


Brief Report

Porcine reproductive and respiratory syndrome virus impacts on gut microbiome in a strain virulence-dependent fashion

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Summary

Porcine reproductive and respiratory syndrome (PRRS) is a viral disease defined by reproductive problems, respiratory distress and a negative impact on growth rate and general condition. Virulent PRRS virus (PRRSV) strains have emerged in the last years with evident knowledge gaps in their impact on the host immune response. Thus, the present study

examines the impact of acute PRRS virus (PRRSV) infection, with two strains of different virulence, on selected immune parameters and on the gut microbiota composition of infected pigs using 16S rRNA compositional sequencing. Pigs were infected with a low virulent (PRRS_3249) or a virulent (Lena) PRRSV-1 strain and euthanized at 1, 3, 6, 8 or 13 days post-inoculation (dpi). Faeces were collected from each animal at the necropsy time-point. Alpha and beta diversity analyses demonstrated that infection, particularly with the Lena strain, impacted the microbiome composition from 6 dpi onwards. Taxonomic differences revealed that infected pigs had higher abundance of *Treponema* and *Methanobrevibacter* ($FDR < 0.05$). Differences were more considerable for Lena- than for PRRS_3249-infected pigs, showing the impact of strain virulence in the intestinal changes. Lena-infected pigs had reduced abundances of anaerobic commensals such as *Roseburia*, *Anaerostipes*, *Butyrivibrio* and *Prevotella* ($P < 0.05$). The depletion of these desirable commensals was significantly correlated to infection severity measured by viraemia, clinical signs, lung lesions and immune parameters (IL-6, IFN- γ and Hp serum levels). Altogether, the results from this study demonstrate the indirect impact of PRRSV infection on gut microbiome composition in a strain virulence-dependent fashion and its association with selected immune markers.

Introduction

Porcine reproductive and respiratory syndrome (PRRS) is one of the infectious diseases with the highest impact on the modern swine industry. The wide diversity among PRRS virus (PRRSV) strains is reflected by the classification of the two genotypes of the virus, PRRSV-1 and PRRSV-2, as two independent viral species, *Betaarterivirus suis 1* and *Betaarterivirus suis 2* respectively (Gorbalenya, 2018). Furthermore, each of these viral species has a broad genetic variability, with strains being

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assigned to different subtypes, clades and lineages (Shi *et al.*, 2010; Stadejek *et al.*, 2013; Balka *et al.*, 2018).

Since 2006, the heterogeneity among PRRSV strains has been reinforced by the identification of virulent strains in Asia (PRRSV-2) and Europe (PRRSV-1) (Tian *et al.*, 2007; Karniychuk *et al.*, 2010). These virulent strains induce high fever and severe respiratory problems complicated with secondary bacterial co-infections, leading to an increase in morbidity and mortality rates (Tian *et al.*, 2007; Karniychuk *et al.*, 2010; Senthilkumar *et al.*, 2018; Rodríguez-Gómez *et al.*, 2019). Among several virulent PRRSV-1 strains, the subtype 3 PRRSV-1 Lena strain is considered to be the prototype (Karniychuk *et al.*, 2010; Morgan *et al.*, 2013; Frydas *et al.*, 2015; Sinn *et al.*, 2016; Canelli *et al.*, 2017; Stadejek *et al.*, 2017). The emergence of these virulent strains has given more complexity to the control of this disease, which represents a cornerstone in the management of the porcine respiratory disease complex.

PRRSV is characterized by a peak of virus replication in the lung and the onset of fever during the first week post-infection; with earlier and higher fever when pigs are infected by virulent strains (Karniychuk *et al.*, 2010; Stadejek *et al.*, 2017; Rodríguez-Gómez *et al.*, 2019). The main clinical signs developed by infected animals consist of dyspnoea and respiratory distress, which may be also associated with poor body condition and diarrhoea according to the virulence of the strain (Sinn *et al.*, 2016; Senthilkumar *et al.*, 2018). Therefore, it is clear that PRRSV, besides affecting the respiratory tract, impacts on the general health status of the animal, which may be reflected in growth retardation and gastroenteric clinical signs.

Despite the gut–lung axis by the interaction of the microbiota and respiratory infections has been already postulated (Budden *et al.*, 2017; Niederwerder, 2017), it is scarcely studied in pigs yet. Recent studies have demonstrated that PRRSV infection, either on its own (Jiang *et al.*, 2019) or together with circovirus infection, modifies the host intestinal microbiota (Niederwerder *et al.*, 2016; Ober *et al.*, 2017). For instance, these studies report a decrease of desirable gut inhabitants such as *Prevotella* (Jiang *et al.*, 2019) or a lower diversity of the overall faecal microbiome linked to a worse clinical outcome in PRRSV and porcine circovirus type 2 (PCV2) dually infected pigs (Ober *et al.*, 2017). In addition, microbiota changes in dual infections, increased *Methanobacteriaceae* species and reduced *Ruminococcaceae* and *Streptococcaceae* species, were associated with low growth rate pigs (Niederwerder *et al.*, 2016; Ober *et al.*, 2017). These studies should inspire new designs which further explore the changes in the microbiome in the respiratory and intestinal tract after PRRSV infection, including factors such as the immune

response, not analysed so far (Jiang *et al.*, 2019). According to the emergence of virulent PRRSV strains, the modulation of the immune response by this virus and the potential interplay between the microbiome and the immune response, there is a clear gap in the scientific literature of previous studies addressing all these issues along PRRSV infection.

In this context, virulent PRRSV strains may directly affect the gut microbiome, leading to a lower diversity than low virulent strains as well as proliferation of pathogenic species, which would relate to the clinical outcome and modulation of different immune mediators. Considering the paucity of studies focused on the role of the faecal microbiome during PRRSV infection as well as the marked interest in deciphering the pathogenesis of PRRSV strains of different virulence, the goal of the present study was to analyse and determine the changes in the composition and diversity of the faecal microbiome in response to infection with two PRRSV-1 strains of different virulence as well as their association with the host immune response.

Results and Discussion

PRRSV infection contributes to a number of immunological outcomes which alter host health, increasing for instance the susceptibility to secondary infections by opportunistic pathogens (Gómez-Laguna *et al.*, 2013). Similarly, the infection may be able to alter the gut microbiota, which is essential in host physiological processes (Xiao *et al.*, 2016; Zhang and Wang, 2018; Pearson-Leary *et al.*, 2019). So far, few studies have analysed the impact of PRRSV on the gut microbiome, and the other way around, (Niederwerder *et al.*, 2016; Ober *et al.*, 2017; Jiang *et al.*, 2019) and none of them have investigated the changes occurring through the infection process from a PRRSV virulence perspective. To assess the goal of this study, an experimental setting was carried out with seventy animals randomly distributed in three experimental groups (Lena- and PRRS_3249- infected groups and control group) and housed in separate pens at containment facilities.

Clinical signs, gross pathology of the lung and viremia are influenced by the virulence of PRRSV strain

The main clinical signs and pulmonary gross lesions observed in this study are described in detail in Rodríguez-Gómez *et al.* (2019). Briefly, pigs infected with the virulent strain Lena showed severe respiratory and systemic signs (i.e. lethargy, anorexia, diarrhoea and pyrexia) as well as marked lung lesions (tan-mottled areas, atelectasis and areas of consolidation), which

occurred earlier and more severely than in pigs from the PRRS_3249 group.

PRRSV was detected by RT-qPCR in the sera of all Lena-infected pigs as early as 1 dpi, whereas four out of five PRRS_3249-infected pigs were simultaneously PRRSV positive. Viraemia was always higher in Lena than PRRS_3249-infected pigs from 1 to 13 dpi ($P < 0.01$ at 1, 3, 6 dpi; $P < 0.05$ at 8 and 13 dpi), reaching the highest level at 6 dpi (1.89×10^7 eq CPD50 per ml) (Fig. 1). PRRSV-specific antibodies were first detected at 8 dpi in sera from both PRRSV-infected groups, with slightly higher sample to positive ratio (S/P) values in case of Lena-infected pigs (File S1).

PRRSV infection alters gut microbiome diversity

Alpha (number of taxa and abundance of these taxa in a particular sample) and beta (differences in taxa between samples) diversity metrics allow evaluating global microbiome composition (Kim and Isaacson, 2015a). Structural analysis of vector spatial distribution regression ($P < 0.05$) and PERMANOVA ($P < 0.001$) tests showed the influence of PRRSV strain virulence on the ordination of samples (File S2). A result in line with the observed differences for Chao1 alpha diversity index in the Lena-infected group compared to the control group ($P < 0.05$; File S3 and S4). Further analyses were performed after splitting samples by day of sampling, the factor that had the strongest effect on the spatial distribution of samples ($P < 0.01$), with a greater effect in

control pigs compared to infected pigs (Fig. 1A). Interestingly, both diversity metrics were impacted by PRRSV infection, particularly from 6 dpi onwards (Fig. 1A, C and D; File S2, sheet 3), when the peak of viraemia was observed, and the clinical signs were more severe. Thus, significant differences were observed in sample ordination among experimental group categories at 6 dpi ($R^2 = 0.25$; $P < 0.05$) and 13 dpi ($R^2 = 0.19$; $P < 0.01$) and differences were close to significance for samples collected at 8 dpi ($R^2 = 0.22$; $P = 0.06$). Similarly, in alpha diversity analyses, at 6 dpi, there were significant differences in alpha diversity values among experimental groups for Shannon ($P < 0.05$) and Simpson ($P < 0.05$) indexes (Fig 1C and D). At 13 dpi, the last time-point of the study, Shannon and Chao1 indexes differed significantly among experimental group categories ($P < 0.01$ and $P < 0.01$ respectively; Fig 1B and C).

The higher diversity of species as well as the increased microbiome evenness and richness in infected pigs, and particularly in the Lena-infected group, together with the spatial differences between infected and control pigs, which matched with the viraemia peak in Lena-infected group, show that PRRSV infection impairs microbiome composition, allowing the emergence or growth of bacteria present at low proportions in healthy animals, and shifting the gut microbiome composition by strain virulence. Previous microbiome PRRSV studies linked increased diversity to better growth and moderate disease outcomes (Niederwerder *et al.*, 2016; Ober *et al.*, 2017). Differences in study design, such as co-infection

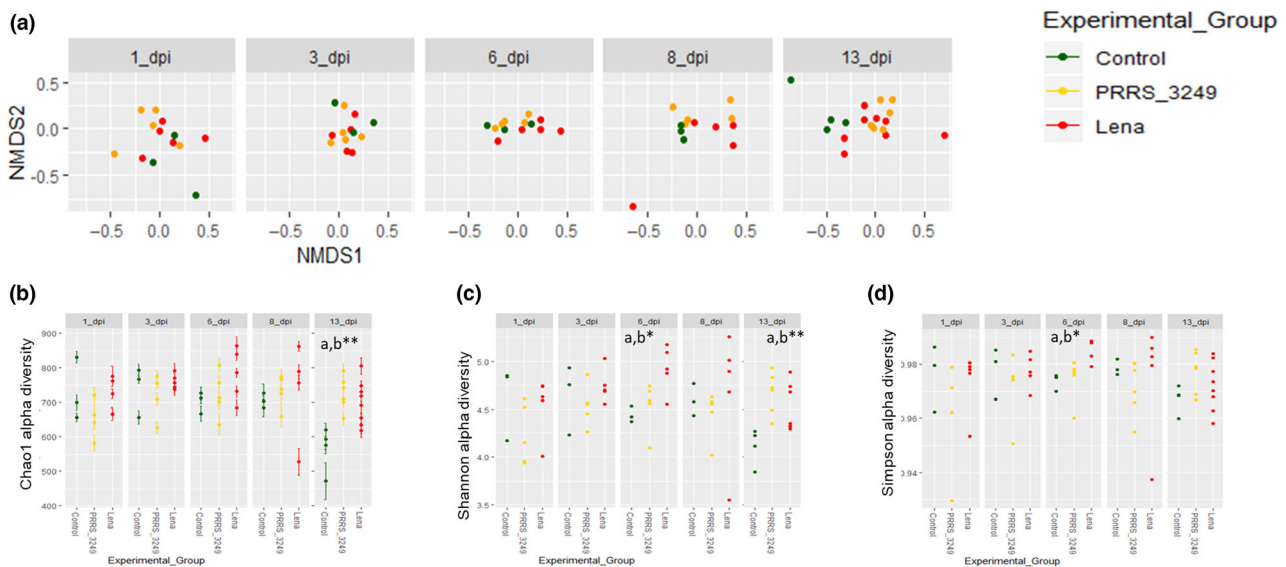


Fig. 1. Diversity analyses in faecal samples from PRRSV-infected pigs. A. Beta diversity estimated by Bray–Curtis distance and non-metric multidimensional scaling of samples at each sampling time-point by experimental group variable. B–D, Chao1, Shannon and Simpson alpha diversity indexes, respectively, by sampling time-point. Statistically significant differences were estimated by ANOVA with Tukey correction and were denoted with (*): * $P < 0.05$; ** $P < 0.01$; and *** $P < 0.001$ and 'a' Lena vs control, 'b' among the three categories.

with PCV2 together with the lack of non-infected pigs to compare with and/or the methodology used to analyse the microbiome, are potential reasons for the observed discrepancies.

Changes in relative abundance are associated with the infection process and PRRSV strain virulence

16S rRNA compositional sequencing allows the estimation of relative abundance of different groups of bacteria (Kim *et al.*, 2015b). By detailed analysis of differences in abundance, we aimed to decipher the particular groups of bacteria involved in the shift observed in the microbiome after PRRSV infection (Fig. 2A; File S5). Changes at taxonomic level were influenced by strain virulence and particularly linked to Lena strain infection. This infected group showed an increased abundance of genera *Treponema* and *Methanobrevibacter*, as was the case for *Treponema* in comparison with PRRS_3249 group. In contrast, the genera *Anaerostipes*, *Prevotella* and *Butyrivococcus* abundance in Lena-infected pigs were significantly lower compared to the other two groups. We finally observed a decreased abundance of *Roseburia* in Lena-infected pigs as well as a decrease in *Mycoplasma* abundance in both Lena- and PRRS_3249-infected pigs compared to controls. The increased abundance of *Treponema* and *Methanobrevibacter*, a bacterium of the phylum *Euryarchaeota*, herein observed has been previously reported in low growth PRRSV-infected pigs (Ober *et al.*, 2017). The role of this sort of methanogens in health and disease is still under discussion (Chaudhary *et al.*, 2018) and may require specific designed amplicons for an accurate characterization (Klindworth *et al.*, 2013).

The analysis of differential abundance by sampling day (File S5) revealed a higher abundance of *Succinivibrio* in infected pigs compared to controls at 6 dpi, coinciding with the viraemia peak. At 8 dpi, a higher abundance of *Treponema* and *Bacteroides* and lower abundance of two *Prevotellaceae*, the genera *Prevotella* and *Alloprevotella*, and two *Clostridiales*, *Roseburia* and *Clostridium* cluster XIVa, was observed. Finally, at 13 dpi, we also observed a higher abundance of *Treponema* in both infected groups. The clustering of samples by relative abundance of genera at targeted days post-infection (6, 8 and 13 dpi) revealed an influence of PRRSV experimental group variable in pig clustering by taxa abundance (Figs. 2B–D). The observed decrease in abundance of anaerobe saccharolytic inhabitants of the distal gut (*Anaerostipes*, *Prevotella* and *Alloprevotella*) as well as in other members of the class *Clostridia* such as *Roseburia* and *Clostridium* cluster XIVa, both from the family *Lachnospiraceae*, and *Butyrivococcus* (*Clostridiaceae*) illustrates the displacement of anaerobes from

the gut in Lena-infected pigs. Furthermore, increased abundance of *Proteobacteria* and *Spirochaetes* in faeces from Lena-infected pigs is indicator of potential favourable conditions for pig enterobacteria such as *E. coli*, *Salmonella* or the two pathogenic spirochaetes *Brachyspira hyodysenteriae* and *B. pilosicoli*. This finding may explain the on-farm link between PRRSV infection and enteric diseases and highlights the relevance of PRRSV control for successful treatment and control of other diseases, including enteric pathogens (Beloeil *et al.*, 2007).

Association between factors linked to PRRSV disease and microbiome abundance

In this study, respiratory and systemic clinical signs, lung lesions, viraemia and several immune markers were measured and correlated to abundance of gut bacteria to establish potential indirect relationships between infection and microbial abundance. An immunologic link between the gastrointestinal tract and respiratory tract mucosa is postulated via mesenteric lymph nodes and thoracic duct connection (He *et al.*, 2017). Following this premise, we looked for potential correlations among studied immune factors or acute-phase proteins and gut microbiome abundance.

Significant correlations between host disease analyses and taxa abundance are detailed in table 2 at phylum, family and genus levels. The relative abundance of six genera was significantly correlated with changes in variables under study (Fig. 3; File S6). Clinical signs and cumulative gross lesions score were positively correlated with the relative abundance of *Methanobrevibacter* and *Spirochaeta*, result which was also observed for the family *Spirochaetaceae* and the phylum *Spirochaetes* (Table 1). In contrast, the score of these two variables was negatively correlated with the relative abundance of genera from the phylum *Firmicutes*, including two *Lachnospiraceae* (*Blautia* and *Roseburia*) and *Faecalibacterium* as well as the *Prevotella* from the phylum *Bacteroides*. These correlations demonstrate the link between disease severity and depletion of anaerobic commensals. A similar depletion of anaerobes from class *Clostridia* is observed in *Salmonella* infection as a consequence of mucosal inflammation (Rivera-Chávez and Bäumlér, 2015; Argüello *et al.*, 2018). Accordingly, to the virulence of PRRSV strain and the severity of clinical signs, including marked inappetence and severe respiratory signs, anorexia or fasting may be the principal motive of the microbial changes observed. Indeed, a study performed in fasted healthy adult horses describes a reduction of class *Clostridia* as occurs in our study (Schoster *et al.*, 2016). Other correlate of disease, such as the viraemia, was strongly correlated with the relative abundance of *Micrococcaceae* and to a lesser extent

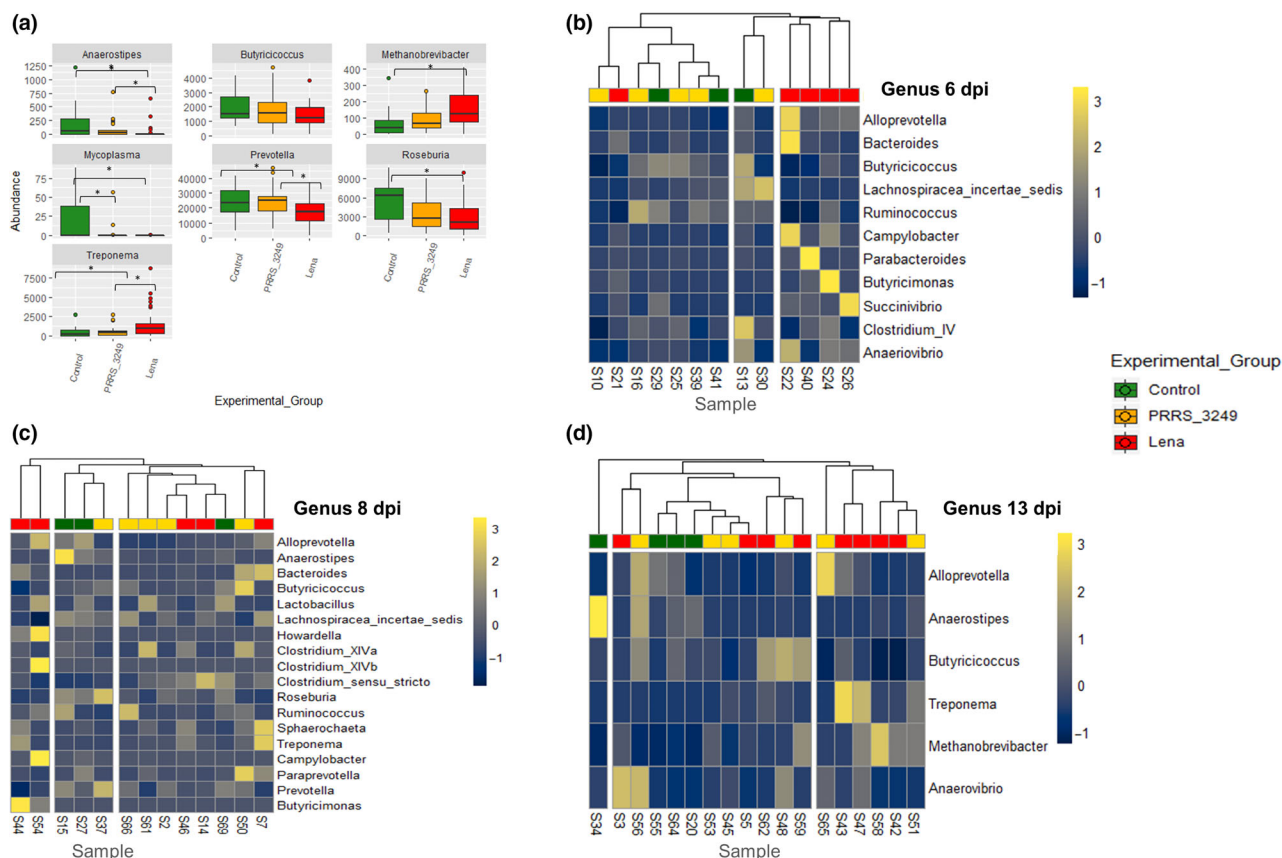


Fig. 2. Genera abundance analyses in experimental groups. A. differentially abundant genera established by *fitzsig* function in Metagenomeseq package among Lena, PRRS_3249 and control groups. Whiskers denote the significant differences among experimental groups and * denotes $FDR < 0.05$. B–D. represent heatmaps of the log-transformed relative temporal abundance of genera for which $P < 0.01$ (Shade *et al.*, 2013) illustrating the relative counts of genera in samples from 6 dpi (B), 8 dpi (C) and 13 dpi (D). The dark blue colour indicates low relative abundance taxa, while light yellow represents those at high relative abundance. The dendrogram was built using Euclidean distances and Ward's method with red colour for pig from Lena-infected group, yellow for PRRS_3249-infected group and green for control group respectively.

with the relative abundance of *Spirochaetaceae*, while we observed a negative correlation to the phylum *Firmicutes* (Table 1). In this sense, the depletion observed in strict anaerobes may provide an opportunity for other families, such as *Spirochaeta*, *Helicobacteriaceae* or *Campylobacteriaceae* to proliferate, as observed here. As above mentioned, these groups include pathogenic bacteria which may co-infect PRRSV-infected pigs.

From the set of immunity markers tested, serum level of IL-6 in PRRS_3249 group showed maximum levels at 6 dpi (mean of $350 \pm SD$ pg ml⁻¹), dropping at 8 dpi, whereas the Lena group reached the highest IL-6 level at 8 dpi (mean of $480 \pm SD$ pg ml⁻¹). Serum IL-6 level was correlated with the relative abundance of *Micrococcaceae*. A significant increase in IFN- γ serum levels was detected at 6 and 8 dpi in Lena-infected pigs (maximum mean level of $234 \pm SD$ pg ml⁻¹ at 6 dpi) compared to control ($P < 0.05$) and PRRS_3249 groups ($P < 0.01$). Serum concentration of IFN- γ was negatively correlated with the relative abundance of *Streptococcaceae*

(Table 1). Finally, for IL-10, Hp and LBP, no differences between groups were observed throughout the study. Among these immune markers, Hp levels were positively correlated with the relative abundance of *Lachnospiraceae* and negatively correlated with the relative abundance of *Helicobacteriaceae* (File S6).

The correlations observed between immune mediators, such as IFN- γ , IL-6 or Hp, and the changes in the gut microbiome, highlight the potential impact of the immune modifications prompted by PRRSV infection in the lung (Gómez-Laguna *et al.*, 2013; Rodríguez-Gómez *et al.*, 2019) on the intestine. Ultimately, the gut-lung immune response axis is complex (He *et al.*, 2017) and further research is needed to determine the exact mechanisms that result in shifts in the gut microbiome.

Altogether, the results from this study demonstrate the impact of PRRSV infection on gut microbiome composition. Microbiome changes were clearly influenced by strain virulence and were more evident from viremia peak onwards. PRRSV infection, principally associated

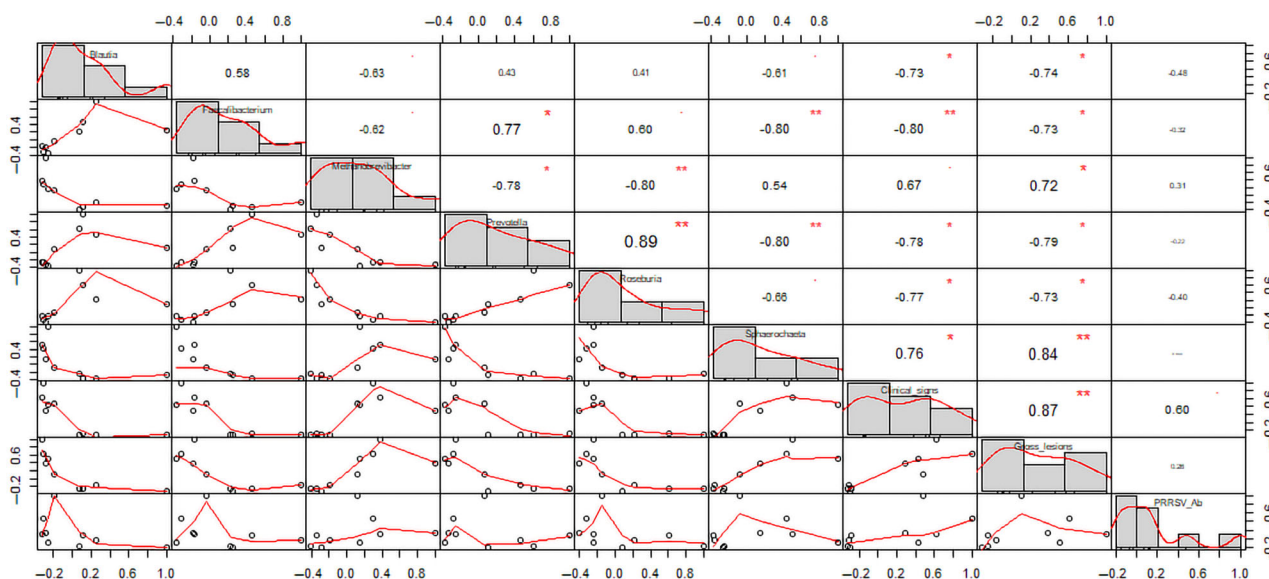


Fig. 3. Correlation matrix between abundance of the main genera and the PRRSV infection parameters under study. Spearman correlations among significant genera abundance and disease parameters.

Table 1. Significant correlations observed between taxa relative abundance and host parameters along PRRSV infection.

Taxa	Host factors					
	Clinical signs	Gross lesions	Viraemia	IFN- γ	IL-6	Hp
Phyla						
<i>Firmicutes</i>	-0.69*	-0.78**	-0.70*	-0.69*	NS	NS
<i>Spirochaetes</i>	NS	0.67*	NS	NS	NS	NS
<i>Proteobacteria</i>	NS	0.71**	NS	NS	NS	NS
Familiae						
<i>Spirochaetaceae</i>	0.72**	0.78**	NS	NS	NS	NS
<i>Helicobacteriaceae</i>	0.61*	0.71**	NS	NS	NS	NS
<i>Campylobacteriaceae</i>	0.52*	0.64*	NS	NS	NS	NS
<i>Clostridiaceae</i>	0.57*	NS	NS	NS	NS	NS
<i>Lachnospiraceae</i>	-0.77**	-0.82***	NS	NS	NS	NS
<i>Prevotellaceae</i>	-0.56*	-0.60*	NS	NS	NS	NS
<i>Micrococcaceae</i>	NS	NS	0.80***	NS	0.63*	NS
<i>Spirochaetaceae</i>	NS	NS	0.52*	NS	NS	NS
<i>Lachnospiraceae</i>	NS	NS	NS	NS	NS	0.54*
<i>Streptococcaceae</i>	NS	NS	NS	-0.55*	NS	NS
Genera						
<i>Methanobrevibacter</i>	0.67*	0.72*	NS	NS	NS	NS
<i>Spirochaeta</i>	0.72**	0.84***	NS	NS	NS	NS
<i>Blautia</i>	-0.73**	-0.74**	NS	NS	NS	NS
<i>Faecalibacterium</i>	-0.80**	-0.73**	NS	NS	NS	NS
<i>Prevotella</i>	-0.78**	-0.79**	NS	NS	NS	NS
<i>Roseburia</i>	-0.77**	-0.73**	NS	NS	NS	NS

IFN, interferon; IL, interleukin; Hp, haptoglobin.

*. P -values and Rho values were established by Spearman rank correlation ($P < 0.05$); * ($P < 0.05$); ** ($P < 0.01$); and *** ($P < 0.001$).

with Lena strain, displaced desirable gut anaerobes and allowed colonization by microbial groups such as *Proteobacteria* or *Spirochaetes* which are indicative of favourable conditions for usual enteric pathogens. These microbial changes were associated with disease severity, but further research is needed to disclose the gut-lung axis relationships in PRRSV infection or secondary factors that prompt the observed changes.

Experimental procedures

Ethics statement

All the animal experiments described in the present study were conducted in strict accordance with the guidelines of the European Union (Directive 2010/63/EU). All animal studies were conducted under protocols

approved by the IRTA Ethics Committee and by the Catalan Autonomous Government (Project 3647; FUE-2017-00533413). All efforts were made to minimize suffering and ensure the highest ethical and humane standards. Accordingly, euthanasia of the animals at the different time-points was performed by initial sedation with azaperone (4 mg kg⁻¹; Stresnil, Ecu-phar Veterinaria, S.L.U., Barcelona, Spain), followed by anaesthesia with ketamine (15 mg kg⁻¹; Ketaset, Zoetis Spain S.L., Madrid, Spain) and a lethal dose of 5 % sodium thiopental (Tiobarbital, Braun Vet Care S.A., Barcelona, Spain).

Animals and experimental design

Animals and samples used in this study were part of a project to elucidate the pathogenesis of PRRSV strains of different virulence (Rodríguez-Gómez *et al.*, 2019). Seventy piglets (Landrace × Large White, four-week-old) were randomly distributed in three different groups and housed in separate pens at the Biosafety Level 3 containment facilities of Centre de Recerca en Sanitat Animal (IRTA-CReSA, Bellaterra, Barcelona, Spain). Pigs were obtained from a high health status farm, historically negative for PRRSV and also confirmed as negative for PCV2 and *Mycoplasma hyopneumoniae* [IDEXX PRRS X3 Ab Test; (Mattsson *et al.*, 1995; Sibila *et al.*, 2004)]. After a 7-day acclimation period, pigs were intranasally inoculated as follows: (i) PRRS_3249 group, 26 pigs were inoculated with 2 ml (1.0 ml per nostril) of 1×10^5 TCID₅₀ of the subtype 1 PRRSV-1 3249 strain; (ii) Lena group, 28 pigs were inoculated at the same conditions with the subtype 3 PRRSV-1 Lena strain; and (iii) control group, 16 pigs were inoculated with porcine alveolar macrophages supernatant diluted in RPMI at the same conditions. Three control pigs and five infected pigs from each group were euthanized on days 1, 3, 6 and 8 post-inoculation (dpi). At 13 dpi, four control pigs, six pigs from PRRS_3249 group and eight pigs from the Lena group were humanely killed.

Clinical signs and samples collection

Rectal temperature and clinical signs, such as abnormal behaviour, anorexia, dyspnoea, cough, skin lesions, presence of diarrhoea and faeces consistency, were daily monitored along the study. Blood samples were collected at 0, 1, 3, 6, 8 and 13 dpi and routinely processed to analyse the viraemia and perform serological studies. At necropsy, immediately after the euthanasia, rectal faeces from each animal were aseptically collected into sterile containers and freeze at -80°C until processing. Gross lung lesions were recorded as previously reported (Rodríguez-Gómez *et al.*, 2019).

Viraemia, detection of antibodies and serological analyses

Sera were collected to evaluate the viraemia, the presence of specific antibodies and the serum concentration of the cytokines IFN- γ , IL-6 and IL-10 as well as the acute-phase proteins haptoglobin (Hp) and lipopolysaccharide-binding protein (LBP). RNA was purified from sera using NucleoSpin RNA virus (Macherey-Nagel, Düren, Germany) according to manufacturer's instructions. Viraemia for either PRRS_3249 strain or Lena strain was quantified by RT-qPCR using LSI™ VetMAX™ PRRSV EU/NA 2.0 kit (Life Technologies, Carlsbad, CA, USA). Serial dilutions of PRRS_3249 strain or Lena strain (known viral titres) were used to determine genome quantification. RT-qPCR reactions were carried out with the QuantStudio 5 Real-time PCR System (Life Technologies, Carlsbad, CA, USA) for 5 min (min) at 50°C, 10 min at 95°C followed by 40 cycles of 3 s (s) at 95°C and 30 s at 60 °C. Viraemia results are showed in equivalent (eq) cytopathic dose 50 (CPD₅₀) per mL. PRRSV-specific antibodies were detected using IDEXX PRRS X3 ELISA test (IDEXX laboratories, Barcelona, Spain) following manufacturer's instructions. For cytokines and acute-phase protein detection, different commercially available ELISA and colorimetric assays were used in accordance with manufacturer's guidelines (IFN- γ , IL-6, IL-10 [Invitrogen, Carlsbad, CA, USA]; LBP, [Hycult Biotech, Uden, Netherlands] and Hp, [Tridelta Development Limited, Kildare, Ireland]). The results were expressed in pg/mL for IFN- γ , IL-6 and IL-10; ng ml⁻¹ for LPB, and mg ml⁻¹ for Hp. The minimum detectable concentrations were 2 pg ml⁻¹ for IFN- γ , 45 pg ml⁻¹ for IL-6, 3 pg ml⁻¹ for IL-10, 1.6 ng ml⁻¹ for LBP and 0.005 mg ml⁻¹ for Hp.

16S rRNA sequencing and bioinformatic processing analysis

The microbiota composition of faecal samples was established by amplicon sequencing. The V3-V4 variable region of the 16S rRNA gene was amplified from each extracted DNA sample according to the 16S metagenomic sequencing library protocol (Illumina Inc., San Diego, CA, USA), following manufacturer's instructions and subsequently sequenced on the Illumina MiSeq platform using v3 sequencing chemistry with 2 × 3000 pb paired-end reads according to the manufacturer's guidelines. The Illumina reads were filtered on the basis of quality (removal of low quality nucleotides at the 3' end, and remove windows 20 nt with a low average quality) and length (removal of sequences with <300 pb) with prinseq (Schmieder and Edwards, 2011), and the paired-end reads with a minimum overlap of 20 bp were joined using Fastq-join (Aronesty, 2013). Finally, all single files

were processed to a final filtering sequence mean quality score > 25. In addition, the sequences were cleaned of dereplicates, elimination of unique sequences and chimeras against gold database using UPARSE-OTU algorithm with Usearch v8.0 algorithm (Edgar, 2010). The resulting sequences were clustered with 97 % identity to obtain operative taxonomic unit (OTUs) using UPARSE-OTU algorithm with Usearch v8.0 algorithm (Edgar, 2010). The taxonomic assignment of these OTUs was obtained against the Ribosomal Database Project (RDP) (Cole *et al.*, 2017).

Statistical analysis

Statistical analyses were performed in R v3.4.2. Microbiota, and study variables (infection, experimental group and sampling day) were included in the estimation of alpha diversity richness (Shannon, Simpson and Chao1 indexes) by the Vegan and Phyloseq R packages (McMurdie and Holmes, 2013). For diversity values, assumption of normality was checked using the Shapiro–Wilk test. Potential differences in richness of factors included in the study were estimated by repeated measures analysis of variance (ANOVA), using sampling time-point as a co-factor and a Tukey multiple comparison test. Dissimilarities in beta diversity between pairs of samples were therefore estimated with the Bray–Curtis dissimilarity index and weighted unifracs index and analysed with non-metric multidimensional scaling (NMDS) in Vegan (Bray and Curtis, 1957; Lozupone *et al.*, 2007). The Vegan *envfit* function, which fits environmental vectors or factors onto an ordination, was used to evaluate whether the factors sampling day and infection status were associated with the (NMDS) ordinations; the significance of the fitted factors was estimated using 999 permutations. Analysis of similarities (Vegan *anosim* function) and Permutational Multivariate Analysis of Variance Using Distance Matrices (Vegan *adonis* function) were also used to establish the influence of the variables under study into the sample ordination. Differences in taxa abundance for 33 experimental groups (Control vs Infected, Control vs Lena/3249 or Lena-infected vs 3249-infected) were analysed using a zero-inflated gaussian model by the *fitZig* function of the metagenomeSeq R package (Paulson *et al.*, 2013), and significance was established with a false discovery rate (FDR) correction with a threshold of 0.05. Heatmaps of genera for which $P < 0.01$ were built using their relative temporal abundance as previously recommended (Shade, *et al.*, 2013), clustered using Euclidean distances and Ward's method using Pheatmap package. Spearman correlations were performed among animal parameters and microbial relative abundance using R core functions and represented by Corrplot and

Performance Analytics packages. For viraemia and serological parameters, statistical analyses were performed in GraphPad Prism 7 software (GraphPad software Inc., La Jolla, CA, USA) using the Mann–Whitney Test ($P < 0.05$).

Sequences accession number

The full data sets have been submitted under BioProject accession number PRJNA596172.

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Conflict of interests

The authors declare no conflict of interest regarding the results of this research.

Ethical approval

All the animal experiments described in the present study were conducted in strict accordance with the guidelines of the European Union (Directive 2010/63/EU). All animal studies were conducted under protocols approved by the IRTA Ethics Committee and by the Catalan Autonomous Government (Project 3647; FUE-2017-00533413).

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Supporting information

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

File S1. PRRSV viral load and antibodies in sera from infected groups. PRRSV genome load was quantified in sera by RT-qPCR (primary axis). Antibodies against PRRSV-N-protein were detected in sera using IDEXX PRRS X3 ELISA test (secondary axis). All data are reported as the median with range of results obtained for the pigs in the Lena group (filled red diamonds for viremia; unfilled red diamonds for antibodies) or in PRRS_3249 group (filled green triangles for viremia; unfilled green triangles for antibodies). Statistically significant differences are indicated with (*): * $P < 0.05$; ** $P < 0.01$ (Mann-Whitney Test; GraphPad Prism 7 software, GraphPad software Inc., La Jolla, CA, USA). S/P: Sample to positive ratio. ELISA cut-off value of 0.4 was applied according to manufacturer's instructions.

File S2. Analysis (*envfit* function of Vegan) of the influence of different factors on the ordination of samples. Significance was established at $\alpha = 0.05$. Results of the permutation multivariate ANOVA test performed in the ordination analysis.

File S3. Alpha diversity values in fecal samples from PRRSV-infected pigs. Figure 3A, Chao1 index values in fecal samples by experimental groups Lena, PRRS_3249 and control.

File S4. Alpha-diversity estimations (Observed OTUs, Shannon, inverted Simpson, Simpson, Chao1) and P -values obtained when alpha-diversity by the estimators selected was compared by the factors under study were compared.

File S5. Differentially abundant genus among pigs infected with differentially virulent PRRSV strains. Difference in mean of \log_2 normalized counts among Lena-infected, PRRS_3249-infected and control groups.

File S6. Correlation matrix between abundance of the main genera and the PRRSV infection parameters under study. Spearman correlations among significant phyla (A) and families (B) abundance and disease parameters.