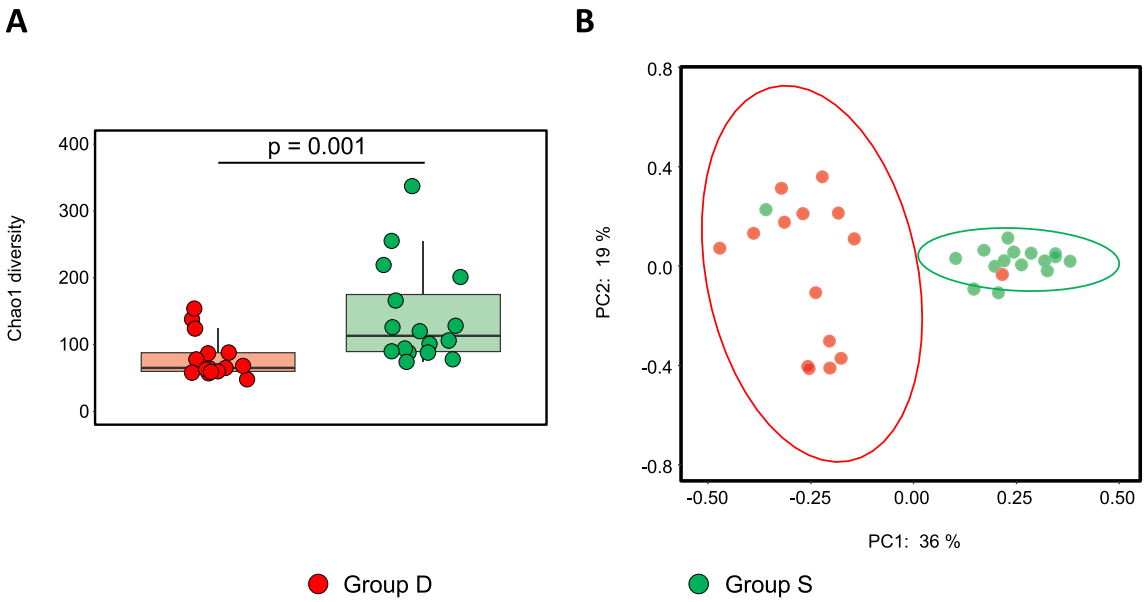

Fig. 1 **A** Alpha diversity measured with chao1 index of S (green) and D (red) groups. **B** Principal Coordinate Analysis of S (green) and D (red) groups using Bray–Curtis dissimilarity index. Ellipses of confidence (95%) are calculated assuming a multivariate t-distribution of distances within the samples of each group

all piglets survived and four mixed litters (with piglets either dying or surviving). The percentage of explanation of the variable "litter of origin" was similar to the previously quantified outcome effect ($R^2 = 7.6\%$ for Jaccard and $R^2 = 17.8\%$ Bray–Curtis, respectively, $P = 0.001$, supplementary Fig. 1), indicating that this variable is a major driver of the difference between clinical outcomes. To assess the bias caused by the selection of litters, the litter was used as a nested variable in the analysis. Interestingly, after accounting for such bias, the outcome of the PRRSV infection was still significantly associated with the microbial composition (R^2 of 5.4% and 10.2% in Jaccard and Bray–Curtis analyses, respectively, $P < 0.006$). This combination of outcome and litter effects would help to explain outlier samples, some of them clustering with animals from the same litter rather than with other piglets that had a similar outcome (such as samples #108 and #93, see supplementary Fig. 1), and vice-versa, i.e. animals clustering by outcome instead of litter (#87, #101 and #105). It was also examined whether pigs that died had higher viral loads for either PRRSV or influenza A virus. Average Ct values for influenza A virus were 23.51 ± 6.26 in the influenza positive animals in D while the average Ct value was 29.1 ± 3.65 in the S animals ($P = 0.553$, Mann–Whitney test), For PRRSV, average Ct obtained were 24.72 ± 4.21 in D animals versus 24.13 ± 4.61 in S animals ($P = 0.612$, Mann–Whitney test).

To unveil the microbial composition, the features found in the microbiota were studied at different taxonomic levels. The nasal microbiota of the piglets from group S was dominated by *Weeksellaceae* (22.59% ± 10.64, *Neisseriaceae* (19.75% ± 10.31), *Moraxellaceae* (19.55% ± 16.20) and *Pasteurellaceae* (18.12% ± 11.54), in similar proportions (supplementary Fig. 2A), while the nasal microbiota from group D was dominated by *Enterobacteriaceae* (28.57 ± 24.75%) and *Moraxellaceae* (28.06% ± 18.00). At genus level (supplementary Fig. 2B), the nasal microbiota of the piglets from group S was dominated by taxa commonly found in the respiratory tract of pigs, such as *Bergeyella* (22.57% ± 10.65), *Neisseria* (originally classified as *Eikenella*, but confirmed as *Neisseria* in other databases, i.e. NCBI and Greengenes 13_8) (19.73% ± 10.33), *Glaesserella* (15.20% ± 11.7) and *Moraxella A* (12.99% ± 16.88). In the case of piglets from group D, the most relatively abundant genera were *Escherichia* (28.17% ± 24.47) and *Moraxella* C (22.92% ± 18.65), followed by *Bergeyella* (11.66% ± 4.85). The complete list of microbiota composition at all taxonomic levels can be found in supplementary Table 1.

To study in more detail the taxonomic differences between groups S and D, we performed a differential ranking model (Songbird). Eighty-seven features with more than 0.01% mean relative abundance in at least one group (considered non-artifacts) were identified by the model as associated with either group S or group D (supplementary Table 2). Among all the significant differential features, forty-eight presented more than 1 $\log_{10}$-fold change between groups, (Fig. 2A), where features classified as *Mesomycoplasma hyorhinis* (present only in 4 animals from group S and totally absent in group D), *Caryophanon latum* and an unclassified *Acinetobacter* species showed the highest association with group S, while *Suipraeoptans intestinalis*, *Escherichia_710834* and *Prevotella heparinolytica* were the most associated with group D. Regarding the most abundant differential taxa (Fig. 2B), several features from common nasal colonizers were found in higher relative abundances in group S compared to D, such as *Bergeyella*, *Glaesserella parasuis*, *Moraxella A* and *Neisseria* (despite some of these presented a negative fold change towards group S). Conversely, two features classified as *Escherichia* were associated with group D (25.03% and 3.65% mean relative abundance, respectively) together with *Moraxella* C and *Rothia*, all of them more abundant in this group. When the same Songbird model was computed at family and genus level, most of the taxa associated to either S or D groups were the families and genera corresponding to the features indicated above (see supplementary Table 2). Moreover, a biplot analysis showed that *Moraxella* C and *Escherichia* were the most informative taxa for group D in both the quantitative and qualitative beta diversity analysis. On the other hand, another member of the *Moraxella* genus *(Moraxella* A), together with *Neisseria, Glaesserella* and *Pasteurella,* contributed the most to the clustering of group S (supplementary Fig. 3A and B). Similar results were obtained using ANCOM-BC and dsf-dr, which also reflected most of the same features, genera and families as differentially abundant (supplementary Table 3), reinforcing the results found by the Songbird model.

To study in more detail the different communities found in the two groups, a functional inference of the predicted metagenomic composition was performed. The principal component analysis of the predicted functions from the KEGG public database showed clustering according to the outcome of the infection (PERMANOVA using Bray–Curtis distance matrix $P = 0.001$, Fig. 3A), suggesting a different abundance of the functional capabilities for the nasal microbiota of piglets from each group. Moreover, group D exhibited a higher level of dispersion in the main PCA space compared to group S (PERMDISP using Bray–Curtis distance matrix $P = 0.047$), showing that the microbiota was quantitatively less similar within group D. Then, the predicted microbial capabilities of communities S and D were qualitatively assessed by comparing the presence and absence

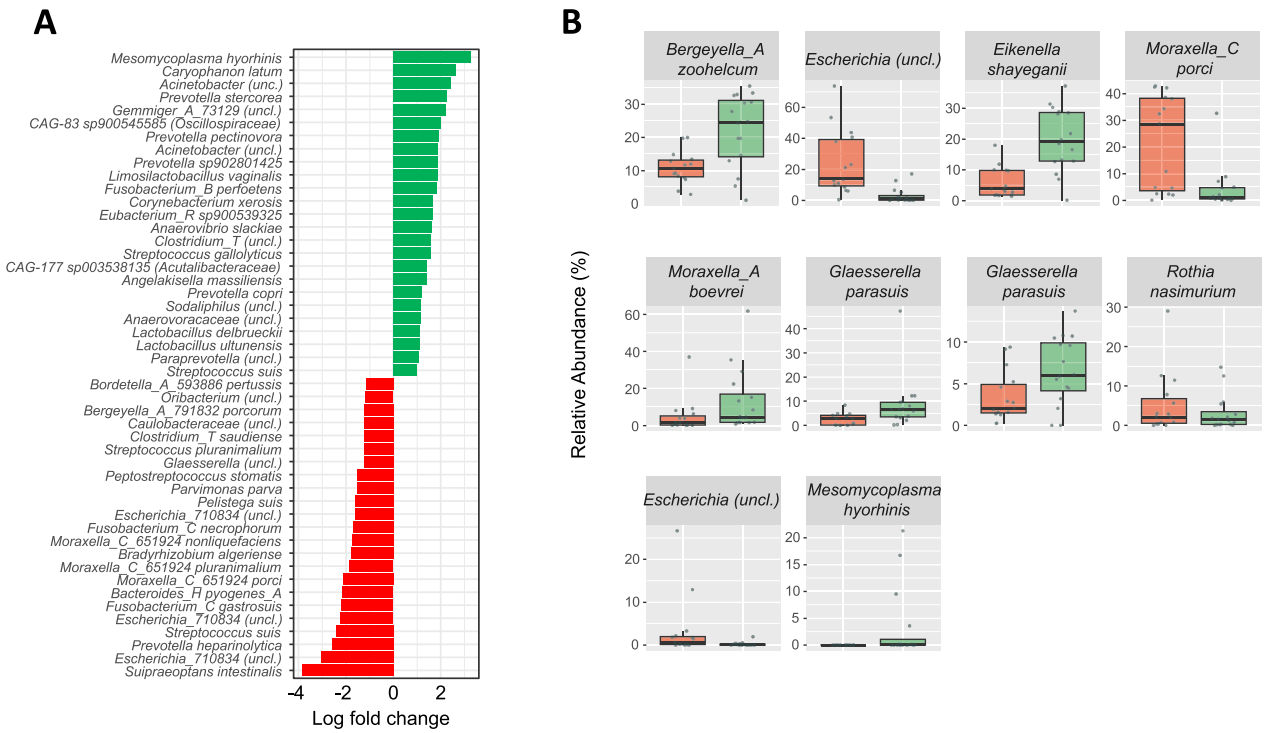

Fig. 2 Songbird model differentially abundant taxa associated to group S (green) or D (red). **A** Features with fold change > 1. **B** Abundance of the 10 features identified by the model that are most relatively abundant (globally). Each dot represents a sample

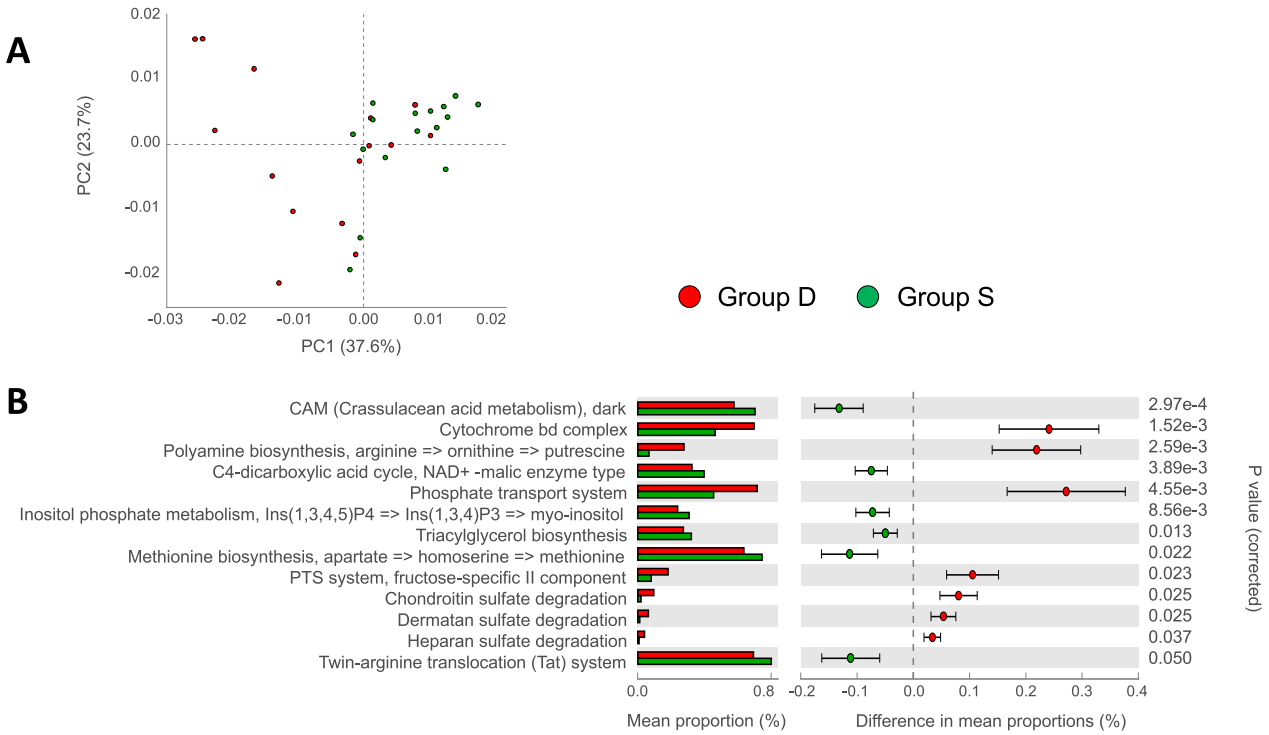

Fig. 3 PICRUSt2 functional prediction of group S (green) or D (red). **A** Principal Component Analysis of the functional KEGG orthologs database modules predicted from the inferred metagenome composition. **B** Differentially abundant modules found between the two study groups

of KEGG genes and modules. A similar number of KO genes was predicted for groups S and D (6,319 and 6,273, respectively), which made up an almost equal ensemble of modules (supplementary Table 4). In agreement, no statistically significant differences were detected between the two groups (PERMANOVA and PERMDISP using Jaccard distances $p > 0.05$). Only a few amino acid and glycan metabolism modules, as well as methicillin resistance were missing or showed to be incomplete in the microbiome of D group, while two multidrug resistance efflux pumps and shiga toxin were missing or showed an incomplete module in group S. Quantitative analyses were performed by submitting these inferred modules to differential analysis, where thirteen of these were found as significantly different between the two study groups; seven of them enriched in group D and six in group S (Fig. 3B).

Microbiota-related susceptibility existed in piglets before PRRSV-1 outbreak

For comparative purposes, samples from same aged piglets that died or survived before the outbreak caused by the new PRRSV strain were also analysed (S_0, n = 4; and D_0, n = 4), which showed similar dynamics in the diversity analysis (Fig. 4). In the beta diversity analysis (Fig. 4B), these samples clustered with the corresponding outcome group, i.e. S_0 with S, and D_0 with D. (PERMANOVA $P = 0.001$, with an estimated effect size of 7.44% and 20.08% for Jaccard and Bray–Curtis indexes, respectively). When the analysis was performed including only S_0 and D_0, despite the low number of samples prevented to find significant differences in alpha diversity, these groups showed to form two separated clusters in the beta diversity analysis (Jaccard PERMANOVA $P = 0.022$, and a tendency using Bray–Curtis $P = 0.082$). When the different tests were run to find differentially abundant taxa (the Songbird differential ranking model was not run due to the low number of samples), only ANCOM-BC reported a few low-abundant taxa (supplementary Table 5). Nevertheless, the two groups reported a similar composition to S and D groups, respectively (supplementary Fig. 4).

Also, samples from sows were included in the study (n = 8 and n = 4, after and before the outbreak, respectively). The nasal microbiota of sows giving birth to healthy or mixed litters reported a higher richness than sows giving birth to piglets that died (Chao1 index $P < 0.05$, supplementary Fig. 5A), suggesting that the differences observed in richness could be traced back to the sows. No significant differences in beta diversity were detected between any of these groups of sows. However, when the communities of the two timepoints were compared (before and after the entrance of the virulent PRRSV strain), the microbiota significantly varied (Jaccard and Bray–Curtis PERMANOVA $P < 0.006$, Adonis R^2 effect size of 11.7% and 16.4% respectively, supplementary Fig. 5B). Among the differences found in the sows

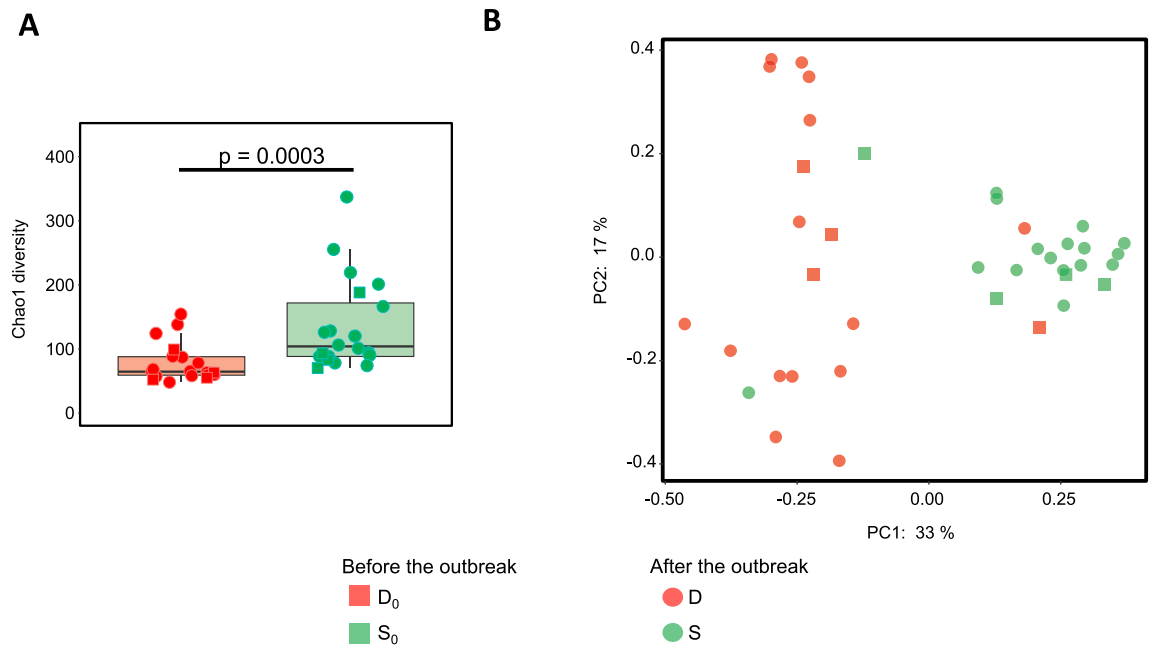

Fig. 4 **A** Alpha diversity measured with Chao1 index of S_0 (green) and D_0 (red) groups, in squares, and S (green) and D (red) groups, in spheres. **B** Principal Coordinate Analysis of S and S_0 (green) with D and D_0 (red) groups using Bray–Curtis dissimilarity index. Shape corresponds to the moment of sampling (before or after the virulent PRRSV-1 outbreak in squares or spheres, respectively)

between these two timepoints (supplementary Table 6), several taxa found were previously identified discriminating S or D groups, such as *Rothia*, *Neisseria*, *Moraxella C* or *Bergeyella*, more relatively abundant in sows before the outbreak; or *Fusobacterium* and *Bacteroides*, which were increased in sows during the outbreak.

Discussion

In this study, the composition of the nasal microbiota in a cohort of piglets infected by a highly virulent PRRSV-1 strain was analyzed. Despite being equally infected by the same PRRSV-1 strain, only a group of piglets had a fatal outcome in the end of the follow-up period. Our results indicated that survival was related to a higher species richness in the nasal microbial community as well as with the dominance of certain bacterial taxa. We also investigated the presence of other possible coinfecting agents (results not shown), but did not find any statistically significant associations with the groups under study.

Several studies have reported that healthy animals have higher bacterial diversity compared to diseased ones (9, 11, 50–52) and, in general, it is considered that lower bacterial diversity is a marker for impaired health [53, 54]. In the present case, lower richness was associated with a fatal outcome despite all the animals were infected by PRRSV. Previous studies targeting the swine fecal microbiota role in PRRSV and PCV2 infections outcome [11, 12] yielded similar results.

Besides, changes in the microbiota composition were also related with the outcome of the disease, especially regarding its' most abundant members, as shown by a major significance level in the quantitative analysis compared to the qualitative one. Similarly, changes in the fecal microbiota populations were also related with PRRSV and PCV2 outcome [11, 12]. In our study, surviving piglets reported a high abundance of genera frequently associated with a healthy swine respiratory tract such as *Bergeyella*, *Neisseria* (*Eikenella*), *Glaesserella* and *Moraxella* (*Moraxella*_A) [50, 53, 55–58]. In agreement, most of these taxa were found to contribute the most in the clustering of group S samples in the PCoA biplot analysis. On the contrary, *Escherichia* was significantly more abundant in piglets that died. Previous studies reported that taxa commonly found in the fecal microbiota or from the environment can be present in the pig nasal microbiota [53, 58, 59], and become predominant when the professional colonizers are not present [60, 61]. Our results are compatible with a dysbiosis in the nasal microbiota of the piglets that died, which resulted in an increased relative abundance of fecal bacteria, especially *Escherichia*. However, also a different clade of Moraxella (*Moraxella*_C) and *Rothia*, common colonizers of healthy piglets, were among the most abundant taxa associated with the fatal outcome. The presence of these common colonizers of the pig respiratory tract [53, 58] may indicate that these genera are more resilient to the disturbance of the nasal microbial community and proportionally increase when other taxa decreased. A previous study focusing on the nasal microbiota changes after challenging the animals with PRRSV and IAV [14] associated *Weeksellaceae* and *Neisseriaceae* with PRRSV infected groups while *Enterobacteriaceae* with uninfected. Despite these results may seem opposite to ours', Chrun et al. focused on immediate microbiota changes after challenge in PRRSV-free animals, while in this study two groups with different outcome in a virulent PRRSV-1 endemic farm were analyzed. Most of the taxa with the highest fold change between the two study groups were found in low relative abundance (*Mesomycoplasma hyorhinis*, *Caryophanon latum* and *Acinetobacter* associated with group S, and *Suipraeoptans intestinalis*, *Prevotella heparinolytica* with group D). Despite most of these taxa have a relationship with the swine nasal and/or gastrointestinal microbiota [52, 58, 59, 62], the biological significance of these changes in the present study is still unknown and deserve further investigation. Also, features from other taxa frequently found in the swine respiratory tract, such as *Lactobacillus*, *Streptococcus* or *Prevotella* [53, 58, 59] were associated with both disease outcomes, suggesting that different strains from the same genera could play different roles and be involved in disease outcome. The functional analysis also reflected differences between the examined groups of pigs, especially quantitatively, in agreement with the beta diversity analysis. Despite few differences were found in the qualitative comparison of the inferred functions, group D exhibited less glycan and amino acid metabolic modules (more incomplete or absent), suggesting some loss of functions in the microbiota of this group. Moreover, quantitative PCA analysis of functional modules revealed a bigger dispersion in group D pointing towards a less-uniform functionality in a more unstable (or unspecific) microbial community. In agreement with our findings, group dissimilarity can be related to dysbiotic microbiotas in unhealthy animals [63]. We could not relate the outcome of the infection (D or S) to higher viral loads for PRRSV or influenza A virus. In this later case, the number of observations was probably too low to raise any conclusion.

In this study, samples collected before the introduction of the new PRRSV strain were also analyzed. Despite the reduced number of samples examined, animals that died or survived before the outbreak had a similar microbiota composition compared with animals sampled after the outbreak. This finding suggests that this highly virulent PRRSV strain was not the cause of

the changed microbiota composition between the different outcome groups. In fact, it is more likely that the microbiota would be a predisposing factor for survivability or susceptibility while the virulence of the new PRRSV strain would be responsible for the increased mortality. The microbiota composition as a factor of susceptibility has been highly studied because the huge impact it can have on host immunity and pathogen exclusion [64]. In pigs, this microbiota-related susceptibility has already been demonstrated in the case of bacterial and viral pathogens [11, 12, 50, 52, 65–67]. The findings in this study reported a different microbiota in animals with good outcome which could be protective for PRRSV but also in secondary infections that may arise in diseased animals. Nevertheless, the examination of the underlying mechanisms explaining the predictive value of the nasal microbiota composition is beyond the scope of the present study but merits further investigation.

Finally, it is worth noting that belonging to a given litter had a strong influence on the composition of the nasal microbiota and thus, in the outcome of the animal. This is in agreement with studies that highlighted the role of the sows in the piglets' microbiota development [60, 68, 69]. In fact, there were two samples from piglets clustering with their siblings rather than with piglets with the same clinical outcome (one animal clustering with the survivors, died, and vice versa). This can be simply explained by the individual (diverse) resilience of the animals or because some animals may have died from causes other than the PRRSV infection. However, there was still an outcome effect acting independently of the litter, as shown by animals from mixed litters whose samples clustered by outcome and detected in the litter-nested beta diversity analysis. Interestingly, the differences observed in the alpha diversity of the piglets were a reflection of the differences observed in their dams, since sows giving birth to surviving and mixed litters reported a higher microbial richness than those to a litter with fatal outcome. The idea that sows with a rich microbiota transfer "more" than sows with a less rich microbiota is in agreement with previous studies discussing the sow-to-piglet microbiota transmission [60, 69, 70]. Moreover, the sows' microbiota showed substantial changes before and after the PRRSV outbreak. However, these variations could be more likely related to analyzing a low number of samples from different sows or changes in environmental and/or farming conditions. Further studies may aid confirming whether these changes observed in sows are caused by this new virus variant and more importantly, whether these variations can be related to an increase in mortality between the two timepoints.

Conclusion

In conclusion, this study suggests that the nasal microbiota could be closely related to virulent PRRSV-1 survival. Specifically, we show that high species richness and the presence of common nasal colonizing bacteria may be the key to a good outcome, while dysbiosis seems to be related to a bad outcome. At last, the analysis of samples taken before the outbreak indicates that such differences between piglets were already present, and the increased PRRSV-1 virulence triggered a fatal outcome in susceptible animals. Future studies may help explaining the connection between such microbiota characteristics and PRRSV susceptibility/resistance as well as investigating whether this microbiota-related susceptibility also exists in other viral respiratory infections. Moreover, this investigation serves as starting point towards exploring the potential of the nasal microbiota in the prevention of viral respiratory infections.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s42523-024-00371-y.

Supplementary figure 1. Principal Coordinate Analysis of Sand Dgroups using Bray-Curtis dissimilarity index. Different colours correspond to the litter of origin. Outlier samples to outcome or litter effect are shown specifically

Supplementary figure 2. Most relatively abundant families and genera in S and D groups

Supplementary figure 3. Biplot Analysis of S and D groups with the four genera contributing the most to sample ordination shown in arrow susing Jaccard and Bray-Curtis dissimilarity indexes

Supplementary figure 4. Most relatively abundant generaand features in S_0 and D_0 groups.

Supplementary figure 5. Diversity analysis of the sows giving birth to the litters under study. A) Alpha diversity measured with Chao1 index of sows giving birth to dying, survivingand mixedlitters. Shape corresponds to the moment of sampling: prioror afterthe arrival of the virulent PRRSV-1 strain. B) Principal Coordinate Analysis of the sows using Bray-Curtis dissimilarity index. Different colours represent the moment of sampling: prioror afterthe arrival of the virulent PRRSV-1 strain. Ellipses of confidenceare calculated assuming a multivariate t-distribution of distances within the samples of each group

Supplementary table 1. Microbiota compositionat all taxonomic levels of the samples of piglets under study. Colour of cells corresponds to groups. D = red, D_0 = purple, S = green, S_0 = light green. Taxonomic classification and amplicon sequences are provide for the feature level.

Supplementary table 2. Differentially abundant taxa at feature, genus and family level found between S and D groups using Songbird differential ranking method. Taxonomy, fold change, mean abundances in each group, and individual abundances in each sample are provided. Colour code corresponds to fold change Sor Dassociation

Supplementary table 3. Differentially abundant taxa at feature, genus and family level found between S and D groups using ANCOM-BC and ds-FDR. Taxonomy, significance, mean abundances in each group, and individual

abundances in each sample are provided. The abundances of the differential features in D_0 and S_0 groups are shown as well.

Supplementary table 4. KEGG modules found in S and D groups. The level of completeness for each metabolic module is indicated: complete, 1 module missing, 2 modules missing, incomplete and not present. Modules more complete in D group are highlighted in red. Modules more complete in S group are highlighted in green

Supplementary table 5. Differentially abundant taxa at feature, genus and family level found between S_0 and D_0 groups using ANCOM-BC. Taxonomy, significance, mean abundances in each group, and individual abundances in each sample are provided.

Supplementary table 6. Differentially abundant taxa at feature, genus and family level found between sow groups using ANCOM-BC. Taxonomy, significance, mean abundances in each group, and individual abundances in each sample are provided. Colour code corresponds to timepoint: samples taken before in purple, and in orange, samples taken after PRRSV-1 outbreak

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Author contributions
POG performed the analysis. FCF and VA supervised the analysis. FCF, VA, MC and EM contributed to the discussion and interpretation of the results. GEMV, MC and EM designed the study. GEMV and HC performed the sampling of the animals and performed the viral detection. POG drafted the manuscript with contributions from all authors. All authors approve the final submitted version.

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Availability of data and materials
The raw data used in this study are available at SRA database (NCBI) under BioProject ID PRJNA1132954. Processed data is available as supplementary tables.

Declarations

Ethics approval and consent to participate
The present project was carried out after approval by the Ethics in Human and Animal Experimentation Committee (no. 5691) and followed all national and EU regulations. The farm consented the participate in the study after being informed of the procedures to be carried out.

Competing interests
The authors declare no competing interests.

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