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Differences in enteric pathogens and intestinal microbiota between diarrheic weaned piglets and healthy penmates

Biel Garcias [a,*], Lourdes Migura-Garcia [b,c], Noemí Giler [b,c], Marga Martín [a], Laila Darwich [a]

[a] Departament Sanitat i Anatomia Animals, Veterinary Faculty, Universitat Autònoma de Barcelona (UAB), Cerdanyola del Vallès 08193, Spain
[b] IRTA-UAB Mixed Research Unit in Animal Health, Centre de Recerca en Sanitat Animal (CReSA), Barcelona, Spain
[c] IRTA Animal Health Programme, CReSA, WOAH Collaborating Centre for the Research and Control of Emerging and Re-emerging Swine Diseases in Europe, Campus de la Universitat Autònoma de Barcelona, Spain

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ABSTRACT

Postweaning diarrhea (PWD) is a multifactorial disease caused by different aetiological agents, like viruses or bacteria and where the role of the microbiota remains unclear. The aim of this study was to assess differences between healthy and diarrheic weaned pigs concerning the prevalence of pathogens and changes in the intestinal microbiota. Eighteen farms with PWD were selected and 277 fecal samples were collected (152 diarrheic vs 125 healthy). Presence of Rotavirus A (RVA), B (RVB), C (RVC) and Porcine Epidemic Diarrhea Virus (PEDV), virulence factors of *Escherichia* coli and *Clostridioides difficile* were analyzed by PCR. Finally, the microbiota composition was also study by 16 S rRNA sequencing on 148 samples (102 diarrheic vs 46 healthy). RVA (53.95 % vs 36 %, $p=0.04$) and RVB (49.67 % vs 28.8 %, $p<0.001$) were more frequent in diarrheic animals. Furthermore, RVA viral load was higher in diseased animals. VT2 toxin was significantly associated with diarrhea, whereas other virulence factors were not. Presence of *C. difficile* and PEDV was almost negligible. Regarding microbiota changes, Fusobacteriota phylum was more frequent in diarrheic samples and Ruminococcaceae family in healthy penmates. During the first week postweaning, Enterobacteriace and Campylobacteria were enriched in animals presenting diarrhea. Furthermore, *Lactobacillus* was detected in those individuals with no RVA infection. In conclusion, RVA seems to play a primary role in PWD. Classic *E. coli* virulence factors were not associated with diarrhea, indicating the need for revising their implication in disease. Moreover, *Lactobacillus* was found frequently in animals negative for RVA, suggesting some protective effect.

1. Introduction

Weaning represents a critical phase for animal health since it is generally a very stressful time for the animals. Under natural conditions, piglets are gradually weaned between 10 and 20 weeks of age. However, in conventional farming, piglets are weaned after 3 or 4 weeks of age. This circumstance causes stress and leads to anorexia that generates intestinal inflammation and increases the susceptibility to pathogens. Furthermore, a disruption in the microbiota occurs after the sudden change of feed, making animals prone to enteric pathogens (Gresse et al., 2017).

The environmental and husbandry conditions during this phase favor the emergence of postweaning diarrhea (PWD), which has become a serious problem in swine industry in terms of animal health and economic impact. In addition to viral or bacterial agents known to cause diarrhea, other factors such as nutrition, poor husbandry practices or different stressors can trigger the occurrence of PWD (Rhouma et al., 2017). This multifactorial aetiology impers a definitive diagnosis. Moreover, at this age piglets are very vulnerable, and they need a fast treatment to survive, which usually consists of empiric antibiotic administration. Thus, weaning is a hotspot of antimicrobial consumption in intensive farms, generating high levels of antimicrobial resistance, worsening the impact on animal and public health (Kyung-hyo et al., 2020).

Regarding viral agents, Rotavirus has been considered the main causative agent of diarrhea in young animals. Different species of Rotavirus such as A (RVA), B (RVB), C (RVC), E (RVE) and H (RVH) have been detected in swine (Vlasova et al., 2017). High prevalence of RVA has been found as causative agent of PWD (Eriksen et al., 2021)The rest of rotavirus species have been less studied, even though they have been

* Corresponding author.
E-mail address: biel.garcias@uab.cat (B. Garcias).

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found when targeted (Molinari et al., 2016). Coronavirus can also generate PWD. The classical ones are porcine epidemic diarrhea virus (PEDV) and transmissible gastroenteritis virus (TGEV), but other such as swine enteric coronavirus (SeCoV), porcine deltacoronavirus (PDCoV) and swine enteric alphacoronavirus (SeACoV) can also generate diarrhea. In Spain, PEDV re-emerged in 2014 creating serious issues to porcine farms (de Nova et al., 2020).

Escherichia coli (*E. coli*) is also considered one of the main causes of PWD. There are different pathotypes involved. Historically, the most frequent pathotype has been enterotoxigenic *E. coli* (ETEC), with F4 or F18 fimbriae like, which facilitates the entry of bacteria into the enterocytes. Toxins such as LT, STa, or STb that alter the electrolytic equilibrium of the gut cells and provoke the excretion of water to intestinal lumen are also a common cause (Dubreuil et al., 2016). Other pathotypes such as enteropathogenic *E. coli* (EPEC) or verotoxigenic *E. coli* (VTEC) can also be involved in PWD outbreaks (Ercoli et al., 2016; Lecce et al., 1982). In consonance, virulence factors such F4, F18, LT, STa, STb, VT1, VT2 or eae are targeted by diagnosis and vaccines. However, since they were first described, swine genetics, farm conditions and vaccines have greatly improved.

Up to date, a high number of studies have been done on the causative agents of PWD, but most of them focused mainly on diseased animals and on a single pathogen (Yang et al., 2019; Renzhammer et al., 2020). Thus, there is a lack of knowledge about the presence of different enteric pathogens in healthy animals from farms with recurrent problems of PWD.

On the other hand, intestinal dysbiosis has been associated with the occurrence of PWD (Gresse et al., 2017). Some studies have tried to better understand which microbiota composition predisposes to the development of disease and which can be considered protective. However, they have been performed in experimental conditions or only assessing individual farms (Karasova et al., 2021; Gryaznova et al., 2022).

In the present research, we have studied the prevalence of enteric pathogens involved in PWD, as well as the intestinal microbiota composition of weaned piglets with diarrhea and healthy penmates under field conditions.

2. Material and methods

2.1. Sampling

Samples from eighteen conventional pig farms (14 farms with active and recurrent PWD outbreaks and 4 farms with no records of PWD outbreaks in the last 12 months) were analysed between February 2020 and December 2021. A total of 277 fecal samples were collected from 3 to 5 weeks old piglets: 152 out of them were diarrheic animals and 125 were healthy penmates that were considered controls.

2.2. Microbiological diagnosis

Presence of *E. coli* and *Clostridioides difficile* (*C. difficile*) was assessed in stool samples Briefly, for *E. coli* isolation, stool samples were cultured on Columbia blood agar (BD GmBh, Germany) and MacConkey agar (Oxoid, UK) and were incubated aerobically for 24 hours at 37 °C. A pure culture was obtained for most of the samples, but, in those cases were two or more colony morphologies were found, the most abundant one was selected. The strains were confirmed as *E. coli* using conventional biochemical tests (oxidase, catalase, TSI, SIM, urease, citrate, and methyl red) and the API system (bioMérieux, Marcy l'Etoile, France). For *C. difficile* detection, samples were cultured on a selective medium *C. difficile* agar base (Conda Laboratorios, Spain) and incubated anaerobically for 48 hours at 37 °C. All bacterial colonies collected were confirmed as *C. difficile* using a molecular diagnosis.

2.3. Molecular diagnosis of bacterial agents

DNA was extracted by boiling the growth from MacConkey agar and *C. difficile* selective plates. Briefly, all the bacterial growths were diluted in 600 μL of sterile distilled water, and 200 μL of the dilution was then transferred to a new tube. Two-hundred microliters of sterile distilled water was added to each tube. The tubes were boiled at 100 °C in a water bath for 10 min, and then centrifuged at 16,000 g for 5 min. After centrifugation, the supernatant was recovered and stored at −20 °C until processing.

Conventional PCR was performed, as previously reported by Vidal et al., (2019) to detect *E. coli* toxins (LT, STa, STb, VT1, VT2 and EAST1) and adhesins (F4, F18 and eae) and *C. difficile* toxins (TcdA and TcdB). Analysis of the amplified products was performed on 1.5 % agarose gel by electrophoresis.

2.4. Molecular diagnosis of viral agents

RNA was extracted from fecal samples using MagMAX™ CORE Nucleic Acid Extraction kit (ThermoFisher Scientific, USA) following the manufacturer's instructions. Detection of RVA, RVB, RVC and PEDV was done using the AgPath-ID™ One-Step RT-PCR kit (Applied Biosystems, ThermoFisher, USA) following the protocol of RT-PCR described by Marthaler et al. (2014).

2.5. Microbiota analysis

Eleven farms with active PWD outbreaks were selected for the analysis of the microbiota. A total of 148 faecal samples, 102 from diarrheic and 46 from healthy piglets were selected. DNA was extracted with QIAamp PowerFecal Pro DNA Kit (Qiagen) following the manufacture's recommendations. Briefly, for each sample, 250 mg of stool and 800 μl of buffer were homogenised with a TissueLyser. After following the manufacturer's instructions, DNA was eluted in 50 μl of the final solution buffer. DNA was quantified by Qubit DNA broad range fluorometer (Thermo Fisher Scientific, MA, USA) and shipped frozen to the sequencing provider (Novogene, Cambridge, UK).

Amplicon library preparation and sequencing was performed by Novogene in a NovaSeq platform to generate 250 bp paired-end raw reads. The region targeted to perform the 16 S amplification was spanning the V3 and V4 region of 16 S rRNA gene.

Paired-end reads were assigned to samples based on their unique barcodes and truncated by cutting off the barcode and primer sequences. Paired-end reads were merged using FLASH (V1.2.7). Quality filtering on the raw tags were performed following Bokulich et al., (2013) approach, with the default parameter settings (Phred >=Q20) to obtain the high-quality clean tags, according to the Qiime (V1.7.0) quality-controlled process. The tags were compared to SILVA138 Database using UCHIME algorithm to detect chimera sequences, and then the chimera sequences were removed. Sequences analyses were performed by UPARSE software using all the effective tags. Sequences with ≥97 % similarity were assigned to the same Operation Taxonomic Units (OTU). For each representative sequence, Qiime in Mothur method was performed against the SSUrRNA database of SILVA138 Database for species annotation at each taxonomic rank (kingdom, phylum, class, order, family, genus, species).

Alpha-diversity was analyzed using Qiime2 studying four metrics: number of observed species, phylogenetic diversity index, Shannon and Simpson. Beta-diversity was studied with generalized UniFrac distances matrices to do Principal Components Analysis (PCoA) with Qiime2 and were displayed with WGCNA and ggplot2 packages. Statistical differences regarding taxonomy were calculated by method of permutated test and False Discovery Rate according Benjamini and Hochberg method.

2.6. Statistical analysis

Statistics were applied to detect differences in pathogens frequencies between groups using Chi-square Test and were visualised using R (version 4.2.1, (R Core Team, 2014)) with ggplot2 package. Odds ratio was calculated using R function glm().

3. Results

3.1. Prevalence of enteric pathogens

All farms were positive for RVB, 72 % for RVA and 55 % for RVC (Table 1). PEDV was only detected in one farm just in one animal (0.4 % of pigs).

The most frequent classical virulent factors detected for *E. coli* isolates at farm level were EAST1 (83 %), F4 (78 %), eae (72 %), STa (55 %) and STb (50 %). The prevalence in infected pigs was also remarkable for EAST1 (49 %), eae (29 %), F4 (25 %), STa (21.1 %) and STb (15 %). Other factors such as F18 (5.4 %), VT1 (7.5 %) and VT2 (8.6 %) had lower frequencies in pigs and were less distributed among farms (Supplementary Table 1). Toxigenic *C. difficile* was only found in one pig.

Comparing the prevalence of viral pathogens between healthy and diarrheic animals, RVA (OR=1.78, p= 0.04) and RVB (OR=2.41, p= 0.02) were the most prevalent, being present in more than half of diarrheal cases and statistically related with disease presentation in PWD outbreaks. The same relationship was found analysing the set of farms. Conversely, no statistical differences were found between groups for RVC (Fig. 1).

Regarding *E. coli* virulence factors in farms with active PWD outbreaks, F4 (OR=2.32, p=0.02), STb (OR=4.81, p=0.04) and VT2 (OR=2.62, p=0.05) were associated with the presence of PWD. Moreover, despite being at a low prevalence, F18 and LT were only present in diarrheic animals sampled on farms with active PWD outbreaks. On the other hand, eae (OR= 0.33, p<0.001) and EAST1 (OR= 0.53, p=0.02) were found more frequently in healthy animals. However, when the overall group of farms were included in the analyses (Figs. 1B and 1D), only VT2 was found to be related with diarrhea (OR=3.25, p= 0.03) and frequencies of F18 and STa were higher in healthy animals. Thus, *E. coli* isolates with virulence factors were also present in healthy animals and farms without PWD problems.

The analysis of Ct values of rotavirus infections between healthy and diarrheic piglets showed that RVA viral load was higher in diarrrehic pigs than in healthy penmates (p= 0.002), indicating its correlation with diarrhea (Supplementary Figure 1). However, no differences were found for RVB and RVC. Moreover, the Ct values were higher for RVB and RVC than for RVA, so their role in the presentation of PWD was not conclusive.

3.2. Microbiota composition in PWD and healthy penmates

A total of 148 faecal samples from 11 farms with active outbreaks of PWD were analysed: 102 samples from diarrheic pigs and 46 from healthy penmates. A total of 11,264,394 reads were obtained, representing a mean of 76,111 reads per sample. The sample with the highest coverage had 96,056 reads and the lowest 58,509 reads.

Alpha-diversity metrics showed that the number of observed species was statistically higher in healthy animals (p= 0.04). However, considering more sophisticated indexes such as phylogenetic diversity, Shannon and Simpson, no alpha-diversity differences were found between diarrheic and healthy pigs (Supplementary Figure 2).

Moreover, Principal Coordinate Analysis (PCoA) clustering was used to elucidate differences between the microbiota of PWD and healthy animals. However, no clear cluster was found (Fig. 2a). De novo clustering following Rhea pipeline (Lagkouvardos et al., 2017) was performed and three clusters were encountered. The two biggest ones were formed by equally proportions of diarrheic and heathy samples and few conclusions could be extracted. The smallest one (n=12) was mostly formed by diarrheic samples (n=10), specially from animals weaned two weeks ago, but it was not exclusively. Additional metadata such diarrhea severity, vaccine status of the piglets, feeding behaviour or previous infections would be needed to shed light on the matter, but it was not available for this study.

Thus, further analyses were performed to find taxa abundance differences that were not affecting PCoA. Firstly, phyla abundance comparation between groups was performed (Fig. 2b), being Bacillota (previously Firmicutes,48.7 % in healthy animals vs 44.6 % in diarrheic animals), Bacteroidota (29.6 % vs 28.4 %) and Pseudomonadota (previously *Proteobacteria*, 7.6 % vs 10.6 %) the most frequent phyla detected in both groups. In a second step, a t-test approach was used to compare taxa between groups considering significant differences those with a False Discovery Ratio lower than 0.1. At phylum level, Fusobacteriota was more represented in diarrheic animals whereas Oscillospirales and Ruminococcaceae were more abundant in healthy piglets (Fig. 2c).

3.3. Changes in the microbiota and enteric pathogens according to the age

Taxonomic relative abundance comparison between PWD and control animals was performed individually for each farm. However, a high heterogenicity of results was found at farm level, being very difficult to find a defined pattern (Supplementary Spreadsheet file). When samples were re-analysed according to the age of the animals presenting PWD (first or second week postweaning), significant differences were found between diarrheic and healthy pigs (Table 1). For animals presenting diarrhea the 1st week postweaning, *Euryarchaeota* and *Clostridia* class *were* found to be more abundant in healthy animals, being especially important families such Oscillospiraceae and Ruminococacceae. Conversely, Pseudomonadota (especially Enterobacteriaceae) and Campylobacterota were enriched in pigs showing diarrhea during the first week postweaning.

Animals presenting diarrhea during the second week exhibited taxas *Spirochaetaceae* and *Clostridia vadin* BB60 group which were missing in healthy animals (Table 2).

The frequency of enteric pathogens in animals presenting PWD also

Table 1
Significant differences in taxonomic abundance between healthy and diarrheic animals during the first week post-weaning. Taxa with FDR<0.1 and a difference between groups of animals bigger than 1 %.

Taxonomy	Control	Difference (%)	FDR	PWD	Difference	FDR
Phylum	*Euryarchaeota*	3.4	0.1	*Campilobacterota*	2.8	0.09
	-	-	-	*Pseudomonadota*	4.8	0.07
Class	*Clostridia*	7.2	0.08	*Campylobacteria*	2.8	0.1
	-	-	-	*Gammaproteobacteria*	4.9	0.08
Order	*Oscillospirales*	4.8	0.02	-	-	-
Family	*Oscillospiraceae*	2.2	0.06	*Enterobacteriaceae*	4.5	0.06
	Ruminococcaceae	2.1	0.06	-	-	-
Genus	-	-	-	-	-	-
Species	-	-	-	-	-	-

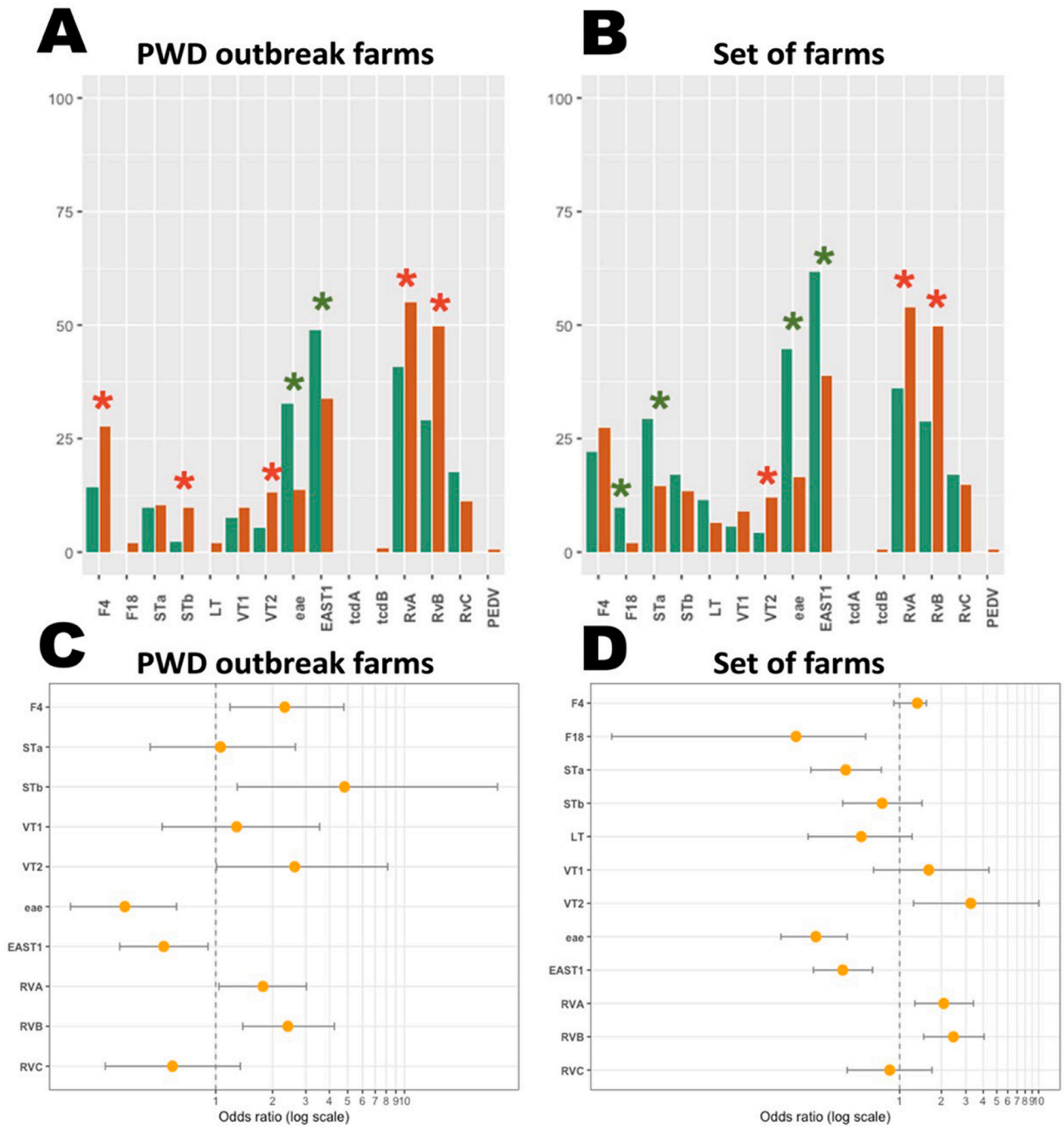

Fig. 1. : Prevalence of enteric pathogens in healthy and diarrheic animals. Red: diarrheic piglets. Green: healthy piglets. Asterisk: statistical significance (p-value <0.05) A: Barplot comparing frequencies in farms with active PWD outbreaks (n=14). B: Barplot comparing frequencies in the set of farms (n=18). C: Odds ratio in farms with active PWD outbreaks. D: Odds ratio in all studied farms.

differed between ages (Supplementary Table 2). Regarding *E. coli* virulence factors, F18 (9.7 % at 1st week postweaning vs 0.9 % at 2nd week), VT1 (9.7 % vs 0.9 %), eae (35.2 % vs 18.3 %) and EAST1 (57.9 % vs 38.3 %) were more frequently found during the first week after weaning in healthy animals. Interestingly, prevalence of RVA was significantly higher the second week postweaning (64.2 %) compared to the first week (29.1 %). Moreover, although the RVA mean Ct values (Supplementary Table 3) were similar during first week in diarrheic (27.24) and healthy pigs (27.52), the second week postweaning, RVA viral load was significantly higher in diarrheic animals (Ct=24.34) compared to the healthy group (Ct=28.94), indicating that RVA is especially relevant the second week after weaning.

3.4. *Microbiota associated with Rotavirus A infection*

Considering the presence or absence of RVA in the sample, no significant differences were found between groups. However, considering the viral load, samples with high RVA viral load (Ct-value <25) presented differences of bacterial abundance compared with lower or negative viral load (Table 3). Thus, *Sphaerochaeta* genus was associated with high viral load of RVA whereas Bacillota phylum and *Lactobacillus* genus were more abundant in samples with negative or low RVA load, 8.7 % and 5.2 % of abundance respectively.

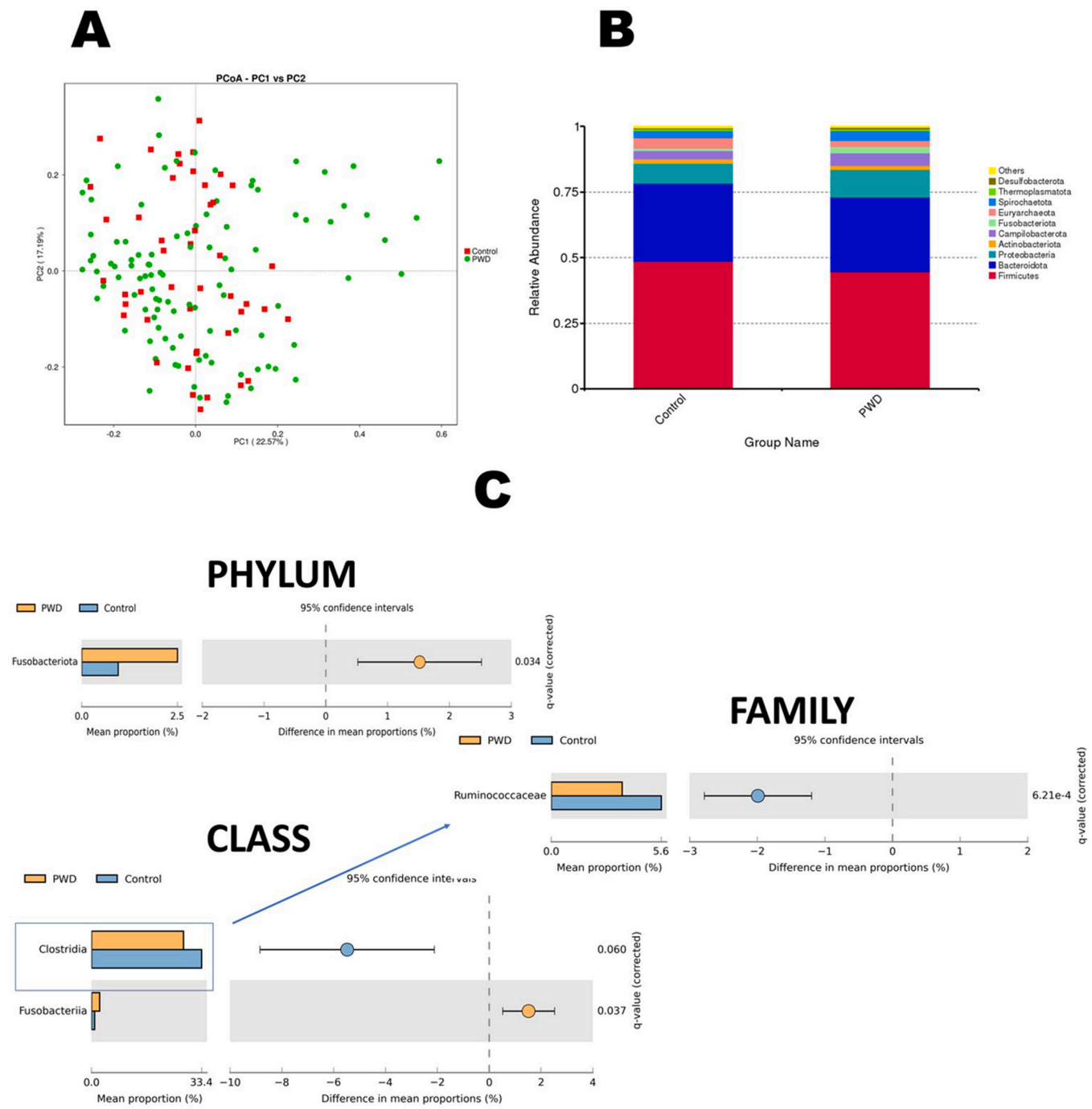

Fig. 2. : Taxonomic abundance differences between groups. A: Principal Coordinate Analysis (PCoA). B: Phylum taxonomic relative abundance comparation between healthy and diarrheic piglets. C: Statistically taxonomic relative abundance differences (FDR>0.1) between groups for phylum, class and family (no differences were observerd for order, genus or species).

4. Discussion

This study contributes to a better understanding of the epidemiology of enteric pathogens involved in PWD. Frequency of pathogens was compared between diarrheic and non-diarrheic weaned pigs. Moreover, the intestinal microbiota associated with diarrhea was analysed. Up to date, most of studies focused on PWD associated microbiota are based on a low number of farms (Karasova et al., 2021; Ren et al., 2022). Herein, we present a study design considering the individual factors of each farm with many samples analysed, contributing to improve our understanding of intestinal microbiota changes under field conditions.

In general, RVA and RVB were detected in almost all farms and in half of samples, being the most frequent viral agents circulating in the farms. *E. coli* isolates, as expected, were also found in all farms with a variable combination of virulence factors. Specifically, F4, EAST1 and eae were the most frequent virulence factors and were ubiquitously found. Moreover, the microbiota composition was very heterogenous between farms and animals, showing the complexity of PWD.

RVA has been described as the causative agent of diarrhea in neonatal piglets in Spain and other regions in the world (Kongsted et al., 2018; Mesonero-Escuredo et al., 2018; Vidal et al., 2019). In the present study, RVA was also detected in faecal samples of diarrheic weaned pigs,

Table 2
Significant differences in taxonomic abundance between healthy and diarrheic animals during the second week post-weaning. Taxa with FDR<0.1 and a difference between groups bigger than 1 %.

Taxonomy	Control	Difference (%)	FDR	PWD	Difference (%)	FDR
Phylum	-	-	-	*Spirochaetota*	2.8	0.04
Class	-	-	-	*Spirochaetia*	2.7	0.07
Order	-	-	-	*Spirochaetales*	2.8	0.08
	-	-	-	*Clostridiales vadin BB60* group	2.5	>0.001
Family	-	-	-	*Spirochaetaceae*	2.7	0.09
	-	-	-	*Clostridiaceae vadin BB60* group	2.3	0.01
Genus	-	-	-	*Clostridia vadin BB60* group	2.5	0.02
Species	-	-	-	-	-	-

Table 3
Significant differences in taxonomic abundance between healthy and diarrheic animals according the RVA infection status. Taxa with FDR<0.1 and a difference between groups bigger than 1 %.

Taxonomy	Negative/Low load RVA	Difference (%)	FDR	High load RVA	Difference (%)	FDR
Phylum	*Bacillota*	8.7	0.006	*Spirochaetota*	3.3	0.02
Class	-	-	-	*Spirochaetia*	3.3	0.05
Order	*Lachnospirales*	2.9	0.02	*Spirochaetales*	3.3	0.02
Family	*Lacnhospiraceae*	2.9	0.02	*Spirochaetaceae*	3.4	0.03
	Ruminococcaceae	1.4	<0.001	-	-	-
	Lactobacillaceae	5.3	0.06	-	-	-
Genus	*Lactobacillus*	5.2	0.06	*Sphaerochaeta*	3.2	0.01
Species	*Lactobacillus reuterii*	3.2	0.08	*Fusobacterium mortiferum*	3.1	0.04

as recently described (Eriksen et al., 2021). Moreover, RVA was present with higher prevalence and viral load in diseased animals than in healthy penmates, especially for piglets exhibiting diarrhea during the second week postweaning. Weaning is a stressful period for piglets that can lead to an impairment of the immune system during the first week of the transitions, increasing the risk to be infected by pathogens that are circulating in the farm. Whereas RVB was present in most of the farms and almost half of the animals, RVC was less frequent. This situation differs from other studies done in suckling piglets (Vidal et al., 2019), where RVC was more frequent while RVB was almost absent. These results indicate that rotavirus epidemiology is different between suckling and weaning pigs, as reported by Marthaler et al. (2014) where RVB was more prevalent in weaned animals. RVB has already been described as primary agent of diarrhea in both, neonatal piglets (Miyabe et al., 2020) and postweaned pigs (Marthaler et al., 2014; Molinari et al., 2016). However, our results evidence that there is a lack of knowledge about its presence in healthy animals, and our study is not conclusive about RVB role. Despite its higher prevalence in diarrhea cases, no significant differences were found in the viral load between healthy and diseased animals. Thus, further studies should be done to clarify RVB implication in PWD.

Other pathogens studied herein, such as PEDV and *C. difficile* were almost absent in the farms. In the case of PEDV, a decreasing trend has been observed, since 2014 when re-emerged causing severe problems to the swine industry. *C. difficile*, despite being commonly found in neonatal diarrhea (Vidal et al., 2019), its presence in PWD was residual. This finding agrees with Arruda et al. (2013), where a drop of prevalence with age is described.

E. coli has been historically considered the main cause of PWD leading to a high antibiotic use during the transition stage (Fairbrother et al., 2005). Virulence factors implicated in *E. coli* causing diarrhea have been described long ago and are still being targeted by vaccines and diagnostic purposes. However, farm conditions have changed during the last decades and bacterial pathogens have evolved. In this study, few differences were found between diarrheic and control animals. Thus, information about virulence factors should be updated. In this line, some genome wide association studies have identified new virulence genes in avian *E. coli* (Mageiros et al., 2021) or *Streptococcus suis* (Guo et al., 2021). Thus, similar approach should be explored in PWD caused by *E. coli* infections.

Regarding intestinal microbiota, although many differences were detected between distinct farms, some trends were observed. Fusobacteriota was found in higher prevalence in diarrheic animals and Ruminococcaceae family was more frequent in healthy animals. These findings agree with previous studies (Dou et al., 2017; Sun et al., 2019; Kong et al., 2022) reinforcing their contribution in the development of diarrhea.

Interestingly, the microbiota associated with health and disease varied with the age of the animals. During the first week postweaning, animals had a significant increase in *E. coli*. Moreover, at this age, Campylobacteria was associated with diseased piglets. Despite being described as a causative agent of diarrhea (Thompson et al., 2019), Campylobacteria have also been found in healthy piglets (Ruiz et al., 2016). The association with diarrhea in our study could be more related with the weaning stress (one of the main non-infectious contributors to PWD), as *Campylobacter* shedding is increased by stress (Whyte et al., 2001). During the second week postweaning, only Spirochaetaceae family and *Clostridium vadin BB60* group were more frequent in diarrheic animal. Since there are no previous reports in the literature about the role of these bacteria in the emergence of PWD, further studies are needed to confirm this association.

Microbiota changes have been associated to infections caused by other enteric porcine virus such as PEDV (Yang et al., 2020). However, these types of associations have not been studied for porcine RVA. Additionally, there is a lack of treatment for this pathogen, therefore studies on the microbiota associated to its infection could be useful to explore new alternatives to prevent PWD. It is especially remarkable the fact that Bacillota and *Lactobacillus* were less abundant in infected animals. Despite the lack of studies in pig production, *Lactobacillus* has been linked with beneficial effects such as alleviation of diarrhea in gnotobiotic pigs infected with human rotavirus (Liu et al., 2013), enhancement of mucosal B cell responses (Kandasamy et al., 2015) and an improvement of the vaccine efficacy (Parreno et al., 2022). For these reasons, *Lactobacillus* probiotics have been proposed as a treatment for acute rotavirus gastroenteritis in humans, showing promising results (Lee et al., 2015; Park et al., 2017). In consequence, *Lactobacillus* administrated as probiotics could be an option to explore to prevent or to reduce the incidence of diarrhea and reduce antimicrobial use. However, further studies are needed to really assess their efficacy in pig production, as probiotics are not always the best solution often are

washed out. Other alternative to study could be the use of prebiotics that increase the *Lactobacillus* concentration.

Although this study improves our understanding of PWD, there are some limitations that need to be considered. First, the farms enrolled for the study were those reporting recurrent cases of PWD, where field veterinarians needed assistance to confirm the diagnostics. Therefore, it is probable that previous batches of animals would have been treated with antimicrobials, resulting in under-detection of other bacterial aetiologies involved in the disease. Second, this is a cross-sectional study where sampling was conducted at one time point in each farm, without following up sampled animals. Thus, the disease evolution of healthy pen-mates could not be registered and therefore the implication of the presence of viral agents in the future development of diarrhea is unknown. However, this is not usually the case for PWD, which usually occurs during the first two weeks of the transition and at the same timepoint for the whole batch. Finally, it needs to be stated that they existed better parameters than OTUs such Amplicon Sequence Variants (ASV) or zero radius OTUs (zOTUs). However, we were trying to get a comparation with the previous studies done in the field and we decided to use OTUs. Nonetheless, use of ASV or zOTUs should be implemented for the future studies done in the field.

5. Conclusion

RVA was the main pathogen involved in PWD, being especially relevant during the second week postweaning. Classic *E. coli* virulence factors were not exclusively related with diarrheic cases. In consequence, virulence factors information should be updated. The microbiota associated to clinical cases of PWD was composed by Enterobacteriaceae and Campylobacteria during the first week postweaning, and Spirochaetaceae and *Clostridium vadin BB60* group in the second week after weaning. In contrast, *Ruminococcaceae* was more frequent in healthy animals with *Lactobacillus* being associated with a lower prevalence of RVA infections. These results can be useful to explore the use of *Lactobacillus* as alternative treatment to prevent PWD and reduce the antimicrobial use in conventional pig farms.

CRediT authorship contribution statement

Noemí Giler: Methodology, Investigation. **Marga Martín:** Writing – review & editing, Supervision, Formal analysis, Conceptualization. **Laila Darwich:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Formal analysis, Conceptualization. **Biel Garcias:** Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Lourdes Migura-Garcia:** Writing – review & editing, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare to not have competing interests.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.vetmic.2024.110162.

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