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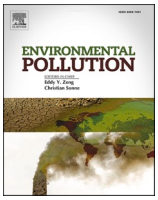

Metagenomic and HT-qPCR analysis reveal the microbiome and resistome in pig slurry under storage, composting, and anaerobic digestion☆

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ABSTRACT

Direct application of pig slurry to agricultural land, as a means of nutrient recycling, introduces pathogens, antibiotic resistant bacteria, or genes, to the environment. With global environmental sustainability policies mandating a reduction in synthetic fertilisation and a commitment to a circular economy it is imperative to find effective on-farm treatments of slurry that maximises its fertilisation value and minimises risk to health and the environment. We assessed and compared the effect of storage, composting, and anaerobic digestion (AD) on pig slurry microbiome, resistome and nutrient content. Shotgun metagenomic sequencing and HT-qPCR arrays were implemented to understand the dynamics across the treatments. Our results identified that each treatment methods have advantages and disadvantages in removal pollutants or increasing nutrients. The data suggests that storage and composting are optimal for the removal of human pathogens and anaerobic digestion for the reduction in antibiotic resistance (AMR) genes and mobile genetic elements. The nitrogen content is increased in storage and AD, while reduced in composting. Thus, depending on the requirement for increased or reduced nitrogen the optimum treatment varies. Combining the results indicates that composting provides the greatest gain by reducing risk to human health and the environment. Network analysis revealed reducing Proteobacteria and Bacteroidetes while increasing Firmicutes will reduce the AMR content. KEGG analysis identified no significant change in the pathways across all treatments. This novel study provides a data driven decision tree to determine the optimal treatment for best practice to minimise pathogen, AMR and excess or increasing nutrient transfer from slurry to environment.

1. Introduction

Antibiotic use in human and veterinary medicine plays a key role in antibiotic resistance (AMR) dissemination. In 2015 the World Health Organisation released their Global Action Plan on AMR to tackle the AMR crisis in both human and animal health. The European Medicines Agency encourages the careful use of antibiotics in humans and animals and started a programme to collate data concerning antibiotic use. Globally, tetracyclines, penicillins and macrolides are the most commonly utilised antibiotics in pig production (Lekagul et al., 2019). The presence of antibiotics at very low concentrations (up to several hundred-fold less than the breakpoint concentrations for pathogens) can select for antibiotic resistance (Gullberg et al., 2011). The antibiotic resistance genes (ARGs) present on mobile genetic elements (MGEs) in antibiotic resistant bacteria (ARB) can be transferred to antibiotic susceptible bacteria within a wide range of biomes (Rasschaert et al., 2020).

The use of animal slurry as organic fertilisers has been shown to impact the soil microbial communities and be the main factor shaping the antibiotic resistome therein (Jechalke et al., 2014; Udikovic-Kolic et al., 2014). According to the One Health concept, environmental biomes connect with human and animal biomes, thus the ARB and ARGs in slurry can transmit to humans and animals and transfer to

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environmental niches when slurry used as soil fertilisers. High abundances and diversities of ARGs have been detected in pig slurry (Zalewska et al., 2021). The application of pig slurry may result in serious environmental issues such as the risk of introducing pathogens, ARB, and ARGs to the soil, crop, and increased nutrients such as nitrogen in water sources (Peu et al., 2006; Rasschaert et al., 2020; Zhu, 2000). Slurry treatment methods such as composting, anaerobic digestion (AD) and storage have been used to reduce the antibiotic residues, ARB, and ARGs in slurry before application to soil as organic fertilisers (Li et al., 2020; Tran et al., 2021; Zhou et al., 2019). The microbial community dynamics and the fate of ARGs were strongly associated with the initial inoculum in the treatment materials and different conditions set up during compost, AD, or storage treatment processes (Cao et al., 2020; Hwang et al., 2016; Lim et al., 2018; Selvam et al., 2012; Zhi et al., 2021).

Previous studies of microbiota and resistome changes in pig slurry were explored either within one treatment method, within modified treatment conditions or without any treatments. However, a comparative study across different treatment methods of the same initial slurry has not been previously undertaken. Here, we present the first study globally comparing the impact of three treatments (storage, compost, and AD) of pig slurry on the microbial community dynamics and resistome. We hypothesised that different treatments applied to pig slurry would result in the substantial changes in the human pathogen content, resistome, microbial community composition, functional pathways, and nutrient content in pig slurry. We used a combination approach of -omics and molecular biology techniques (shotgun metagenomic sequencing and HT-qPCR arrays) to explore and compare the effect of storage, compost, and AD treatments on the dynamics of microbial community composition, functional and metabolic pathways, and resistance gene profiles of the same initial pig slurry. Correlation analysis was applied to identify the links within microbial communities, between the microbial taxa, functional pathways, and resistance genes in slurry samples. We aimed to identify the most applicable and effective treatment currently available to farmers to reduce AMR transmission and minimise the onward risk to the environment, animals, and humans. Based on the data generated we aimed to also provide a matrix decision tree to determine the optimum treatment depending on the most significant problem to be addressed or by addressing a combination of all problems, but with the chance of compromise increasing with each additional problem tackled.

2. Materials and methods

The pig slurry samples were collected from a commercial pig farm in Ireland in agreement with the farm owner. The history of antibiotic use in the farm was provided by the farm owner. The samples were subjected to three treatments, storage for 4 months, compost for 8 weeks, and AD for 90 days. Six potential pathogens (*E. coli*, *Klebsiella* spp., *Acinetobacter* spp., *Pseudomonas* spp., *Enterococcus* spp., and *Staphylococcus* spp) were enumerated from samples collected every two weeks during all treatments. The dry matter, organic matter, and nutrient analysis was carried on samples prior to treatment and products of each treatment.

The total genomic DNA extracted from collected samples were sent for metagenomic sequencing and HT-qPCR for further analysis of the microbiome and resistome. Details of microbiological and molecular biology testing, and data analysis are available in Supplementary information.

3. Results and discussions

3.1. Microbial analysis of pig slurry during different treatments

In this study, *E. coli, Klebsiella* spp., *Acinetobacter* spp., *Pseudomonas* spp., *Enterococcus* spp., and *Staphylococcus* spp. were detected in the pig slurry prior to treatment and a reduction in abundance was observed in all treatments over the experimental period, to varying extents (Fig. 1). Previous works detected major food-borne pathogens such as *Campylobacter, Salmonella,* and *Listeria* in pig slurry by culture-based techniques (Farzan et al., 2010; Mieszkin et al., 2009; Peu et al., 2006). In addition to analysis of faecal indicators, we also enumerated other critically important pathogens which pose risks to human and animal health (*Acinetobacter*, *Klebsiella*, *Pseudomonas*, and *Staphylococcus*). To the best of our knowledge the enumeration of these bacteria has not been previously reported in pig slurry under different treatments.

Enterococcus spp. were the only bacterial species to persist to the final timepoints during storage and compost treatment. *Acinetobacter* spp., *Staphylococcus* spp., and *Klebsiella* spp. were detected in storage samples collected until week 12, while *E. coli* was detected until week 10, and *Pseudomonas* spp. was detected in all samples until week 8 (Fig. 1a). The significant decrease of faecal indicator bacteria (enterococci and *E. coli*) during storage was observed during the storage of pig slurry previously (between storage tank and the pond, and in batch studies) (Munch et al., 1987; Olsen, 1988; Peu et al., 2006). Storing waste in general reduced the abundance of enteric bacteria and could also significantly alter the compositions of enteric bacterial populations (Duriez and Topp, 2007).

Among compost samples, *Klebsiella* spp., *Acinetobacter* spp., *Pseudomonas* spp., and *Staphylococcus* spp. were all absent in the week 6 sample. *E. coli* was detected in the week 2 sample only (Fig. 1c). Most bacterial pathogens analysed were not detected at week 6 of compost except for *Enterococcus* spp. Microbial analysis of compost at different time points in the work of McCarthy et al. (2011) showed that *E. coli* decreased below the limit of detection by day 14 and remained under detection limits until day 56, while enterococci were found to day 28 and below the limit of detection by day 56 (Mc Carthy et al., 2011). In the compost procedure, temperature plays an important role in the product sanitation to ensure it is generally regarded as safe to use (Malińska et al., 2014). The temperature in all compost treatment replicates was above 50 °C for more than 10 consecutive days, indicating a thermophilic phase. In this phase, the thermophilic microorganisms take part in the degradation of complex compounds such as cellulose, lignin, and fats (Bernal et al., 2009).

The AD treatment led to a rapid (week 1), substantial initial reduction of all analysed bacterial species in pig slurry, compared to the control samples. However, unlike in the storage and compost treatments, the six bacterial pathogens were present in all samples collected during AD treatment, including the final timepoint at week 16, meaning that the AD treatment had the highest putative pathogen load of all treatments post treatment. There was little reduction in the cfu/g of each pathogen in the AD samples from week 1 to week 16 (Fig. 1b). At week 16 there was a minimum of 10^3 cfu/g of each pathogenic species detected in the samples. Few bacterial colonies were detected on selective agars that were supplemented with antibiotics, indicating a low level of AMR in the species tested present in the slurry initially and throughout the treatments. AD treatment has been reported to result in significant reductions of bacteria counts including coliforms (Costa et al., 2017).

Our results showed the reduction in bacterial counts in all treatments, which agreed with previous findings for faecal indicators and food-borne pathogens (Costa et al., 2017; Demirel and Scherer, 2008; Mc Carthy et al., 2011; Olsen, 1988; Zhu, 2000). The greater reduction of enumerated bacteria was found in storage and compost samples compared with AD samples, considering the bacterial counts in control and in the final treated products in our work. It was also noted that the levels of AMR in the bacteria tested were extremely low in all sample types, indicating that the detected potential pathogenic species were antibiotic susceptible.

3.2. Microbial composition changes due to treatments

Previous studies on pig manure treatment mainly focused on analysing the microbial communities under one treatment, such as different

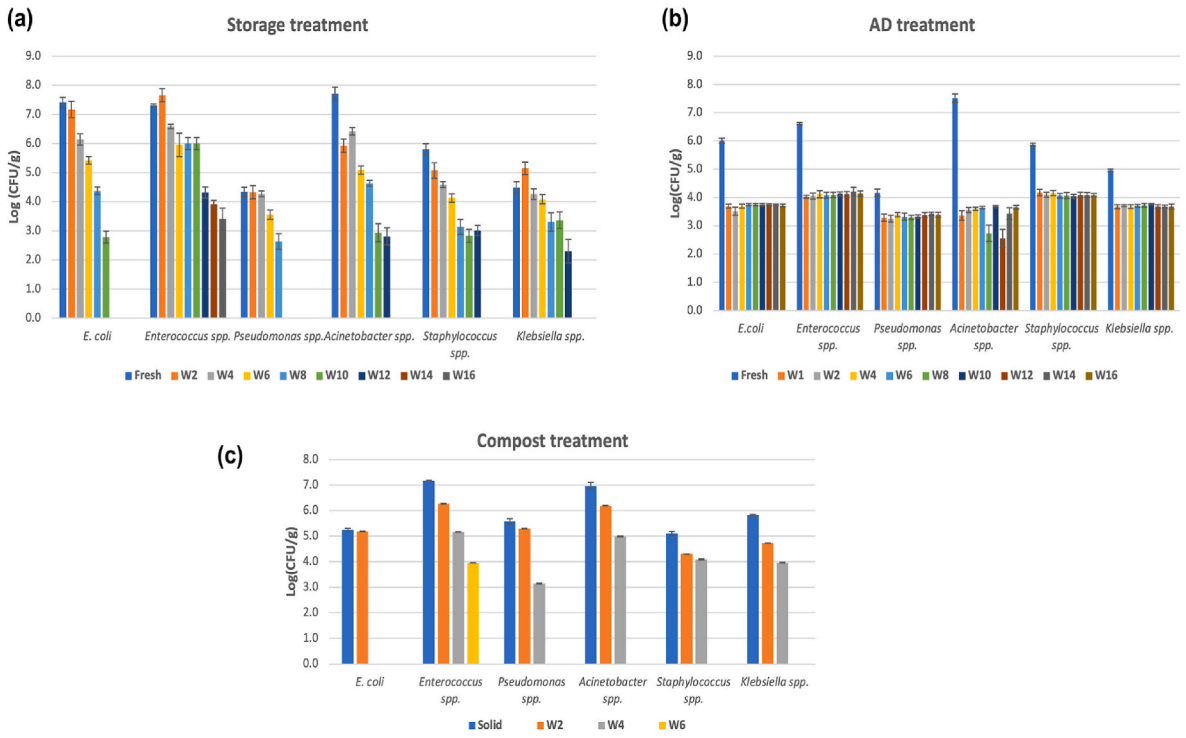

Fig. 1. Bacterial enumeration during storage (a), AD (b), and compost (c) treatments of pig slurry. The bacteria were enumerated on agar without selective antibiotics. Fresh: raw slurry before treatments; Solid: solid fraction of pig slurry, which was used for composting with sawdust; W2–W16: weeks when the samples were collected e.g. W2 = week 2.

storage conditions (Lim et al., 2018) or using different raw material contents, or at different stages of AD or composting procedures (Demirel and Scherer, 2008; Liu et al., 2020; Partanen et al., 2010; Ros et al., 2017). Here, we presented a first study investigating the dynamics of microbial communities during different treatments (storage, composting, and AD) on the same pig slurry. A major expectation of this study was to compare the microbial response across all treatments to address the efficiency and molecular basis behind the result of each treatment process.

After quality trimming and assembling, 40.34–78.44% of the metagenomic sequencing reads could be assigned to Bacteria, Archaea, Virus and other unclassified organisms with Kaiju (Table S1). The composition of microbial communities was assessed at the phylum level for all samples (Fig. 2a). The three phyla Firmicutes, Bacteroidetes, and Proteobacteria, were dominant in all samples. These phyla were the most abundant in the gut microbiome of pigs (Tang et al., 2020; Wang et al., 2019a). The phylum Firmicutes was the most abundant phylum among control (30.67%–55.75%), and storage (30.62%–38.8%) samples, followed by Bacteroidetes (from 19.72% to 53.56% across control and storage samples), Proteobacteria (5.07%–67.25%), and Actinobacteria (2.2%–8.3%) (Data S1). The dominance of these phyla detected during storage was reported previously (Kumar et al., 2020). In AD samples, Firmicutes was the most abundant phylum (49.99%–60.34%), followed by Bacteroidetes (11.4%–20.56%), Proteobacteria (6,26%–8.54%), and Euryarchaeota (3.55%–9.56%). These phyla were commonly detected during AD in different works (Di Maria and Barratta, 2015; Ros et al., 2017; Stolze et al., 2015). Similar to other studies (Jiang et al., 2020; Liu et al., 2020; Zhang et al., 2018), the most abundant phylum in the compost samples was Proteobacteria (48.5%–67.2%), followed by Bacteroidetes (10.24%–41.2%), Firmicutes (5.26%–18.98%), and Actinobacteria (1.27%–3.49%).

Different profile in microbial communities of pig slurry under tested treatments indicating that each treatment created distinct microbial communities in slurry. In storage, relative abundances of some phyla were decreased such as Firmicutes, Spirochaetes, and Euryarchaeota; while other phyla showed an increase (Bacteroidetes, Proteobacteria) or were consistent throughout (Actinobacteria, Spirochaetes) (Data S1). The reduction in relative abundances of Firmicutes and the increase of Bacteroidetes were previously detected in other studies of pig slurry during storage and chicken guts at different growing time (Kumar et al., 2020; Mohd Shaufi et al., 2015).

The decrease in relative abundances was found for Bacteroidetes and Actinobacteria during AD, while Firmicutes and Euryarchaeota were increased. Firmicutes were reported as the dominant bacteria in the mesophilic reactor at 37 °C (Zamanzadeh et al., 2017). This phylum consists of bacteria involved in the degradation of various volatile fatty acids detected in AD and activated sludge systems (Garcia-Peña et al., 2011). The increase of Firmicutes and the decrease in relative abundance of Bacteroidetes, Proteobacteria, and Actinobacteria detected here was also reported by different authors when studying AD (Di Maria and Barratta, 2015; Nelson et al., 2011; Ros et al., 2017; Stolze et al., 2015). The Euryarchaeota was the most abundant Archaea, which was also frequently identified in other AD studies (Leclerc et al., 2004; Pampillón-González et al., 2017; Rabii et al., 2019).

The bacterial community composition underwent a succession from Firmicutes dominance before composting to an abundance of Proteobacteria and Bacteroidetes within the first two weeks. Proteobacteria, Bacteroidetes, and Actinobacteria have been reported to play important roles in the degradation of organic matter in composting (Awasthi et al., 2017), while Firmicutes participate in the decomposition of lignin, cellulose, and hemicellulose (PANDEY et al., 2013). The decrease in the relative abundance of Firmicutes along with the increase of Proteobacteria and Bacteroidetes in our work was in line with previous studies (Jiang et al., 2019; Li et al., 2020). Firmicutes can grow at high temperatures, thus these bacterial phyla may dominate in the beginning of composting. Proteobacteria and Bacteroidetes can likely exist at lower temperatures in the late composting stages.

The heat map of the genus profile, based on their relative abundance, also revealed the prevalence of a distinct pattern based on the treatment groups (Fig. S1a). In control and storage, *Prevotella* and *Bacteroides* were

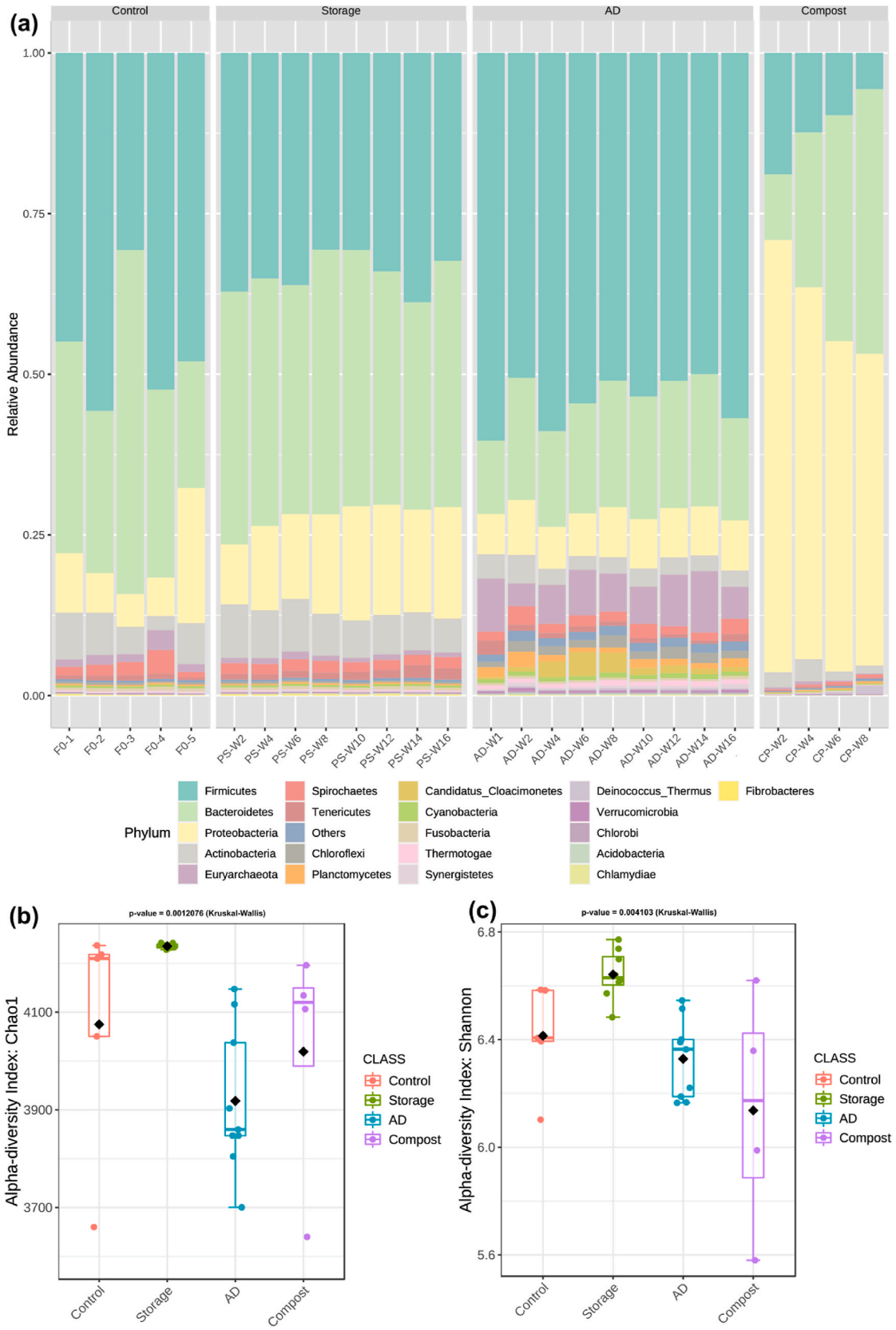

Fig. 2. Microbial composition at phylum level (a) and Chao 1 (b) and Shannon (c) indexes of microbial communities in control, storage, AD, and compost treatments. Samples were divided into 4 groups: Control (F0-1, F0-2. F0-3, F0-4, and F0-5) contained raw pig slurry samples collected before any treatment; Storage (PS–W2 to PS-W16): samples collected during the storage treatment at weeks 2, 4, 6, 8, 10, 12, 14, and 16; AD (AD-W1 to AD-W16): samples collected every 2 weeks during 16 weeks of AD process; and Compost (CP–W2 to CP-W8): samples collected during pig slurry composting at weeks 2, 4, 6, and 8.

the most prevalent. These bacteria were previously reported in pig slurry under storage conditions (Chen et al., 2017; Hwang et al., 2016; Peu et al., 2006; Snell-Castro et al., 2005; Zhu, 2000). *Hungateiclostridium* and *Syntrophomonas* were dominant among AD samples, while *Pseudomonas* was dominant in compost samples. *Pseudomonas* was found to be highly productive at different composting stages. These bacteria dissolve minerals, produce nutrients, and play an important role in lipid degradation. Thus, they contribute to the quality of the compost product as soil fertilisers.

A linear discriminant analysis (LDA) effect size (LEfSE) was performed to characterise the microbiota of pig slurry in different sample groups. The LDA model predicts the features most likely different between treatment samples groups, based on their abundances and estimates the effect size of significant different features (Segata et al., 2011). The microbial taxa at the genus level with LDA score [log 10] > 2 among 50 of the most prevalent taxa were calculated (Fig. S1b). LEfSE identified 49 representative genera, which displayed statistically significant differences of microbiota between different sample groups. The control group was characterised by 11 genera with the most enriched genera being *Prevotella*, *Lactobacillus,* and *Clostridium*. Among 12 representative genera in the storage sample group, *Bacteroidetes*, *Parabacteroidetes*, and *Corynebacterium* were the most abundant. *Syntrophomonas*, *Hungateiclostridium*, and *Methanobacterium* were the most enriched among 15 genera designated to AD samples, while the compost samples indicated 10 differential genera with the most abundance of *Pseudomonas*, *Alcaligenes*, and *Brevundimonas*. These data identify the specific genera changes due only to the treatments applied and the resulting fingerprint of the treated slurry samples.

The microbial community composition of the AD samples at the genus level was dominated by 3 bacteria genera *Syntrophomonas*, *Hungateiclostridium*, *Clostridium* (belonging to Firmicutes), and 2 Archaea genera *Methanoculleus*, and *Methanobacterium* (belonging to Euryarchaeota). These bacterial phyla participate in syntrophic metabolism, substrate hydrolysis, and fermentation (Bosshard et al., 2002; Chen et al., 2010; Vanwonterghem et al., 2014). *Clostridium* is known to dominate the hydrolytic and acidogenic stages of AD (Fontana et al., 2016). It is important to address the presence of any potential pathogenic species in this genus in AD products, which could pose risks to the agricultural environment, humans, and animals. Both archaea genera are hydrogenotrophic methanogens (Ros et al., 2017).

The LEfSE analysis identified the microbial fingerprints in our data of genus abundances for each treatment sample group. These fingerprints can describe the treatment-specific taxa by comparing the taxa abundances between different sample types (Segata et al., 2011). The LEfSE results indicated that the microbial taxa present could be used to differentiate treatment samples from each other and from control groups. These also determined different responses of microbial communities to different conditions between control and all treatment groups, establishing the unique characteristic microbiota in each sample group. These highlighted the important role of treatment-specific taxa as major factors driving the microbiome functions in each treatment type.

3.3. Microbial diversity

The microbial diversity of all samples was analysed based on relative abundances of identified taxa with detailed taxonomic paths. In total 7776 taxa were assigned across all samples. The richness and diversity of the microbial communities were assessed using Chao 1 and Shannon indexes (Fig. 2b and c). These alpha diversity indexes can be used to identify the variation of microbial diversity between samples. The Chao 1 indexes showed significantly higher richness of the microbial community in the pig slurry control and storage sample groups, in comparison with AD and compost samples ($p < 0.05$). The relatively rich nutrient environment in the storage samples would be responsible for the increase of microbial richness by promoting copiotrophic microorganisms (Medina-Sauza et al., 2019).

The AD and compost treatments led to a decrease in the Shannon diversity indexes. The decrease of Shannon diversity was also reported by Wan et al., (2021) (Wan et al., 2021) when analysing the microbial composition during composting, and Zealand et al. studying anaerobic co-digestion of dairy manure with rice straw (Zealand et al., 2018). In contrast, the Shannon index increased in storage, and this value was higher in the storage sample group than those in the control. The difference in alpha diversity across different sample groups may be due to the availability of nutrient contents (Shehata et al., 2021). Indeed, the increase of Shannon index in the storage is likely due to the rich nutrient contents during the treatment, while the lowest alpha diversity indexes in compost samples could relate to the lowest nutrient contents in this process.

The microbial communities from all samples were also visualised by principal coordinate analysis (PCoA) based on the Bray-Curtis dissimilarity matrices (Fig. S2a). The PCoA plot displayed 4 differential clusters formed by 4 sample groups, indicating the significant difference in the microbial community structures across sample groups. This confirmed the individual microbial profile of pig slurry in each treatment, which aligned with the relative abundance of microbial taxa. There is an overlap only between 2 clusters of control and storage samples, confirming the prevalent order of microbial phyla within the samples. The PCoA result again demonstrates the unique microbial populations within the AD and compost treated samples and the similarity between the stored and control slurry. These results indicated that the sample classification can be established based on the microbial community in the sample and that the treatments each alter the microbial content of the slurry in a unique manner, except storage.

3.4. Characteristics of pig slurry resistome under different treatments

A total of 181 genes comprising 154 ARGs and 27 MGEs were detected across all samples. The detected ARGs included 11 main resistance groups: aminoglycoside, beta-lactam, MDR, macrolide-lincosamide-streptogramin B (MLSB), polymyxin, phenicol, quinolone, sulfonamides, tetracycline, trimethoprim, and vancomycin. Four identified MGE groups were integrons, transposons, insertional sequences, and plasmid-associated genes. The total detected genes (both ARGs and MGEs) in pig slurry increased during storage and compost treatments (Fig. 3). In control samples, the most abundant ARGs were tetracycline, aminoglycosides, sulfonamides, and MLSB (Data S2). These ARG classes were also dominant among storage samples. The relative abundance of the total detected genes in pig slurry decreased during AD treatment. In comparison with the control samples, the AD final product showed a decrease in the relative abundance of analysed resistance gene classes (except polymyxin resistance genes, which showed an increase from 0.0004 to 0.0008 and trimethoprim resistance genes remained at the same level of abundance). The greatest abundances were identified across aminoglycoside, tetracycline, vancomycin, and beta-lactam ARGs in the AD sample group.

The most prevalent gene classes in all compost stages were resistance to sulfonamides, followed by aminoglycoside, and tetracycline. The increase in total ARGs during pig slurry composting was reported previously. Cao et al. identified an increase by 0.19–1.62 logs of the relative abundance of total ARGs after composting (Cao et al., 2020). Most of the gene classes (sulfonamides, aminoglycosides, trimethoprim, MLSB) were increased at week 2 and week 4 of composting compared with control samples, then reduced toward the end of the process, but remained at a higher level than the other treatments. Similar results were also reported by other authors in studying pig slurry and sewage sludge composting (Cao et al., 2020; Su et al., 2015; Wang et al., 2015). However, few other studies showed decrease in ARGs in pig slurry composting (Chen et al., 2007; Selvam et al., 2012). The effect of compost procedures on ARG removal efficiency has differed among many studies (Chen et al., 2007; Selvam et al., 2012; Wang et al., 2015; Zhang et al., 2017). The fate of different kinds of ARGs varied and the

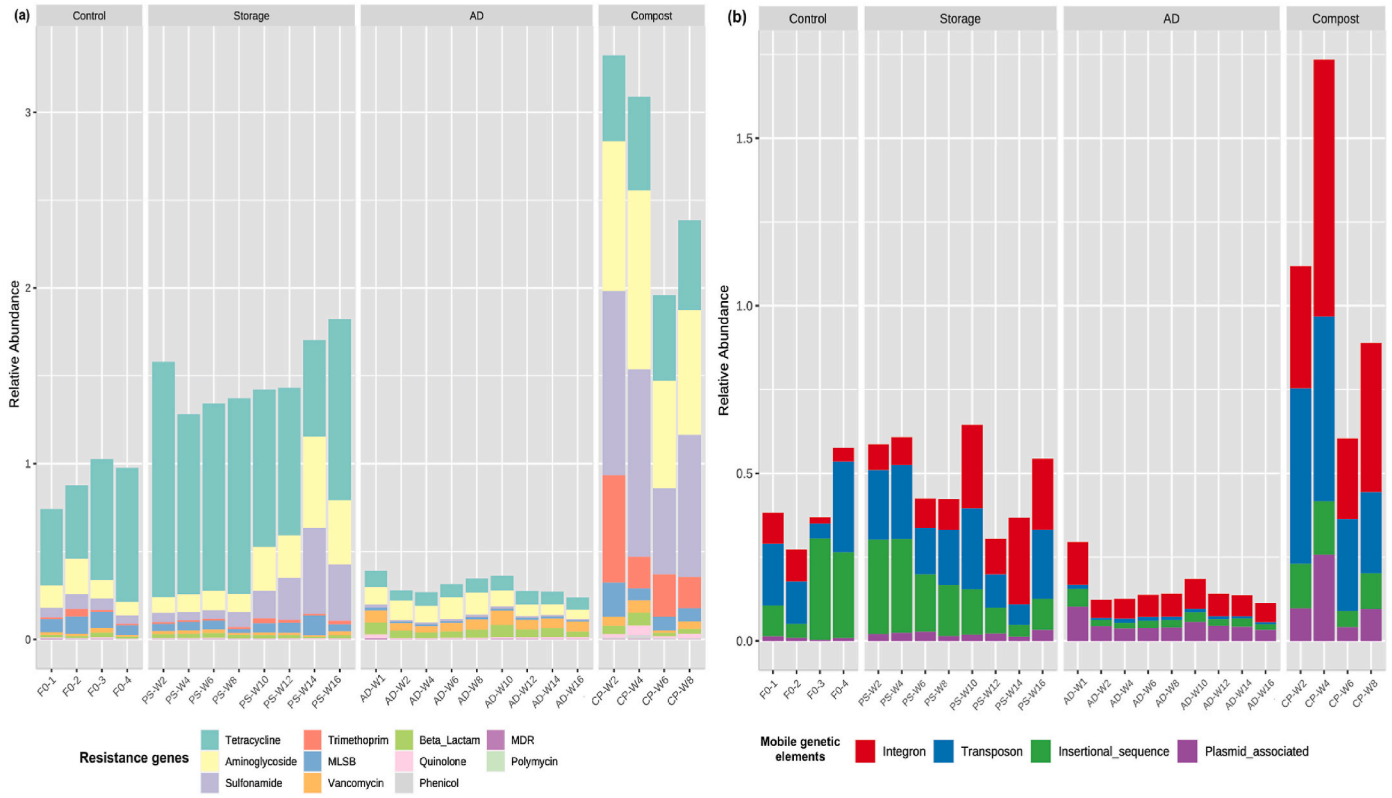

Fig. 3. Gene relative abundance detected in pig slurry under different treatments for (a) resistance gene and (b) mobile genetic elements. The data is presented as the sum of relative abundances ARGs or MGEs. Samples were divided into 4 groups: control contained slurry samples collected before treatment (F0-1, F0-2, F0-3, F0-4); Storage (PS–W2/W16): samples collected every 2 weeks during storage; AD (AD-W1 to AD-W16): samples collected every 2 weeks during anaerobic digestion; and Compost (CP–W2 to CP-W8): samples collected during composting process at weeks 2, 4, 6, and 8.

abundances of some ARGs increased while others decreased during/-after composting (Zhang et al., 2017). The behaviour of ARGs in the same class was also different, for instance, *tetM*, *tetO*, *tetQ*, and *tetW* were decreased while *tetA*, *tetC*, *tetG* and *tetL* increased after pig slurry composting (Wang et al., 2015). The increase of aminoglycoside and sulfonamide resistance here is also in agreement with other studies (Cao et al., 2020; Wang et al., 2015). It is important to note that, the compost in this study was kept at 50 °C for over 10 days, but higher temperatures were applied by other authors, which may alter the ARG removal efficiency. The efficiency of compost in removing ARGs was not always satisfactory; it strongly depends on the properties of the compost mixture and the control conditions.

The relative abundance of detected MGEs varied in all samples. The MGEs were found at a different level across control samples. The most prevalent MGE was insertional sequences, followed by transposons, integrons, and plasmid-associated genes. A decrease of total detected MGEs were found at weeks 6, 8, 12, and 14 among storage samples. The relative abundance of insertion sequences decreased after 16 weeks of the storage, while an increase was found for other MGE classes. These data suggest that, with a reduction in insertion sequences, a concurrent increase in transposon and integrons occurs at week 16. The MGE trend in storage samples does not mirror the ARG data, suggesting that the MGEs contribute only partially to the resistome and ARGs could also be carried on non-mobile elements in these samples. AD led to a decrease in total MGE relative abundance in pig slurry compared with the storage and control sample groups. Among AD samples, the most prevalent MGE class was integrons, followed by plasmid-associated genes, insertional sequences, and transposons.

Composting of pig slurry resulted in an increase in total MGE relative abundance compared with other sample groups. The MGEs' relative abundance increased from week 2 to week 4, then decreased in the later timepoints, but remained relatively high. The composition of MGEs in compost samples was dominant by integrons, followed by transposons, insertional sequences, and plasmid-associated genes (Data S2). The resistome data of AD and compost samples (weeks 6 and 8) mirror the MGE data suggesting an important role for the MGEs in the resistome of these samples. This contrasts with the storage samples where more fluctuation in the MGE data occurred in comparison with the resistome data.

The richness (Chao1) of ARGs decreased in AD and storage samples (Fig. S3a). However, the differences in ARGs richness were not statistically significant ($p > 0.05$). The Shannon indexes showed significant distinction among sample groups ($p < 0.05$), with an increase of ARG diversity in compost and AD samples and a decrease in storage samples in comparison with the control group (Fig. S3b). A significant reduction in the MGE richness was observed in all treatment groups (Fig. S3c) ($p < 0.05$). The MGE Shannon indexes were also significantly different between all group samples (Fig. S3d) ($p < 0.05$).

The relative abundance of both ARGs and MGEs was lowest in AD treatment, indicating the highest efficiency in removing antibiotic resistance. These also resulted in the low richness (Chao1) of ARGs and MGEs in AD sample groups comparing with control and other sample groups. However, the highest Shannon diversity index of ARGs in AD indicates a high diversity of ARGs and MGEs present.

The composition of ARGs and MGEs was analysed through PCoA analysis based on Bray-Curtis dissimilarity (Figs. S2b and c, PERMANOVA test, $p < 0.05$). In the ARG PCoA plot, control, compost, and AD clusters separated from each other, while the storage cluster overlapped with control and compost clusters (Fig. S2b). The MGEs of the AD sample group also formed a cluster separated from other sample groups. The MGEs of the control, storage and composted samples overlapped with each other (Fig. S2c). These results identify the differences in the

profiles of ARGs and MGEs between all sample groups. The storage samples displayed the most similar profile to control in comparison with other treatments, which was also indicated by the overlap between ARG and MGE clusters of two sample groups.

A core resistome containing 89 genes was identified based on the presence of these genes across all sample groups (Fig. S2d). These genes included resistance to tetracycline (n = 17), aminoglycosides (n = 16), beta-lactam, (including bla_{NDM}) (n = 12), sulfonamides (n = 5), trimethoprim (n = 2), MLSB (n = 4), vancomycin (n = 6), phenicol (n = 1), MDR (n = 2), polymyxin (*mcr1*) (n = 1), quinolone (n = 1) and MGEs (n = 22) (Table S2). This lists the genes which were not removed by any of the slurry treatments and thus persisted regardless of treatments to the final product. The core ARGs include bla_{NDM} and *mcr1*, both mobile ARGs to the last line of defence antibiotics; carbapenems and colistin, respectively. The core resistome of pig slurry among all sample groups, with a high abundance of tetracycline and aminoglycoside resistance genes, was consistent with the core resistome of gut microbiota in industrialised feedlot pigs and laboratory pigs (Looft et al., 2012; Wang et al., 2019b).

The network analysis based on strong and significant Spearman's correlations ($|r| > 0.85$, $p < 0.05$) between ARGs and MGEs was employed to understand the co-occurrence of ARGs and MGEs across all samples (Fig. 4a, Data S3). The network contains 126 nodes and 489 edges formed by 93 negative (blue edges) and 395 positive (red edges) correlations. The MGEs showed a higher degree of interactions with ARGs, indicating their central role in the network formulation. The network displayed two large clusters C1 and C2. In cluster C1, the *repA* and Tn5 genes displayed the most interaction with ARGs, followed by Tp614 and IS631. They formed the main hubs in this cluster. While *repA* displayed mostly positive interactions (red lines), Tn5 displayed mostly negative interactions (blue lines) with the ARGs. The interaction in C1 mainly formed between beta-lactamase resistance genes and some others including tetracycline, aminoglycoside resistance, *sul* and, *mcr-1* genes and MGEs. The main hubs in cluster C2 were formed by *tnpA* and *intI* genes (Fig. 4a). The C2 cluster included the interaction between MGEs mainly with aminoglycoside resistance, *sul*, and *tet* genes. The high degree of positive correlations with various ARGs was found for the *intI1* integron, which is a proxy for anthropogenic gene pollution (Gillings et al., 2015).

A high number of genes within the tetracycline, aminoglycoside, and sulfonamide resistance groups positively interacted with MGEs in all sample. This result indicates the strong dissemination of these genes via horizontal gene transfer. This was also reported in previous works analysing pig slurry (Cao et al., 2020; Wang et al., 2021). The integrons and transposons formed the main large hubs in the network, determining their essential role in ARG dissemination.

The relationship between microbial phyla and ARGs were investigated with the network based on Spearman's correlation analysis (Spearman's $|r| > 0.7$, $p < 0.05$) (Fig. 4b). The network consists of 61 nodes (from 7 microbial phyla and 54 ARGs), and 163 edges (built from 113 negative (blue lines) and 50 positive (red lines) correlations). Proteobacteria had the most positive interactions with 20 ARGs, followed by Bacteroidetes having positive interactions with 7 ARGs. Actinobacteria had positive correlations with *tetW* and $bla_{CTX\text{-}M\text{-}6}$ only (Fig. 4b, Data S4). These results indicated their role as primary ARG hosts, consistent with previous findings (Qian et al., 2021; Wang et al., 2021). When these data are compared with the relative abundance data of phyla and ARGs across samples (Figs. 2 and 4a) we can identify the trend of increasing relative abundance of Proteobacteria and Bacteroidetes, and ARGs in compost samples. Whereas increases in Firmicutes in the AD samples did not result in increases in ARGs. Bacteroidetes were strongly correlated to *tet* genes, which was also previously reported, and we know that many members of the Bacteroidetes phyla contain *tet* genes on their chromosomes (Wang et al., 2017). Firmicutes were found to be important in ARG dissemination (Song et al., 2017). This phylum showed positive interactions with aminoglycoside resistance genes, while negative interactions with others. These bacterial phyla were also reported as the ARG hosts in soil microbiota (Qian et al., 2021). Euryachaeota and Candidatus Cloacimonetes held a high degree of negative interactions with ARGs.

The network was also built for microbial families and ARGs on similar parameters. This network mirrors the interaction between microbial phyla and ARGs at the family level with confirming the role as primary ARGs hosts of families from Proteobacteria, Bacteroidetes, Actinobacteria, and Firmicutes. Among them, *Alcaligenaceae* had the most positive interactions with 19 ARGs and formed the biggest node in the network, followed by *Pseudomonadaceae*, *Bacteroidacease* (both have 5 positive interactions), and *Brucellaceae* (4 positive interactions) (Fig. S4, Data S4).

3.5. KEGG functional annotation

We identified 354 KEGG pathways across all samples. In control and storage sample groups, the largest group of annotated genes was assigned to ABC transporters, followed by two-component systems and methane metabolism. The methane metabolism was dominated in the AD sample group, followed by ABC transporters and ribosome. The dominance of methane metabolic pathways in the AD treatment was consistent with previous findings (Guo et al., 2015; Ma et al., 2021). This is directly linked to the high abundance of methanogenic archaea in the microbial community. Among the detected pathways in compost samples, the most abundant was two component systems, followed by ABC transporters and purine metabolism (Fig. S5a). The ABC transporters was previously reported within high abundance species in microbial communities (Campanaro et al., 2016).

The LEfSE analysis among 50 of the most abundant pathways revealed the characteristics of each sample group (Fig. S5b). The control group was characterised by 13 pathways. Among them, the most enriched pathways were ribosome, starch and sucrose metabolism, and pyrimidine metabolism. The storage sample group was represented by 6 pathways with the most enrichment of ABC transporters, phosphotransferase system PTS, and sulfur metabolism. The compost sample group was recognised by the overrepresentation of two component systems, benzoate degradation, and bacterial secretion system over 13 representatives. Methane metabolism, terpenoid backbone biosynthesis, and DNA replication were the most enriched among 14 marker pathways of the AD sample group.

All samples formed 4 separate clusters in the PCoA plot, where the control sample cluster overlaps the other three (Fig. S6c), confirming the variation of the difference in profile and composition of detected pathways in all sample groups. Across all samples, the KEGG pathway distributions did not varying greatly. The overall pattern of KEGG pathways was maintained across treatments and time. Thus, while the microbial populations and resistomes varied the major KEGG pathway genes remained stable. The richness of detected pathways was higher in all treatment sample groups compared with the control group (Fig. S6a). A statistically significant difference in Shannon indexes was found between all sample groups (Fig. S6b).

3.6. Effect of treatments on pig slurry physio-chemical properties

The concentrations of main crop nutrients (N, P, K), elements (Ca, Mg, S, Na, C), dry matter (DM), and organic matter (OM) contents in the control and final treated samples were measured to identify if treatment resulted in a change of nutritional value for fertilisation (Table S3). The DM content decreased after storage, while it largely increased after pig slurry composting and remained almost the same in the AD system. The organic matter (OM) content increased after all the treatments. In comparison with the control slurry, the storage treatment showed enrichment of N and K, AD treatment revealed an increase in the N concentration, while composting led to a reduction in concentration of all the main nutrients. The storage also led to an increase of Na, S, and

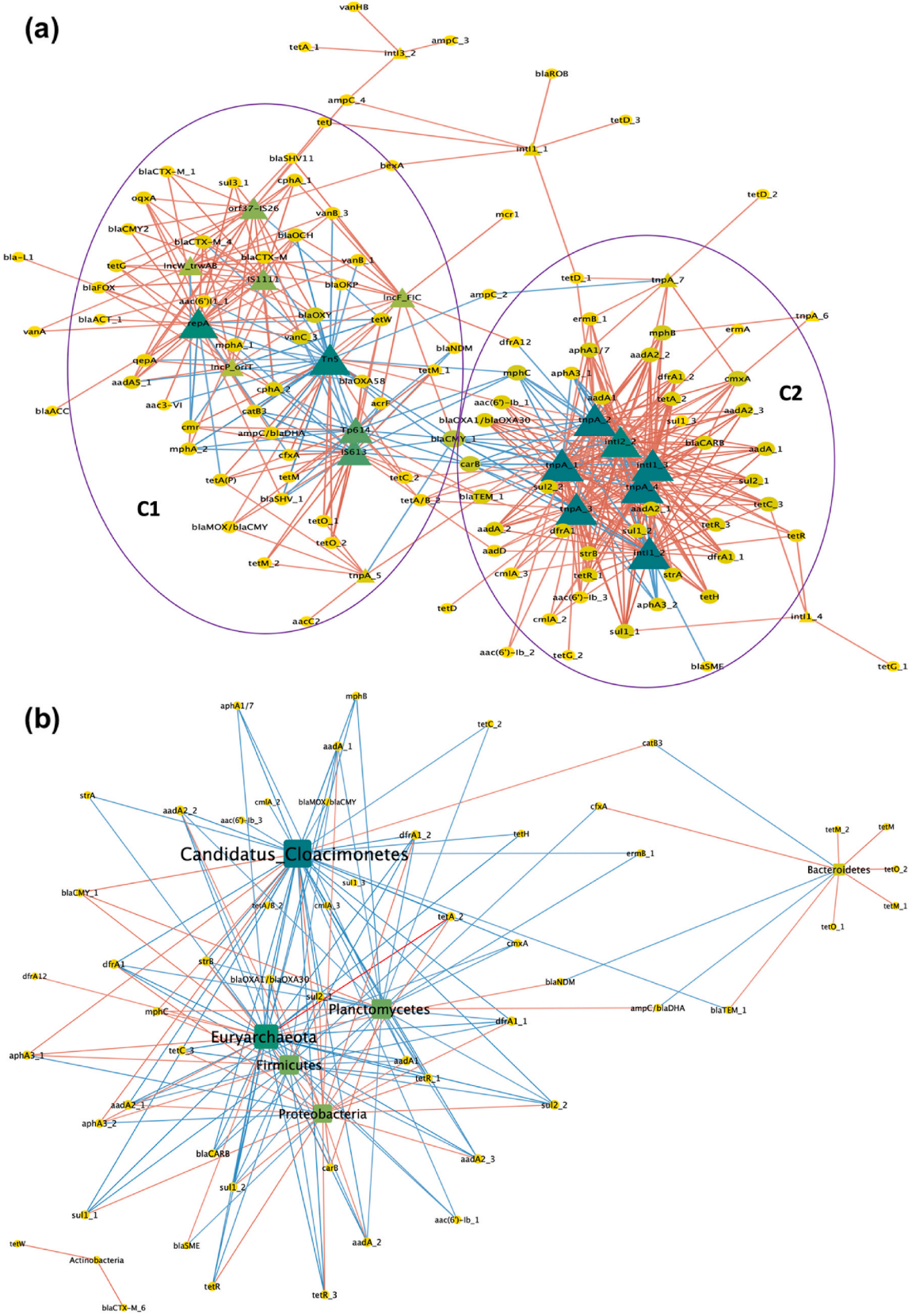

Fig. 4. ARG and MGE interaction network (a) and ARG and microbial phylum interaction network (b) presented in "organic layout". A connection shows a strong and significant correlation based on Spearman's rank analysis ($|r| > 0.85$, $p < 0.05$). The red and blue edges indicated the indexes of positive and negative correlations, respectively, between ARGs and MGEs. The size and colour (ranging from yellow to dark green) of the nodes showed degree of the interactions. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Mg elements, while reduced others. Pig slurry composting resulted in an increase of carbon with a decrease in other elements. The reduction in N, P, K after pig slurry composting could make the compost product suitable to sustainable environmentally friendly agricultural land spreading. The increase in N concentration after storage and AD may cause problems with further use as fertilisers in relation to water pollution and climate change policy implementation in the future.

3.7. Data derived decision tree

Using the data derived from this study we generated a simple decision tree matrix (Fig. 5). Each treatment was assigned a positive integer where the outcome was positive and a negative integer where the outcome was negative. The tree is based on three problems: AMR, pathogens, and Nitrogen. As the requirement for nitrogen may be either positive, in terms of maintaining the nitrogen content of the slurry or negative in terms or requiring the reduction of nitrogen to minimise pollution this was factored in two separate equations. The optimal treatment for AMR reduction was AD, for pathogen reduction were storage or composting, and for nitrogen reduction was composting but increasing nitrogen content were storage and AD. Overall, where nitrogen reduction was required, composting was the recommended treatment but where nitrogen increase was preferred then storage or AD were recommended.

4. Conclusion

This is the first study to provide a direct comparison of different treatments on the same pig slurry microbiome and resistome. Our results determined the effectiveness of storage, composting, and AD on reducing/removing potential pathogens by culture-based methods. The storage is considered the simplest technique to treat pig slurry in comparison to composting and AD. However, composting and AD showed better capacities to decrease the diversity of microbial communities, especially AD which also showed the best efficiency at reducing the microbial load, ARGs and MGEs in pig slurry. The link between microbial composition and resistome is well demonstrated in our study via the compost samples, where the change in microbial composition resulting in Proteobacteria and Bacteroidetes dominating, is mirrored by a large increase in the relative abundance of ARGs and MGEs. This indicates that the changes in microbial population composition correlate with specific ARG and MGE changes in the populations. Such data is only possible due to the extensive sequencing and data analysis performed in this study. Similar microbial changes have been observed in previous studies demonstrating the reliability of these data.

Although the treatments in our work were designed on small scale and more data should be obtained for the nutrient quality, the outcomes can be used to design an optimal low-cost treatment adapted to actual conditions on different pig farms. We also found that each of the treatment methods had advantages and disadvantages, depending on the parameter measured e.g., reduction in ARG or MGE content or microbial diversity or pathogens. However, the implications of the changes within the microbiome are not yet known. Our data derived decision tree provides a structure for the determination of optimal strategies for slurry treatment. While this is a simple model of decision additional factors, such as cost, or time may be added to determine the optimal recommendation. The most important component of this tree is that the data is comparable as all treatments were performed on the same initial slurry samples, thus the only changing component was the treatment. Future decision trees and models may be generated from these data to model different input data such as higher or lower pathogen content or different AMR gene or nutrient content.

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Author contributions

TTD and FW: Design and/or interpretation of the reported experiments, Acquisition and/or analysis of data, Drafting and revising the manuscript, Administrative, technical or supervisory support. **SN**: Acquisition and/or analysis of data, Drafting and revising the manuscript. **NH**: Acquisition and/or analysis of data. **VOF**: Administrative, technical or supervisory support. **FB and CB:** Drafting and revising the manuscript, Administrative, technical or supervisory support.

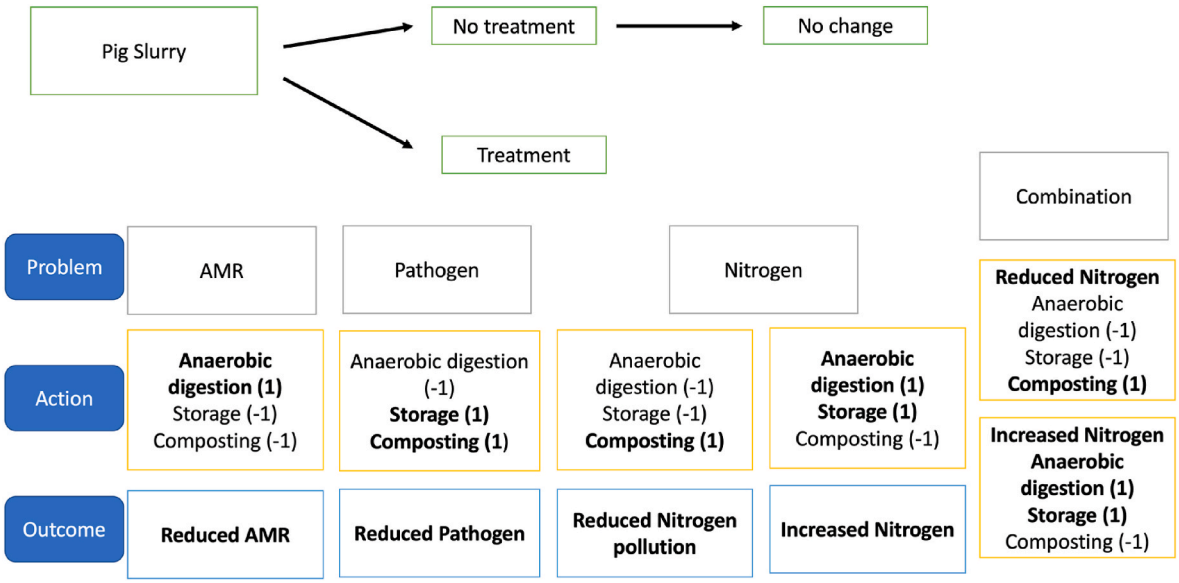

Fig. 5. Slurry treatment decision tree. The tree is based on three problems: AMR, pathogens, and Nitrogen. Each treatment was assigned a positive integer where the outcome was positive and a negative integer where the outcome was negative.

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.envpol.2022.119271.

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