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Nanopore *versus* Illumina to study the gut bacterial diversity of sows and piglets between farms with high and low health status

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Abstract

Background Antibiotics are used in animal husbandry to control infectious diseases. Different stressors can compromise animal health, leaving piglets vulnerable to pathogens, especially enterotoxigenic *Escherichia coli* (ETEC), which causes post-weaning diarrhoea (PWD), the major source of mortality and morbidity in swine production. Furthermore, PWD is a recurrent disease for certain farms, suggesting a link between gut microbial composition and animal health. The aim of this study was to identify the intestinal microbiota of pigs on farms with high health status (HHS) and low health status (LHS) to determine the relationships between sanitary status and gut health. Therefore, three pig farms with LHS presenting recurrent problems of PWD and three farms with HHS were selected to characterise the intestinal microbiome of sows and their piglets. 16 S rRNA gene sequencing technology was used to determine the associations of the gut microbiome with health. With the aim of bringing the MinION Nanopore device to the field for its portability and taxonomic resolution, the results obtained with Illumina were compared to those obtained with Nanopore.

Results Overall, the results indicated remarkable differences in intestinal microbial communities between animals from LHS farms and those from HHS farms, suggesting that the microbiomes of LHS animals were enriched with potential pathogenic microorganisms, mainly from the Pseudomonadota phylum, such as the genus *Escherichia-Shigella*, and their associated related species. Moreover, animals from HHS were enriched with beneficial microorganisms, such as *Lactobacillus* spp., *Christensenellaceae* R7 group, *Treponema*, *Acetitomaculum* and *Oscillospiraceae* UCG-005.

Conclusions This study identifies potential microorganisms that may contribute to health and disease in pig farms with HHS and LHS, suggesting that tracking their occurrence might provide insight into sanitary conditions. Moreover,

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this research highlights the compatibility between Illumina and Nanopore sequencing platforms, justifying the use of MinION Nanopore device in field applications for in situ studies of PWD. This application has the potential to enhance sustainable economic growth in swine farms by enabling more effective monitoring and management of animal health.

Keywords Antimicrobial resistance, Post-weaning diarrhoea, Nanopore sequencing, Illumina sequencing, Pigs, Swine, Intestinal microbiome

Introduction

During the last century, the pig industry faced the need to increase animal production to sustain a growing human population. This intensification of livestock farming systems, from small farms to large-scale production where animals are housed in high density, increased the emergence and spread of foodborne zoonosis [1]. In this context, antimicrobial agents have been extensively used to prevent and control diseases, contributing to the emergence of antimicrobial-resistant (AMR) bacteria. According to recent data, 73% of the antimicrobials consumed worldwide are used in animal husbandry, and in many countries, this consumption is still justified to prevent disease or promote growth [2]. In the European Union (EU), efforts to mitigate the impact of AMR have resulted in strict regulations, such as the prohibition of growth promotors in 2006 and the more recent ban on the prophylactic use of antimicrobials or the therapeutic use of zinc oxide in swine production [3]. Additionally, many countries have established antimicrobial stewardship programmes prioritising animal health and welfare while reducing the use of antimicrobials [4, 5].

In Spain, implementation of the National Action Plan, alongside European legislation, resulted in a 69% reduction in antimicrobial sales for food-producing animals from 2014 to 2022 [6]. The country is the leading producer of swine and pork products in the EU. According to data provided by the Spanish Agency for Medicines and Medical Devices (https://www.resistenciaantibioticos.es/es/lineas-de-accion), this sector purchases the highest amount of antimicrobials expressed in mg/PCU (population correction unit). Generally, one of the most critical phases of the rearing cycle for the swine industry is the post-weaning period. At that stage, 21- to 28-day-old piglets encounter different stressors, including transport to a different facility, changes in diet, mixing of litters and a reduction in maternal immunity. All these factors can compromise animal health and well-being, leaving piglets vulnerable to pathogens, especially enterotoxigenic *Escherichia coli* (ETEC), which can cause PWD. PWD outbreaks often require the use of antimicrobials to control the disease, although different management practices, such as strict biosecurity measures, cleaning and disinfection procedures, and the formulation of new diets, have been applied to reduce the risk of PWD [7]. Interestingly, in Spain, swine production is integrated, with a low number of companies holding the majority of the country's herds. These companies tend to standardise all these management practices within their commercial farms. However, there are certain farms that, despite implementing similar management practices, including diets and the replacement of animals by the same multiplication farms, are recurrently affected by PWD episodes, representing farms with LHS. In contrast, their counterparts never suffer from PWD, indicating a HHS of animals.

Some of the differences in health status can be influenced by environmental conditions, which are difficult to control between different commercial farms. However, it is currently well established that the gut microbiota plays an important role in the development of PWD from early life. For instance, seven-day-old piglets displayed lower evenness and higher abundance of *Prevotellaceae*, *Lachnospiraceae*, *Ruminocacaceae* and *Lactobacillaceae* compared to piglets who developed PWD later in life [8]. Recent advances in sequencing technology have provided the opportunity to explore these differences in microbiome abundance and composition leading to health or disease. In general, most current studies on microbiota use the Illumina platforms (MiSeq and HiSeq), which present limited read lengths and amplify a small region of the 16 S rRNA gene [9]. The development of long-read sequencing technology, such as Nanopore, has allowed the sequencing of the full-length, 1,500 bp 16 S rRNA gene, enabling better taxonomic resolution, even at the species level [10]. Furthermore, moving Illumina technology to the field is not simple; instead, the portability of the Oxford Nanopore Technologies MinION device and its relatively low cost make this device an ideal tool for in situ sequencing on farms. However, one of the drawbacks was the accuracy of the reads, which has improved over the years, with current estimates suggesting a raw read accuracy of >95% and a >99% consensus accuracy for amplicon sequencing [11]. This study aimed to determine differences in the gut microbiota composition between animals from LHS farms suffering from recurrent cases of PWD and those from HHS farms. In addition, with the aim of bringing this technology closer to the farm and implementing it for real-time diagnostics, 16 S rRNA gene results obtained by Illumina were compared with those obtained with Nanopore.

Materials and methods

Study design

From March to June 2023, six pig farms belonging to one of the main Spanish swine producers were recruited to participate in the study [12]. By selecting the same producer, we reduced the bias of microbial changes due to nutritional programs, since Spanish producers tend to standardise these programs within the majority of their farms. Briefly, three of these farms were classified as LHS, involving farms with recurrent problems of PWD confirmed in the laboratory to be caused by *E. coli* within two weeks after weaning and at least 10–15% morbidity. The remaining three farms were classified as HHS and met the following criteria: (i) An outstanding production performance in terms of average daily gain and feed conversion rate falling within the best 25% of the whole swine population. (ii) Outstanding health records, including mortality (from weaning to slaughter maximum 4%), percentage of substandard pigs during the rearing cycle (maximum 3%) and antimicrobial treatment cost (1-1.5 € per pig), reaching at least the best 10% of the whole swine population. Additionally, based on the records provided by the companies participating in the study, management practices were standardized across all the farms. Nevertheless, both HHS and LHS farms successfully passed the Biocheck UGent survey. However, HHS farms achieved higher scores in certain aspects of both external and internal biosecurity, particularly in areas such as "purchase of breeding pigs," "personnel and visitors," "measures between compartments," "working lines and equipment usage," and "cleaning and disinfection." In addition to the Biocheck UGent survey, the company developed its own risk index for major swine pathogens, which was based on their occurrence on these farms over the past ten years. HHS farms were selection and multiplication farms free of the following swine pathogens: porcine reproductive and respiratory syndrome virus (PRRSV), swine dysentery and *Mycoplasma hyopneumoniae* [12].

In each farm, at the nursery facility, 10 multiparous sows of 3 to 5 parities were selected. Faecal samples were collected individually in anaerobic tubes directly from each sow and three of their 15-day-old piglets to make a total of 40 samples per farm. Samples were transported to the laboratory at 4 °C and processed within the next 18 h of sampling. Once in the laboratory, equal contents of faecal material from the three siblings were pooled into a single sample, making a total of 20 faecal samples per farm (10 from the sows and 10 pools from three piglets per sow). Faecal samples were mixed with DNA/RNA shield (Zymo Research) and stored in a freezer at -80 °C until further use (Supplementary Fig. 1).

Genomic DNA extraction

For each sample, 0.25 g was centrifuged at 10.000 xg for 1 min. The pellet was washed with 1 mL of sterile phosphate buffer saline (PBS) and DNA was extracted using the PowerFecal Pro DNA Kit (Qiagen) according to the manufacturer's protocol. DNA quality was assessed using a Nanodrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA), while DNA quantity was measured with the Qubit™ dsDNA High Sensitivity (HS) Assay Kit (Qubit 2.0 Fluorometer, Thermo Fisher Scientific). The DNA was frozen at – 20 °C until use.

Illumina library preparation, sequencing and bioinformatics analysis

For Illumina sequencing, DNA samples were sent to the Instituto de Investigación Sanitaria y Biomédica de Alicante—ISABIAL (Alicante, Spain). A total of 12.5 ng of DNA from each sample, quantified using Qubit, was used to prepare the amplicon libraries targeting the 16S rRNA gene according to the 16S Metagenomic Sequencing Library Preparation protocol for the Illumina MiSeq System (Illumina®, San Diego, CA, USA). Primer sequences cover the V3–V4 regions of the 16S rRNA gene [13]. The primers used for amplification include the Illumina adapters: 16S Amplicon PCR Forward Primer = 5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3'; and 16 S Amplicon PCR Reverse Primer = 5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATC-3'. Sequencing of the prepared libraries was conducted on the MiSeq system (Illumina) using a 2 × 300 bp format. The quality of the raw reads was assessed using FastQC software (v. 1.0.0) [14]. Initial processing of the raw sequencing data was conducted using QIIME2 v2023.9. The DADA2 pipeline incorporated into QIIME2 was employed for sequence denoising, filtering and chimera removal, resulting in the clustering of reads into Amplicon Sequence Variants (ASVs). To ensure data quality, forward and reverse reads were trimmed to 290 and 215 bp, respectively, based on a quality-score acceptance rate of 30 or higher. Primer sequences were removed from all reads. Taxonomy classification was assigned to the ASVs at genus level, leveraging the SILVA v138 database taxonomic training data optimised for DADA2 (99% 16 S full-length) [15, 16]. Reads not assigned to any genus or classified as Eukaryote or Archaea or present in less than 20% of the samples in both groups were removed from further analysis. The 16 S rRNA gene amplicon sequencing results are available at NCBI (BioProject PRJNA1123624).

Nanopore library preparation, sequencing and bioinformatics analysis

The entire 16S rRNA gene (V1-V9; ~1500 bp) was targeted for sequencing using the Nanopore MinION sequencing device and platform. For each sample, 10 ng of genomic DNA was transferred into a DNA LowBind tube, and the final volume was adjusted to 10 µL with nuclease-free water (Sigma–Aldrich). PCR amplification was prepared using the 16S Rapid Barcoding Kit SQK-16S024 (Oxford Nanopore Technologies) from barcode multiplexing 1–24, containing forward 27 (5'-AGAGT TTGATCCTGGCTCAG-3') and reverse 1492 (5'-TAC CTTGTTACGACTT-3') primers [17]. The PCR reaction mix was performed in 50 µL for each sample, which consisted of 5 µL of H_2O, 25 µL of LongAmp Hot Start Taq DNA Polymerase (New England Biolabs), 10 µL of each barcode containing also 16 S forward and reverse primers and 10 µL of template DNA. For negative and positive controls, 1 µL of nuclease-free water or ZymoBIOMICS Microbial Community DNA Standard (Zymo Research) was used, respectively. Cycling conditions for PCR were initial denaturation (95 °C; 1 min); amplification (35 cycles) comprising denaturation (95 °C; 20 s), annealing (55 °C; 30 s) and extension (65 °C; 2 min); and a final extension (65 °C; 5 min). The resulting amplicons were purified with AMPure XP Beads (Beckman Coulter) magnetic beads and quantified by a Qubit™ dsDNA HS Assay Kit for DNA library preparation with an equimolar pool of amplicons. For library preparation, total DNA was incubated with 1 µL of Rapid adapter (RAP) following the manufacturer's protocol. The library was loaded onto a MinION flow cell FLO-MIN106D-R9 (ONT) with Flow Cell Priming Kit (EXP-FLP004) following the manufacturers' protocol. Sequencing was performed for ~24 h at voltage of – 180 V. Reads were basecalled with MinKNOW software (v. 21.05.25) using Guppy's fast basecalling model, and sequences with Q < 7 (default threshold implemented in MinKNOW) were discarded. On average, Nanopore amplicon libraries in this study contained a minimum of 100 K read counts per barcode. Nanopore raw reads were analysed with Spaghetti, a custom pipeline for automatic bioinformatic analysis of Nanopore sequencing data [18]. The Spaghetti pipeline consisted of the following steps: (i) removal of primers and adapters with Porechop (v 24.5.0), (ii) filtering reads shorter than 1,200 bp or longer than 1,800 bp with Nanofilt (v 2.8.0), (iii) quality check with Nanostat (v 1.6.0) [19], (iv) chimera removal with yacrd (v 1.0.0) [20], (v) mapping long reads to the SILVA database with minimap2 (v. 2.26) [21, 22] and (vi) filtering and alignment with python scripts (included in the pipeline) for obtaining taxonomy and abundance tables of single reads [18]. A detailed explanation of the pipeline and the specific commands that were used can be found on Spaghetti's GitHub repository (https://github.com/adlape95/Spaghetti). To minimise the effects of ONT sequence, Nanopore data were collapsed to the final assignments and filtered with a minimum of 10 sequences represented in 20% of the samples.

Microbiome data analysis

Data visualisation and statistics were done using software R version (v. 4.3.1). Alpha and beta diversity from Illumina and MinION data analysis was determined by the phyloseq package (v. 1.46) [23]. Before estimation of the alpha diversity indexes, samples were rarefied to a depth of 86,770 reads to correct for the sequencing depth. Statistical differences were evaluated using the alpha diversity index with Pairwise Wilcox Test, with R packages ggpubr (v. 0.6) and vegan (v. 2.6-4). Beta diversity was represented by Principal Coordinates Analysis (PCoA) performed using the Bray-Curtis dissimilarity between samples. Permutational multivariate analysis of variance using distance matrices (PERMANOVA) test was also performed to study significant differences between microbial communities, using the previously cited software packages. Pearson correlation between the relative abundance of taxa in the Illumina and Nanopore datasets was calculated using the "cor" function in R. Correlations between sequencing methodologies were considered (i) high if Spearmans rho (r_s) was +/−0.9 to 1, (ii) strong if r_s was +/−0.7 to 0.9, (iii) moderate if r_s was +/−0.5 to 0.7, (vi) weak if r_s was +/−0.3 to 0.5, (v) or negligible if r_s was +/−0.0 to 0.3 and if $p < 0.05$ [24, 25]. Core microbiome taxa shared by samples at the genus level was identified using a Venn diagram obtained through https://www.interactivenn.net. Health status-associated microorganisms were differentially identified by using linear discriminant analysis (LDA) effect size (LEfSe) with the microbiomeMarker (v. 1.8) [26] and tidyverse (v.2.0) packages, with an LDA score of 2 and a Kruskal-Wallis' cut-off of $P = 0.05$ [27]. Custom figures were created using ggplot2 (v. 3.5.1) [28], ggrepel (v. 0.9.5), gridExtra (v. 2.3), plotly (v. 4.10.4) and ggsignif (v. 0.6.4) [29].

Results

Swine gut microbiome from high health status (HHS) and low health status (LHS) determined by two sequencing platforms

Short-read sequencing samples through the Illumina MiSeq pipeline generated 6,189,306 sequencing reads after denoising, removing chimeras and filtering low-quality sequences. Reads were processed, yielding a total of 2,165 reads for taxonomic assignment. Finally, 221 taxa collapsed at genus level after processing the raw data. A total of 183 taxa were present among samples with a frequency greater than ten and a prevalence of 20% at the genus level. For Illumina dataset, samples G3L10

and G6L9 were excluded, as they seem outliers in the following analysis (Supplementary Table 1). Long-read sequencing samples using the Nanopore MinION platform produced an average of 4,790,000 raw reads (average sequence length of 1,700 bp) above average quality of 10.08 threshold. After aligning the reads against the SILVA reference database, we obtained 2,914 taxa collapsed at genus level. Furthermore, by filtering with a frequency greater than ten and 20% prevalence among samples, a total of 214 taxa were obtained at genus level. Piglet samples G2L3 and G2L6 were removed from the Nanopore analyses, as they appeared to be outliers in the following analysis (Supplementary Table 1).

To better understand gut microbiota dynamics, we evaluated the alpha diversity at genus level between samples (mothers and piglets) in different farm status (HHS vs. LHS). Independently of the sequencing technique (Illumina vs. Nanopore), the overall Observed Features and Shannon indices were significantly higher ($P \leq 0.001$) for mothers in comparison to piglets in both HHS and LHS farms (Fig. 1). Nevertheless, only significant differences ($P = 0.03$) were observed in mothers from Illumina sequencing when comparing same animals in the different health status (HHS vs. LHS) (Fig. 1A).

Shifts in community membership were studied using on PCoA plots based on Bray-Curtis distance at the genus level (Fig. 2). No significant changes in dispersion

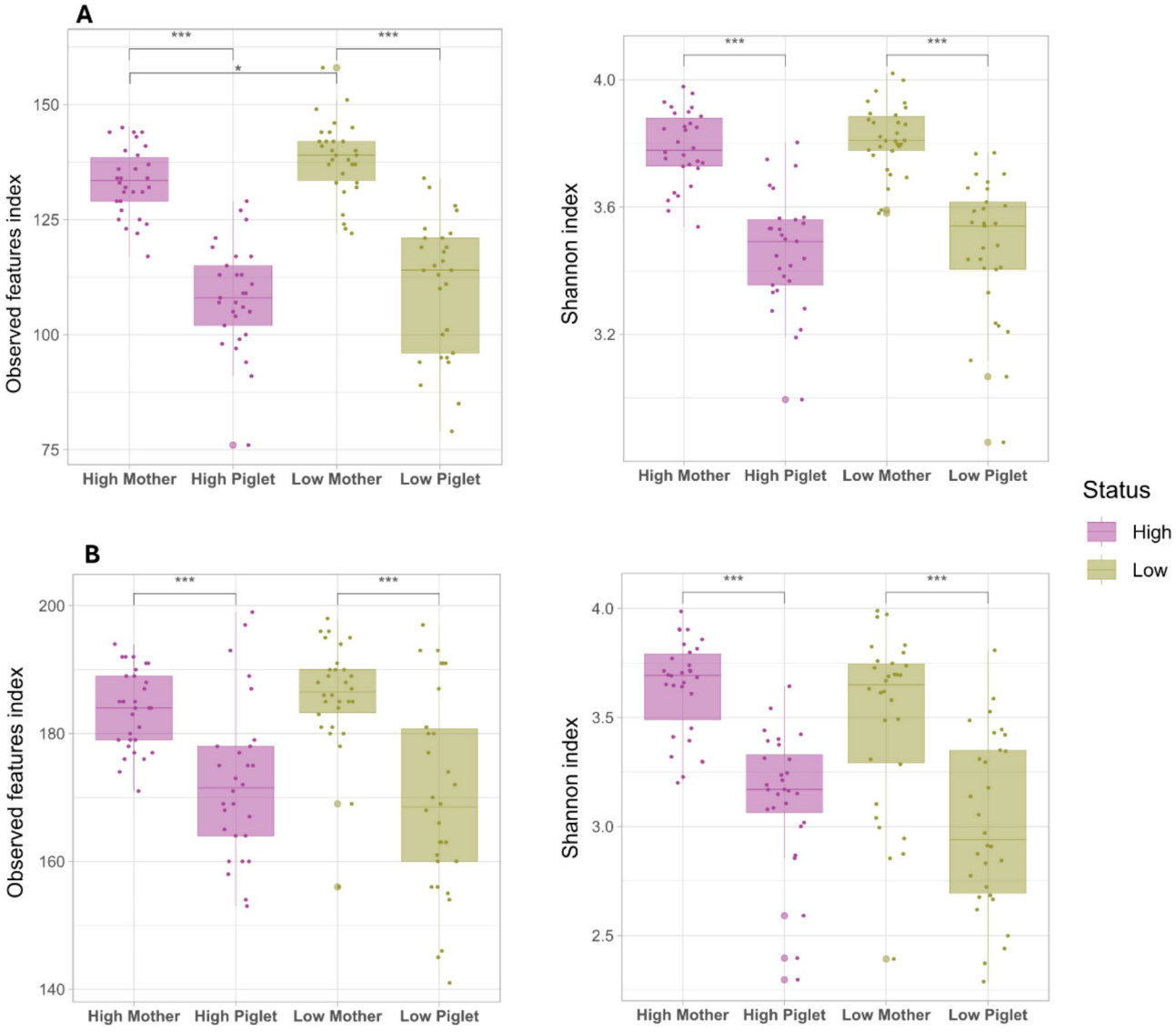

Fig. 1 Comparison of alpha diversity. Alpha diversity at genus taxonomic level in swine gut microbiome community richness (Observed features index) and evenness (Shannon index) between mothers and piglet samples from HHS (purple) and LHS (yellow) sanitary status for (**A**) Illumina and (**B**) Nanopore sequencing method. Means with "*", "**" and "***" are significantly different in 0.05, 0.01, and 0.001 (Pairwise Wilcoxon test)

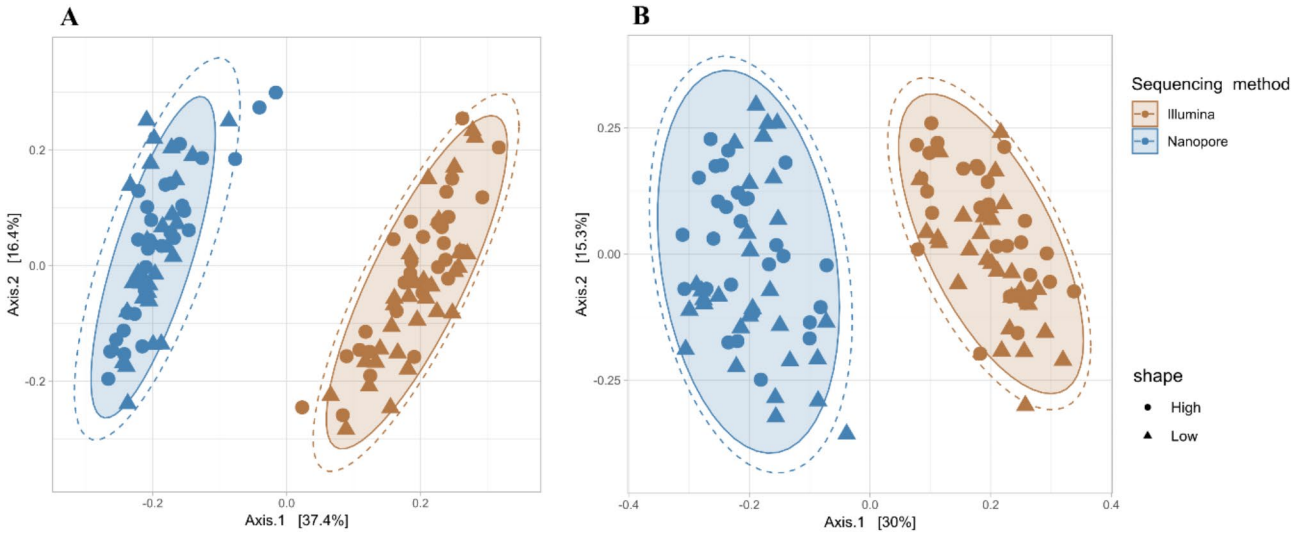

Fig. 2 Comparison of beta diversity. PCoA of beta diversity plot at genus taxonomic level in faecal samples of (**A**) mothers and (**B**) piglets between HHS (round) and LHS (triangle) sanitary status in different Illumina (brown) and Nanopore (blue) sequencing methods. Significant differences between sequencing method (Illumina vs. Nanopore) in mothers (PERMANOVA, $P = 0.0001$) and piglets (PERMANOVA, $P = 0.0001$) and piglets in HHS vs. LHS for Illumina (PERMANOVA, $P = 0.0226$) and Nanopore (PERMANOVA, $P = 0.0201$) sequencing method

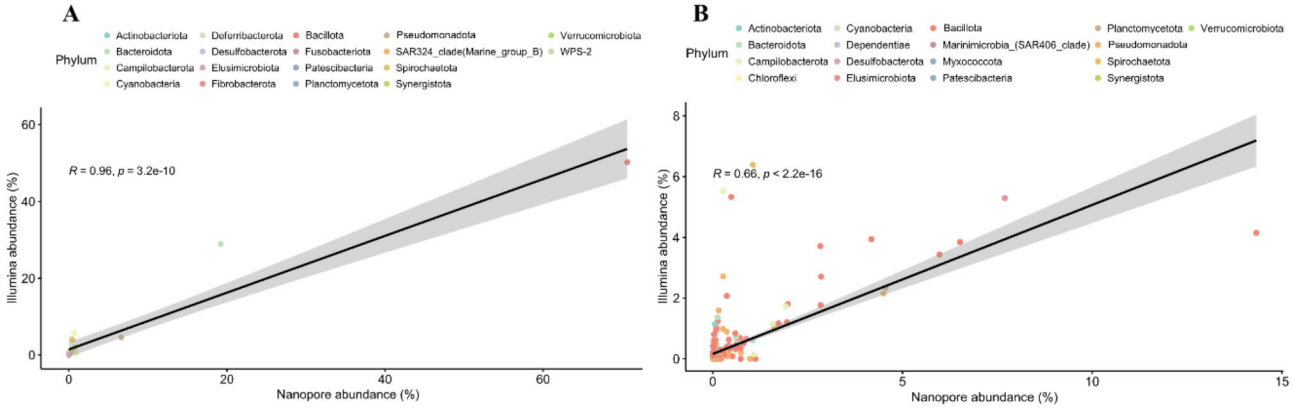

Fig. 3 Correlation study between sequencing platforms. Correlations between the relative abundances of the most abundant taxa from the Illumina and Nanopore datasets. (**A**) Phylum level (Pearson's r: 0.96, $P \leq 3.2 \times 10^{-10}$) and (**B**) genus level (Pearson's r: 0.66, $P \leq 2.2 \times 10^{-16}$). Legend with Phylum taxonomic rang. Black line corresponds to a 1:1 ratio, and the grey line shows the confidence interval between the two sequencing methods

or evenness were observed between mothers in different sanitary status (HHS vs. LHS) (Fig. 2A). Conversely, significant changes were determined in piglets from HHS and LHS with Illumina (PERMANOVA, $P = 0.0226$) and Nanopore (PERMANOVA, $P = 0.0201$) platforms (Fig. 2B). Comparing sequencing platforms (Illumina vs. Nanopore), significant shifts were noted between them in mother (PERMANOVA, $P = 0.0001$) and piglet (PERMANOVA, $P = 0.0001$) samples.

Correlation between Illumina and Nanopore sequencing platforms

Agreement between Illumina and Nanopore datasets to provide a depth comparison between each sequencing strategy was also studied. We compared the correlations at different taxon levels between the two sequencing techniques (Fig. 3, Supplementary Fig. 2). Overall, there was high correlation between the relative abundances obtained by Illumina and Nanopore at high taxonomic levels, such as the phylum level (Pearson's $r = 0.96$, $P \leq 3.2 \times 10^{-10}$) and order level (Pearson's $r = 0.9$, $P \leq 2.2 \times 10^{-16}$) (Fig. 3A, Supplementary Fig. 2A). This correlation decreased and remained strong at family level (Pearson's $r = 0.73$, $P \leq 2.2 \times 10^{-16}$) and moderate at genus level (Pearson's $r = 0.66$, $P \leq 2.2 \times 10^{-16}$) (Fig. 3B, Supplementary Fig. 2B). There were some differences between the sequencing technologies in terms of relative abundance for certain genera, which was consistent across all the taxonomic levels studied. For example, *Symphothece* PCC-7002, *Muribaculaceae* and *Prevotella* genera had higher abundances in samples sequenced with Illumina technology. In contrast, *Rikenellaceae* RC9 group had

higher relative abundance in samples sequenced with Nanopore technology.

Comparison of the microbial composition of mothers and piglets from high and low sanitary status determined by two sequencing platforms

To examine the existence of an identifiable common core microbiome and heritability shared between sows and their piglets, a Venn diagram was created (Fig. 4). Using the Illumina methodology, we detected 163 genera shared by mothers and piglets from HHS farms, whereas only 16 genera were unique to mothers, and 11 were unique to piglets. Similarly, 168 genera were shared by mothers and piglets from LHS farms, whereas only 15 genera were unique to mothers and 7 were unique to piglets (Fig. 4A). Interestingly, the majority of the genera that differed between mothers and piglets were detected on both HHS and LHS farms. In contrast, the same analyses performed with Nanopore sequencing revealed that a total of 212 and 213 genera were shared between mothers and piglets from HHS and LHS, respectively. Only one genus was unique to piglets in both health status groups, corresponding to *Clostridium innocuum* group for HHS and *Bifidobacterium* for LHS (Fig. 4B).

We next compared the microbial composition of sows and piglets from high- and low-health status farms using the two sequencing platforms. The top phyla are represented in Table 1. Overall, the most abundant phyla in sanitary status from both mothers and piglets across sequencing methods were Bacillota and Bacteroidota, followed by Pseudomonadota.

According to Illumina sequencing, Bacillota, Spirochaetota and Thermodesulfobacteriota phyla were more abundant in mothers from HHS. In contrast, Cyanobacteria and Pseudomonadota were found in a higher proportion in LHS. The same tendency was observed for the piglet samples. However, Cyanobacteria was enriched in piglets from HHS, and Synergistota and Fusobacteriota were enriched in piglets from LHS. When we compared mothers and piglets in both sanitary states, Cyanobacteria, Spirochaetota, Fusobacteriota, Pseudomonadota and Synergistota had higher abundance in piglets. In contrast, Bacillota and Bacteriota were more represented in mothers.

For Nanopore sequencing, Campylobacterota, Bacillota, Spirochaetota, Thermodesulfobacteriota and Verrucomicrobiota were enriched in mothers from HHS, whereas Bacteroidota, Fusobacteriota and

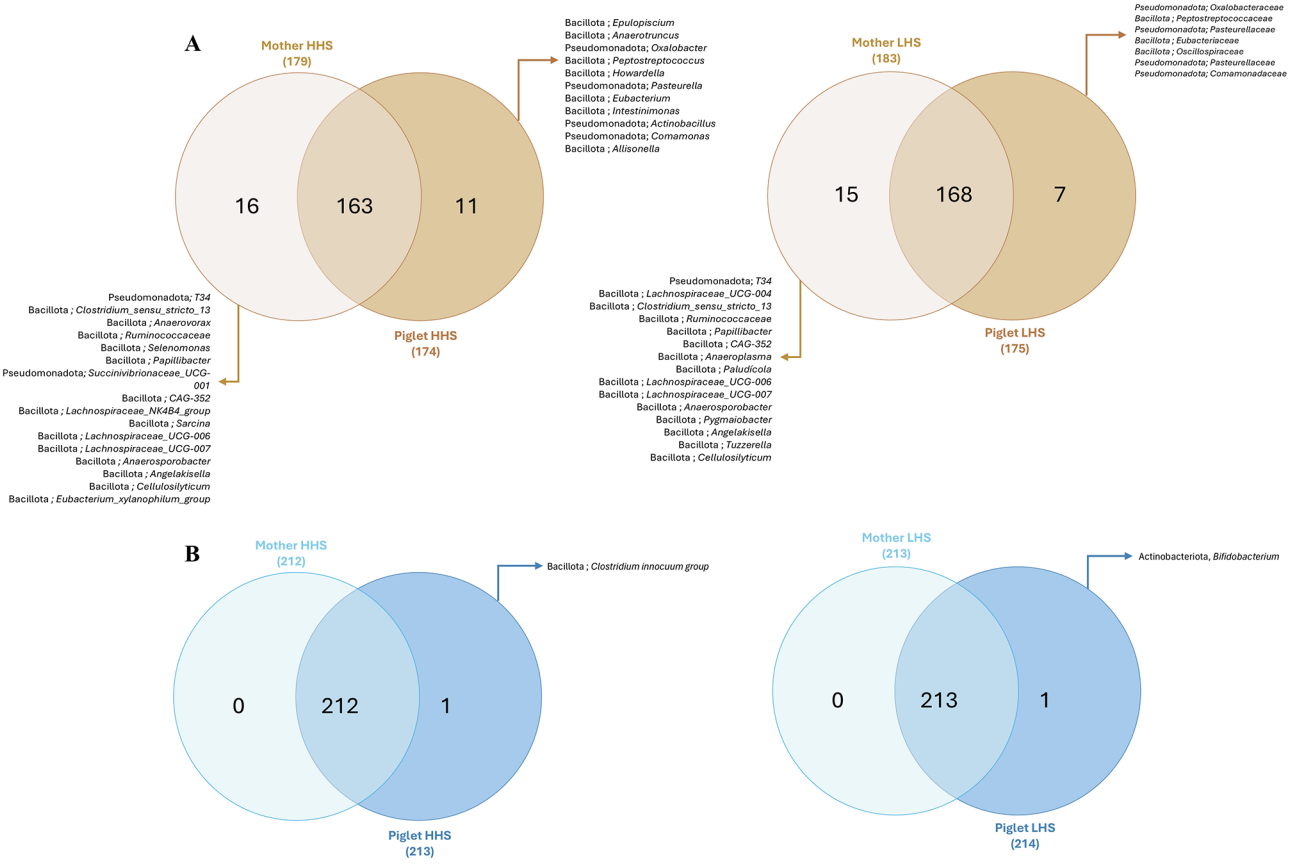

Fig. 4 Venn diagram analysis. Representation of shared and unique genera in mothers and piglets under different sanitary status conditions (HHS vs. LHS) (https://www.interactivenn.net/) for (**A**) Illumina sequencing platform (brown) and (**B**) Nanopore sequencing platform (blue)

Table 1 Relative abundance (%) of the top bacterial phyla in the faecal samples from mothers and piglets of high and low health status comparing illumina and nanopore results

Phylum name	Illumina				Nanopore			
	High mother (%)	High piglet (%)	Low mother (%)	Low piglet (%)	High mother (%)	High piglet (%)	Low mother (%)	Low piglet (%)
Fibrobacterota	0.72	0.01	0.76	0.00	0.05	0.001	0.04	0.01
Cyanobacteria	2.20	9.22	3.12	8.88	0.54	0.82	0.54	0.59
Bacillota	53.45	49.31	52.15	49.27	80.22	62.23	77.91	61.57
Spirochaetota	6.44	1.87	5.88	1.67	0.77	0.20	0.52	0.14
Thermodesulfobacteriota	0.64	1.35	0.46	1.23	0.13	0.11	0.07	0.10
Bacteroidota	32.38	25.67	32.68	25.73	14.53	23.70	15.39	23.61
Verrucomicrobiota	2.28	1.47	2.43	1.85	1.25	0.41	0.81	0.71
Fusobacteriota	0.00	1.40	0.00	2.69	0.00	0.46	0.08	0.54
Pseudomonadota	1.78	7.06	2.42	7.59	1.87	10.31	4.07	11.48
Synergistota	0.10	2.62	0.10	1.09	0.04	0.34	0.06	0.16
Campylobacterota	0.27	0.64	0.22	0.61	0.64	1.41	0.54	1.11

Table 2 Relative abundance (%) of the top bacterial genera in the faecal samples from mothers and piglets of high and low health status comparing illumina and nanopore results

Genus name	Illumina				Nanopore			
	High mother (%)	High piglet (%)	Low mother (%)	Low piglet (%)	High mother (%)	High piglet (%)	Low mother (%)	Low piglet (%)
Symphothece PCC-7002	1.91	9.08	2.80	8.62	0.04	0.66	0.09	0.34
Muribaculaceae	4.84	6.07	5.10	5.39	0.48	0.54	0.45	0.48
Prevotella	7.80	4.88	6.94	5.92	2.26	1.20	1.21	1.28
Treponema	4.76	0.82	4.16	1.02	0.57	0.05	0.38	0.07
Lactobacillus	4.99	4.75	3.95	7.59	7.23	5.01	7.48	11.02
Oscillospiraceae UCG-002	2.10	5.56	2.04	6.25	2.39	6.10	2.92	5.52
Clostridium sensu stricto 1	4.37	1.01	6.97	1.25	7.65	1.51	11.26	3.33
Rikenellaceae RC9 group	4.49	3.86	4.19	4.10	8.46	18.96	10.57	19.90
Phascolarctobacterium	2.82	5.00	2.71	4.43	2.26	3.88	2.24	3.08
Christensenellaceae R7 group	3.32	5.89	2.74	3.53	6.60	9.19	4.65	5.93
Escherichia-Shigella	0.13	4.91	0.23	4.30	0.29	8.00	1.66	8.60
Clostridia vadinBB60 group	1.41	4.41	1.06	4.08	1.31	5.22	1.47	3.63
Oscillospiraceae UCG-005	3.58	1.18	3.14	0.64	7.35	2.79	5.85	1.97
Terrisporobacter	3.23	0.10	3.57	0.06	4.99	0.16	5.13	0.96

Pseudomonadota were more abundant in LHS. The same tendency was observed in piglets, except for Verrucomicrobiota, which was lower for HHS piglets. Additionally, Cyanobacteria and Synergistota were more abundant in piglets from HHS. Comparing mothers and piglets in both the HHS and LHS, Bacillota, Verrucomicrobiota and Spirochaetota were more abundant in mothers, while Cyanobacteria, Fusobacteriota, Pseudomonadota, Synergistota and Campylobacterota were more represented in piglets.

Comparison between Nanopore and Illumina sequencing platforms were also performed. Higher proportions of Cyanobacteria, Spirochaetota, Fibrobacterota, Thermodesulfobacteriota and Bacteroidota were observed in samples obtained from Illumina, whereas Pseudomonadota, Bacillota and Campylobacterota were more abundant in samples obtained from Nanopore. Despite the differences in abundance mentioned above, proportional relationships between phylum, sample type

(mothers and piglets) and farm status (HHS vs. LHS) seemed to be maintained.

The top most abundant genera are represented in Table 2. According to Illumina sequencing, *Prevotella*, *Treponema*, *Lactobacillus* and *Christensenellaceae* R7 groups exhibited greater abundances among mothers from HHS, whereas *Symphothece* PCC-7002, *Muribaculaceae* and *Clostridium sensu stricto* 1 were more abundant in LHS. In contrast, piglets displayed an opposite trend, where only *Clostridium sensu stricto* 1 and *Christensenellaceae R7* group genera were maintained. When comparing mothers and piglets in both HHS and LHS, *Prevotella* and *Treponema* were more abundant in mothers, while *Symphothece* PCC-7002, *Oscillospiraceae* UCG-002 and *Clostridium sensu stricto* 1 were enriched in piglets. Interestingly, in HHS *Christensenellaceae* R7 group had more representation in piglets in comparison to mothers, whereas in LHS *Lactobacillus* was more represented in piglets unlike in mothers.

Nanopore sequencing revealed that *Christensenellaceae* R7 group and *Oscillospiraceae* UCG-005 were more abundant in HHS mothers. In contrast, *Clostridium sensu stricto* 1 and *Escherichia-Shigella* had more representation in LHS. In piglets, some differences were observed, with *Phascolarctobacterium*, *Christensenellaceae* R7 group and *Clostridia vadinBB60* group being enriched in HHS farms. On the contrary, *Lactobacillus*, *Clostridium sensu stricto* 1 and *Terrisporobacter* were more abundant in LHS farms. Comparing mothers and piglets, in both sanitary status *Clostridium sensu stricto* 1, *Oscillospiraceae* UCG-005 and *Terrisporobacter* were enriched in mothers, whereas *Rikenellaceae* RC9 group and *Escherichia-Shigella* were more abundant in piglets. As previously observed, for LHS, *Lactobacillus* was more represented in piglets than in mothers.

Comparison between Illumina and Nanopore sequencing methods revealed that the genera *Symphothece* PCC-7002, *Muribaculaceae*, *Prevotella* and *Treponema* were overrepresented in the Illumina dataset. Conversely, *Oscillospiraceae* UCG-005, *Escherichia-Shigella*, *Clostridia vadinBB60* group, *Terrisporobacter* and *Rikenellaceae* RC9 group exhibited elevated abundances within the Nanopore dataset. Despite the variation in abundance noted, proportional relationships between genus, sample type (mothers and piglets) and farm status (HHS vs. LHS) were maintained among sequencing platforms, as observed for phylum composition.

At the species level, only a few taxa were identified with the Nanopore sequencing platform (Supplementary Table 2). Some of the most remarkable species were represented in a plot showing their relative abundance between samples (mothers and piglets) and sanitary

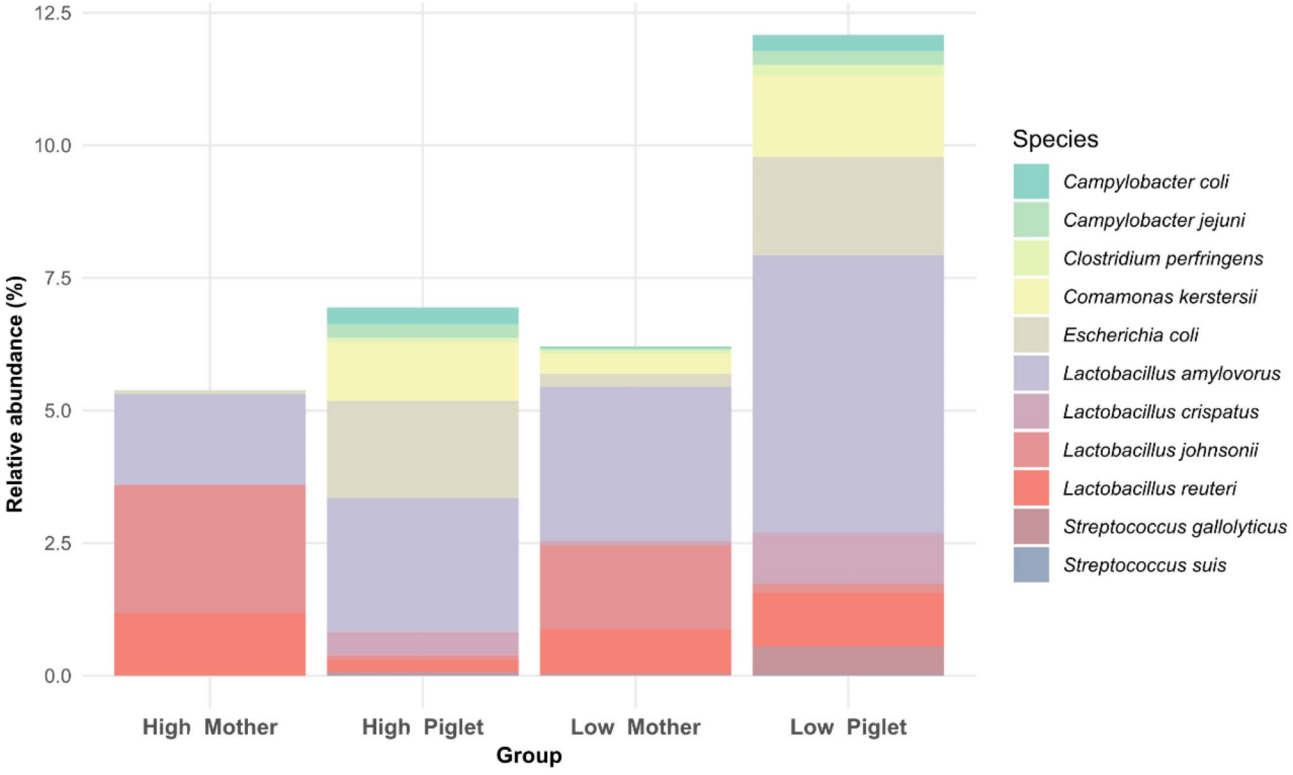

Fig. 5 Bar chart of species abundance. Relative abundance (%) of the remarkable species identified in mothers and piglets from HHS and LHS farms from the Nanopore dataset. Each colour represents a specific species

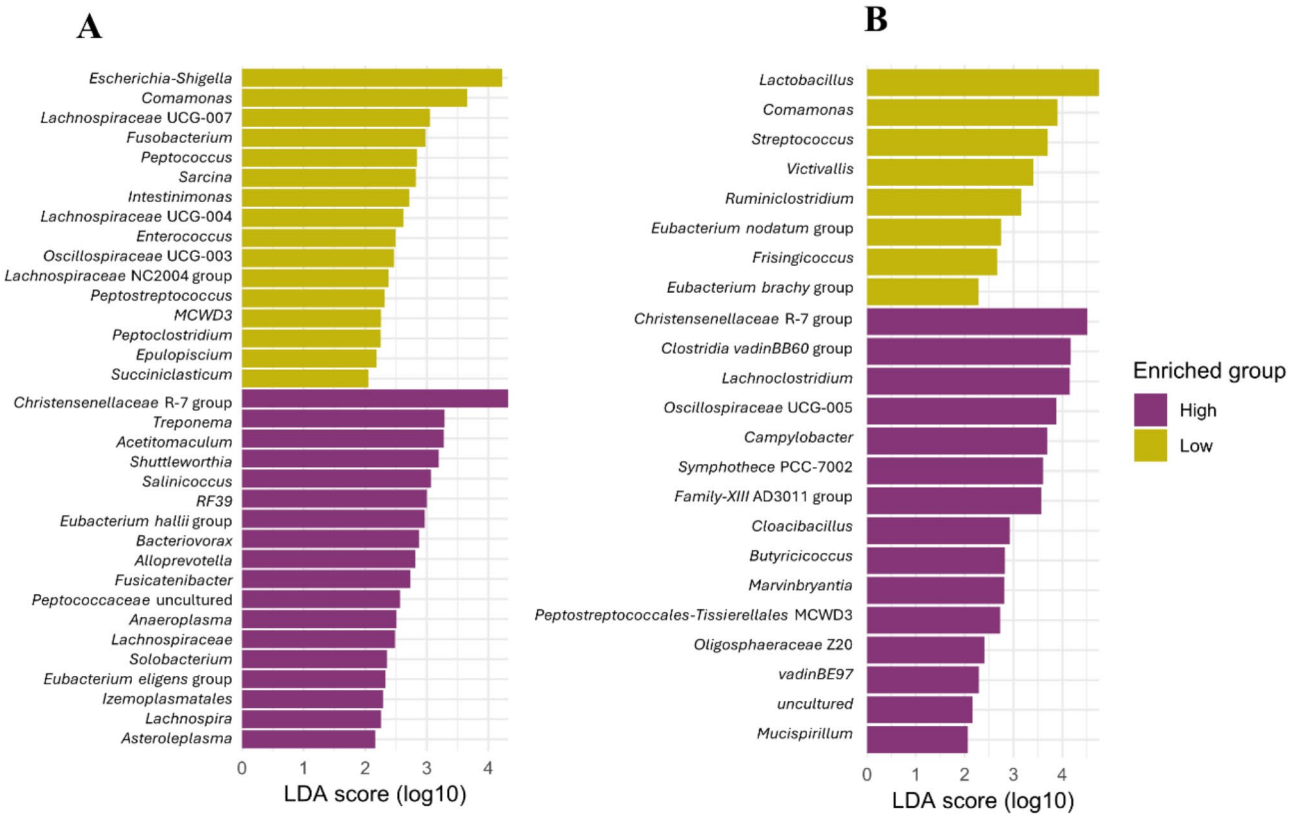

Fig. 6 Histogram of linear discriminant analysis (LDA) effect size (LEfSe) computed for bacterial taxa at genus level. Differentially abundant among HHS (purple) and LHS (yellow) groups for mothers (**A**) and piglets (**B**) from Nanopore dataset. *Kw cutoff* = 0.05 and *LDA cutoff* = 2

status (HHS vs. LHS) (Fig. 5). In general, a higher abundance of *Lactobacillus johnsonii* and *Lactobacillus reuteri* was detected in HHS mothers compared to LHS. These species were also present in piglets but in lower abundances. In contrast, *Lactobacillus amylovorus* and *Lactobacillus crispatus* were more represented in piglets, where *L. amylovorus* was strongly present in the LHS. On the contrary, *Campylobacter coli*, *Campylobacter jejuni*, *Clostridium perfringens*, *Comamonas kerstersii*, *Escherichia coli*, *Streptococcus gallolyticus* and *Streptococcus suis* were more abundant in piglets than in mothers, especially in LHS. Interestingly, the abundance of these species was also higher in mothers from LHS in comparison to HHS.

We then identified sanitary status-associated genera by using LEfSe analyses of the Illumina (Supplementary Fig. 3, Supplementary Tables 3–4) and Nanopore datasets (Fig. 6, Supplementary Tables 5–6), confirming most of the observations mentioned above. For example, *Christensenellaceae* R7 group was one of the most relevant genus for both mothers and piglets belonging to HHS farms, whereas *Escherichia-Shigella* was differentially represented in mothers of LHS farms. In contrast, *Treponema* was one of the most relevant genera for mothers of HHS, as was *Acetitomaculum*, *Shuttleworthia* and *Salinicoccus* (Fig. 6A). *Comamonas* was the only genus present in both mothers and piglets in LHS farms, whereas *Streptococcus* and *Lactobacillus* were some of the top genera differentially expressed in piglets from LHS farms. Conversely, *Clostridia vadinBB60*, *Lachnoclostridium*, *Oscillospiraceae* UCG-005 and *Campylobacter* were some of the most represented genera in HHS piglets (Fig. 6B).

Discussion

Recent studies suggest that the contribution of the microbiome to pathogenicity and health in swine can be attributed to specific bacterial microorganisms [30]. To investigate the relationship between animals (piglets and sows) from HHS and LHS farms, 16 S rRNA amplicon sequencing approaches (Illumina vs. Nanopore) were applied to infer microbial communities associated with health and disease, especially PWD.

Both sequencing methodologies enabled accurate characterization of the intestinal microbiome on both HHS and LHS farms. However, Nanopore technology allows the identification of the taxonomic level of the species involved [17]. Independently of the sequencing technique employed, the alpha diversity index of the gut microbiota was lower for piglets compared to their mothers,

suggesting an increase in alpha diversity during the initial phases of the growing period, despite the sanitary status (HHS and LHS). This observation shows that mothers have higher microbial richness and evenness, proposing a more mature and stable microbiome, as high diversity of the gut microbiota is generally considered beneficial for host health [31]. This is remarkable, as it has been demonstrated that during the weaning period, piglets are more vulnerable to colonisation by opportunistic infections from their own microbiome and environment [32, 33].

In addition, it is worth noting that more than 90% of the genera were shared between mothers and piglets, supporting the hypothesis that the intestinal microbiome is vertically transmitted from sows to their litter, influencing the development of the piglet microbiome. To this end, we observed that Bacillota and Bacteroidota were the core phyla in both mothers and piglets regardless of age, followed by Pseudomonadota [34]. Bacillota was the most dominant phylum in faecal samples, exhibiting an increasing abundance tendency towards the growing phase. This can be attributed to changes associated with a complex diet, environment and maturation of the gut microbiota structure of the animal [31]. In the case of Bacteroidota, we observed a further stabilisation during animal growth because of its high presence in maternal milk and metabolic activity during early stages of piglet development [35]. Conversely, Pseudomonadota demonstrated the opposite trend, with greater abundance in young piglets, as a consequence of immature microbiome and immune system [36, 37]. Additionally, our results showed that Pseudomonadota and Fusobacteriota were more abundant in animals from LHS. Interestingly, these phyla are associated with pathogenicity and incidence of diarrhoea in pigs [38, 39]. Moreover, this study also revealed that Spirochaetota phylum was more abundant in animals from HHS, which is described as a biomarker of health in pigs for fermenting carbohydrates and providing energy [40].

The results obtained at the genus level have also shown differences in both mothers' and piglets' samples between HHS and LHS farms, independent of the sequencing technique applied. Gut microbiota of LHS animals (mothers and piglets) was enriched with potential pathogenic genera, such as *Clostridium sensu stricto* 1 [41] and *Escherichia-Shigella* [42]. For example, *Clostridium sensu stricto* 1 is associated with intestinal inflammation, while *Escherichia-Shigella* is described as an indicator of diarrhoea [43], both of which compromise the health of the intestinal microbiome. In the case of the Nanopore analyses and farms with LHS, our investigations allowed us to infer more deeply at the species level, observing that the species of *Escherichia-Shigella* were significantly enriched in mothers. Nevertheless, in adult life, the concentration of this genus is balanced in the microbiome [27]. However, our study suggests that diarrhoeic episodes in piglets could be related to an increase in the concentration of this genus in the mothers. Indeed, some research has shown that some *E. coli* pathotypes, such as ETEC and enteropathogenic *E. coli* (EPEC), are causative agents of neonatal disease and PWD, leading to significant economic losses in the swine industry [44, 45]. Moreover, we also detected a significant abundance of opportunistic pathogens such as *Comamonas* in both piglets and sows, as well as *Streptococcus* in piglets from LHS. Whereas some species of these genera, observed by Nanopore, are common colonisers of the gut microbiome, others can potentially cause severe illness in swine. For example, *Comamonas* causes bacteriemia and abdominal infection [45, 46], while *S. suis* and *S. gallolyticus* mainly cause meningitis and endocarditis, respectively [47, 48].

Otherwise, our study, based on both sequencing techniques, revealed that *Lactobacillus* spp. had more representation in mothers from HHS, suggesting that these animals are more protected from recurrent diarrhoea and other gastrointestinal infection. The use of *Lactobacillus* spp as probiotics in swine is proofed to play a role in gut health and immune system [49, 50]. According to literature, fermenting *Lactobacillus* spp. increase acetate and butyrate concentrations in the intestine improving metabolism and nutrient absorption. Furthermore, certain strains improve pig growth performance [51]. Additionally, *Lactobacillus* is known for regulating the immune system reducing inflammatory markers and producing antibacterial substances, such as bacteriocins [49]. In contrast, the opposite trend was observed in piglets, probably because of immature microbial communities, which are likely to be replaced in adult life [52]. Additionally, *Christensenellaceae* R7 group was significantly enriched in animals (mothers and piglets) from HHS, suggesting a healthier gut status, as this genus is also involved in maintaining the structure and function of the host intestinal tract and lipid metabolism [53, 54]. Specifically, species of the family *Christensenellaceae* participate in carbohydrate degradation into short-chain fatty acids (SCFAs), which provide energy to the host and have an anti-inflammatory effect [55]. Our research suggests that this genus may serve as a reliable indicator of a healthy microbiome, preventing episodes of diarrhoea or other enteric diseases. Moreover, we also detected a significant abundance of other beneficial genera in mothers from HHS, including *Treponema*, which has been reported to reduce diarrhoea in pigs [56], and *Acetitomaculum*, which is associated with the conversion of lactate to acetate [57]. Additionally, piglets from HHS farms were enriched with *Oscillospiraceae* UCG-005, one of the most important producers of SCFAs in the gut

microbiome [58], particularly involved in the production of n-butyric acid [59].

Nevertheless, there was a significant presence of the *Campylobacter* genus in piglets from HHS, probably as a result of a premature intestinal microbiome, which balances during the life of the growing piglet [60]. Indeed, *Campylobacter* genus has been the leading cause of human foodborne diarrhoea in EU since 2005 [61], considering pigs as a natural reservoir [62, 63]. Curiously, the results at the species level obtained by Nanopore revealed a notable presence of *C. coli* and *C. jejuni* in mothers from LHS compared to HHS. In accordance with previous statements, our investigation suggests that animals from LHS farms have a greater risk of pathogenicity and incidence of diarrhoea, supported by the presence of both species, as previously observed [64].

All the results obtained highlight the importance of studying microbial diversity, composition and function to understand health status and to make field decisions as promptly as possible. Although Illumina sequencing has been used for a long time, working with this platform normally requires externalisation of the sequencing and high specialisation [65]. Instead, Nanopore technology is portable and provides effective taxonomic resolution in quick real-time sequencing and analysis [66], being able to achieve results comparable with those obtained with Illumina. While Nanopore's portability is a key strength, there are some technical challenges that might arise during on-site use. An example would be a higher error rate due to low accuracy ~ 95% during base calling as a result of nucleic acid identification in current alterations through the nanopore [67] compared to Illumina with an accuracy ~ 99.9% [68]. Several strategies have been developed to enhance these limitations, such as optimization of nanopores and motor proteins [69], performing multiple sequencing or updating the base-calling algorithm [67], all offering an accuracy closer to Illumina sequencing > 99%.

However, there are still some differentiations in bacterial proportions between methods, mainly caused by primer bias. Nevertheless, Nanopore allowed to achieve species taxonomic-level resolution, as reported above [70]. This is remarkable since we could differentiate specific species representing a potential health risk and identify taxa that can be used as potential probiotics to prevent PWD. Hence, the accuracy of Nanopore chemistry is improving, and consequently, taxonomic resolution at higher levels is expected [71].

Conclusions

In conclusion, this study has identified potential microorganisms that may contribute to health and disease in farms with HHS and LHS. Interestingly, our data is consistent with our previous study [12] where we also observed notable differences in environmental microbial communities between the same HHS and LHS farms, which in some cases agree with the results obtained in the intestinal microbiome of the animals. This relationship highlights the importance of considering both, the gut environment and the external environment in the holistic perspective of One Health when studying microbiome dynamics and their impact on health in swine farming.

In addition, our research suggests that Nanopore technology, specifically MinION device, has the potential to be applied in farms as a quick diagnostic tool for specific pathogens, and identifying biomarkers of health status, which could help producers to implement interventions to improve gut health and prevent disease outbreaks. In the future, rapid sequencing technologies may become an approach to help design intervention strategies in real time to prevent episodes of diarrhoea as well as other diseases, supporting better animal welfare and economic viability in farming operations. However, more studies are needed at the field level to standardise on-farm sequencing techniques and applications, as well as to explore how the two platforms could be used synergistically to balance accuracy and practicality.

Abbreviations

ETEC	Enterotoxigenic *E. coli*
EPEC	Enteropathogenic *E. coli*
PWD	Post-weaning diarrhoea
AMR	Antimicrobial resistant
EU	European Union
HHS	High health status
LHS	Low health status
LEFSE	Linear discriminant analysis Effect Size
LDA	Linear discriminant analysis
PERMANOVA	Permutational analysis of variance
PCOA	Principal coordinate analysis
SCFA	Short-chain fatty acids

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12917-025-04693-0.

Supplementary Material 1

Supplementary Material 2

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Author contributions

CT-M: Data curation, Formal analysis, Investigation, Methodology, Software, Visualization, Writing original draft, review & editing. LL-R: Data curation, Formal analysis, Investigation, Methodology, Software, Visualization, Writing review & editing. LM-D: Investigation, Writing review & editing. SV: Investigation, Writing review & editing. JR: Investigation, Writing review &editing. M-PV: Investigation, Writing review & editing. MP-G: Investigation, Writing review & editing. JG-M: Software, Visualization, Writing review & editing. NG-B: Methodology, Writing review & editing. AM-F: Investigation, Writing review & editing. GD: Methodology, Writing review & editing. CM: Conceptualization, Data curation, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing

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Data availability
Sequence data that support the findings of this study have been deposited in the National Center for Biotechnology Information with the primary accession code PRJNA1123624. Access link: https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1123624.

Declarations

Ethical approval
The present study was approved by the Ethics Committee for Animal Experimentation from the Universitat Autònoma de Barcelona and the Animal Experimentation Commission from the local government (Dpt. de Medi Ambient i Habitatge from the Generalitat de Catalunya; Reference 12234). Informed consent was obtained from the producers.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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