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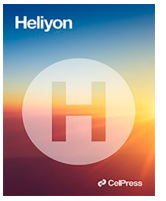

Research article

Associative analysis of sludge microbiota and wastewater degradation efficacy within swine farm sludge systems

Cheng-Han Cai [a,1], Chee Kin Then [b,1], Yan-Ling Lin [a], Cheng-Chun Shih [a], Chih-Chieh Li [a,1], Tzu-Sen Yang [c,d,e,f,g,*,1]

[a] *Industry Technology Research Institute, Nantou City, Taiwan*
[b] *Department of Radiation Oncology, Shuang Ho Hospital, Taipei Medical University, New Taipei City, Taiwan*
[c] *Graduate Institute of Biomedical Optomechatronics, Taipei Medical University, Taipei, 110, Taiwan*
[d] *International Ph.D. Program in Biomedical Engineering, Taipei Medical University, Taipei, 110, Taiwan*
[e] *School of Dental Technology, Taipei Medical University, Taipei, 110, Taiwan*
[f] *Research Center of Biomedical Device, Taipei Medical University, Taipei, 110, Taiwan*
[g] *TMU Research Center of Cancer Translational Medicine, Taipei Medical University, Taipei, 110, Taiwan*

A R T I C L E I N F O

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A B S T R A C T

Industrial wastewater management is a significant global challenge. Sludge microbiota from swine farms may play a crucial role in enhancing wastewater treatment processes, thereby reducing water pollution from industrial activities. A deeper understanding of this complex community could lead to innovative approaches for improving wastewater treatment methods. Sludge samples were collected from the anaerobic, sedimentation, and thickening tanks of ten swine farms. The microbiota communities were analyzed using 16S rRNA full-length sequencing on the PacBio platform, with subsequent data analysis conducted on the QIIME2 platform utilizing the SILVA database. Compared to anaerobic and thickening tanks, the sedimentation tanks exhibited a unique profile of sludge microbiota, with higher abundances of the phyla Proteobacteria, Bacteroidota, and Caldatribacteriota. Additionally, sludges from farms already utilized in processing industrial water—specifically farms B, G, and J—contained higher concentrations of bacteria (>20 ng/μL), indicating the robustness of the bacterial load for practical industrial use. Furthermore, sludge from farms with higher alpha diversity, such as E, G, I, and J, exhibited enriched degradation profiles, including the degradation of aromatic compounds, polymers, industrial compounds, toluene, and vanillin. The farms were categorized based on wastewater ammonia nitrogen degradation levels, revealing a clustering effect of the microbiota from the sedimentation tanks in the Principal Coordinates Analysis (PCoA) plot. A higher relative abundance of the families *Rhodocyclaceae*, *AKYH767*, and *Comamonadaceae*, and a lower abundance of the families *Anaerolineaceae* and *Christensenellaceae*, were found in groups with high ammonia nitrogen reduction, suggesting potential targets for bioaugmentation strategies. In conclusion, this study underscores the critical role of microbial abundance, composition, and biodiversity in optimizing wastewater treatment and advocates for comprehensive microbiota analysis to identify suitable sludge for industrial applications.

* Corresponding author. Institute of Biomedical Optomechatronics, Taipei Medical University No.301, Yuantong Rd., Zhonghe Dist., New Taipei City 235, Taiwan.
E-mail address: tsyang@tmu.edu.tw (T.-S. Yang).
[1] These authors have contributed equally to this work.

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1. Introduction

The management of industrial wastewater represents a significant global challenge, with far-reaching implications for environmental sustainability and public health [1,2]. The United Nations, in its World Water Development Report, highlights that over 80 % of wastewater globally is discharged into the environment without adequate treatment [3]. In developing countries, only 8 % of industrial and municipal wastewater undergoes treatment of any kind, exacerbating environmental and health issues [4]. Focused research efforts are now being directed towards understanding the complex processes involved in wastewater treatment [5].

Ammonia nitrogen decomposition is a critical step in neutralizing one of the most harmful pollutants in wastewater, making it central to industrial wastewater treatment [6]. Ammonia nitrogen is a major contributor to aquatic toxicity and eutrophication, making its effective removal a priority in wastewater treatment [7]. Ammonia nitrogen wastewater can be treated using physical, chemical, and biological methods. Physical treatment processes alter the physical state or properties of the wastewater to facilitate the separation of ammonia (NH_3-N), utilizing techniques such as air stripping, adsorption, and membrane filtration. Chemical methods rely on reactions that transform ammonia nitrogen into less harmful or more easily removable forms [8]. Although physical and chemical methods are straightforward, stable, and efficient, biological treatments provide additional economic and environmental benefits [8]. Biological methods involve the metabolism of microorganisms to convert ammonia nitrogen (NH_3-N) into nitrate (NO_3^-), nitrite (NO_2^-), or nitrogen gas (N_2) through processes such as nitrification, denitrification, and anaerobic ammonium oxidation [9].

Managing wastewater is crucial on swine farms to minimize environmental impact [10]. The swine wastewater treatment process typically involves several stages, including solid-liquid separation, followed by treatment in anaerobic tanks, aeration tanks, and finally, sedimentation ponds [11,12]. The initial step of solid-liquid separation plays a key role in reducing the wastewater's organic load, setting the stage for subsequent biological treatments. These treatments leverage anaerobic and aerobic processes to not only lessen the sludge volume but also transform polluted substances into safer compounds.

Sludge seeding is employed to accelerate the startup of biological wastewater treatment systems or to restore efficiency when these systems have degraded or completely failed [13,14]. Swine sludge, a nutrient-rich byproduct, is typically used as fertilizer. To enhance the resource utilization of swine sludge, some studies have explored the potential of this biological treatment enhancement technology in industrial wastewater treatment. Jheng et al. demonstrated that seeding with sludge from swine farms improved the COD (Chemical Oxygen Demand) removal efficiency of petrochemical wastewater treatment systems [15]. The study further indicated that the specific types of microbes present in swine sludge can significantly influence the composition of the microbial community within the wastewater treatment system.

The study aims to achieve three objectives: (1) to investigate the composition and diversity of sludge microbiota on swine farms, (2) to identify characteristics or factors of the microbiota associated with improved functional outcomes in wastewater treatment, and (3) to provide an overview of the functional capabilities of these microbial communities. The findings of this study are intended to contribute to the optimization of wastewater treatment processes, promoting more efficient and sustainable practices within the industry.

2. Materials and methods

2.1. Sludge sample collection and characteristic testing

Samples were collected using a sludge core sampler from ten swine farms, focusing on three types of tanks in each farm's wastewater treatment system: anaerobic tank, sedimentation tank, and thickening tank. Three samples were taken from each tank to conduct biological repeatability tests. The samples were transported to the Human Microbiome Lab at Taipei Medical University under controlled conditions of 4 °C within 24 h. The total solids (TS) and volatile solids (VS) were determined according to the methodology outlined by the National Environmental Research Academy in Taiwan [16].

Table 1
Influent volume and the hydraulic retention time of wastewater treatment process.

Farm	Influent volume (CMD)	Hydraulic retention time of anaerobic tanks (hours)	Hydraulic retention time of aerobic tanks (hours)	Hydraulic retention time of sediment tanks (hours)
A	707	1328.5	289	22.9
B	223.9	404.7	110.8	57.2
C	335.3	348.7	22.63	11.6
D	85	634.2	67.7	20.1
E	352	1139.9	142.9	17.9
F	419.6	303.3	103.4	29.9
G	80	421.6	330.5	33.8
H	348.5	1961.7	49.8	7.9
I	251.5	480	20	18
J	60	1140.6	146.6	18.7

2.2. Water quality testing

The wastewater treatment processes of each farm, including hydraulic retention times of anaerobic tanks, aerobic tanks, and sediment tanks, along with the influent volume of each farm, are listed in Table 1. Biochemical Oxygen Demand (BOD), Suspended Solids (SS), Chemical Oxygen Demand (COD), and ammonia nitrogen in wastewater samples collected from the wastewater treatment plants of each farm were tested. All methods followed the environmental analysis standard methods established by the National Environmental Research Academy in Taiwan [16–19].

2.3. Sludge and water quality reports

Fig. 1 illustrates the wastewater treatment process in swine farms, highlighting the different tanks involved: the anaerobic, aeration, and sedimentation tanks. Initially, the wastewater undergoes solid-liquid separation. Subsequently, it is treated in a series of tanks: anaerobic, aerobic, and sedimentation, resulting in the production of effluent. A thickening tank is utilized to concentrate the sludge by reducing its water content, preparing it for further industrial applications. It also presents the sludge quality in these tanks, specifically Total Solids (TS) and Volatile Solids (VS) as detailed in Table 2. The anaerobic tank exhibited an average TS of 5.15 % w/w and VS of 2.59 % w/w. In comparison, the sedimentation tank had average values of 5.36 % w/w TS and 2.47 % w/w VS. The thickening pond recorded the highest averages, with 6.27 % w/w TS and 3.48 % w/w VS.

Regarding the water quality analysis, BOD, SS, and COD were evaluated in the wastewater after treatment in the solid-liquid separation, anaerobic, and sedimentation tanks (Table 3). A notable reduction of more than 90 % in all these parameters was observed across all farms, with the exception of the SS levels in farm H. However, missing values were noted for farm A. Additionally, the levels of ammonia nitrogen were assessed before and after treatment in the aeration and sedimentation tanks, revealing a decrease ranging from 7.8 % to 87 %.

2.4. Microbial DNA extraction and preservation

Microbiota samples from the sludge were collected from the anaerobic, sedimentation, and thickening tanks. Upon arrival, the sludge samples were immediately processed for microbial DNA extraction using the QIAamp DNA Microbiome Kit (QIAGEN 51704) according to the manufacturer's instructions. The DNA concentration was measured using a NanoDrop spectrophotometer (Thermo Scientific, San Jose, CA, USA). All DNA samples were stored at −80 °C until they were sent for quality assessment, library preparation, and sequencing at the Genomics commercial genome sequencing company.

2.5. Quality assessment result

Due to the possible presence of impurities in the sludge that could inhibit PCR, only 21 samples initially passed the quality check and showed clear bands in PCR (see Supplementary Table 1). After adjusting the PCR conditions from 25 to 30 cycles, another 44 samples also successfully produced bands. An additional 25 samples showed bands in PCR after a DNA purification step. Thus, an optimized process for extracting microbial DNA from swine farm sludge was established, which involves adjustments to PCR conditions and DNA purification (DNA clean-up) to overcome the impact of excessive sludge impurities on subsequent PCR, library construction, and sequencing processes.

2.6. Generation of amplicon

The full-length 16S rRNA genes were amplified using specific primers that included barcodes. The PCR reactions were conducted in 25 μL volumes, utilizing 2X KAPA HiFi HotStart ReadyMix PCR Reagent (Kapa Biosystems, Woburn, MA, USA), 2.5 μM of both forward

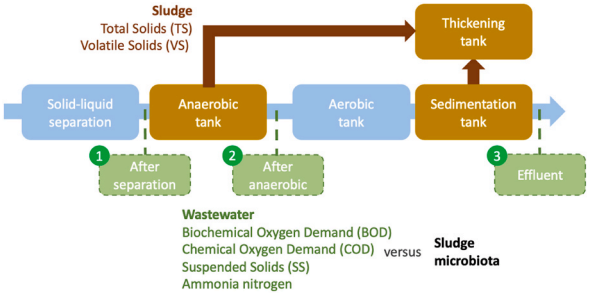

Fig. 1. The wastewater treatment process in swine farms. Wastewater first undergoes solid-liquid separation, then passes through anaerobic, aerobic, and sedimentation tanks to produce effluent. Sludge is concentrated in a thickening tank. The total solids (TS) and volatile solids (VS) levels of the sludge (indicated in blue), along with biochemical oxygen demand (BOD), chemical organic demand (COD), suspended solids (SS), and ammonia nitrogen concentrations in the wastewater (indicated in brown) were monitored.

Table 2
Sludge quality report on TS and VS from anaerobic, sedimentation, and thickening tanks across ten swine farms.

Farm	Anaerobic tank		Sedimentation tank		Thickening pond	
	TS (% w/w)	VS (% w/w)	TS (% w/w)	VS (% w/w)	TS (% w/w)	VS (% w/w)
A	6.12	2.94	4.01	1.70	9.18	4.59
B	3.75	2.41	1.93	1.12	3.90	2.48
C	4.30	2.98	16.14	6.13	7.68	5.31
D	9.67	3.86	1.41	0.77	7.18	4.29
E	8.80	4.26	5.77	2.59	7.60	5.86
F	4.27	2.25	5.20	2.42	7.98	3.75
G	6.63	2.85	4.71	2.70	4.68	2.46
H	2.33	1.30	2.93	1.69	0.58	0.34
I	1.22	0.60	9.57	4.44	11.56	4.37
J	4.45	2.44	1.90	1.09	2.35	1.35
Average	5.15	2.59	5.36	2.47	6.27	3.48

Table 3
Water quality report on BOD, SS, COD, and ammonia nitrogen levels in wastewater after treatment in solid-liquid separation, anaerobic, and sedimentation tanks across ten swine farms.

		After solid-liquid separation			After anaerobic tank				After sedimentation tank			
		BOD(mg/L)	SS(mg/L)	COD(mg/L)	BOD(mg/L)	SS(mg/L)	COD(mg/L)	Ammonia nitrogen(mg/L)	BOD(mg/L)	SS(mg/L)	COD(mg/L)	Ammonia nitrogen(mg/L)
A	Sep 12						1232				243	
B	Aug 22	1,500	2,300	3,760	45.1	51.7	338	302	31.7	126	259	264
C	Sep 22	9,060	12,600	18,800	190	292	914	516	87.4	136	585	476
D	Jul 6	4,310	4,550	8,690	152	219	793	822	55.4	90.3	403	727
E	Sep 18	7,060	6,050	12,700	84.2	241	498	535	65.5	15.3	227	448
F	Sep 18	18,300	67,200	55,100	112	452	896	681	81.4	215	543	623
G	Sep 14	6,390	8,950	12,900	74.1	175	536	482	30.5	99	252	217
H	Sep 14	2,100	655	3,260	104	165	508	406	36.8	13.2	246	52.8
I	Sep 12	3,570	6,800	9,750	31.4	134	258	214	44.3	149	249	181
J	Sep 21	820	1,030	1,390	20.4	35.8	111	87.8	16.4	18.5	83.3	21
Average		5,901	12,237	14,039	90	196	539	450	50	96	316	334

		After solid-liquid separation			After anaerobic tank				After sedimentation tank			
		BOD(%)	SS(%)	COD(%)	BOD(%)	SS(%)	COD(%)	Ammonia nitrogen(%)	BOD(%)	SS(%)	COD(%)	Ammonia nitrogen(%)
A	Sep 12										-80.3	
B	Aug 22				-97.0	-97.8	-91.0		-29.7	143.7	-23.4	-12.6
C	Sep 22				-97.9	-97.7	-95.1		-54.0	-53.4	-36.0	-7.8
D	Jul 6				-96.5	-95.2	-90.9		-63.6	-58.8	-49.2	-11.6
E	Sep 18				-98.8	-96.0	-96.1		-22.2	-93.7	-54.4	-16.3
F	Sep 18				-99.4	-99.3	-98.4		-27.3	-52.4	-39.4	-8.5
G	Sep 14				-98.8	-98.0	-95.8		-58.8	-43.4	-53.0	-55.0
H	Sep 14				-95.0	-74.8	-84.4		-64.6	-92.0	-51.6	-87.0
I	Sep 12				-99.1	-98.0	-97.4		41.1	11.2	-3.5	-15.4
J	Sep 21				-97.5	-96.5	-92.0		-19.6	-48.3	-25.0	-76.1
Average					-97.8	-94.8	-93.5		-33.2	-31.9	-37.3	-32.2

The top section presents the raw data, while the bottom shows the percentage reduction compared to the previous tanks.

and reverse primers, and approximately 10 ng of DNA template. The thermal cycling conditions for amplification were as follows: an initial denaturation at 95 °C for 3 min; followed by 25 cycles of denaturation at 95 °C for 30 s, annealing at 57 °C for 30 s, and elongation at 72 °C for 1 min; and a final extension at 72 °C for 1 min.

The sequences of the primers targeting the full length of the 16S rRNA gene are as follows [20]:

27F: AGRGTTYGATYMTGGCTCAG.

1492R: RGYTACCTTGTTACGACTT.

2.7. Quality control and PCR optimization before sequencing

For quality assessment, the concentrations of the microbial DNA samples were determined using a Qubit 2.0 Fluorometer (Thermo Scientific, Waltham, MA, USA), and their purity was assessed by measuring the A260/280 ratios using a NanoDrop spectrophotometer. The physical characteristics of the DNA samples, including color, sediment volume, and viscosity, were evaluated. PCR reactions were conducted under standard conditions; if unsuccessful, conditions were optimized on an individual basis. Qualitative analysis of the PCR products was performed using agarose gel electrophoresis on 0.8 % agarose gels to verify the correct product size, with the intensity of the product peak being stronger than the 1500bp marker signal. The qualified PCR products were mixed in equal amounts and purified via AMPure PB beads (PacBio, Menlo Park, CA, USA).

2.8. Library preparation and bacterial 16S rRNA sequencing

A total of 600 ng of the pooled 16S amplicons was utilized as templates to construct the SMRTbell libraries using the SMRTbell Prep Kit 3.0 (PacBio, Menlo Park, CA, USA), following the manufacturer's protocol. The library preparation included steps for repairing damaged DNA and end-repairing before ligating the DNA inserts to the adapters. Subsequent sequencing was conducted on the SMRT Sequel IIe system, using an 8M Cell and version 2 chemistry (PacBio, Menlo Park, CA, USA). Initial data filtering was performed on the Sequel IIe system, and comprehensive secondary analysis was executed using version 11.0.0 of the SMRT Link analysis pipeline.

2.9. Microbiota analysis by using QIIME2

After receiving the raw sequencing data, the analysis was conducted using the QIIME2 platform, LEfSe, and various R packages. All 16S rRNA gene-based metagenomic analyses were performed on the QIIME2 platform [21]. The quality-filtered sequences were clustered with greater than 97 % identity into groups known as Operational Taxonomic Units (OTUs) via open-reference OTU picking. The relative abundance of each OTU across all samples was then determined. In taxonomic analysis, the microbiota were classified at multiple hierarchical levels—phylum, class, order, family, genus, and species—referencing the SILVA 138 database. The relative abundance of each taxon was calculated as its percent composition relative to the total number of organisms.

The analysis pipeline is outlined as follows.

i. The primers were removed from the sequences.
ii. Sequencing errors were de-noised using the "DADA2" plugin within QIIME2.
iii. Taxonomic assignment was performed with the SILVA 138 database using the "feature-classifier" command in QIIME2.
iv. The LEfSe method was utilized to identify features that characterize differences between groups. Validation was done via the Kruskal-Wallis test with an alpha of 0.05, and effect sizes were estimated by LDA based on significantly different vectors from the comparison of abundances.
v. A phylogenetic tree was generated using the "phylogeny" plugin in QIIME2.
vi. Alpha and beta diversity were investigated using QIIME2's diversity commands "alpha-group-significance" and "beta-group-significance" to obtain metrics such as observed features, Shannon's and Simpson's index, and Bray-Curtis dissimilarity. PERMANOVA was conducted to test within-group sample similarity, and PCoA plots were generated with the Emperor Tool.
vii. The OTU table was rarefied using the "alpha-rarefaction" command in QIIME2, illustrating sample richness with increasing sequence counts.
viii. Functional metagenome profiles were predicted with PICRUSt2 using KEGG, Tax4Fun, and MetaCyc databases by collapsing the predicted functions (KEGG Orthology; KO) into broader categories (KEGG pathways) post-OTU picking and normalization. Furthermore, an ADONIS test was employed to statistically assess significant differences in microbial community composition among various dietary groups. Additionally, the correlations between environmental factors (such as region, rainfall in the past three days, temperature, humidity, etc.) and microbial flora were investigated.

Furthermore, an ADONIS test was employed to statistically assess significant differences in microbial community composition among various dietary groups. Additionally, the correlations between environmental factors (such as region, rainfall in the past three days, temperature, humidity, etc.) and microbial flora were investigated.

2.10. Statistics

All results are presented as means ± standard deviation, and a P-value of less than 0.05 is considered to indicate statistical significance. All statistical analyses were performed using GraphPad Prism version 10.0 (La Jolla, CA). To compare data from more than two groups, a one-way ANOVA followed by Dunnett's multiple comparison test was utilized. For comparing two groups, a two-tailed *t*-test was employed. The LEfSe (LDA Effect Size) method was applied to identify significant differences in taxa, using the Kruskal-Wallis test for initial comparisons. Taxa that were found to be significantly different in the Kruskal-Wallis test were then subjected to Linear Discriminant Analysis (LDA), which generated an LDA score to quantify the effect size of each differentially abundant feature.

3. Results

3.1. Baseline environmental factors

Among these ten swine farms in Taiwan, four are located in Changhua, while two each are situated in Yunlin, Chiayi, and Tainan (Supplementary Table 2). A clustering effect of the sludge microbiota based on their regional area was observed in the PCoA plot (Supplementary Fig. 1). On the day of collection, the weather was sunny at all locations, though four farms had experienced rain in the three days preceding the collection. Notably, only farms F and J operate as finishing farm and sow farms, while the remaining farms are farrow-to-finish operations. The environmental conditions were consistent across these sites, with temperatures ranging between 28.9 °C and 32.9 °C, and humidity levels varying from 52 % to 92 %. Samples from all farms were collected and dispatched between September 12th and 21st.

3.2. Sludge DNA concentration and microbial community from anaerobic, sedimentation, and thickening tanks, pooled from ten swine farms

Significant differences in bacterial DNA amounts were observed among various treatment system areas after extracting DNA from similar quantities of sludge samples, as shown in Fig. 2A. The DNA levels varied in descending order: anaerobic, sedimentation, and thickening tanks. Furthermore, no significant difference in alpha diversity was observed in the three tanks, depicted in Fig. 2B. The PCoA plot, utilizing Bray-Curtis dissimilarity, revealed a distinct clustering of all sludge microbiota from the sedimentation tank (Fig. 2C). Fig. 2D shows the microbiota composition in each tank, with the top ten family taxa, ranked by relative abundance, being *Anaerolineaceae, Christensenellaceae, JS-1, Hungateiclostrideaceae, Rikenellaceae, Candidatus Pacebacteria, Smithellaceae, GWA2-38-13b, Clostridiaceae*, and *Anaerovoracaceae*. Additionally, the bacterial taxa at lower taxonomic levels predominantly enriched in the sedimentation tank included the Gammaproteobacteria class, Burkholderiales order, and Bacteroidia class, as illustrated in Fig. 2E. Supplementary Figs. 2 and 3 show the composition profile at the species level and the profile of each swine farm at the family level, respectively.

3.3. Sludge DNA concentration and microbial community by swine farms

Fig. 3A and Supplementary Fig. 4 displays the sludge DNA concentration for individual farms, identifying farms B, D, G, and J as the four farms with the highest levels, where concentrations remained within 20 ng/μL as determined by NanoDrop and Qubit measurements. Except for farm J, the sludge from the other three farms are utilized in several industrial wastewater system (Table 4) and sludge seeding do enhance the removal efficiency of these wastewater treatment systems. This suggests that bacterial abundance is crucial for its suitability for industrial applications. To accurately depict the microbiota situation in swine farms, Fig. 3B and Supplementary Fig. 5 presents the top twenty bacteria at the family level found in the sludge from these farms. The top ten bacteria taxa include *Anaerolineaceae, Christensenellaceae, JS-1, Hungateiclostrideaceae, Rikenellaceae, Candidatus Pacebacteria, Smithellaceae, GWA2-38-13b, Clostridiaceae*, and *Anaerovoracaceae*. These bacteria account for approximately forty to eighty percent of the total bacterial population, highlighting significant variation in the bacterial community composition across swine farms. Notably, *Anaerolineaceae* and *Christensenellaceae* are the most prevalent, constituting fifteen to fifty percent of the bacterial population in the sludge of every farm.

Supplementary Fig. 6 illustrates the top ten bacterial taxa at the species level. There are up to 1900 bacterial species present in the sludge of swine farms, with five common species varying across different farms. These include the uncultured *Longilinea* from the family *Anaerolineaceae*, the R-7 group from the family *Christensenellaceae*, an uncultured bacteria from the *Anaerolineaceae* family, an uncultured organism from the JS1 group, and an uncultured bacterium from the family *Anaerolineaceae*. Furthermore, more than half of the bacteria in each sample constitute less than one percent of the total bacterial population, underscoring the rich biodiversity in swine farm sludge. This diversity demonstrates the complex and varied bacterial communities that support sludge metabolic functions.

3.4. Biodiversity of microbial communities in farm sludge

In Fig. 3C and Supplementary Fig. 7, the α-diversity of microbes in the sludge is assessed using three indicators: species richness (i.e., the number of taxonomic groups observed, referred to as observed features) and the evenness of group abundance distribution, as measured by the Shannon and Simpson indices. These indicators reveal a consistent trend: the farms with the highest alpha diversity are farms E, G, I, and J. Previous studies have suggested that higher species diversity is associated with enhanced industrial applications. Therefore, the correlation between α-diversity and industrial utility is explored to identify factors that could improve microbial diversity and evenness. Fig. 3D presents the results of the β-diversity analysis, conducted using Bray-Curtis dissimilarity. These analyses consistently demonstrate significant clustering among samples from the ten individual farms, indicating distinct microbial compositions for each farm. Integrating ninety samples from anaerobic, sedimentation and thickening tanks, the Adonis test revealed that the individual farm was the most significant factor influencing the composition of sludge microbiota ($R^2 = 0.1637$), with a clustering effect observed in the PCoA plot.

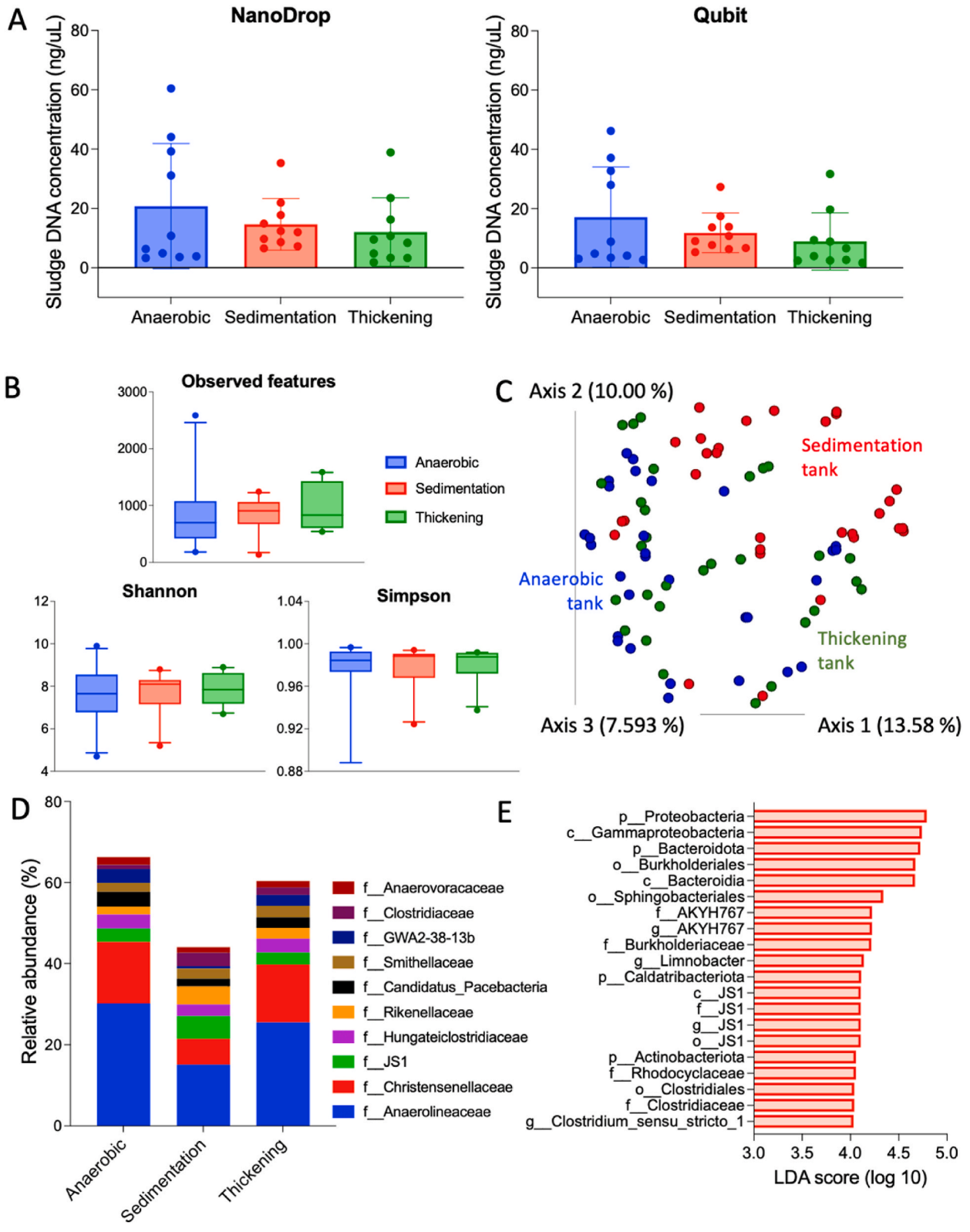

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Fig. 2. Concentration of sludge microbial DNA and microbial analysis from anaerobic, sedimentation, and thickening tanks, pooled from ten swine farms. (A) The bacterial load was estimated by the DNA concentration in sludge extracted from a similar amount of sludge, as determined by NanoDrop and Qubit. (B and C) Diversity analysis of the sludge microbiota by tank types, namely anaerobic, sedimentation, and thickening tanks. (D) Taxonomic composition profiles at the family level. (E) LEfSe analysis investigated the microbiota enriched in specific tanks.

3.5. Functional prediction of degradation and decomposition profiles microbial communities in swine farm sludge

To assess the potential of sludge in industrial wastewater treatment, functional predictions were performed using 16S rRNA sequencing results. Three databases were employed: MetaCyc (Fig. 4), KEGG (Kyoto Encyclopedia of Genes and Genomes, Supplementary Fig. 8) Pathway, and Tax4Fun (Supplementary Fig. 9), to deduce the biochemical pathways and metabolic networks of sludge microbiota in swine farms, with a focus on their degradation or decomposition capabilities. Each database offers unique features for functional prediction from 16S rRNA sequencing data. MetaCyc contributes a curated compilation of experimentally validated pathways, enhancing the functional interpretation of 16S rRNA gene sequencing data. KEGG provides extensive gene and pathway annotations for predicting metabolic functions from gene sequences. Tax4Fun, tailored for 16S rRNA data, matches sequence information to the functional profiles of KEGG, facilitating functional predictions without the need for whole-genome data.

In Fig. 4 and Supplementary Figs. 8 and 9, the profile encompasses degradation pathways for amino acid derivatives, aromatic compounds, carbohydrates, polysaccharides, nucleotides, nucleosides, other organic compounds, polymers, industrial compounds, steroids, nitrogenous compounds, toluene, and vanillin. Farm E maintained enriched profiles in most pathways, while farm G was notable for carbohydrate and polysaccharide degradation, other organic compound degradation, polymer and industrial compound degradation, and steroid and nitrogenous compound degradation. Farm I excelled in steroid and nitrogenous compound degradation, as well as several organic compound degradations, and farm J was prominent in amino acid derivative degradation, aromatic compound degradation, carbohydrate and polysaccharide degradation, and steroid and nitrogenous compound degradation. Regarding nitrogenous compound degradation, farms E, G, I, and J were all competent, with the ranking being farms $E > G = J > I$.

The left column of Supplementary Fig. 10 lists pathways, including the degradation of synthetic and natural organic compounds, halogenated compounds, biological macromolecules, metabolic processes, and biosynthesis and biodegradation processes. It depicts the various microbial metabolic pathways and their relative abundances across different groups. The analysis indicated that farms E, G, I, and J exhibit the most extensive degradation profiles within the KEGG pathways, while farms D, F, and H show the least. Farm E displayed broader degradation profiles than farm G, but farm G was more adept at degrading glycosaminoglycans, other glycans, and RNA. A notable correlation between these profiles and higher alpha diversity, as discussed previously, highlights the importance of biodiversity in degradation applications. Farm G stands out as a prime candidate for industrial wastewater treatment, and these findings suggest that farm E may also be a viable option.

Supplementary Fig. 11 illustrates the metabolic pathways from the Tax4Fun database and their relative abundance across groups. These pathways encompass the degradation of a variety of substances including pesticides (like atrazine), plasticizers (such as those related to bisphenol degradation), industrial chemicals, volatile organic compounds (VOCs), solvents (including toluene and xylene), halogenated compounds (such as chloroalkanes and chloroalkenes), aromatic compounds, natural organic compounds (e.g., geraniol, limonene, and pinene), steroids, drugs, and xenobiotics (processed by cytochrome P450 enzymes), as well as various metabolic processes (like the synthesis and degradation of ketone bodies and the degradation of amino acids such as valine, leucine, and isoleucine). The trend of higher degradation profiles in farms E, G, I, and J persisted, with farm E leading in nearly all categories, except for nitrogen metabolism in farm J and nitrotoluene degradation in farm H.

3.6. Functional prediction by correlation with the real-world data: sludge microbiota analysis of the sedimentation tanks of swine farms with high and low ammonia nitrogen decomposition

To accumulate higher-level evidence of sludge microbiota in wastewater processing, the association between sludge microbiota and ammonia nitrogen degradation was investigated. The high ammonia nitrogen degradation group (>50 % reduction) included farms G, H, and J, while the low group (<20 % ammonia nitrogen reduction) comprised farms B, C, D, E, F, and I (Fig. 5A). Environmental factors, such as temperature, negatively correlated with humidity ($R^2 = 0.788$, $p = 0.0014$; Fig. 5B). All three farms with high ammonia nitrogen reduction exhibited higher temperatures and lower humidity. There was no difference in alpha diversity between the high and low ammonia nitrogen reduction groups (Fig. 5C); however, the PCoA plot of Bray-Curtis dissimilarity demonstrates a clustering effect within the high or low ammonia nitrogen decomposition groups (Fig. 5D).

3.7. In-depth analysis of the sludge microbial community in swine farms exhibiting high and low ammonia nitrogen decomposition

The presence of different sludge microbiota profiles in the sedimentation tanks was further investigated (Supplementary Fig. 12). The top ten families in the sedimentation tank are *Anaerolineaceae, Christensenellaceae, JS1, Hungateiclostidiaceae, Rikenellaceae, Candidatus Pacebacteria, Smithellaceae, GWA2-38-12b, Clostridiaceae and Anaerovoracaceae*. To identify bacterial taxa that could potentially augment or hinder ammonia nitrogen decomposition, the taxa enriched or depleted in the group with high ammonia nitrogen reduction were examined. In the sedimentation tank, the group with high ammonia nitrogen reduction exhibited lower abundances of the *Anaerolineaceae*, *Christensenellaceae*, and *JS1* families (Fig. 5E). Conversely, there were higher abundances of the *Rhodocyclaceae*, *AKYH767*, *Comamonadaceae*, *Pedosphaeraceae*, *Candidatus Roizmanbacteria*, *Nitrosomonadaceae*, *PHOS-HE36*,

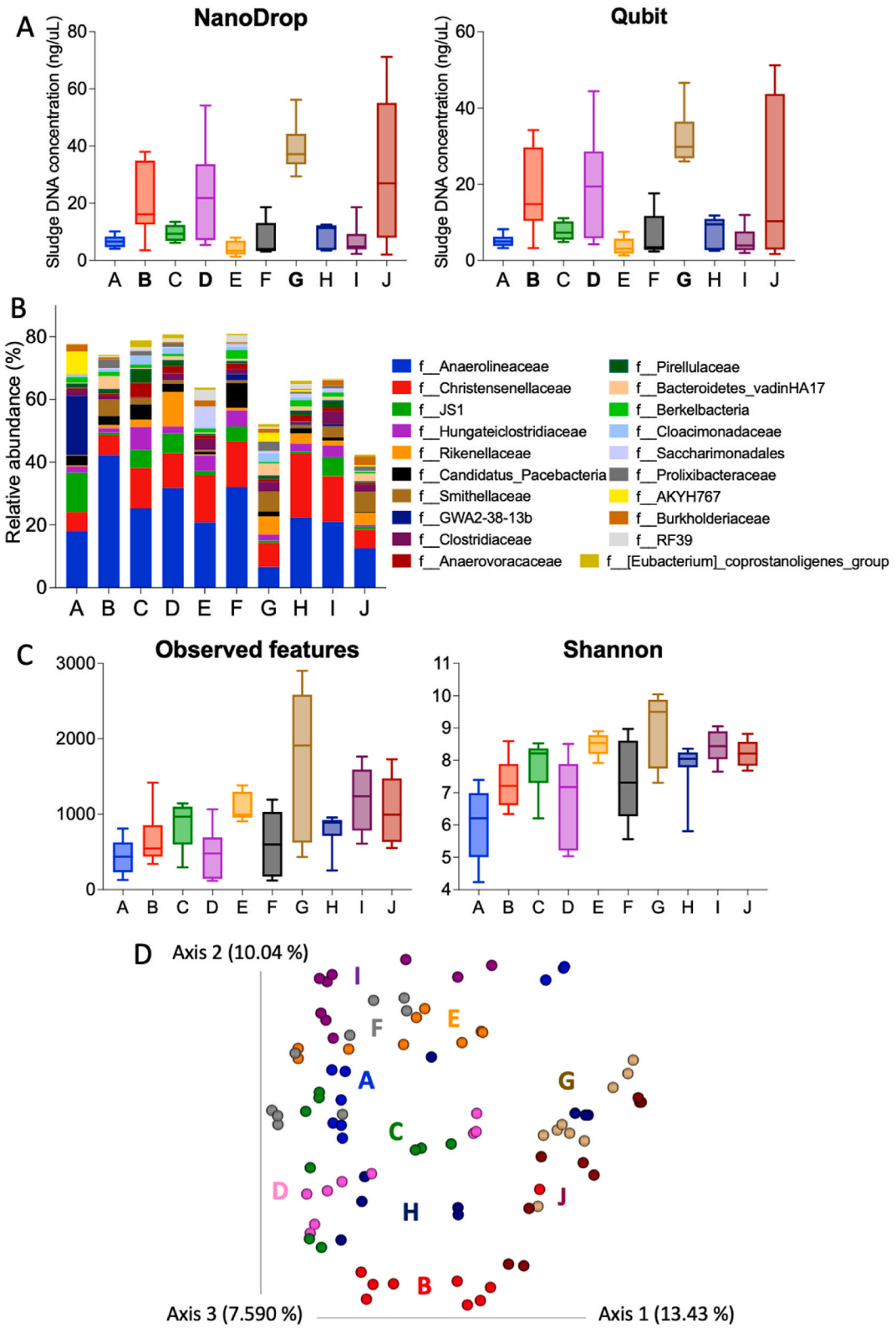

Fig. 3. Microbial analysis of sludge in ten swine farms. (A) Concentration of microbial DNA, (B) composition profiles at the family level, (C) alpha and (D) beta diversity of the sludge microbiota pooled from the anaerobic, sedimentation, and thickening tanks.

Table 4
Utilization of swine sludge in industrial wastewater systems.

Swine farm	Seed sludge utilization
A	N/A
B	Petrochemical industry
C	N/A
D	Petrochemical industry
E	N/A
F	N/A
G	Semiconductors industry
H	Optoelectronics industry
I	N/A
J	N/A

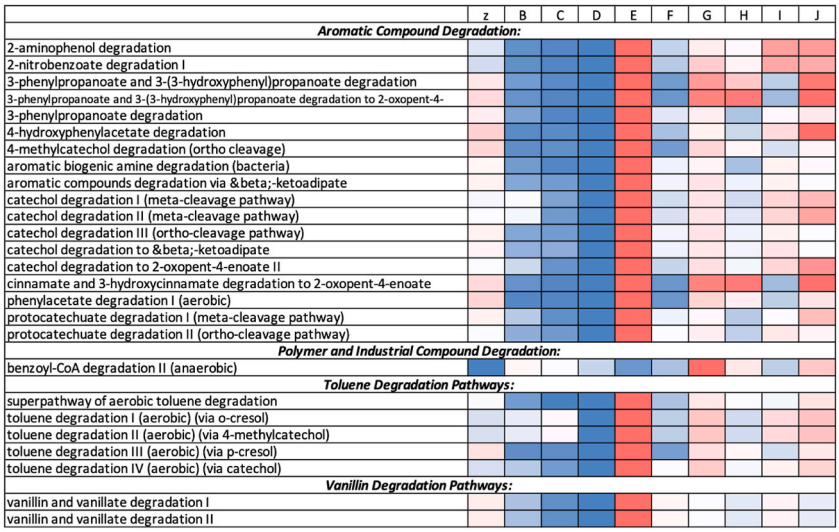

Fig. 4. Functional prediction of degradation-related pathways in sludge microbiota from ten swine farms using MetaCyc.

Hydrogenophilaceae, *Lentimicrobioaceae*, and *Kapabacteriales* families (Fig. 6).

4. Discussion

To the best of our knowledge, this is the first study utilizing full-length 16S rRNA sequencing to comprehensively investigate the sludge microbiota in swine farms. In this study, this study explored the microbiota composition and biodiversity across three processing tanks—anaerobic, sedimentation, and thickening—in ten swine farms that span various regions of Taiwan. Environmental and technical factors that could influence the microbiota community were also examined, and details on sample processing and quality assessment were provided, which are crucial for future studies on sludge microbiota in farms. Additionally, beyond predicting functionality through database mapping, the sludge microbiota results were correlated with real-world ammonia nitrogen reduction in wastewater.

The DNA extraction was performed by taking a similar volume of sludge from each sample, and its concentration was quantified using NanoDrop and Qubit, yielding consistent results. It is important to note that the samples were not precisely weighed or measured; therefore, the DNA concentration can only estimate the abundance of sludge microbiota. Higher DNA concentrations were observed in four groups of sludge samples—namely farms B, D, G, and J. Notably, the first three groups consist of sludge already being utilized in the treatment of industrial wastewater from industries such as petrochemicals, optoelectronics, and semiconductors (Table 4). Sludge seeding has been consistently applied in biological wastewater treatment systems in these instances. This implies that a sufficient amount of sludge bacteria is necessary for effective industrial wastewater processing.

Consistent with a previous study [22], there is a notable trend between the biodiversity profiles of sludge microbiota in swine farms and their functional prediction profiles. Farm E, G, I, and J, which consistently exhibited high richness and evenness of microbiota, also showed high enrichment of degradation profiles in the KEGG and Tax4Fun databases. Moreover, based on the MetaCyc database, farms E and G had unique degradation profiles in different aspects. The former was enriched in amino acid and aromatic compound degradations, while the latter was enriched in carbohydrate and polysaccharide degradations. They also exhibited uniqueness in nitrogenous compound degradation. Interestingly, farm G, which has a high microbiota abundance and biodiversity, is used for industrial wastewater treatment. These findings suggest that both bacterial amount and biodiversity are important factors in determining

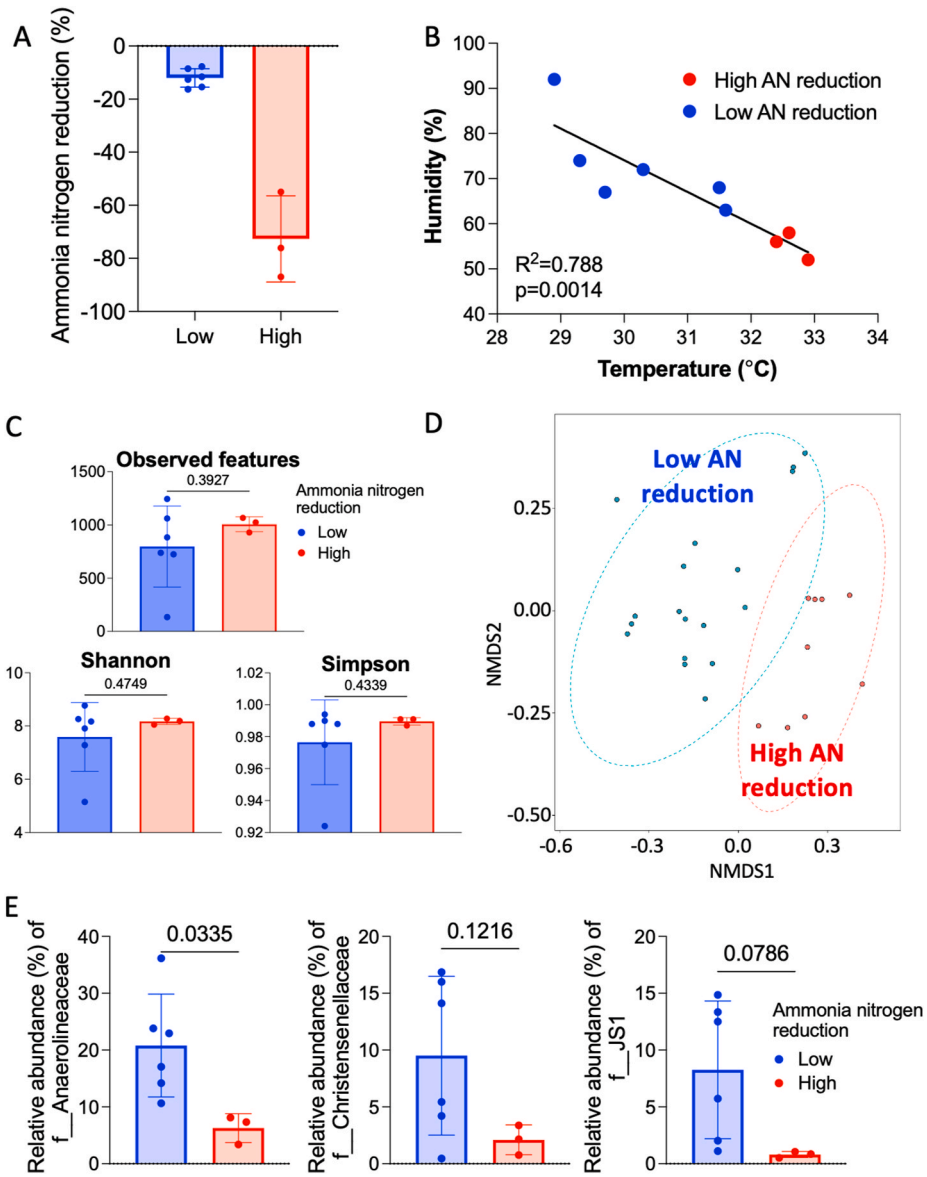

Fig. 5. Analysis of environmental factors and sludge microbiota diversity in relation to ammonia nitrogen reduction levels. (A) The samples were categorized into high and low ammonia nitrogen reduction groups, based on differences observed before and after treatment in aerobic and sedimentation tanks. (B) The correlation between the humidity and temperature at swine farms. (C and D) The diversity analysis of sludge microbiota in the sedimentation tanks of swine farms. (E) The differences in the Anaerolineaceae and JS1 families in sedimentation tanks between swine farms with high and low ammonia nitrogen reduction levels.

their usage in processing industrial wastewater. This highlights the importance of conducting 16S rRNA studies to explore complete microbiota profiles when selecting suitable sludge for wastewater processing in corresponding industrial sectors.

The sludge from three swine farms with higher capabilities in ammonia nitrogen reduction exhibited higher temperatures and lower humidity. A microbiota list enriched in the high ammonia nitrogen reduction group is provided (Fig. 6) [23]. It is important to note that the *Rhodocyclaceae* family is well known for its denitrifying bacteria [24,25]. Along with the *Comamonadaceae* and *Hydrogenophilaceae* families, this group could be utilized to modify the sludge microbiota. However, a major limitation of this study is the absence of a demonstrated causal relationship between specific microbial profiles and their capability to degrade ammonia nitrogen, along with the mechanisms involved. Therefore, conducting in vitro studies using single cultures or cross-feeding/co-culture methods may establish causality and enhance the reliability of the functional prediction profiles.

Several other limitations are noted in this study. The investigation focused solely on the sludge microbiota composition and degradation within the same system. While the functional prediction of sludge microbiota was extended to potential industrial applications, further experiments are required to measure the microbiota and evaluate their efficacy in treating other real-world

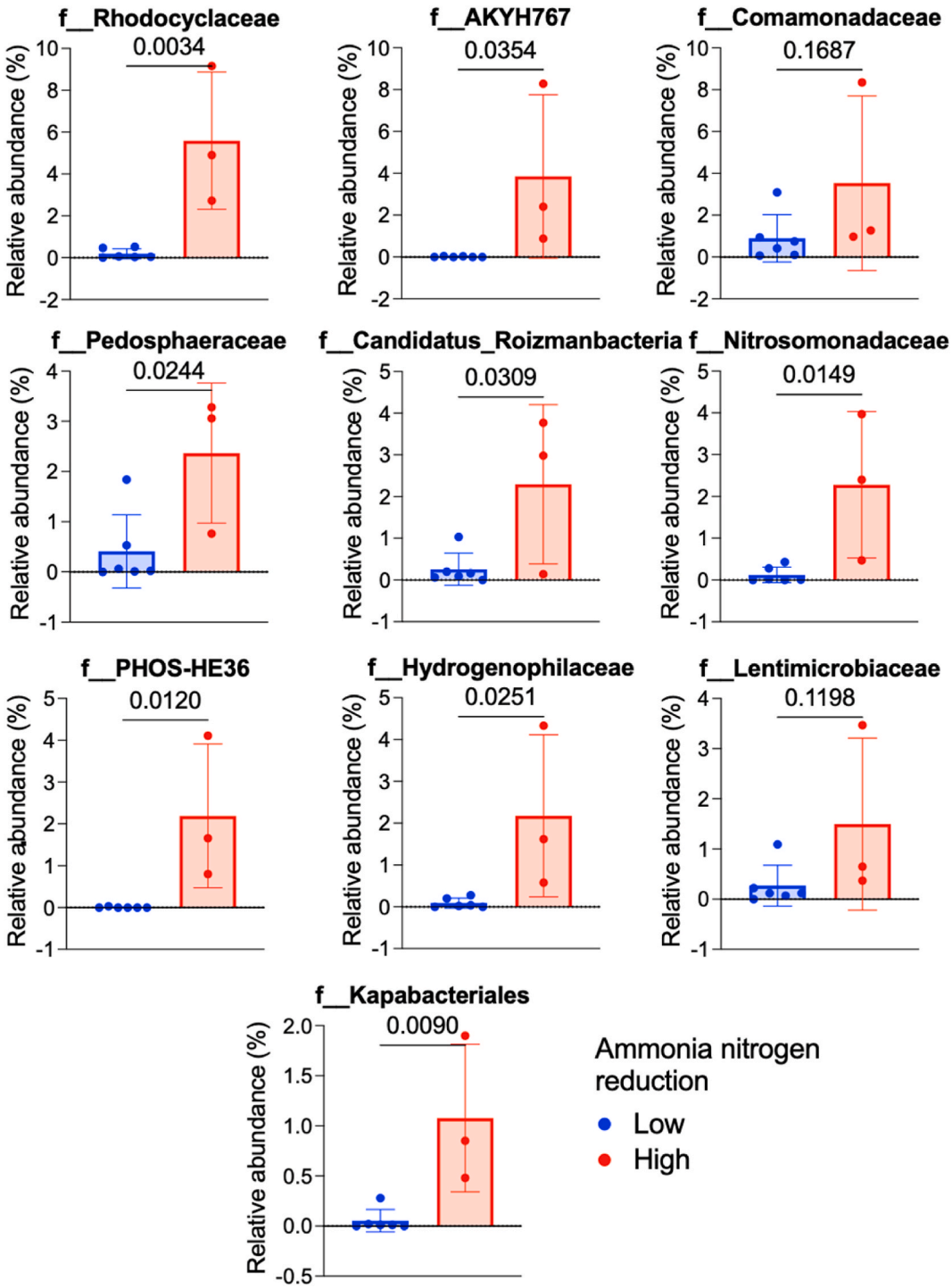

Fig. 6. Bacterial taxa at the family level enriched in the high ammonia nitrogen reduction group within the sedimentation tank.

industrial wastewater, such as tannery, textile, and brewery wastewater. Additionally, to better understand the underlying mechanisms, quantifying functional genes, such as ammonium-oxidizing genes, would provide valuable insights into nitrification processes. Since microbial communities adapt to their environment, long-term sampling is essential to observe changes in microbial communities in response to influent variations, operational parameters, and climatic conditions. Thus, a broader and more in-depth analysis should be conducted in future studies to achieve a comprehensive understanding of the industrial applications of swine sludge microbiota.

The next phase of the experiment will also explore a wider array of decomposition parameters to identify potential correlations. It is

important to note that the associative nature of this study and its reliance on predictive models for functional profiles highlight the need for further research to establish causal relationships through additional experiments or manipulative studies. Furthermore, incorporating a wider range of parameters, such as Chemical Oxygen Demand (COD) [26], along with more environmental factors and pollutant degradation data using analytical techniques like mass spectrometry, could enhance the reliability of the results and help identify more suitable candidates for specific applications.

The analysis showed that sludge microbiota tend to cluster within similar types of ponds, and the factor of individual swine farms overrides the differences in the types of tanks. Therefore, in the future, a single sample from each processing tank in each farm, or just a sludge sample from the sedimentation tank from each farm, would reasonably cover a large scale of swine farms. This approach aims to increase the generality of the study and strengthen the entire database of sludge microbiota in comprehensive regions.

As overall diversity is a critical indicator for the suitability of use in processing industrial wastewater, functional prediction could facilitate precise mapping for suitable sludge in corresponding industrial applications. Therefore, transitioning to short-term Nanopore sequencing could address the time and cost constraints of the current methodology. This methodology, which includes DNA extraction, quality assessment, sequencing, and data analysis, spans approximately two months. The emerging trend of employing Nanopore sequencing, despite its suboptimal accuracy, offers a practical alternative for commercial applications. Alternatively, a rapid test kit or chip that covers the top ten bacterial taxa could be a potential tool to evaluate bacterial abundance and diversity more quickly and in a cost-effective manner.

In the correlation analysis, heatmaps were employed to predictively map degradation pathways or biological processes. This approach can aid in matching specific microbiota to desired outcomes. A predictive model based on an algorithm integrating several critical parameters, including sludge microbiota community information (bacterial load, alpha diversity, and specific microbial profiles), environmental factors such as temperature and humidity, and the biochemical analysis of the sludge or processed wastewater, as well as other characteristics of farms, could be visualized in a radar chart. This visualization makes it easier for farmers or industrial users to interpret and apply the information.

Sludge microbiota is indeed influenced by environmental factors such as temperature. Research has shown that microbial communities in swine manure change in response to temperature variations [27]. Additionally, other studies emphasize the role of temperature in swine wastewater treatment, particularly its impact on microbial activity. Elevated temperatures have also been associated with increased methane metabolism and oxidative phosphorylation [28]. In the current study, a correlation between temperature and humidity has also been observed, which is linked to ammonia nitrogen reduction. Therefore, environmental factors, especially temperature and humidity, should be carefully considered in future studies.

The importance of high microbial diversity underscores the potential application of entire sludge microbiota transplantation, akin to fecal microbiota transplantation in clinical research. Instead of concentrating on augmenting a single or a few bacterial strains, a diverse microbiota is imperative for the effective decomposition or degradation of waste, drawing on the concept of cross-feeding. This emphasizes the necessity of examining the overall microbiota profile to understand their functional outputs, rather than focusing solely on specific bacterial taxa. Additionally, considering the overall bacterial amount through the assessment of absolute abundance, as opposed to only relative abundance, is also crucial in the utilization of sludge microbiota for processing industrial wastewater.

5. Conclusion

The study demonstrates that both the robust bacterial load and higher biodiversity of sludge microbiota are crucial for their suitability in processing industrial wastewater. The families *Rhodocyclaceae*, *AKYH767*, and *Comamonadaceae* are associated with stronger ammonia nitrogen reduction, indicating the potential application of these microbes. A comprehensive microbiota analysis is essential for precise mapping between suitable sludge and their corresponding industrial uses.

CRediT authorship contribution statement

Cheng-Han Cai: Writing – original draft, Formal analysis, Data curation, Conceptualization. **Chee Kin Then:** Writing – original draft, Formal analysis, Data curation, Conceptualization. **Yan-Ling Lin:** Formal analysis, Data curation, Conceptualization. **Cheng-Chun Shih:** Formal analysis, Data curation, Conceptualization. **Chih-Chieh Li:** Writing – review & editing, Supervision, Resources, Funding acquisition. **Tzu-Sen Yang:** Writing – review & editing, Visualization, Supervision, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Consent for publication

Not applicable.

Data availability statement

The datasets generated from sludge samples supporting the conclusions of this article are available in the Figshare repository, https://figshare.com/s/64aa42f3c9b894365fb5. The DOI is 10.6084/m9.figshare.25697355.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e39997.

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