Animal Microbiome

RESEARCH Open Access

Faecal microbiome profiling uncovers putative biomarkers for piglets resilient to post-weaning diarrhoea

Mattia Pirolo[1], Moiz Khan Sherwani[1,2], Carmen Espinosa-Gongora[1], Esben Østergaard Eriksen[3], Clara Tassinato[1], Antton Alberdi[2] and Luca Guardabassi[1*]

Abstract

Background Post-weaning diarrhoea (PWD) is a major health and economic concern in intensive pig production. In this study, we hypothesized that the faecal microbiome, sampled before disease onset, could provide early prognostic markers of PWD risk and applied a machine-learning framework to identify biomarkers predictive of piglet susceptibility or resilience to PWD. At two Danish commercial farms experiencing PWD outbreaks, four pens per farm were monitored for 14 days post-weaning, with daily clinical assessments and rectal swabs collected every other day. In a nested case–control design, we profiled 140 samples from 41 piglets that developed PWD and 82 samples from 16 piglets that remained healthy by 16S rRNA sequencing. Additionally, we performed shotgun metagenomics on 56 pre-diarrhoeic samples from susceptible piglets and 47 from resilient piglets. A random-forest classifier with recursive feature elimination identified metagenome-assembled genomes (MAGs) predictive of resilience or susceptibility, trained and cross-validated independently within each farm. Negative binomial zero-inflated mixed (NBZIM) models assessed associations with known PWD risk factors (e.g. birth/weaning weights, weaning age and dam parity).

Results Prior to diarrhoea onset, microbial community structures differed significantly between resilient and susceptible piglets at both farms (PERMANOVA, $p < 0.05$). Feature-reduced models achieved high accuracy (AUC = 0.94 and 0.82 in Farm A and Farm B, respectively) and identified 10 and 13 MAGs enriched in resilient piglets, and one and two MAGs enriched in susceptible piglets from the two farms, respectively. All MAGs were farm-specific, highlighting the multifactorial aetiology of PWD. NBZIM models indicated that most predictive MAGs were independent of established PWD risk factors. Temporally, these MAGs peaked in relative abundance early after weaning (day 4 in Farm A; day 0 in Farm B). In the farm with unclear aetiology, functional analysis showed that susceptibility-associated MAGs were depleted for arginine/ornithine and vitamin (cobalamin, thiamine) biosynthesis and lactate production traits, suggesting metabolic dysbiosis.

Conclusions Our findings indicate that pre-diarrhoeic faecal microbiome signatures predict PWD risk and provide a foundation for early prognostic tools and targeted interventions, including probiotic development, to mitigate PWD and reduce reliance on antimicrobials in pig production.

*Correspondence:
Luca Guardabassi
lg@sund.ku.dk

Full list of author information is available at the end of the article

Keywords Swine, Enteritis, Machine learning, Prognostic markers, Probiotics

Background

Pigs are the food-producing animal species in the EU/EEA with the highest share of antimicrobial sales, accounting for approximately one third (19,423 PCU) of the total sales (60,287 PCU), as measured in population corrected units (PCU) [1]. Most of the antimicrobials used in pig production are used during the weaning period, especially for treatment of post-weaning diarrhoea (PWD), a common enteric condition occurring within the first two weeks after weaning [2, 3]. Piglets, typically weaned at three to four weeks of age, are vulnerable to PWD due to a combination of factors, including the discontinuation of lactation-mediated immunity, a developing intestinal mucosal barrier, an immature microbiota composition, social stress, and change of diet [4]. All these factors predispose piglets to infection by viral and bacterial pathogens, mainly enterotoxigenic *Escherichia coli* (ETEC) [5]. ETEC-associated PWD is a major cause of economic losses in pig production worldwide due to low growth performance, mortality, and treatment costs [6]. The recent restrictions in the use of colistin and zinc oxide in the EU associated with the steady increase of resistance of ETEC to other antimicrobials [3] pose a major challenge in the management of PWD. Therefore, there is an urgent need for new strategies for prevention and control of PWD.

A recent cross-sectional study integrating 16S rRNA gene sequencing with LC/MS-based metabolomics revealed alterations in the microbiota community structure and faecal metabolic profile of piglets affected by PWD compared to healthy controls [7]. Two previous longitudinal studies, employing 16S rRNA gene sequencing or qPCR assay quantification, reported differential abundance of specific bacterial taxa in the faeces of healthy piglets compared to their PWD-affected siblings [8, 9], suggesting that faecal microbiota diversity and composition may serve as potential indicators of susceptibility to PWD. Given that piglets display different susceptibility to PWD despite sharing the same environment, our hypothesis was that the intestinal microbiome may predict whether piglets develop PWD. Here, we applied a machine-learning approach, integrating shotgun metagenomics with Random Forest (RF) classifier and statistical modelling to identify faecal microbiome biomarkers that distinguish PWD-susceptible from PWD-resilient piglets. In contrast to previous studies conducted on experimental facilities [7, 8] or a single commercial farm [9], which had small sample sizes (8–34 piglets) and were limited to 16S rRNA-targeted methods, our study analysed a larger cohort from two commercial farms and employed shotgun sequencing on selected samples collected prior to the onset of PWD. This approach provided greater statistical power, species-level resolution, and more comprehensive insights into the differences between diarrheic and healthy piglets.

Material and methods

Sample and data collection

The study was carried out as a nested case-control study with retrospective risk-based sampling from a full cohort. The cohort has been comprehensively described elsewhere [4]. Briefly, 300 piglets were followed from birth to 14 days after insertion to the nursery units at two Danish intensive indoor farms [4]. Piglets were weaned without use of in-feed medicinal zinc oxide at 21–27 days of age in Farm A and 20–26 days of age in Farm B and distributed in four pens at each producer, allocating approximately 35 piglets per pen. The piglets remained in the same pen ($n = 4$ per farm) and were clinically examined every day for the first 14 days after insertion to the nursery unit. Faeces were classified as non-diarrhoeic or diarrhoeic based on their appearance on a rectal swab [10], and on spontaneous defecation. Diarrhoea cases were treated individually by oral administration of neomycin sulphate (Neomay, ScanVet, Fredensborg, Denmark) after sample collection. Half of the original cohort (150 piglets) were sampled every second day after the insertion to the nursery, up to day 12, using rectal swabs (ESwab, COPAN Diagnostics, California, USA). Swabs were introduced in Amies medium, placed at 4 °C immediately at the farm, and transported to the laboratory twice weekly, where an aliquot was frozen at −80 °C for detection of rotavirus A and microbiome analysis.

For the present study, 57 of the 150 piglets included in the broader sampling scheme were selected for microbiome analysis based on the availability of faecal material (Fig. 1). This subset comprised all piglets that developed PWD during the study period ($n = 41$, classified as PWD-susceptible) and two randomly selected, clinically healthy piglets per pen that remained diarrhoea-free throughout the observation period ($n = 16$, classified as PWD-resilient). Although rectal swabs were scheduled every second day after insertion into the nursery unit, the final dataset contained an uneven number of samples per piglet, because (i) samples from PWD-susceptible piglets were available only until the onset of diarrhoea, and (ii) some scheduled sampling points were unavailable because the original faecal material had not been stored or retained for microbiome analysis in the previous study (Figure S1). In total, 222 faecal samples were obtained (Supplementary Table S1): 82 from resilient piglets, 110 pre-diarrhoeic samples from susceptible piglets, and 30

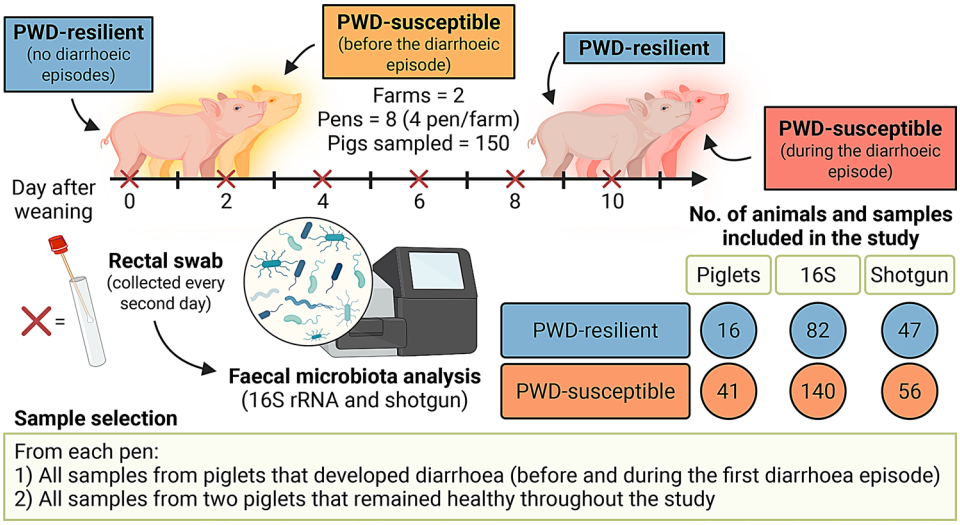

Fig. 1 Schematic overview of the experimental design, sample selection criteria, and the total number of animals and samples included. Only samples from pre-diarrhoeic, post-weaning diarrhea (PWD)-susceptible piglets were selected for shotgun sequencing

diarrhoeic samples collected at the onset of PWD (Figure S1).

DNA extraction and sequencing

High quality DNA was extracted from the final collection of 222 samples using the QIAamp UCP Pathogen Mini kit (QIAGEN, Copenhagen, Denmark), including a mechanical lysis step using Pathogen Lysis Tubes S (QIAGEN, Copenhagen, Denmark). An extraction control was included in each round of DNA extraction ($n = 9$). Libraries were prepared for 16S rRNA amplicon sequencing using the Quick-16S NGS Library Prep Kit (Zymo Research, USA) with Quick-16S Primer Set V3–V4. Amplification was carried out by using the LightCycler 96 System (Roche Life Science). One DNA extraction control was included in each extraction round, and for each of the six sequencing runs we included one PCR negative control and one mock bacterial community (positive control). Sequencing was performed on Illumina MiSeq (2×300 bp paired end reads) using the MiSeq Reagent Kit v3 (600 cycles; Illumina), according to manufacturer's instructions.

To identify bacterial metagenome-assembled genomes (MAGs) associated with resilience or susceptibility to post-weaning diarrhoea (PWD), a subset of samples from 47 PWD-resilient piglets ($n = 20$ from Farm A and $n = 27$ from Farm B) and 56 PWD-susceptible piglets ($n = 19$ from Farm A and $n = 37$ from Farm B) was subjected to shotgun metagenomic sequencing using the NovaSeq PE150 System (Illumina, California, USA), generating approximately 6 GB of raw data per sample. Samples were selected based on DNA quality, ensuring a balanced representation of PWD-resilient and -susceptible samples and pens in each farm.

Sequencing data processing

16S rRNA sequencing data were processed using DADA2 v1.14.1 [11]. Optimal filtering and trimming parameters were identified using FIGARO v3.0 [12]. Taxonomy assignment of amplicon sequence variant (ASV) was performed using the Silva taxonomic database v0.138.1 for DADA2 [13]. Contaminants were removed using decontam v0.1.12.0 on control samples [14]. Sequences not assigned to bacteria were removed. A phyloseq object was constructed from the ASV and taxonomy tables using phyloseq v1.30.0 [15], and ASVs with > 50 unrarefied reads in at least 10 samples were retained for subsequent analysis, a criterion chosen to remove low-prevalence ASVs while preserving the large majority of biologically meaningful signal. Mock bacterial communities were assessed through the complete processing pipeline to confirm expected taxonomic composition, and all profiles were consistent with the reference composition.

Shotgun data were quality controlled using fastp v0.23.1 [16], with the following settings: -trim_poly_g, -trim_poly_x, -n_base_limit 5, -qualified_quality_phred 20, -length_required 35. Following processing of reads, bowtie2 v2.4.4 [17] and samtools v1.12 [18], were used to map reads to the Sus scrofa (Sscrofa11.1) reference genome assembly. Host-removed reads were then taxonomic profiled with MetaPhlAn4 v4.1.1 [19]. MAG reconstruction followed a custom pipeline as previously described [20]. Briefly, host-removed reads were individually assembled using metaSPAdes [21] and binned using MetaWRAP binning module [22]. Bins were dereplicated with dRep [23] into MAGs clusters with > 98% average nucleotide identity (ANI), which was selected for short-reads genomes [23]. MAGs were taxonomically annotated using GTDB-tk [24] and profiled using

CoverM (https://github.com/wwood/CoverM) to generate the final sample count table, with standard parameters. MAG counts were normalized by genome size and filtered by genome covered fraction (≥30%). MAGs were functionally annotated using the DRAM pipeline [25], and functional traits were distilled into Genome-Inferred Functional Traits (GIFTs) using distillR (available at https://github.com/anttonalberdi/distillR), as previously described [26]. GIFTs are scored on a scale of 0 to 1, where 0 indicates the complete absence of genes associated with a given pathway and 1 indicates the presence of all relevant genes. Finally, MAGs were screened for the presence of putative biosynthetic gene clusters (BGCs) using antiSMASH v7.0 [27].

Microbiome profiling and machine-learning prediction of PWD

The α-diversity (Shannon diversity-index) and β-diversity (Bray–Curtis dissimilarity metric) indexes were calculated using R package vegan. The overall difference in the α-diversity was assessed using the Kruskal–Wallis, and differences between groups were calculated using the Wilcoxon Rank Sum with Benjamini–Hochberg correction for multiple testing. The effect of health status on β-diversity was assessed with PERMANOVA ($n = 999$ permutations), with Benjamini–Hochberg correction for multiple testing when pairwise comparison was performed. Significantly enriched ASVs were identified using linear discriminant analysis effect size (LEfSe) analysis [28], where only ASVs with linear discriminant analysis (LDA) score ≥3 and p-value adjusted with the Benjamini–Hochberg correction ≤0.05 were considered significant.

A supervised Random Forest (RF) classifier (RandomForestClassifier, *scikit-learn*) was used independently for each farm to identify MAGs predictive of PWD status. MAGs relative abundances were used as features, and each sample was labelled as PWD-resilient (0) or PWD-susceptible (1). To minimise over-fitting and reduce dimensionality, recursive feature elimination (RFE; KFold from scikit-learn) was applied with three-fold cross-validation at each step [29]. Models were trained and cross-validated independently within each farm to account for farm-specific microbiome variability. The area under the operating curve (AUC) of each model was calculated using roc_auc_score from scikit-learn. At each RFE iteration, model performance (AUC) was averaged across folds to determine the optimal feature set. Feature stability was assessed by examining the recurrence and ranking consistency of selected MAGs across cross-validation splits. In addition, robustness was further evaluated through five independent label-permutation tests, in which class labels were randomly reassigned before refitting the model.

To evaluate the robustness of the predictive MAGs, we assessed whether their association with PWD reflected true disease-related differences rather than correlations with fixed effects, including previously established PWD risk factors [4, 30, 31] or study design covariates. For this purpose, we fitted negative binomial zero-inflated mixed (NBZIM) models [32] to each MAG, using the formula: "y ~ Weaning_weight + Weaning_age + Dam_parity + Sampling_day + Weaning_pen, random = ~1|Pig_no, weights = w", where y denotes the relative abundance of an individual MAG, and w represents a sampling weight defined as the inverse probability of a pig being selected from the underlying full cohort within each health-status group. This weighting approach accounts for the nested case–control sampling design and corrects for the differential sampling probabilities between PWD-resilient and PWD-susceptible piglets in each farm. Random intercepts for pig identity were included to account for repeated measures.

Results

PWD-resilient piglets have a distinct faecal microbiota compared to susceptible ones

After primer removal and quality trimming, 16S rRNA gene sequencing of 222 samples collected from the 57 piglets yielded an average of 1.5×10^5 high-quality reads per sample (range 7.2×10^4 – 4.1×10^5). Following the removal of predicted contaminants and chimeras, 59,358 ASVs were identified, and 1379 ASVs were retained after filtering, with an average read retention rate of 90.4 ± 4.9%.

Members of *Prevotellaceae* (12.8%), *Lachnospiraceae* (11.6%), *Oscillospiraceae* (8.4%), *Erysipelotrichaceae* (7.0%) and *Ruminococcaceae* (6.2%) formed the core faecal microbiome at family level in both farms (i.e., representing ≥5% of the total relative abundance), irrespective on the sampling day and health status (Fig. 2A). β-diversity analysis revealed distinct microbial community structures across farms (PERMANOVA, $p < 0.001$; Figure S2A), despite these showed a similar α-diversity (Shannon index, $p = 0.405$; Figure S2B). Due to these differences in community composition, subsequent analyses were performed separately for each farm.

Health status was associated with the Shannon (α-diversity) index only in Farm B (Kruskal–Wallis test, $p = 0.05$), whereas no significant differences were observed in Farm A ($p = 0.62$). In Farm B, pairwise comparisons showed that the Shannon index was significantly lower in diarrhoeic samples than in both healthy and pre-diarrhoeic samples (Wilcoxon Rank Sum test, $p < 0.05$). β-diversity based on Bray–Curtis dissimilarity revealed that health status was associated with a different community structure in both farms (PERMANOVA, $p < 0.05$; Fig. 2C). In both farms, pairwise comparison between groups

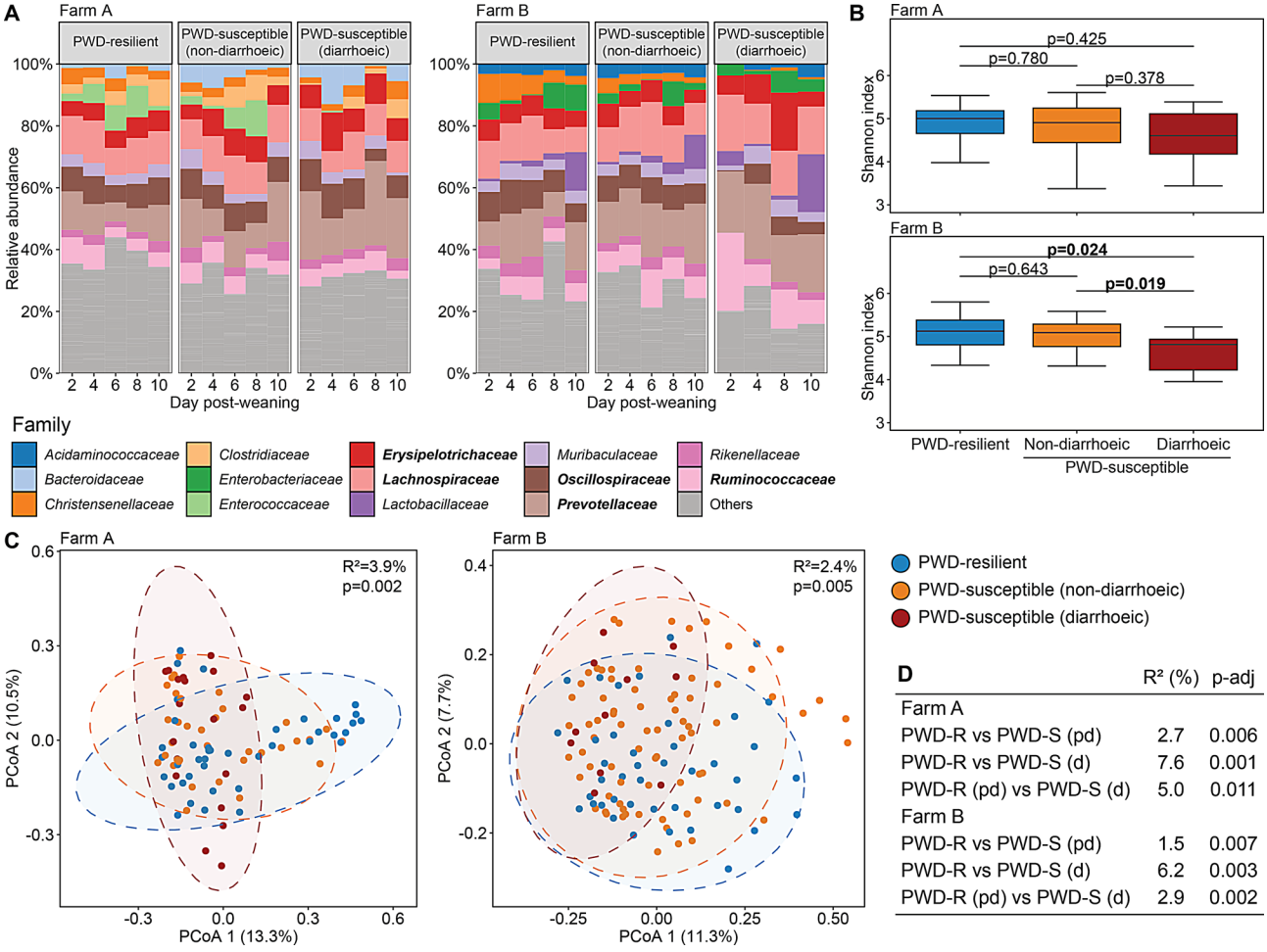

Fig. 2 Longitudinal profiling of the faecal microbiota of PWD-resilient and pre-diarrhoeic and diarrhoeic PWD-susceptible piglets from two commercial farms based on 16S rRNA data. Relative abundance of core families in the faecal microbiota of piglets after weaning (panel A). Bacterial families forming the core faecal microbiome are highlighted in bold. Diarrhoeic piglets showed a significant lower bacterial evenness calculated with the Shannon index (Wilcoxon Rank Sum test, $p = 0.024$; panel B) in Farm B but not in Farm A. Principal coordinate plots based on the Bray-Curtis matrix (panel C) revealed that PWD-resilient piglets host distinct gut microbial communities compared to PWD-susceptible piglets, both before and during the diarrhoea episode (PERMANOVA, $p < 0.05$; illustrated in panel D). Abbreviations: PWD-R, PWD-resilient piglets; PWD-S (d), diarrhoeic PWD-susceptible piglets; PWD-S (pd), pre-diarrhoeic PWD-susceptible piglets

revealed significant differences in community structure between PWD-resilient and susceptible piglets, but also between pre-diarrhoeic and diarrhoeic samples within PWD-susceptible piglets (PERMANOVA, $p < 0.05$; Fig. 2D).

LEfSE analyses comparing the three health groups identified 21 and 18 ASVs significantly enriched (LDA score ≥3, adjusted-$p < 0.05$) in PWD-resilient piglets in Farm A and Farm B, respectively (Supplementary Table S2). These ASVs were predominantly assigned to *Oscillospiraceae* (7 and 5 ASVs in Farm A and B, respectively) and *Lachnospiraceae* (5 and 3 ASVs in Farm A and B, respectively) Pre-diarrhoeic susceptible piglets exhibited 10 discriminant ASVs in each farm, primarily belonging to *Bacteroidaceae*, *Clostridiaceae*, *Erysipelotrichaceae* and *Peptostreptococcaceae* (2 ASVs each) in Farm A, and to *Christensenellaceae* and *Acidaminococcaceae* (2 ASVs each) in Farm B. Diarrhoeic samples showed enrichment for 24 ASVs in Farm A and 12 ASVs in Farm B, dominated by *Prevotellaceae* (9 and 10 ASVs in Farms A and B, respectively) and, in Farm A, *Bacteroidaceae* (6 ASVs).

MAGs identified by machine learning differentiate PWD-resilient piglets from susceptible ones

After quality filtering and host decontamination, 4.7×10^9 reads were obtained from shotgun sequencing, with an average of 4.6×10^7 high-quality non-host reads per sample (range $1.4–7.1 \times 10^7$). Read-based taxonomic profiling was consistent with 16S rRNA results (Figure S3A), except for a higher proportion of reads assigned to *Enterobacteriaceae* in the shotgun data (mean 28.3%) compared with 16S rRNA sequencing (mean 3.8%),

which detected this bacterial family only in Farm B. While no significant differences in bacterial richness or evenness were observed (Figure S3B), community structure analysis confirmed a significant shift in the microbiota structure between PWD-resilient and susceptible piglets (PERMANOVA, $p < 0.05$; Figure S3C).

From assembly of non-host reads, 1156 dereplicated MAGs were obtained, of which 580 (50.2%) showed high completeness (> 90%) and low contamination (< 5%). Profiling of MAGs identified 1056 and 1119 MAGs in Farms A and B, respectively. To determine the MAGs most predictive of pre-disease status, a RF classifier was employed, using a threefold cross-validation strategy with iterative training and testing splits. The classifier achieved an AUC of 0.72 and 0.62 for Farm A and B, respectively (Fig. 3A). Model validation with five iterations of classifying randomly permuted labels resulted in lower performance (AUC 0.44–0.55 for Farm A and 0.32–0.55 for Farm B; Figure S4), supporting that the observed predictive accuracy was not driven by chance. Model performance improved with a reduced set of MAGs selected through cross-validation, identifying 11 predictive MAGs in Farm A (AUC = 0.94) and 15 MAGs in Farm B (AUC = 0.82; Fig. 3A). Notably, none of the predictive MAGs were shared between farms. β-diversity analysis based on these predictive MAGs revealed a significant separation between PWD-resilient and susceptible piglets in Farm A (PERMANOVA, $p = 0.001$) and partial separation in Farm B (PERMANOVA, $p = 0.001$; Fig. 3B).

From the predictive MAGs identified through RF analysis, 10 and 13 were enriched in PWD-resilient piglets from Farms A and B, respectively (Figure S5 and Supplementary Table S3). Conversely, one and two MAGs were enriched in PWD-susceptible piglets from Farms A and B, respectively (Figure S5 and Supplementary Table S3).

Draft genomes of the 26 MAGs selected by the RF classifier were mined for GIFTs and BGCs (Fig. 3C). A comprehensive overview of the identified GIFTs and BGCs is provided in Figure S6 and Figure S7, respectively. In Farm B, GIFT analysis revealed distinct functional profiles between MAGs associated with PWD resilience and susceptibility, whereas no such separation was observed in Farm A (Fig. 3C). Specifically, MAGs from Farm B enriched in PWD-susceptible piglets were depleted for specific traits related to amino acid biosynthesis (i.e., arginine and ornithine), organic anion production (i.e., L-lactate and D-lactate), and vitamin biosynthesis (i.e., cobalamin and thiamine). Additionally, MAGs enriched in PWD-resilient piglets displayed a higher abundance of BGCs compared to those enriched in PWD-susceptible ones, regardless of the farm of origin (Fig. 3Cand Figure S7).

Fixed effect influence on resilience- and susceptibility-associated MAGs differs between farms

NBZIM models were used to assess whether the predictive ability of the 26 MAGs was due to association to known PWD risk factors or study design covariates. In Farm A, six of the 10 MAGs associated with PWD-resilient piglets, as well as the single MAG linked to PWD-susceptible animals (*Parabacteroides goldsteinii*, MAG1114), were not associated with the tested fixed effects (Figure S8 and Supplementary Table S3). The six resilience-associated MAGs comprised four members of the *Oscillospiraceae* family, including three classified as *Faecousia* spp. (MAG0019, MAG0622, MAG1020), and three members of *Lachnospiraceae*, including two classified as *Oliverpabstia* (MAG0001, MAG0882) and one as *Blautia* spp. (MAG1019). The four resilience-associated MAGs showing associations to fixed effects were MAG0052 (*Faecousia* spp.; sampling time and weaning pen), two *Sodaliphilus* spp. (MAG0288 and MAG0743; weaning age) and *Limivicinus* spp. (MAG108; sampling time).

In Farm B, only four of the 15 selected MAGs had no association with fixed effects (Figure S8 and Supplementary Table S3), namely *Enterococcus faecalis* (MAG0439) and an unclassified *Lachnospiraceae* (MAG0677) in resilient piglets, and *Angelakisella* spp. (*Ruminococcaceae*, MAG0373) together with an unclassified *Christensenellales* (MAG0626) in susceptible piglets. Out of the eleven MAGs showing associations with fixed effects, most were affected by sampling time (10/15), following by weaning weight (3/15), weaning age (2/15), weaning pen (1/15), and dam parity (1/15).

Temporal analysis showed that MAGs unaffected by fixed effects ($n = 11$) exhibited distinct abundance trajectories during the early post-weaning period (Fig. 4). In Farm A, resilience-associated MAGs, including *Faecousia* spp., *Oliverpabstia* spp. and *Blautia* spp., generally displayed an early peak around day 4, with consistently higher relative abundances in PWD-resilient piglets than in susceptible ones. In contrast, the susceptibility-associated MAG1114 in Farm A (*P. goldsteinii*) increased sharply in susceptible piglets on day 4 and declined thereafter, remaining nearly absent in resilient animals. In Farm B, *E. faecalis* (MAG0439) and the unclassified *Lachnospiraceae* MAG0677 showed stable or increasing abundances in resilient piglets, whereas susceptibility-associated MAGs (*Angelakisella* spp., MAG0373, and *Christensenellales* MAG0626) displayed early spikes at day 0–2 followed by a marked decline.

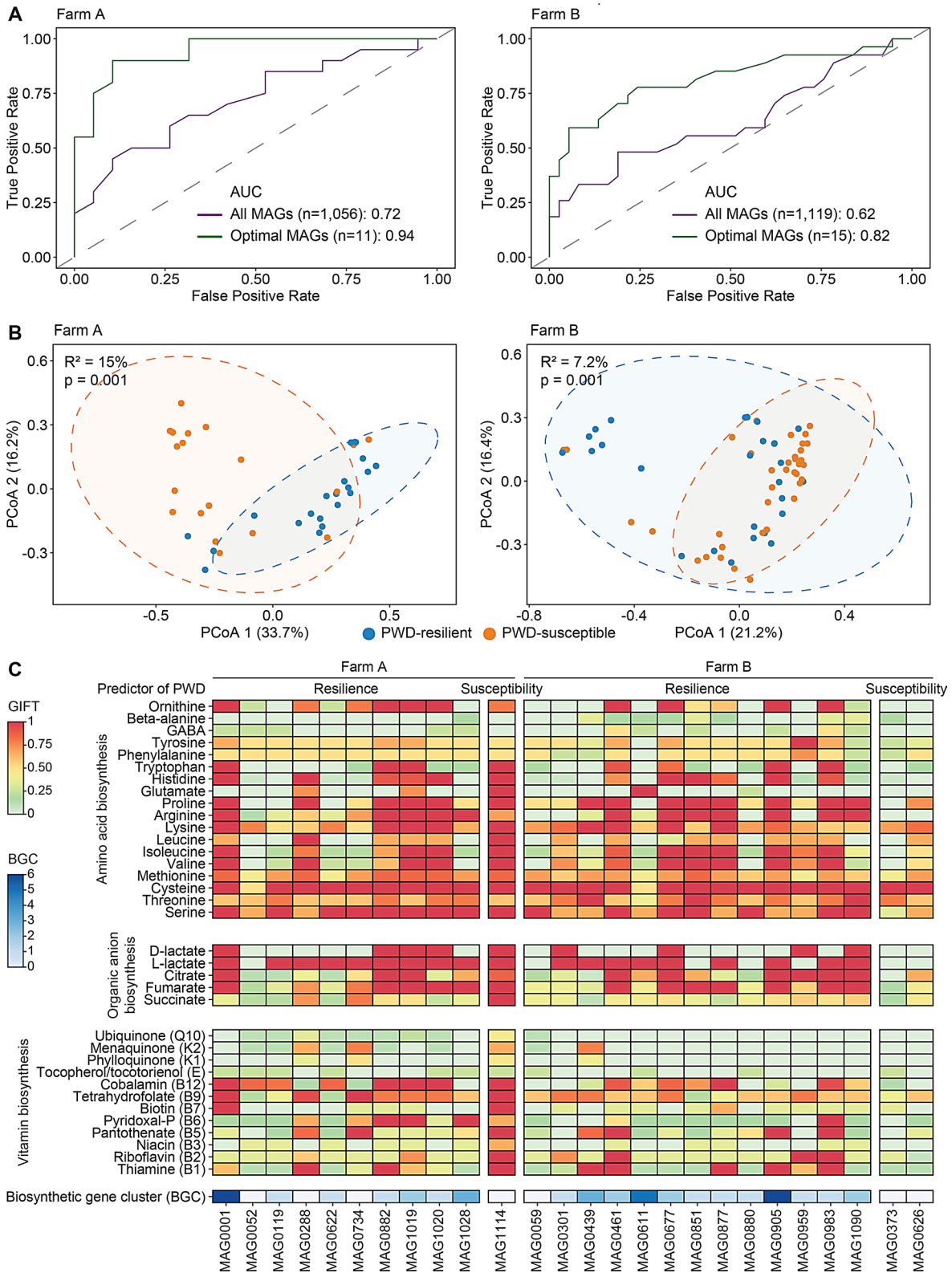

Fig. 3 Metagenome-assembled genomes (MAGs) to predict the health status of post-weaned piglets in two commercial farms. The performance of the random forest algorithm (panel A) was higher when the number of MAGs were reduced by the three-fold cross-validation analysis. The community composition based on Bray-Curtis index (panel B) of the predictive MAGs allowed to discriminate piglets based on their health status (PERMANOVA, $p < 0.05$). Weighed capacities of genome-inferred functional traits (GIFT) and number of biosynthetic gene clusters (BGCs) in MAGs associated with PWD-resilient or PWD-susceptible piglets (panel C)

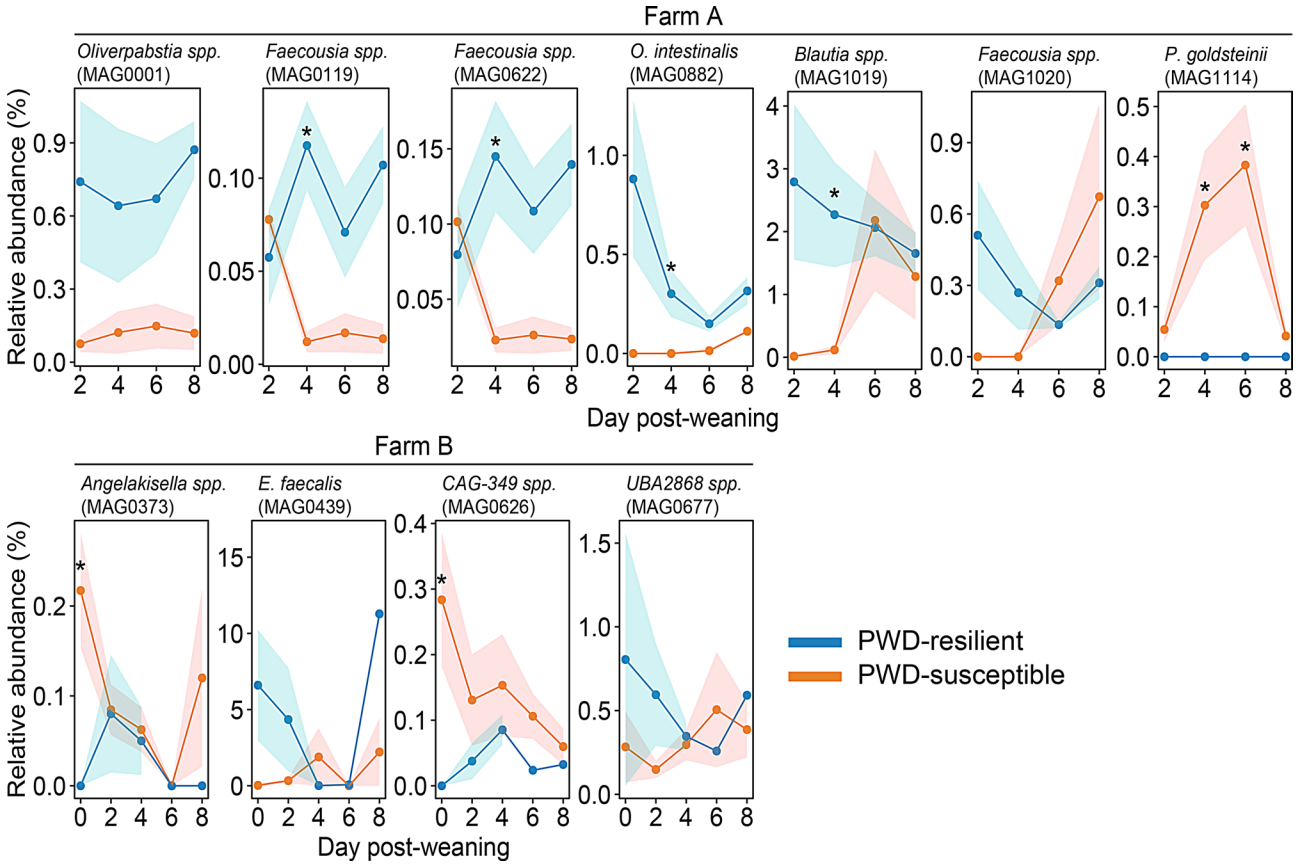

Fig. 4 Temporal and potential probiotic analysis of predictive MAGs. Line plots showing the relative abundances (%) of the MAGs over the sampling days. The asterisk indicates significantly higher abundances ($p < 0.05$) calculated with the Wilcoxon Rank Sum test

Discussion

Using a machine-learning approach that combined shotgun metagenomics data with a RF classifier, we identified specific MAGs that differed significantly in abundance between piglets that develop PWD within the two weeks after weaning and those that remain healthy, even before diarrhoea onset. This early distinction suggests that the relative abundance of these MAGs in faecal samples can serve as prognostic markers of PWD risk. Identifying such markers prior or immediately after insertion into the weaning unit would allow high-risk piglets to be pinpointed before clinical symptoms appear, thereby enabling timely interventions to prevent disease spread. Temporal analysis further revealed that resilience- and susceptibility-associated MAGs peaked very early after weaning (Fig. 4), highlighting a narrow intervention window for risk-based screening and targeted management to separate high- from low-risk piglets.

Despite these promising results, the MAGs associated with PWD resilience and susceptibility were not consistent across farms, complicating their universal application as biomarker candidates. This variability suggests that local farm-specific factors, such as management practices, feed composition, and baseline microbiota differences, also play a significant role in influencing PWD susceptibility. To facilitate the identification of universal biomarkers that predict PWD across diverse farms, future work should adopt multi-farm and multi-cohort strategies. A complementary approach is the use of large-scale meta-analyses integrating publicly available metagenomic datasets with newly generated farm-level data, thereby increasing statistical power and capturing broader environmental and management variability.

Notably, the two farms differed in diarrhoea aetiology: in Farm A, PWD cases were linked to rotavirus A during the first week and ETEC in the second week, whereas in Farm B, rotavirus A was also detected during the first week, but most cases lacked a known aetiological agent despite the presence of haemolytic non-toxigenic *E. coli* strains [4]. Given these differences, the next step is to determine whether the observed variability in biomarkers correlates with the underlying cause of PWD. This will require a larger-scale, multi-farm study where piglets are screened at nursery unit entry using rapid, cost-effective methods (e.g., PCR) targeting all biomarkers identified in this study, alongside individual clinical diagnosis to differentiate between ETEC and non-ETEC cases.

Interestingly, NBZIM models indicated that most MAGs associated with PWD resilience (six in Farm A and two in Farm B) or susceptibility (one in Farm A and two in Farm B) were unrelated with established risk factors [4, 30, 31] or study design covariates (Figure S8–S9 and Supplementary Table S3). In other words, while some MAGs were associated to fixed effects, especially sampling time, the microbiome signatures identified as independent by the NBZIM models appear to provide predictive information beyond conventional risk factors. This independence underscores the potential utility of these MAGs as early, robust biomarkers for identifying piglets at risk of PWD, as they are unlikely to be the result of an association to risk factors of the disease.

Several studies have shown that ETEC can be present in clinically healthy pigs and is not consistently associated with PWD [4, 33, 34]. In PWD outbreaks with an unclear aetiology, such as on Farm B, resilience or susceptibility may instead be linked to functional and metabolic variations within the microbiota. Supporting this, our functional analysis on full set of predictive MAGs identified by the RF model in Farm B, regardless of their association with fixed effects in NBZIM models, revealed a significant depletion of specific genetic traits in MAGs associated with PWD susceptibility, including pathways involved in amino acid biosynthesis (arginine and ornithine), lactate production, and vitamin biosynthesis (cobalamin and thiamine), which may contribute to a metabolically mediated dysbiosis. Arginine and ornithine are critical for maintaining intestinal barrier integrity, promoting mucosal repair, and supporting immune function during the stressful weaning period [35, 36]. Under stressful conditions such as weaning, the endogenous synthesis of arginine may not be sufficient to meet physiological demands, and recent evidence suggests an optimal dietary arginine level (1.5% to 1.9% standardized ileal digestible arginine) during the first three weeks post-weaning significantly improves overall pig growth performance [37]. Given the depletion of microbial genes involved in arginine biosynthesis observed in the MAGs associated with PWD susceptibility in Farm B, the intestinal microbiota's reduced ability to provide adequate arginine could exacerbate the physiological stress during weaning, further compromising intestinal integrity and increasing susceptibility to diarrheal diseases. Likewise, reduced microbial lactate production may impair short-chain fatty acid (SCFA) synthesis, negatively affecting epithelial barrier function and increasing susceptibility to pathogen colonization [38]. Moreover, cobalamin and thiamine deficiencies could exacerbate dysbiosis through altered microbial competition, potentially impacting nutrient absorption and microbial community dynamics [39, 40]. Reduced functional redundancy in these pathways may further limit the microbiota's capacity to buffer perturbations during weaning, thereby increasing susceptibility to dysbiosis [41]. Collectively, these findings suggest that in the absence of ETEC, rotavirus and other known pathogens, PWD may arise from a dysbiotic microbiota that fails to maintain gut homeostasis under the stresses of weaning.

The taxa predictive of a reduced risk of PWD included members of *Lachnospiraceae* (in both farms) and *Oscillospiraceae* (mostly in Farm A), which have been associated to healthy piglets before [8, 42]. These MAGs encoded for different BGCs, which are critical for microbe–microbe and microbe–host interactions and have been implicated in shaping the pig gut microbiome composition [43]. In addition, members of these families are known to produce SCFAs [44]. SCFAs play critical roles in gut health by serving as an energy source for intestinal epithelial cells, promoting their proliferation and differentiation, and exerting systemic metabolic effects [45]. Notably, members of *Oscillospiraceae* have recently gained attention for their ability to regulate chronic inflammation and are considered potential "next-generation probiotics" [46]. Here, we identified three MAGs from this family, which were classified as *Faecousia* spp. Additionally, three health-associated MAGs in Farm A and one in Farm B belong to the *Lachnospiraceae* family, which have been associated with young piglets that do not develop PWD [47] and exhibit high feed efficiency [48]. In Farm B, one MAG was assigned to *Enterococcus faecalis*, a species previously tested as a feed supplement in weaned piglets, though with inconsistent effects on diarrhoea prevention [49, 50], largely attributed to strain-specific properties. These findings warrant further investigation to determine whether these MAGs represent a novel, pig-specific resource of probiotic candidates. Probiotics represent a promising non-antimicrobial strategy for mitigating PWD due to their potential to enhance immunity and resistance to pathogens in pigs [51, 52]. However, most current research focuses on probiotics derived from human studies, such as lactobacilli, *Saccharomyces cerevisiae*, and *Bacillus* spp. Notably, MAGs predictive of PWD-resilient pigs in our study belong to species (e.g., *Oliverpabstia intestinalis*) and genera (*Faecousia* spp. and *Blautia* spp.) that have been successfully isolated from pigs or humans, with established cultivation protocols [53, 54]. While these bacteria exhibit promising probiotic features, the association between their abundance and health outcomes does not establish a causative relationship. Further research is required to determine whether they can be isolated, stabilized, and developed into effective probiotic formulations for PWD prevention. In addition, regulatory approval for next-generation probiotics requires comprehensive safety and efficacy data, which are currently lacking for these taxa.

One MAG associated with PWD susceptibility was identified in Farm A (*P. goldsteinii* MAG1114), and two in Farm B (*Angelakisella* spp. MAG0373 and unclassified *Christensenellales* MAG0626). The enrichment of these taxa in piglets prior to the development of PWD in one of the farms does not imply a causative relationship with the disease. Indeed, very little is known about the prevalence and impact of these bacteria on pig gut health. A single study in weaned pigs linked inoculation of *P. goldsteinii* to anti-inflammatory effects and enrichment of beneficial bacteria, such as *Lactobacillales* and *Butyricimonas* [54]. Based on studies in mice, *P. goldsteinii* and other members of the *Parabacteroides* genus are known to exhibit dual effects, meaning they can have both beneficial and detrimental impacts depending on the host context and specific strains involved [55].

Comparison of 16S rRNA and shotgun metagenomics data revealed consistent patterns in the faecal microbiota composition of PWD-resilient and -susceptible piglets, with both approaches showing significant differences in community structure between groups. Taxonomic profiles at the family level were broadly consistent, although shotgun data revealed a higher proportion of *Enterobacteriaceae*, possibly due to a low resolution of this family with 16S V3–V4 region used in this study [56], a limitation that has been reported for other 16S variable regions [57].

Regarding the machine-learning methodology used, RF classifiers are well-established in human microbiome research for their robustness to high-dimensional and sparse data. In veterinary contexts, they have previously been used to differentiate bacteriophage signatures between healthy and diarrhoeic neonatal piglets [58] and to identify microbiome markers of *Mycobacterium avium* subsp. *paratuberculosis* infection in cattle [59]. Our study extends these applications by combining RF with RFE at the MAG level, offering higher taxonomic resolution than previous genus-level approaches [29]. The models achieved high predictive accuracy (AUC = 0.94 in Farm A and 0.82 in Farm B), with robustness confirmed by permutation testing. Importantly, we also applied NBZIM models to test the association of the selected MAGs with potential study design covariates and risk factors for PWD [4, 30, 31]. This two-step framework not only improved predictive performance but also ensured epidemiological relevance, highlighting the potential of this integrative approach to leverage microbiome data for diagnostic, prognostic, and therapeutic applications.

Some limitations should be considered when interpreting our findings. First, although the sample size for shotgun metagenomics ($n = 103$) exceeds those of previous longitudinal PWD studies using 16S rRNA gene sequencing, it is restricted to only two farms and remains insufficient to achieve optimal statistical power for machine-learning analyses, which require large datasets to robustly detect complex microbial patterns and associations. As a result, some associations may have been missed or overestimated. Second, the modest cohort size constrains the ability to perform comprehensive subgroup analyses, such as stratification by specific PWD aetiology or sampling day. Because piglets contributed multiple samples across the post-weaning period, the dataset also includes repeated measures, which introduces some degree of non-independence. Although the key biomarker analysis focused on pre-diarrhoeic samples to reduce within-animal clustering, repeated sampling may still influence diversity and taxonomic comparisons. Third, the number of MAGs associated with PWD susceptibility was small (one in Farm A and two in Farm B), which limits the breadth of functional inference and warrants cautious interpretation of the functional differences observed. Finally, the differences observed between the two farms further highlight the impact of farm-specific factors, such as diet and management practices, on microbiota dynamics. Given the increasing availability of public metagenomic repositories and associated metadata, it is highly desirable that our findings be validated and expanded using larger, more diverse datasets to improve their robustness and generalizability.

Conclusions

Our study demonstrates that the faecal microbiome of piglets differs significantly before the onset of PWD, offering valuable insights for early prediction of disease risk. While the farm-specific nature of the identified resilience and susceptibility biomarkers highlights the complexity of PWD and limits their immediate generalisability, it also underscores the influence of local factors and disease aetiology. Nonetheless, our integrative machine-learning approach provides a promising framework for advancing microbiome-based diagnostics and prognostics, particularly as larger and more diverse datasets become available. Finally, the identification of novel probiotic candidates supports future strategies aimed at improving animal health and reducing reliance on antimicrobials in pig production.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s42523-026-00522-3.

Supplementary material 1

Supplementary material 2

Supplementary material 3

Supplementary material 4

Supplementary material 5

Supplementary material 6

Supplementary material 7

Supplementary material 8

Supplementary material 9

Supplementary material 10

Acknowledgements
We thank Raphael Eisenhofer (University of Copenhagen) and Håkan Vigre (Technical University of Denmark) for their valuable assistance and discussions on metagenomic data analysis and NBZIM modelling, respectively. We also acknowledge the authors of the Eriksen et al. (2021) for establishing the cohort and collecting data and samples used in this study.

Author contributions
LG, MP, CEG and EØE designed the study. MP and CT did laboratory work. MP, MKS and AA analysed the data. MP wrote the first draft of the manuscript and prepared figures. All authors reviewed and approved the final version of the manuscript.

Funding
Open access funding provided by Copenhagen University. This study received financial support from the European Union´s Horizon 2020 Innovation Action project "Alternatives to Veterinary Antimicrobials" (AVANT, Grant Agreement No. 862829) and from the Joint Programming Initiative on Antimicrobial Resistance (JPIAMR) project "Farm Interventions to Control Antimicrobial Resistance" (FARM-CARE, JPIAMR2021-169).

Data availability
16S rRNA and shotgun sequencing data have been deposited in the NCBI Sequence Data Archive (SRA) under BioProject PRJNA1290942.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
EØE has previously worked in projects financed by the Danish pig production industry and the authorities regulating the industry. The rest of the authors declare no competing interests.

Author details
[1]Department of Veterinary and Animal Sciences, University of Copenhagen, Stigbøjlen 4, Frederiksberg C 1870, Denmark
[2]Center for Evolutionary Hologenomics, GLOBE Institute, University of Copenhagen, Copenhagen, Denmark
[3]Department of Production Animal Clinical Sciences, Norwegian University of Life Sciences, Ås, Norway

Received: 2 September 2025 / Accepted: 16 January 2026
Published online: 24 February 2026

References

1. European Medicines Agency. European Surveillance of Veterinary Antimicrobial Consumption, 2022. Sales of veterinary antimicrobial agents in 31 European countries in 2022 (EMA/299538/2023).
2. Guardabassi L, Apley M, Olsen JE, Toutain P-L, Weese S. Optimization of antimicrobial treatment to minimize resistance selection. Microbiol Spectr. 2018;6:6.3.09.
3. DANMAP. Use of antimicrobial agents and occurrence of antimicrobial resistance in bacteria from food animals, food and humans in Denmark. 2023. ISSN 1600-2032.
4. Eriksen EØ, Kudirkiene E, Christensen AE, Agerlin MV, Weber NR, Nødtvedt A, et al. Post-weaning diarrhea in pigs weaned without medicinal zinc: risk factors, pathogen dynamics, and association to growth rate. Porc Health Manag. 2021;7.
5. Nagy B, Fekete PZ. Enterotoxigenic *Escherichia coli* in veterinary medicine. Int J Med Microbiol. 2005;295:443–54.
6. Nadeau É, Fairbrother JM, Zentek J, Bélanger L, Tremblay D, Tremblay C-L, et al. Efficacy of a single oral dose of a live bivalent *E. coli* vaccine against post-weaning diarrhea due to F4 and F18-positive enterotoxigenic *E. coli*. Vet J. 2017;226:32–39.
7. Zheng X, Nie K, Xu Y, Zhang H, Xie F, Xu L, et al. Fecal microbial structure and metabolic profile in post-weaning diarrheic piglets. Genes. 2023;14:1166.
8. Dou S, Gadonna-Widehem P, Rome V, Hamoudi D, Rhazi L, Lakhal L, et al. Characterisation of early-life fecal microbiota in susceptible and healthy pigs to post-weaning diarrhoea. PLoS ONE. 2017;12:e0169851.
9. Karasova D, Crhanova M, Babak V, Jerabek M, Brzobohaty L, Matesova Z, et al. Development of piglet gut microbiota at the time of weaning influences development of postweaning diarrhea - a field study. Res Vet Sci. 2021;135:59–65.
10. Eriksen EØ, Sejersen MF, Pedersen KS. The cotton swab method: an accurate and less invasive way to assess fecal consistency in weaned pigs. BMC Vet Res. 2024;20:47.
11. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP. DADA2: high resolution sample inference from Illumina amplicon data. Nat Methods. 2016;13:581–83.
12. Sasada R, Weinstein M, Prem A, Jin M, Bhasin J. FIGARO: an efficient and objective tool for optimizing microbiome rRNA gene trimming parameters. J Biomol Tech. 2020;31:S2.
13. Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, et al. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Res. 2013;41:D590–6.
14. Davis NM, Proctor DM, Holmes SP, Relman DA, Callahan BJ. Simple statistical identification and removal of contaminant sequences in marker-gene and metagenomics data. Microbiome. 2018;6:226.
15. McMurdie PJ, Holmes S. Phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. PLoS ONE. 2013;8:e61217.
16. Chen S, Zhou Y, Chen Y, Gu J. Fastp: an ultra-fast all-in-one FASTQ preprocessor. Bioinformatics. 2018;34:i884–90.
17. Langmead B, Salzberg SL. Fast gapped-read alignment with bowtie 2. Nat Methods. 2012;9:357–59.
18. Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, et al. The Sequence Alignment/Map format and SAMtools. Bioinformatics. 2009;25:2078–79.
19. Blanco-Míguez A, Beghini F, Cumbo F, McIver LJ, Thompson KN, Zolfo M, et al. Extending and improving metagenomic taxonomic profiling with uncharacterized species using MetaPhlAn 4. Nat Biotechnol. 2023;41:1633–44.
20. Pirolo M, Espinosa-Gongora C, Alberdi A, Eisenhofer R, Soverini M, Eriksen EØ, et al. Bacterial topography of the upper and lower respiratory tract in pigs. Anim Microbiome. 2023;5:5.
21. Nurk S, Meleshko D, Korobeynikov A, Pevzner PA. metaSpades: a new versatile metagenomic assembler. Genome Res. 2017;27:824–34.
22. Uritskiy GV, DiRuggiero J, Taylor J. MetaWRAP-a flexible pipeline for genome-resolved metagenomic data analysis. Microbiome. 2018;6:158.
23. Olm MR, Brown CT, Brooks B, Banfield JF. dRep: a tool for fast and accurate genomic comparisons that enables improved genome recovery from metagenomes through de-replication. Isme J. 2017;11:2864–68.
24. Chaumeil P-A, Mussig AJ, Hugenholtz P, Parks DH. GTDB-Tk: a toolkit to classify genomes with the genome taxonomy database. Bioinformatics. 2019;btz848.
25. Shaffer M, Borton MA, McGivern BB, Zayed AA, La Rosa SL, Solden LM, et al. DRAM for distilling microbial metabolism to automate the curation of microbiome function. Nucleic Acids Res. 2020;48:8883–900.
26. Koziol A, Odriozola I, Leonard A, Eisenhofer R, San José C, Aizpurua O, et al. Mammals show distinct functional gut microbiome dynamics to identical series of environmental stressors. mBio. 2023;e01606–23.
27. Blin K, Shaw S, Augustijn HE, Reitz ZL, Biermann F, Alanjary M, et al. antiSMASH 7.0: new and improved predictions for detection, regulation, chemical structures and visualisation. Nucleic Acids Res. 2023;51:W46–50.
28. Segata N, Izard J, Waldron L, Gevers D, Miropolsky L, Garrett WS, et al. Metagenomic biomarker discovery and explanation. Genome Biol. 2011;12:R60.
29. Ma T, Villot C, Renaud D, Skidmore A, Chevaux E, Steele M, et al. Linking perturbations to temporal changes in diversity, stability, and compositions of neonatal calf gut microbiota: prediction of diarrhea. Isme J. 2020;14:2223–35.

30. Svensmark B, Nielsen K, Willeberg P, Jorsal SE. Epidemiological studies of piglet diarrhoea in intensively managed Danish sow herds. Acta Vet Scand. 1989;30:55–62.
31. Skirrow S, Buddle J, Mercy A, Madec F, Nicholls R. Epidemiological studies of pig diseases: 2. Post-weaning diarrhoea and performance in Western Australian pigs. Australian Vet J. 1997;75:282–88.
32. Zhang X, Yi N. NBZIMM: negative binomial and zero-inflated mixed models, with application to microbiome/metagenomics data analysis. BMC Bioinf. 2020;21:488.
33. Blirup-Plum SA, Jensen HE, Nielsen SS, Pankoke K, Hansen MS, Pedersen KS, et al. Gastro-intestinal lesions are not relatable to diarrhoea or specific pathogens in post-weaning diarrhoea (PWD) in pigs. Acta Vet Scand. 2023;65:30.
34. Garcias B, Migura-Garcia L, Giler N, Martín M, Darwich L. Differences in enteric pathogens and intestinal microbiota between diarrheic weaned piglets and healthy penmates. Vet Microbiol Microbiol. 2024;295:110162.
35. Tan B, Xiao H, Xiong X, Wang J, Li G, Yin Y, et al. L-Arginine improves DNA synthesis in LPS-challenged enterocytes. FBL. 2015;20:989–1003.
36. Li Y, Shi P, Yao K, Lin Q, Wang M, Hou Z, et al. Diarrhea induced by insufficient fat absorption in weaned piglets: causes and nutrition regulation. Anim nutr. 2024;16:299–305.
37. Perez-Palencia JY, Ramirez-Camba CD, Haydon K, Urschel KL, Levesque CL. Effects of increasing dietary arginine supply during the three first weeks after weaning on pig growth performance, plasma amino acid concentrations, and health status. Transl Anim Sci. 2024;8:txae047.
38. Iraporda C, Errea A, Romanin DE, Cayet D, Pereyra E, Pignataro O, et al. Lactate and short chain fatty acids produced by microbial fermentation downregulate proinflammatory responses in intestinal epithelial cells and myeloid cells. Immunobiology. 2015;220:1161–69.
39. Degnan PH, Taga ME, Goodman AL. Vitamin B12 as a modulator of gut microbial ecology. Cell Metab. 2014;20:769–78.
40. Bek S, Teo YN, Tan X-H, Fan KHR, Siah KTH. Association between irritable bowel syndrome and micronutrients: a systematic review. Gastroenterol Hepatol. 2022;37:1485–97.
41. Moya A, Ferrer M. Functional redundancy-induced stability of gut microbiota subjected to disturbance. Trends Microbiol. 2016;24:402–13.
42. Yang Q, Huang X, Wang P, Yan Z, Sun W, Zhao S, et al. Longitudinal development of the gut microbiota in healthy and diarrheic piglets induced by age-related dietary changes. Microbiologyopen. 2019;8:e923.
43. Wylensek D, Hitch TCA, Riedel T, Afrizal A, Kumar N, Wortmann E, et al. A collection of bacterial isolates from the pig intestine reveals functional and taxonomic diversity. Nat Commun. 2020;11:6389.
44. Umu ÖCO, Frank JA, Fangel JU, Oostindjer M, da Silva CS, Bolhuis EJ, et al. Resistant starch diet induces change in the swine microbiome and a predominance of beneficial bacterial populations. Microbiome. 2015;3:16.
45. Canfora EE, Jocken JW, Blaak EE. Short-chain fatty acids in control of body weight and insulin sensitivity. Nat Rev Endocrinol. 2015;11:577–91.
46. Yang J, Li Y, Wen Z, Liu W, Meng L, Huang H. *Oscillospira* - a candidate for the next-generation probiotics. Gut Microbes. 2021;13:1987783.
47. Pluske JR, Turpin DL, Kim J-C. Gastrointestinal tract (gut) health in the young pig. Anim nutr. 2018;4:187–96.
48. Jiang H, Fang S, Yang H, Chen C. Identification of the relationship between the gut microbiome and feed efficiency in a commercial pig cohort. J Anim Sci. 2021;99:skab045.
49. Bednorz C, Guenther S, Oelgeschläger K, Kinnemann B, Pieper R, Hartmann S, et al. Feeding the probiotic *Enterococcus faecium* strain NCIMB 10415 to piglets specifically reduces the number of *Escherichia coli* pathotypes that adhere to the gut mucosa. Appl Environ Microbiol. 2013;79:7896–904.
50. Hu Y, Dun Y, Li S, Zhang D, Peng N, Zhao S, et al. Dietary *Enterococcus faecalis* LAB31 improves growth performance, reduces diarrhea, and increases fecal *lactobacillus* number of weaned piglets. PLoS ONE. 2015;10:e0116635.
51. Barba-Vidal E, Martín-Orúe SM, Castillejos L. Review: Are we using probiotics correctly in post-weaning piglets? Animal. 2018;12:2489–98.
52. Su W, Gong T, Jiang Z, Lu Z, Wang Y. The role of probiotics in alleviating postweaning diarrhea in piglets from the perspective of intestinal barriers. frontiers in cellular and infection microbiology. 2022;12.
53. Hitch TCA, Masson JM, Pauvert C, Bosch J, Nüchtern S, Treichel NS, et al. HiBC: a publicly available collection of bacterial strains isolated from the human gut. Nat Commun. 2025;16:4203.
54. Deng X, Guo T, He Y, Gao S, Su J, Pan H, et al. *Parabacteroides goldsteinii* alleviates intestinal inflammation in dextran sulfate sodium-treated pigs. Animals. 2025;15:1231.
55. Liu J, Qiu H, Zhao J, Shao N, Chen C, He Z, et al. *Parabacteroides* as a promising target for disease intervention: current stage and pending issues. NPJ Biofilms Microbiomes. 2025;11:137.
56. Jovel J, Patterson J, Wang W, Hotte N, O'Keefe S, Mitchel T, et al. Characterization of the gut microbiome using 16S or shotgun metagenomics. Front Microbiol. 2016;7.
57. Chakravorty S, Helb D, Burday M, Connell N, Alland D. A detailed analysis of 16S ribosomal RNA gene segments for the diagnosis of pathogenic bacteria. J Microbiol Methods. 2007;69:330–39.
58. Wang Z, Li J, Ma L, Liu X, Wei H, Xiao Y, et al. Metagenomic sequencing identified specific bacteriophage signature discriminating between healthy and diarrheal neonatal piglets. Nutrients. 2023;15:1616.
59. Lee S-M, Park H-T, Park S, Lee JH, Kim D, Yoo HS, et al. A machine learning approach reveals a microbiota signature for infection with *Mycobacterium avium* subsp. Paratuberculosis Cattle. Microbiol Spectr. 2023;11:e03134–22.

Publisher's Note