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Potential reservoirs of antimicrobial resistance in livestock waste and treated wastewater that can be disseminated to agricultural land

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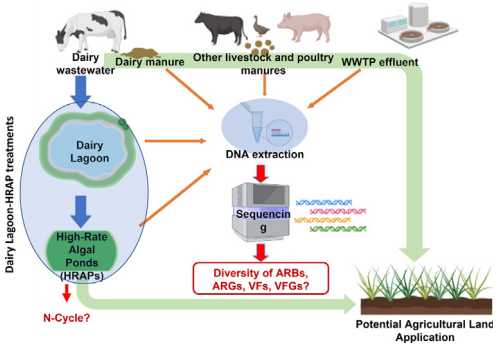
HIGHLIGHTS

- Bacterial diversity from the different microbiomes was not significantly different.
- ARGs and VFGs were significantly different from different microbiomes.
- Aminoglycosides, tetracyclines, beta-lactam, and macrolides were the main ARGs.
- Drug resistance mechanisms were associated with carbapenem, MDR, and efflux pump.
- Bacteroidales was involved in DNRA in dairy lagoon effluent.

GRAPHICAL ABSTRACT



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ABSTRACT

Livestock manure, dairy lagoon effluent, and treated wastewater are known reservoirs of antibiotic resistance genes (ARGs), antibiotic-resistant bacteria (ARB), and virulence factor genes (VFGs), and their application to agricultural farmland could be a serious public health threat. However, their dissemination to agricultural lands and impact on important geochemical pathways such as the nitrogen (N) cycle have not been jointly explored. In this study, shotgun metagenomic sequencing and analyses were performed to examine the diversity and composition of microbial communities, ARGs, VFGs, and N cycling genes in different livestock manure/lagoon and treated wastewater collected from concentrated animal feeding operations (CAFOs) and a municipal wastewater treatment plant along the west coast of the United States. Multivariate analysis showed that diversity indices of bacterial taxa from the different microbiomes were not significantly different based on InvSimpson ($P = 0.05$), but differences in ARG mechanisms

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were observed between swine manure and other microbiome sources. Comparative resistome profiling showed that ARGs in microbiome samples belonged to four core resistance classes: aminoglycosides (40–55 %), tetracyclines (30–45 %), beta-lactam-resistance (20–35 %), macrolides (18–30 %), and >50 % of the VFGs that the 24 microbiomes harbored were phyletically affiliated with two bacteria, *Bacteroidetes fragilis* and *Enterobacter aerogenes*. Network analysis based on Spearman correlation showed co-occurrence patterns between several genes such as transporter-gene and regulator, efflux pump and involved-in-polymyxin- resistance, aminoglycoside, beta-lactam, and macrolide with VFGs and bacterial taxa such as *Firmicutes*, *Candidatus Themoplasmatota*, *Actinobacteria*, and *Bacteroidetes*. Metabolic reconstruction of metagenome-assembled genome (MAGs) analysis showed that the most prevalent drug resistance mechanisms were associated with carbapenem resistance, multidrug resistance (MDR), and efflux pump. *Bacteroidales* was the main taxa involved in dissimilatory nitrate reduction (DNRA) in dairy lagoon effluent. This study demonstrates that the dissemination of waste from these sources can increase the spread of ARGs, ARB, and VFGs into agricultural lands, negatively impacting both soil and human health.

1. Introduction

The rise in antimicrobial resistance (AMR) is a serious public health challenge worldwide (World Health Organization, 2014). The Center for Disease Control and Prevention reported 35,000 deaths a year in the United States, and 700,000 deaths a year worldwide related to AMR (Center for Disease Control, 2013, 2019). The extensive use of antimicrobials in animal husbandry is a major driver of AMR selection and the emergence of antimicrobial-resistant bacteria (ARBs) (World Health Organization, 2014). In 2018 alone, approximately 11.6 million kg of antimicrobials were used in the US for livestock industry (U. S. Food and Drug Administration: Accessed on 8 Oct 2021). As a result of large stocks of antimicrobials prescribed, the livestock and their environment are reservoirs of antimicrobial resistance genes (ARGs) and ARBs (Kimera et al., 2020). The animal source is a significant vector of AMR determinants (such as ARBs, ARGs, VFGs, and MGEs) in the environment as well as a risk factor in the dissemination of antimicrobials (Hendriksen et al., 2019). Virulence factor genes are involved in invading, colonizing, and damaging host cells and contributes to pathogenicity, by enhancing not only the infectivity of pathogenic bacteria but also by exacerbating antimicrobial resistance which in turn restricts treatment options (de Nies et al., 2021). A recent review by He et al. (2020) reports that the number of antibiotic resistance genes (ARGs) is higher by three to five folds in swine and chicken waste than in hospital and municipal waste (He et al., 2020). The abundance of ARGs in cattle and fish waste was about the same to hospital and municipal waste. Therefore, there are considerable differences in the abundance of ARGs among different livestock, possibly due to varying antibiotic usage, dosing patterns, and diet.

Antimicrobials used in human medicine also contribute to the selection of ARGs in bacteria that can subsequently spread in the environment through treated municipal wastewater effluents and biosolids. The collection of data on AMR determinants in animal and human sources is crucial to effectively combat resistant bacterial pathogens that may affect human health not only through the food web of animal origin but also through direct contact with the animal or animal husbandry environments (EF[1]SA, 2012, Walle et al., 2019). Despite these concerns, about 57 million kg of antibiotics were used globally in animal agriculture in 2010 (Van Boeckel et al., 2015).

Application of livestock manure and lagoon effluent to cropland is a common practice to improve the fertility of soils for crop production all over the world. This practice is widespread from developed to developing countries. However, land-applied manure can come from different farm animals ranging from cattle to poultry. Many studies have focused on antibiotic residues and antibiotic resistance genes in manures from individual animal microbiomes subjected to intensive antibiotic use, such as cows, swine, and chickens (Wichmann et al., 2014, Udikovic-Kolic., 2014, Zhu et al., 2013 Ross and Topp, 2015, Noyes et al., 2016, Qian et al., 2016, Eckstrom and Barlow, 2019). These studies suggested the potential for a large amount of antibiotic resistant bacterial and antibiotic resistance genes to be disseminated from animal manure to agricultural soils (Durso et al., 2012). It has been documented that soil is one of the environmental reservoirs for antibiotic resistance genes accounting for about 30 % of ARGs in the public domain (Nesme and Simonet, 2015). Both animal manure and dairy lagoon wastewater are widely used to recycle nutrients for crop production. These may contain ARB and ARGs (Dungan et al., 2018; Dungan and Bjorneberg, 2020) and chemicals of emerging concern (CEC) such as antibiotics and other pharmaceutical compounds (Ashworth et al., 2023). These compounds can impact human health, ecosystem functioning, and metabolic activities such as the nitrogen (N) cycle (Semedo and Song, 2020, Semedo et al., 2018; Semedo and Song, 2022). N cycle is an oxidation-reduction process which microbial communities transform inorganic N using both assimilatory and dissimilatory pathways during nitrification or denitrification processes. Therefore, it is critical to elucidate the impact of ARGs on N-cycle pathways based on metagenomic analysis.

Application of manure fertilization to agricultural soil has resulted in a bloom of ARGs even though the animals that produced the manure had not been treated with antibiotics (Udikovic-Kolic et al., 2014). The most significant aspect of manure application to agricultural soil may be the “farm to fork” effect where fresh produce that is eaten raw or minimally processed is grown in such soil (Guron et al., 2019). The authors concluded that manure fertilization allowed for the enrichment of resident soil bacteria that harbored ARGs. Additional work on antimicrobial-resistant bacterial populations and ARGs obtained from environments impacted by livestock and municipal wastewater showed that antimicrobial resistance is a widespread phenomenon. Higher diversity of antimicrobial resistance genes is present in treated human waste discharged from municipal wastewater treatment plants than in livestock environments (Agga et al., 2015).

In this study, we applied shotgun metagenomic sequencing to assess bacteria and resistance gene diversity through different environmental matrices. The matrices included swine manure, duck effluent, cattle manure, beef manure, dairy manure, dairy lagoon effluent (DLE), DLE treated in four high-rate algae ponds (HRAPs), and treated municipal wastewater (TWW). Shotgun sequencing was used to determine bacterial diversity and ARGs in these matrices as previously described (Bengtsson-Palme et al., 2014; Rowe et al., 2016; Durso et al., 2012; Eckstrom and Barlow, 2019; Wichmann et al., 2014), and in food waste feeding and composting on a poultry farm (Eckstrom and Barlow, 2019; Guron et al., 2019). Our main objectives were to investigate the antibiotic resistome structure and microbial community in different animal manure sources as potential reservoirs of AMR determinants, as well as determine the impact of ARGs on N cycling potential in HRAPs that is used for the treatment of DLE. To accomplish these two goals, manure samples from different livestock and secondary treated municipal wastewater were collected to quantify ARGs, microbial community, and water samples were collected from dairy lagoon effluent (DLE) and four high-rate algae ponds (HRAPs) to quantify the effect of HRAPS on bacterial diversity and antibiotic resistance genes (ARGs). Further analyses from these samples included assessing potential pathogenic species and associated virulence factors from different samples to elucidate the potential mechanisms of resistance transfer within the farm environment. We selected the different manure sources because they have

[1] EMA, EFSA, EMA, EFSA, 2017.

significant differences in their diets. We hypothesized that ARGs and microbial communities should cluster according to the feeding habits of the animals, and that feeding habits of livestock can determine the biochemical and biological properties of manures, and having a predictable effects on microbial community composition and function. Hence manure profiles could potentially be used to steer and manage manure application to agricultural lands.

2. Materials and methods

2.1. Sample collection and processing

Manure samples were collected from dairies at California Polytechnics University campus (San Luis Obispo, CA, USA) dairy farm. The dairy farm was the source of seasonal samples from four high-rate algae ponds

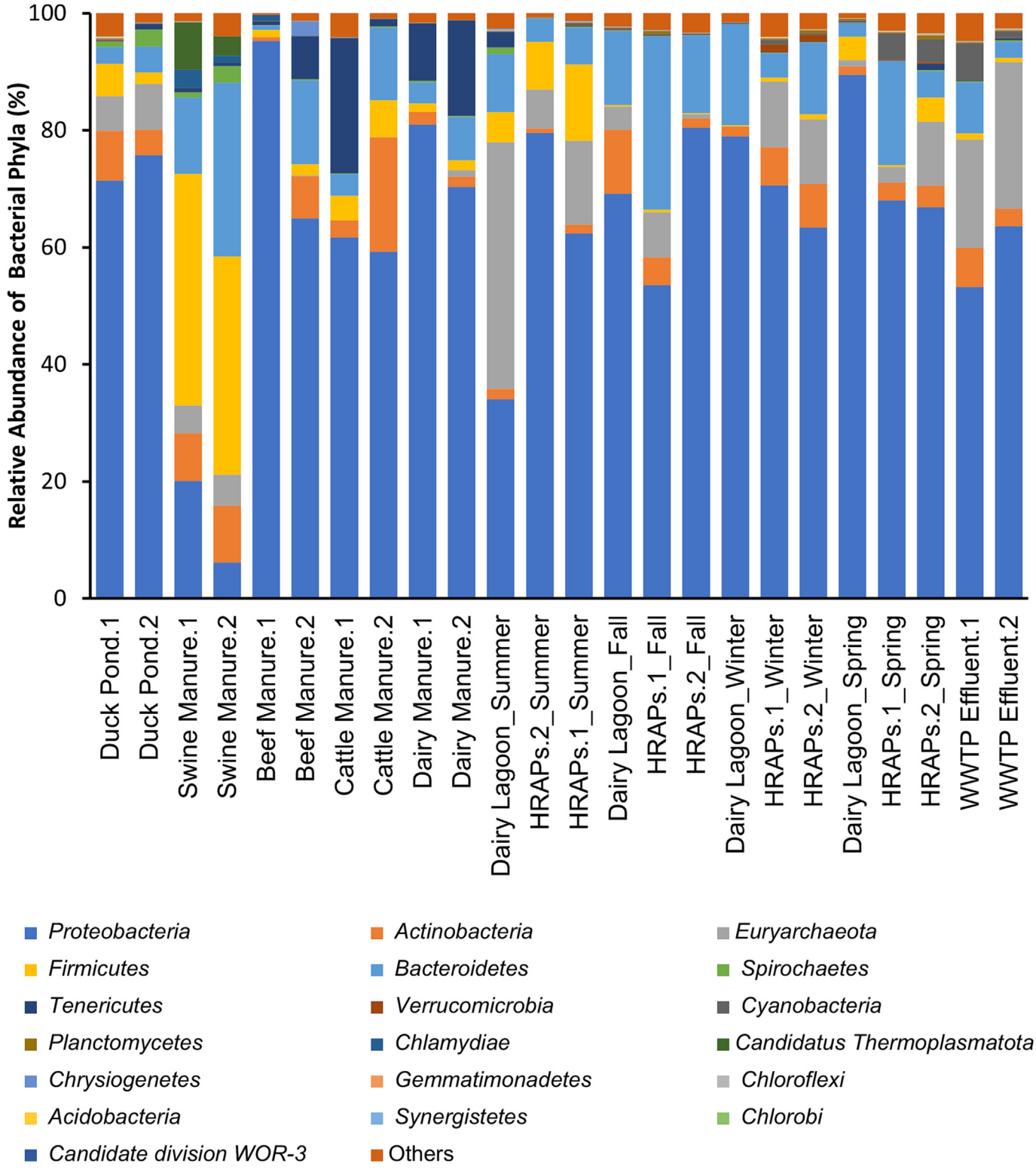
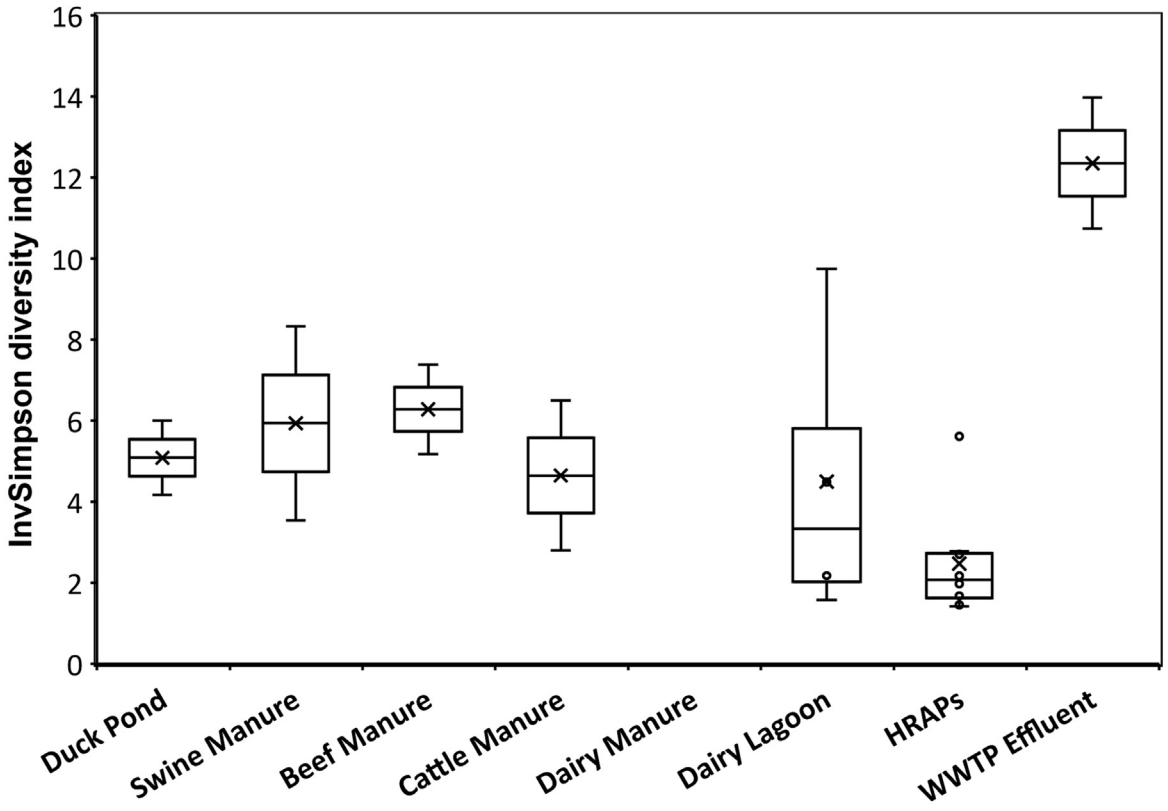


Fig. 1. Bacterial phyla from different microbiomes.



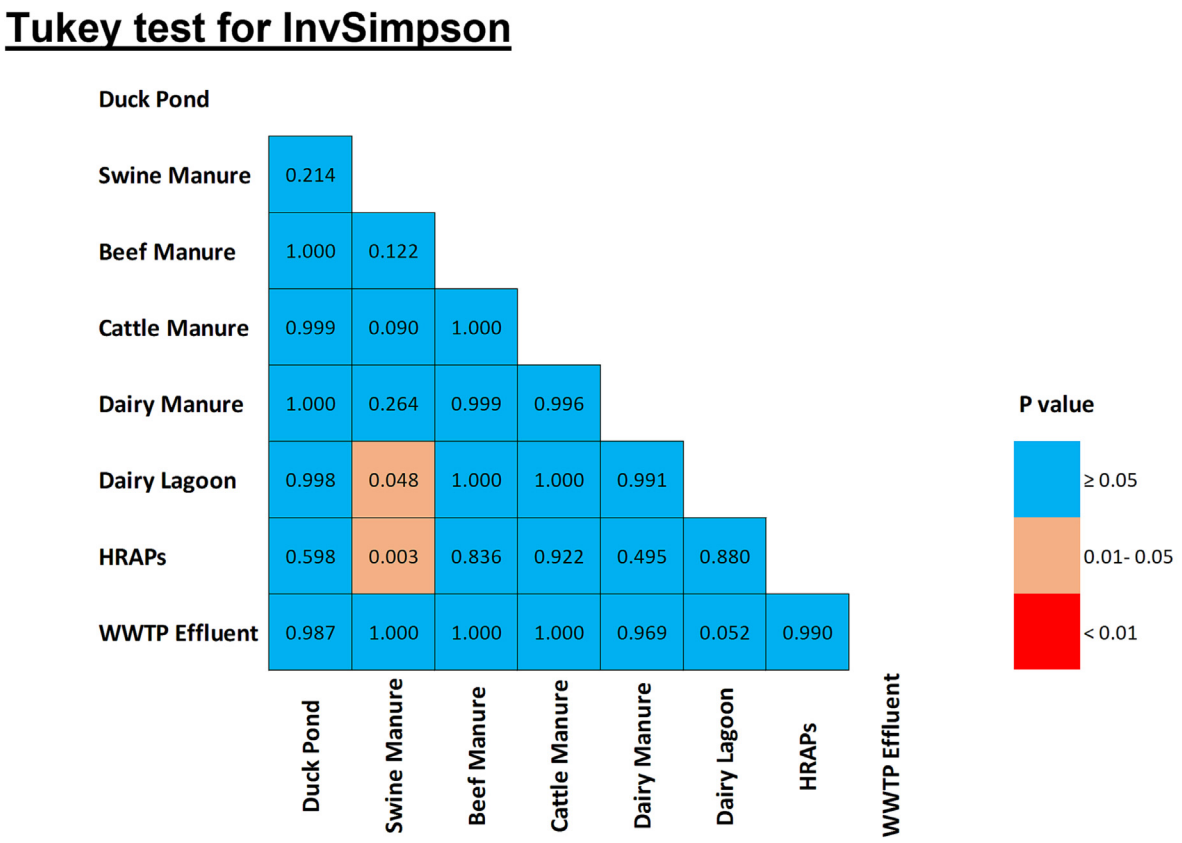
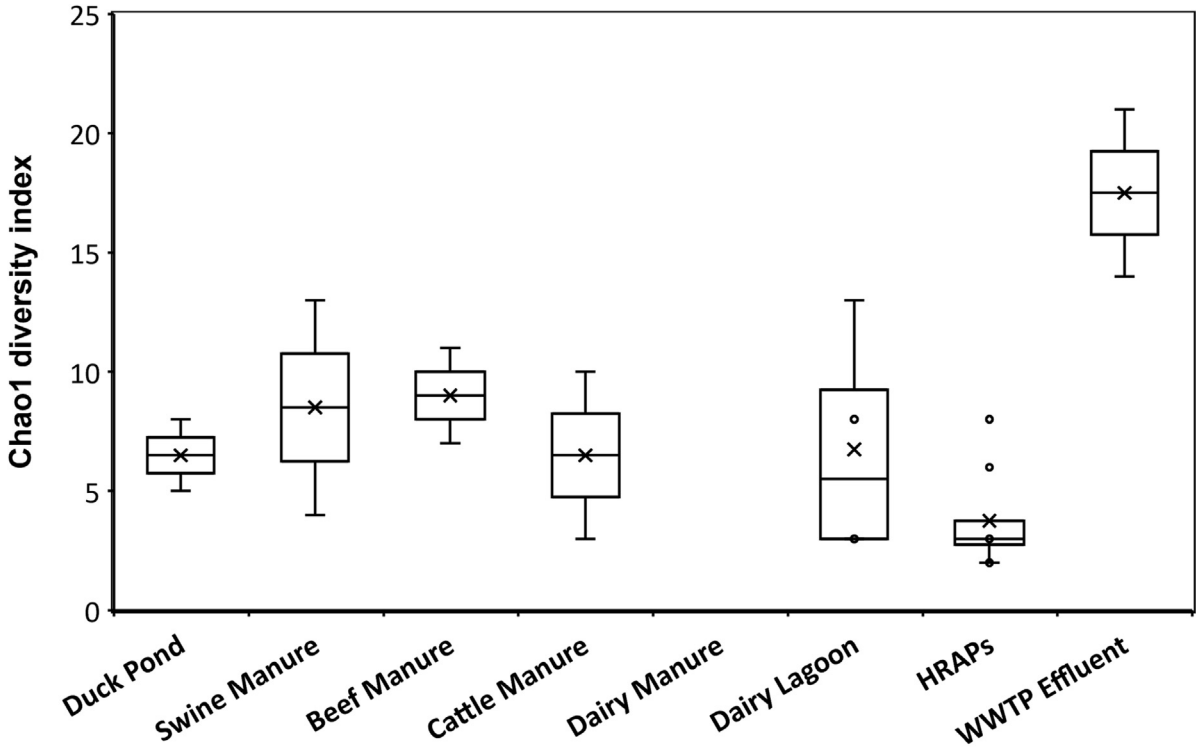
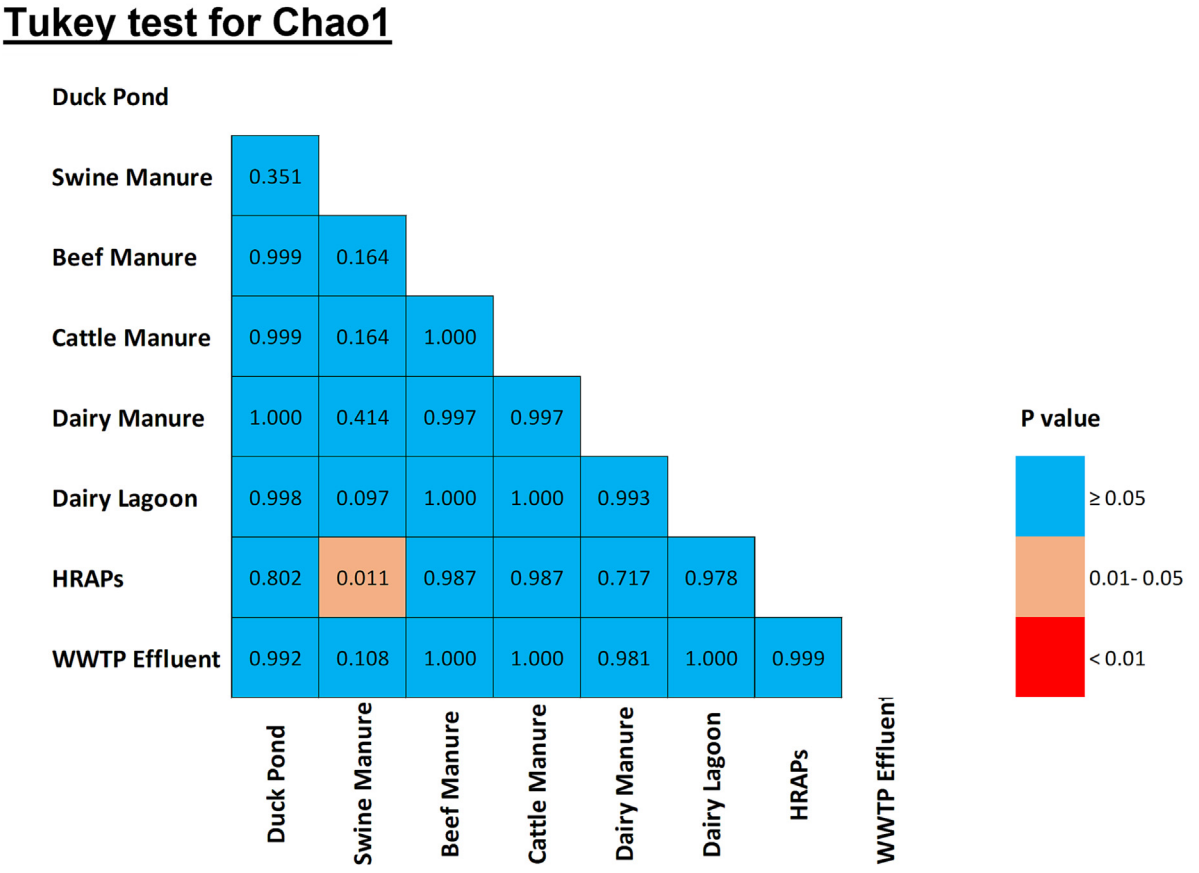


Fig. 2. Reservoirs of virulence factor genes (VFGs). The VFGs from total microbiomes based on InvSimpson diversity test (panel A) and Chao1 diversity (panel B). Distribution of VFGs in microbiome samples (panel C). Reservoirs of VFGs with heatmap representing the number of VFGs in individual samples (panels D&E), and Phyletic (taxonomic) affiliations of the VFGs that were found in the individual samples (panel F).





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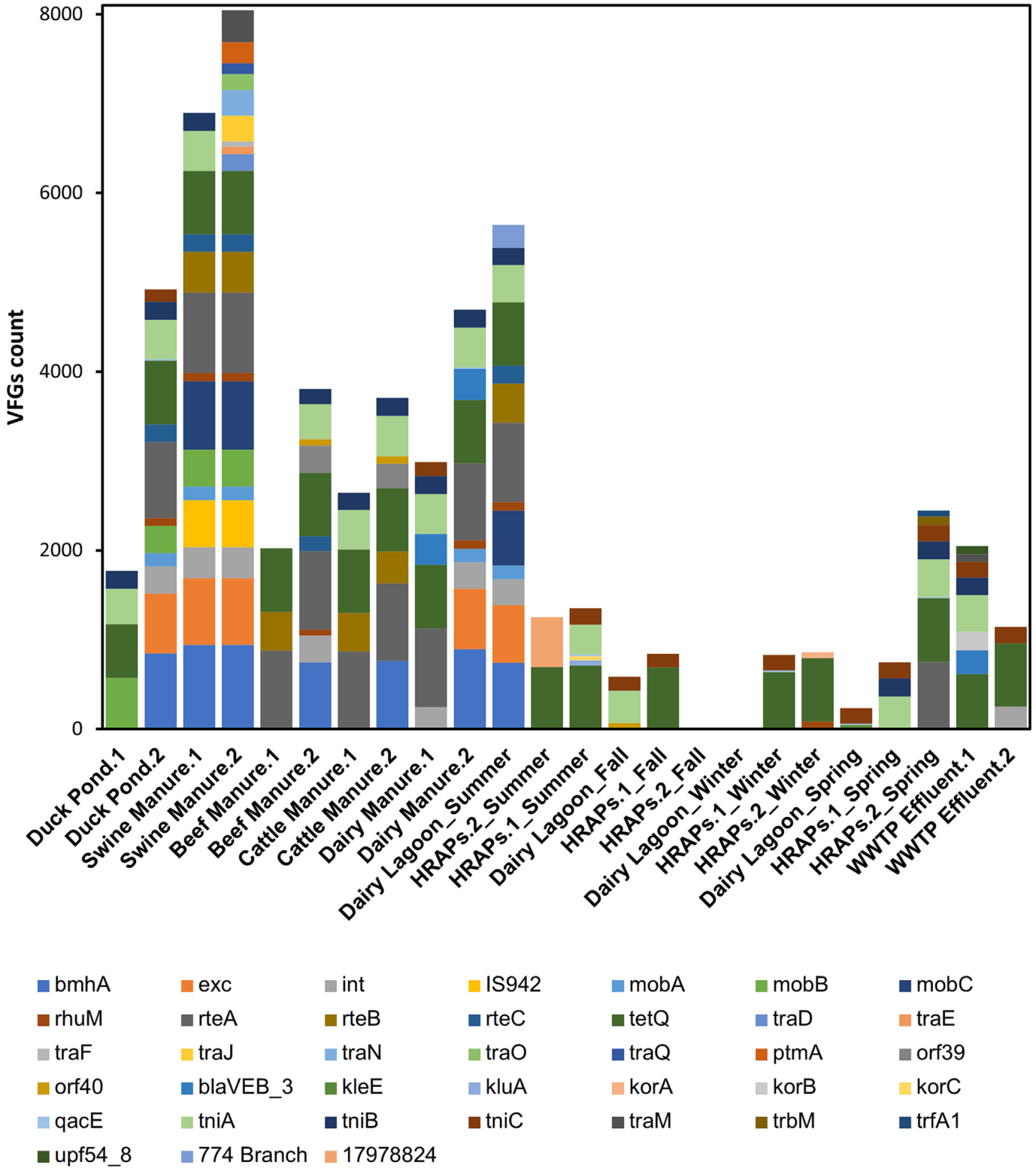
(HRAPs) and dairy lagoon effluent (DLE). The DLE was obtained when dairy flush water was captured and pumped through a screen separator. The liquid then flows into a lagoon and was stored, while the screened solids were composted or used as bedding. New, fresh wastewater was added daily to the lagoon from the milking parlor, and DLE was also applied to multiple crops throughout the year as needed to meet nutrient



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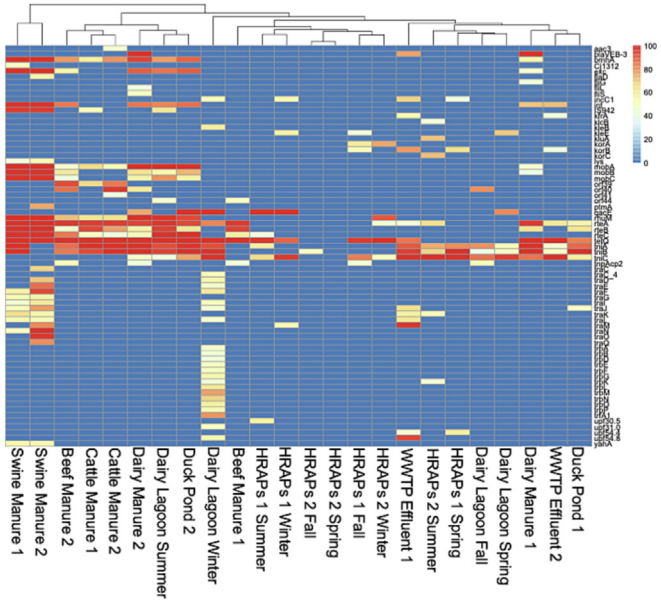
requirements. Four identical three-square meters high-rate algae ponds (HRAP) were installed adjacent to the DLE at the 250-head dairy unit. The HRAPs were paddle wheel-mixed raceway ponds operated at a 30-cm depth, containing a volume of 970 l per reactor (Fig. S1). The reactors were made of a wooden frame with a flexible plastic liner. A variable low-speed motor was used to drive 6–blade plastic paddlewheels. The ponds were operated as individual units to conduct experiments for multiple conditions to determine nutrient removal rates in DLE at various DLE dilution rates and pond HRAPs, seasonally. Physical parameters of the units, such as the relationship between paddle wheel revolutions per minute (RPM) and channel water velocity, as well as cross sectional flow patterns that are critical for developing bio-flocculating algal communities were



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analyzed (Ibekwe et al., 2016; Schwartz et al., 2021). The algae ponds were fed a combination of screened dairy flush water and fresh water to meet the required experimental hydraulic retention time (HRT). Nutrients added to the HRAPs came from either the DLE or water-soluble miracle grow fertilizer (N-P-K: 24–8-16). Nutrient additions were based on a target total available N, which ranged from 1.5 to 2.5 g of N per square meter per day fed to the ponds. Ponds were operated in duplicates, and either received 80–100 % of the nutrients from DLE or 40–50 % of the nutrients from DLE. The remainder was delivered from the miracle grow fertilizer which was added as supplemental nutrients to determine if the units fed DLE were inhibited due to the dark color of the units. Data were collected from the outdoor pilot-scale HRAPs under steady-state conditions during winter, spring, summer, and fall. Treated municipal wastewater effluent was collected from a nearby wastewater treatment plant during the four seasons. Additional manure samples were collected from California Polytechnics University Pomona beef cattle. In addition, manure samples were collected from a commercial beef and dairy farm (cattle) in southern California, U.S.A, as well as swine manure and duck pond effluent from the same farm. All samples were kept on ice and transported to the laboratory, where the liquid effluent was processed within 24 h, and the manure solids were stored at −80 °C until processing.

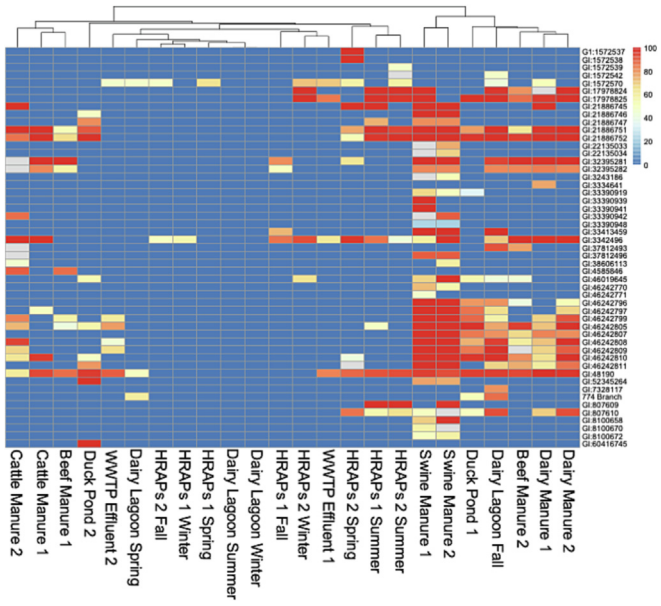
2.2. DNA extraction, library preparation, and shotgun metagenomic sequencing

DNA was extracted from samples using Qiagen PowerSoil Pro Kit (Qiagen, Hilden, Germany) from manure samples and Qiagen DNeasy PowerWater Sterivex Kit for water using sterivex filter units. All extractions followed the manufacturer's recommended protocol. After extraction, all samples were stored at −20° C for quantification using nanodrop 2000 (Nanodrop-ND 2000, Wilmington DE) and Qubit 2.0 Fluorometer dsDNA system (Thermo Fisher, Waltham, MA, USA).

Extracted genomic DNA was sent to CosmosID (Rockville, MD, USA) for sequencing as previously described (Hasan et al., 2014). Each genomic DNA sample was normalized to a final concentration of about 0.5 ng μL^{-1} using a Biomek FX liquid handler (Beckman Coulter Life Sciences, Brea, CA, United States) before library preparation. Libraries were constructed using the Nextera XT Library Prep Kit (Illumina, San Diego, CA, United States) and subjected to 150 bps paired-end sequencing using an Illumina HiSeq 4000 (Illumina Inc., CA, USA) platform. The shotgun metagenomic raw reads were analyzed by CosmosID metagenomic software (CosmosID Inc., Rockville, MD) as previously described (Hasan et al., 2014; Connelly et al., 2018; Kaleko et al., 2016; Zaouri et al., 2020; Ponnusamy et al., 2016) to determine microbial community composition and resistome. The unassembled quality-filtered sequences were mapped to the CosmosID's curated GeneBook databases to elucidate (i) taxonomic profile, (ii) antibiotic resistance genes (ARGs), and (iii) VFGs as described below. Through comparative analysis between designated groups, various statistical analysis results, such as beta-diversity and biomarker discovery, were provided.

2.3. Metagenomic, virulence factor genes, and antibiotic resistance gene profiling

Unassembled sequencing reads were analyzed using CosmosID metagenomic software (CosmosID Inc., Rockville, MD) as described elsewhere (Hasan et al., 2014) to reveal associated microbial community composition. Briefly, the system utilizes a high-performance data-mining k-mer algorithm and highly curated dynamic comparator databases that rapidly disambiguate millions of short reads into the discrete genomes or genes engendering the sequences. The pipeline has two comparators: the first consists of a pre-computation phase for reference database and a per-sample computation. The input to the pre-computation phase is a reference microbial genome, antibiotic resistance and virulence gene database, and its output is phylogeny trees, together with sets of variable length k-mer fingerprints (biomarkers) that are uniquely identified with distinct nodes, branches, and leaves of the tree (Wood et al., 2019; Chalita et al., 2020; Yoon et al., 2019). The second per-sample, computational phase searches millions of short sequence reads against the fingerprint sets. The resulting statistics are analyzed to give fine-grain composition and relative abundance estimates. The second comparator uses edit distance-scoring techniques to compare a target genome or gene with a reference set (Li et al., 2009; Langmead and Salzberg, 2012; Quinlan and Hall, 2010). The algorithm provides similar functionality to BLAST but sacrifices some recall precision for a one or two order of magnitude processing gain. Overall classification precision is maintained through aggregation statistics. Enhanced detection specificity is achieved by running the comparators in sequence. The first comparator finds reads in which there is an exact match with a k-mer uniquely identified with a reference genome



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or antibiotic resistance or virulence gene; the second comparator then statistically scores the entire read against the reference to verify that the read is indeed uniquely identified with that reference. For each sample the reads from a species are assigned to the strain with the highest aggregation statistics. Specifically, virulence factor genes (VFGs) profiles were produced by using a pre-built bowtie2 (Langmead and Salzberg, 2012) database composed of reference factors obtained from the Virulence Factors of Pathogenic Bacteria (VFDB) database (Liu et al., 2019). Each read of the metagenome sample was mapped against these virulence factors using bowtie2 with the option, and the output was then converted and sorted by samtools (Li et al., 2009). Finally, for each virulence factor found, depth and coverage were calculated using samtool's mpileup script. The Antibiotic resistance genes (ARGs) profiles were produced by using a pre-built bowtie2 (Langmead and Salzberg, 2012) database composed of NCBI's National Database of Antibiotic Resistant Organisms (NDARO, www.ncbi.nlm.nih.gov/pathogens/antimicrobial-resistance/) reference genes. Each read of the metagenome sample was mapped against these genes using bowtie2, and the output was then converted and sorted by samtools (Li et al., 2009). Finally, for each gene found, depth and coverage were calculated by using samtool's mpileup script. Alpha diversity and richness of ARGs were estimated with InvSimpson and Chao1, respectively.

2.4. Metagenomic assembly and binning

Briefly, several strategies were employed to analyze the sequencing data. The raw reads were quality filtered with BBMap v38.18 (Bushnell and Rood, 2014). The De novo assemblies were performed with SPAdes v 3.14 (Bankevich et al., 2012). The scaffolds were trimmed to a minimum length of 1500 bps with BBMap v38.18. The per base coverage was calculated by mapping quality filtered reads against the scaffolds using BBMap v38.18. MetaBAT1 v v1.0.18 subsequently used the mapped file to form bins from scaffolds (Kang et al., 2015). CheckM v1.0.18 assessed the qualities of the formed bins with a lineage-specific marker (Parks et al., 2015). The bins with >70 % completeness and <5 % error were designated as metagenome-assembled genomes (MAGs) upon lineage-specific makers. The taxonomic affiliations of the MAGs were delineated using GTDB-Tk v 1.3.0 (Chaumeil et al., 2020).

2.5. Metabolic pathway assessment of MAGs

Prediction of protein and annotations for metagenome-assembled genomes (MAGs) was performed using Prokka v 1.14.6 (Seemann, 2014). The predicted proteins for MAGs were parsed through Microbe-Annotator (Ruiz-Perez et al., 2021) for comprehensive functional annotations. These annotations were summarized in the Kyoto Encyclopedia of Genes and Genomes (KEGG) modules (Kanehisa et al., 2016). Heatmap representing completeness of each KEGG module for individual MAGs were constructed using pheatmap package in R (Kolde and Kolde, 2018).

2.6. Statistical analysis

All statistical analyses were carried out for all microbiome samples and then with microbiome samples along the treatment line from dairy manure, dairy manure effluent, and HRAPs in duplicates. Statistical analyses were conducted by the R studio version 4.0.5 (Kim et al., 2014). Nonparametric Kruskal–Wallis test and the pairwise Wilcoxon rank-sum test were used to assess the differences in microbial diversity, ARGs, VFGs profiles among sample groups by the R statistical package. Alpha diversity was estimated by the Inverse Simpson Diversity, Simpson, Shannon, and ARGs richness indexes. Comparisons in alpha diversity estimates were carried out with the Wilcoxon signed-rank test. The R package was used to perform clusters based on the Bray–Curtis beta-diversity metrics of both the taxonomy profile and ARG profile. Differences in the composition and structure of microbial communities and ARGs profile between clusters were then evaluated using the permutational multivariate analysis of variance test (PERMANOVA) implemented in the "vegan" package in R. The partition with the highest R^2 value (coefficient of determination) was selected to represent the clustering structure. All statistical tests were considered significant at a P-value <0.05. For network analysis, Spearman's correlation coefficient (Rho > 0.6 and $P < 0.05$) was calculated with GraphPad Prism 9.0.0 (Dotmatrics, Boston, MA, USA) to determine the correlation between ARGs, VFGs, and microbial taxa. These strong Spearman's rank correlation coefficients were employed for network visualization using Gephi interactive platform (Gephi version 0.9.6). To understand the interaction of ARGs, VFGs, with microbial taxa in different settings, the network analyses among (a) ARGs vs. VFGs and (b) ARGs vs. phylum levels were performed for manure samples and for dairy lagoon-HRAPs treatments, separately. The alpha diversity and richness were estimated with InvSimpson and

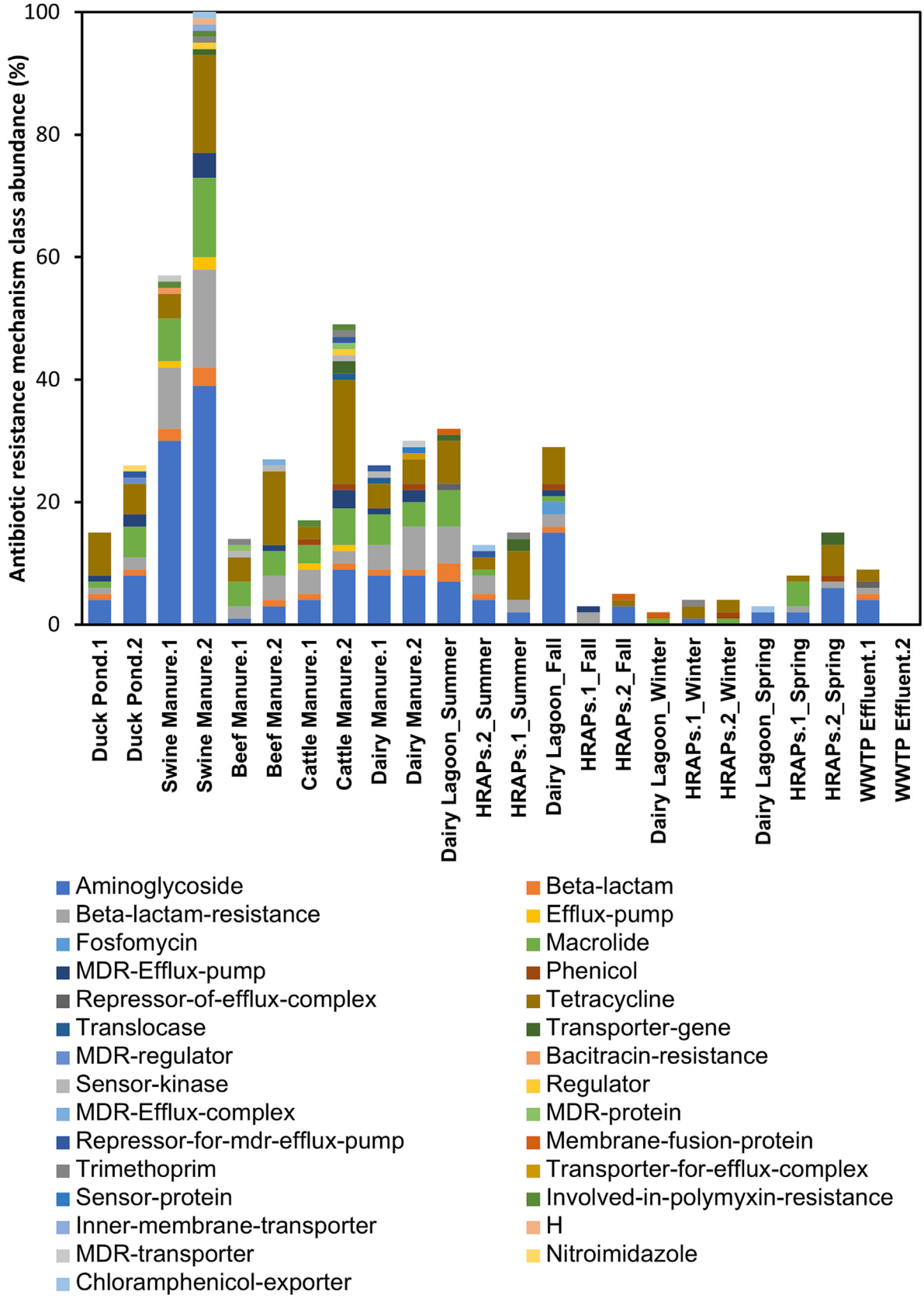


Fig. 3. Reservoirs of antibiotic resistance genes (ARGs) across the samples. ARGs across all microbiomes, with a total of 29 resistance mechanisms in all the samples. The resistance to aminoglycoside was the most prevalent mechanism based on the corresponding ARGs presence in all the samples followed by tetracycline, Beta-lactam resistance, and macrolide-lincosamide-streptogramin (MLS) (panel A).
Diversity of ARGs across samples based on InvSimpson diversity (panel B) and the Chao1 index of diversity (panel C).

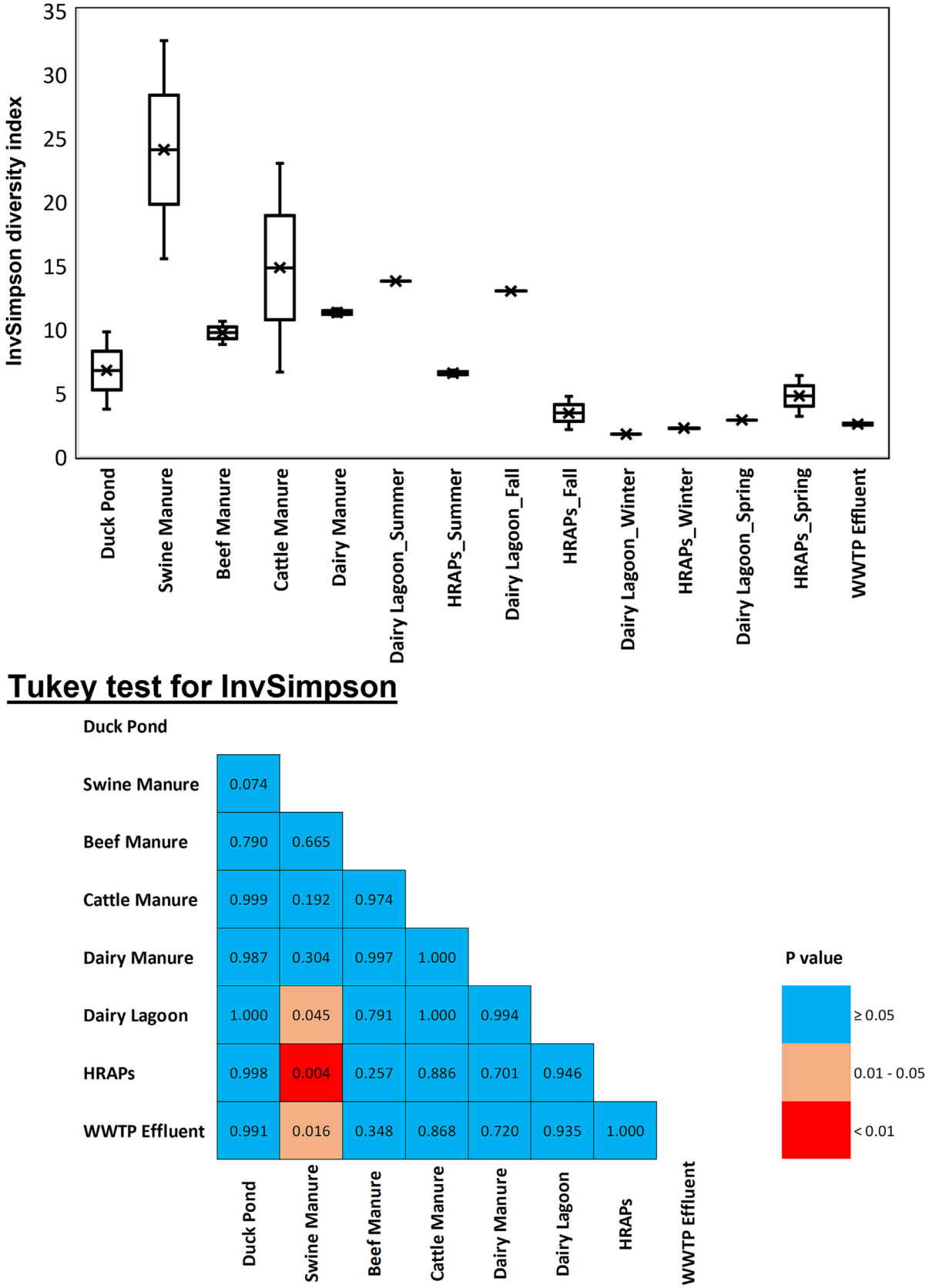
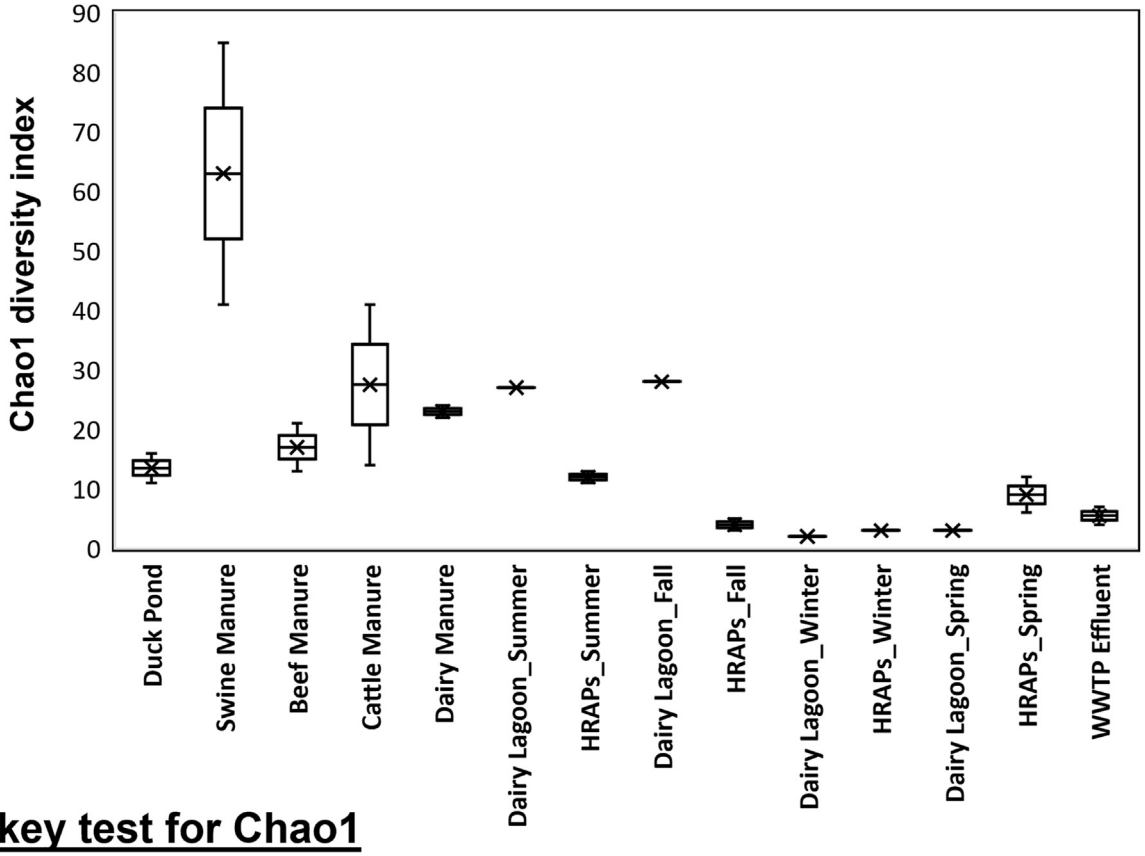


Fig. 3 (*continued*).

Chao1, respectively, using the Phyloseq package (McMurdie and Holmes, 2013). The alpha diversity for each sample type was tested by permutational ANOVA (PERMANOVA) using the adonis function of the vegan package in R (Oksanen et al., 2017). A one-way ANOVA was also performed to test for significant differences sample dissimilarity (Bray-Curtis) between sample types.



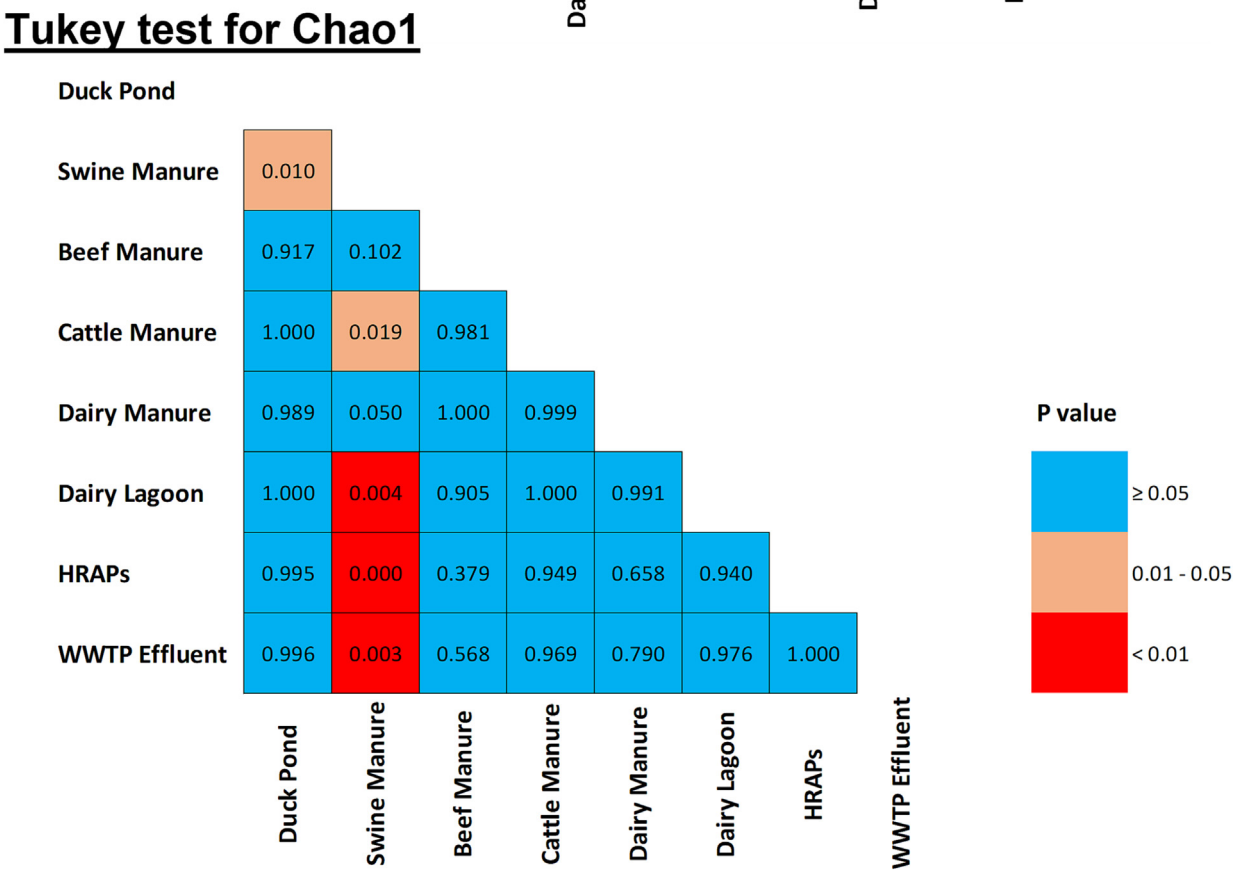


Fig. 3 (*continued*).

3. Results

Shotgun sequencing produced ~2.5 Gbps (Giga base pairs) of raw reads for all metagenomes (Table S1). After assembly, integrated, non-redundant gene catalogs were built for all animal microbiome and wastewater samples. The results showed that the duck microbiome contained the highest number of reads out of all the samples (Table S1). The read statistics range from about 79 M reads (dairy lagoon effluent) to about 140 M reads (duck pond). Bacterial phyla from all the samples showed that *Proteobacteria*, *Actinobacteria*, *Firmicutes*, and *Bacteroidetes* were the most dominant phyla in the samples (Fig. 1). *Proteobacteria* dominated all samples except swine microbiome that *Firmicutes* dominated. However, the

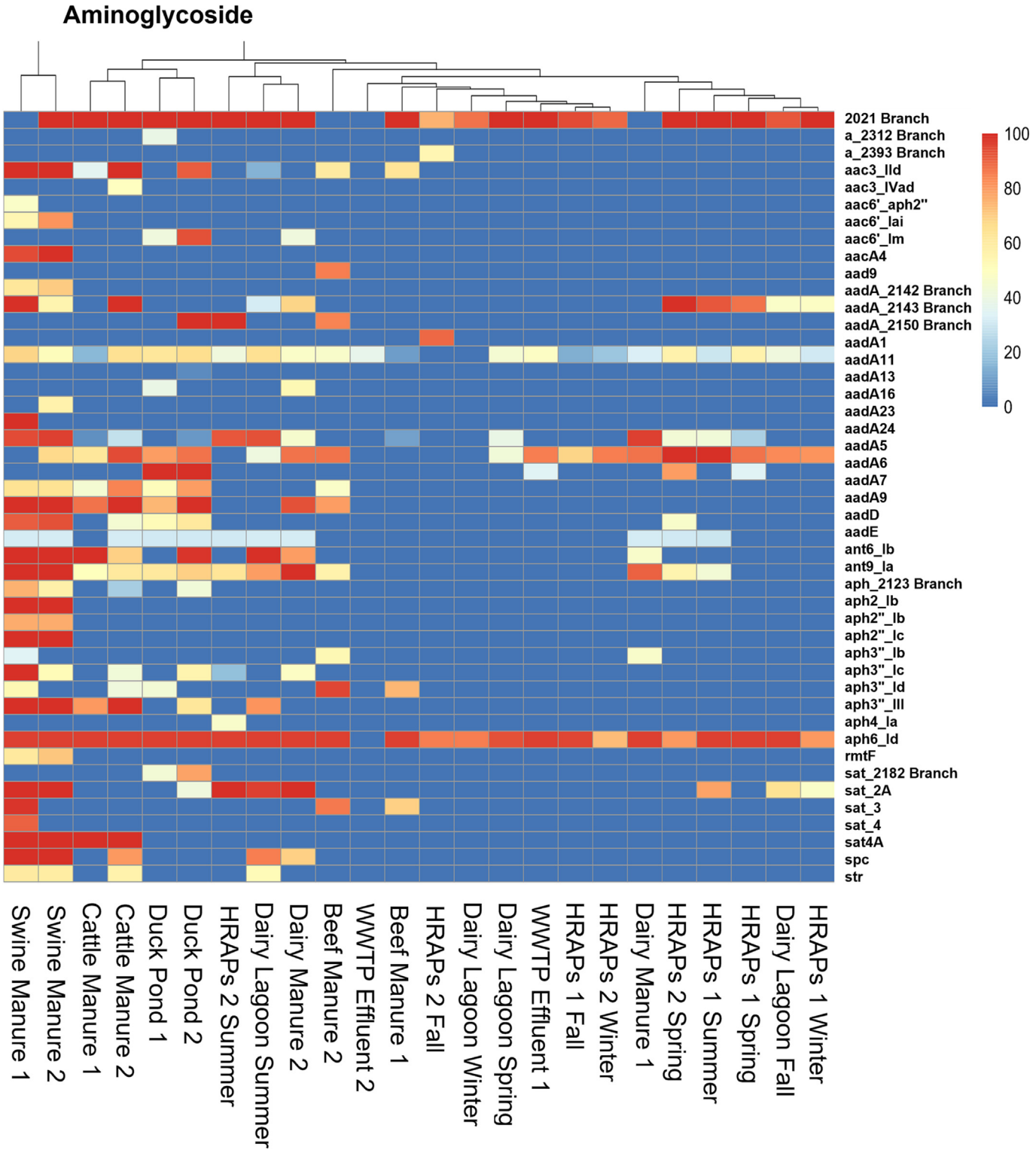
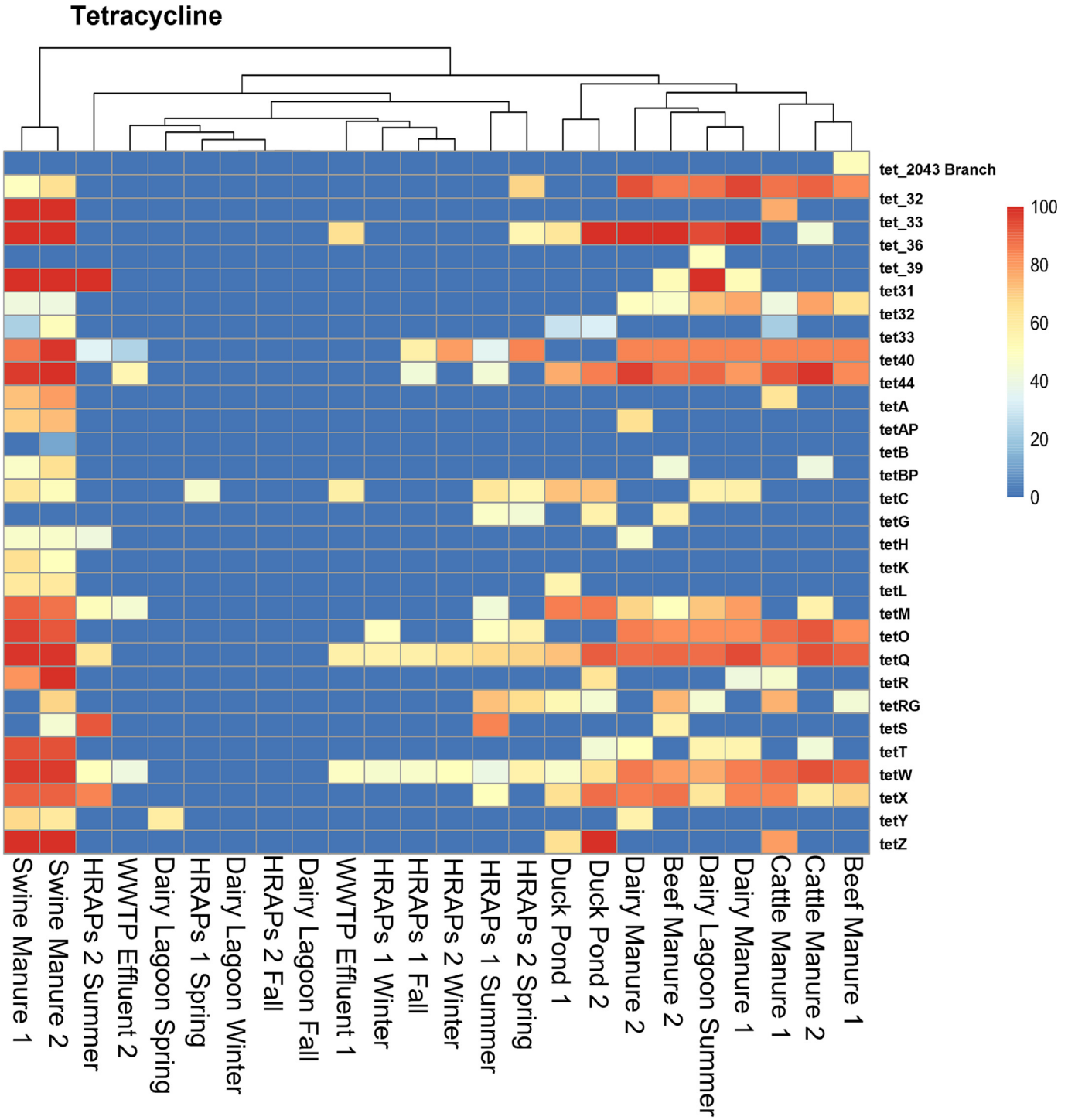


Fig. 4. Reservoirs of antibiotic resistant genes (ARGs) across the samples. ARGs present in the samples belonged to a core resistome comprised of four main resistance classes: panel A: Aminoglycosides (40–55 %), panel B: Tetracyclines (30–45 %), panel C: Beta-Lactam-resistance (20–35 %), panel D: Macrolides (18–30 %), panel E: multi-drug resistant group (MDR) and efflux pumps, and panel F: other additional and unclassified ARGs such as Fosfomycin, Bacitracin-resistance, Trimethoprim, Nitroimidazole.



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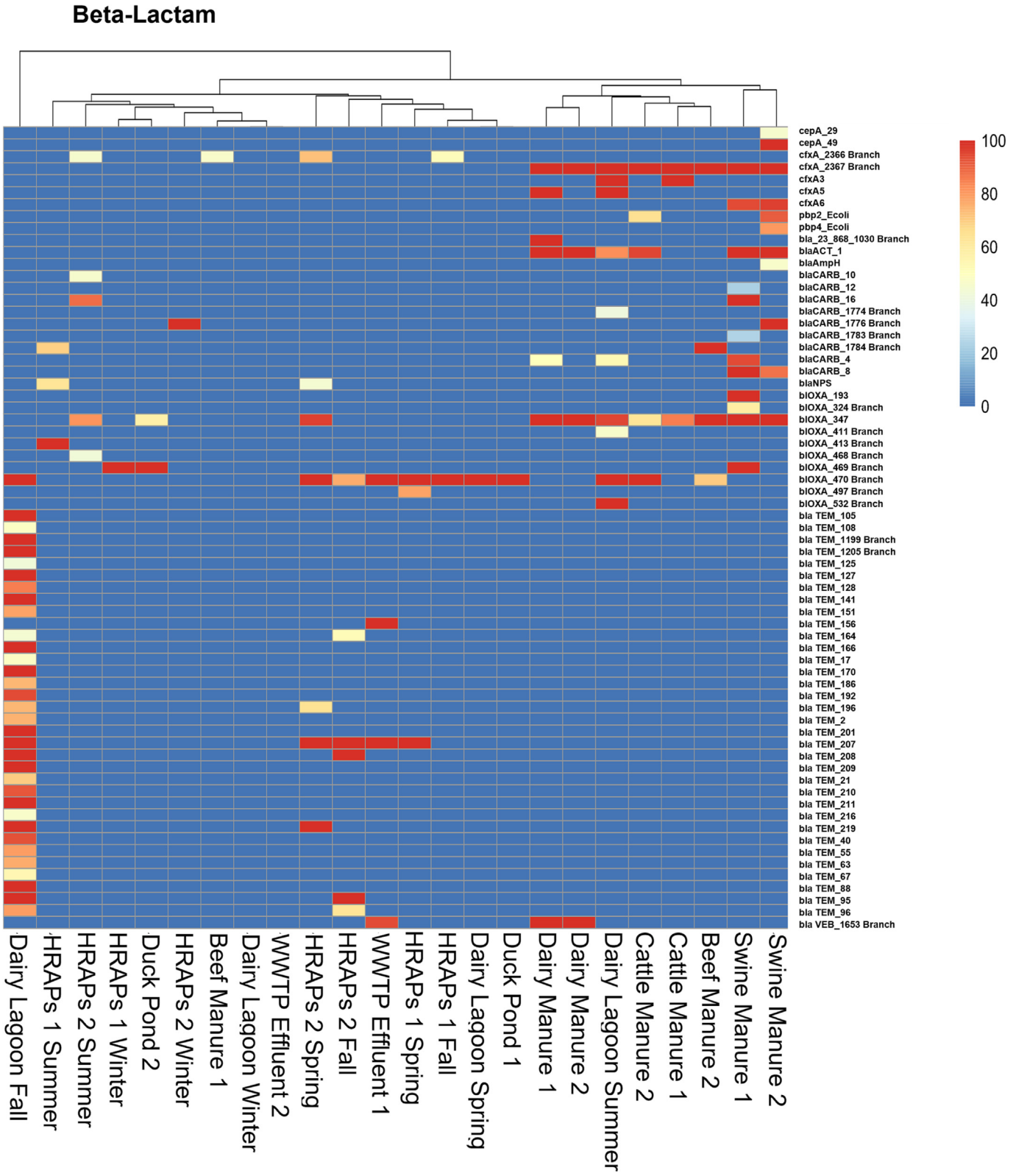
diversity index based on InvSimpson (Fig. S2A) and Chao1 test (Fig. S2B) suggests that bacterial diversity was not significantly different from each other ($P = 0.05$) but varied among various microbiome sources, with the lowest diversity associated with cattle manure, dairy manure, and dairy lagoon effluent (Fig. S2A).

3.1. Reservoirs of virulence factor genes (VFGs)

The quality-filtered reads from 24 samples (microbiomes) were individually mapped to the virulence factor gene database (CosmosID's curated database) to identify reservoirs of VFGs. The animal manure from swine, cattle, beef, dairy, and duck harbors many VFGs among their microbiomes. The VFGs from swine manure microbiomes were significantly ($P = 0.03$) higher in diversity than HRAPs and dairy lagoon effluent (0.048) based on the InvSimpson diversity test (Fig. 2. A), but not significantly different from other samples. Also, Chao1 diversity was significantly higher in swine manure samples ($P = 0.011$) than HRAPs, but not any other samples sources (Fig. 2B). A total of 24 and 28 VFGs in swine manure microbiome were recorded (Fig. 2C), and this was followed with the summer dairy lagoon effluent, duck pond, and dairy manure. One of the most dominant



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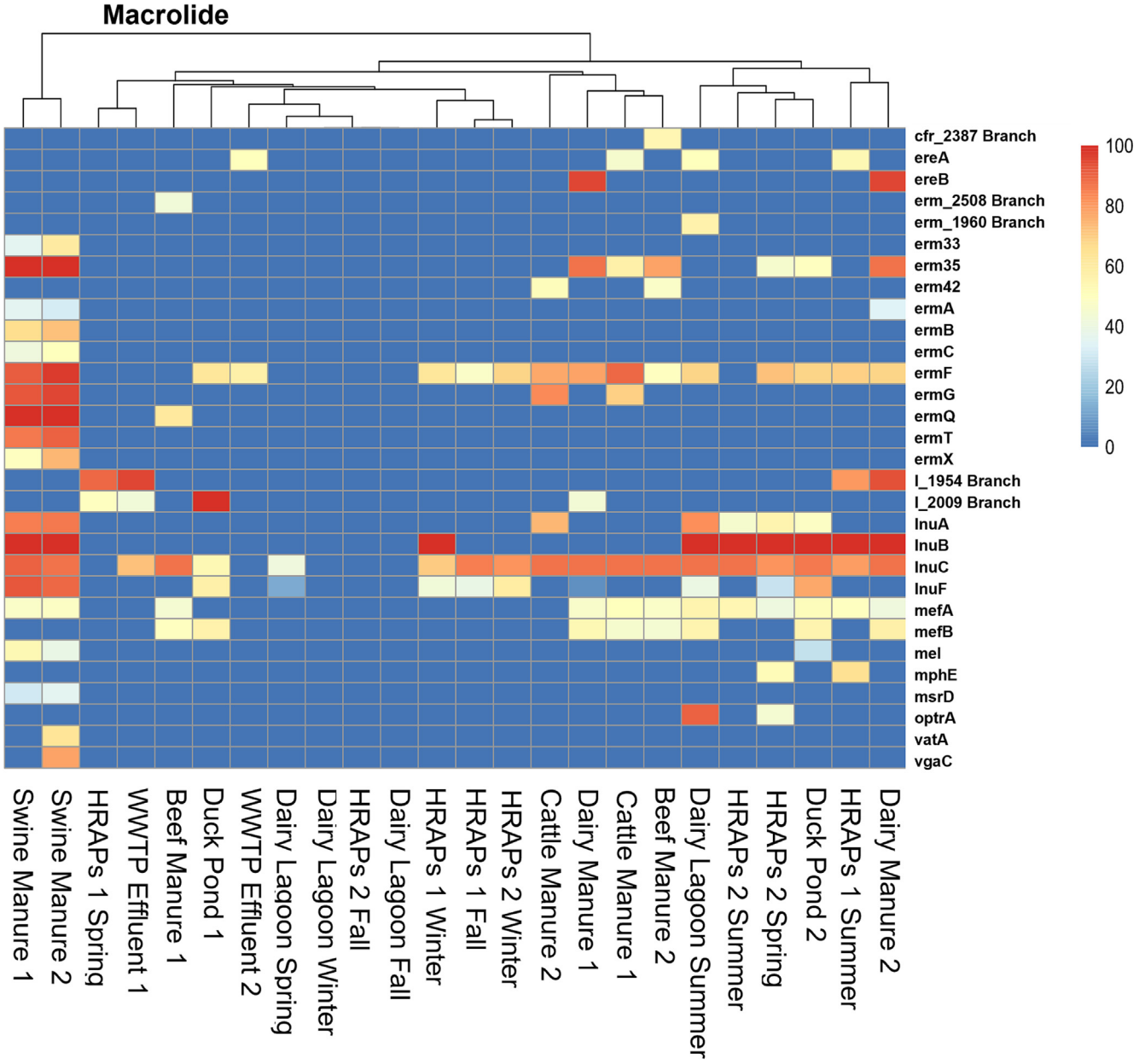
VFGs was bmhA with frequencies in summer DLE, swine manure, duck pond, beef, cattle, and dairy manure. This VFG was not identified in any of the HRAPs. Another VFG, tetQ, was identified in 18 out of the 24 samples, while tniA and tniB showed high occurrences in most of the samples. Altogether, the frequency of VFGs was highest in all the animal samples.

More than 50 % of the VFGs that the 24 microbiomes harbor were phyletically affiliated with two bacteria, *Bacteroidetes fragilis* and *Enterobacter aerogenes* (Fig. 2 D–F). Other bacteria that contributed VFGs in the microbiome included *Campylobacter jejuni*, *Citrobacter freundii*, *Enterococcus faecium* and *faecalis*, *Escherichia coli*, and others, as shown in Fig. 2D–F.



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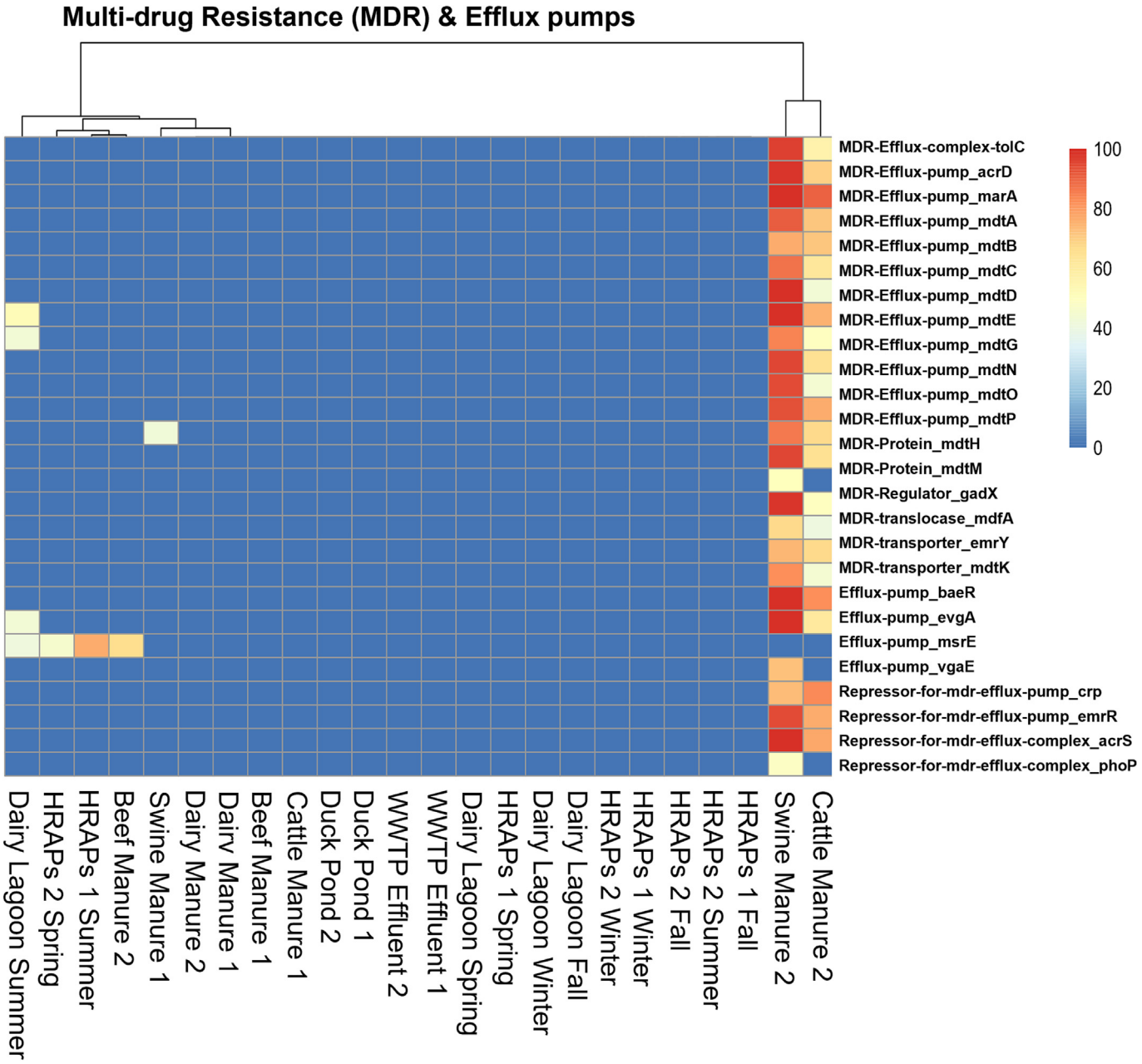
3.2. Occurrence and diversity of ARGs in metagenomes

A total of 177 ARGs were identified in the microbiome samples and classified into 29 different resistance mechanisms from the different metagenomes. These were associated with major resistance mechanisms such as antibiotic efflux pump, MDR-efflux-pump, MDR-regulators, transporter genes, antibiotic inactivation, antibiotic target protection, antibiotic target alteration, etc. The 29 antibiotic resistance mechanisms were found in all the samples. The resistance to aminoglycoside was the most prevalent mechanism based on the corresponding ARG presence in all the samples and their relative abundance, followed by tetracycline, Beta-lactam resistance, and macrolide-lincosamide-streptogramin (MLS) (Fig. 3A).

The ARG patterns were significantly different between swine manure and wastewater effluent ($P = 0.016$), swine manure and HRAPs ($P = 0.004$), and swine manure and dairy lagoon effluent ($P = 0.045$). No significant differences were found among the rest of the samples (Fig. 3B) based on the InvSimpson diversity test. Also, there were no significant differences in ARG diversity in the HRAPs during the four seasons based on the InvSimpson test. The Chao1 index of diversity (Fig. 3C) was used to test for richness, and swine manure was significantly more diverse than duck pond ($P = 0.010$), WWTP ($P = 0.003$), HRAPs ($P = 0.0001$), and dairy lagoon effluent ($P = 0.004$).

Of the 177 ARGs types frequently identified, 157, 68, 57, and 53 types were detected in swine, cattle, dairy, and beef, respectively, after comparative resistome profiling was conducted. This data showed that ARGs present in the samples belonged to a core resistome comprised of 4 main resistance classes: aminoglycosides (40–55 %), tetracyclines (30–45 %), beta-lactam-resistance (20–35 %), macrolides (18–30 %). The remaining major ARGs were part of the multi-drug resistant group (MDR), and efflux pumps or other additional and unclassified ARGs such as fosfomycin, bacitracin-resistance, trimethoprim, nitroimidazole, and others (Fig. 4). The most prevalent ARG class conferred resistance to aminoglycoside. The highest proportion of aminoglycoside resistance genes was observed in swine



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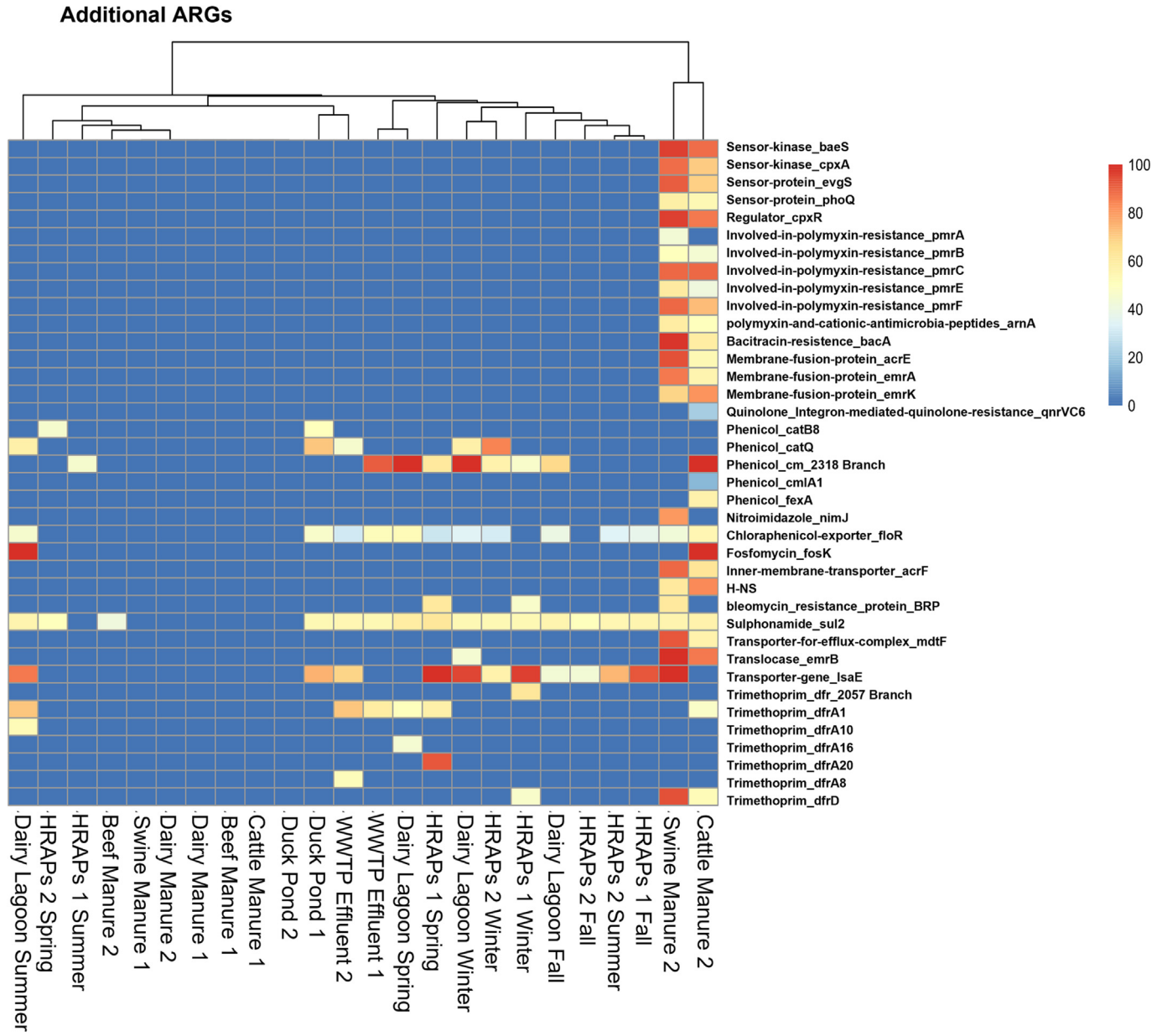
microbiome, followed by the duck pond, cattle manure, and dairy manure (Fig. 4A). In fact, aminoglycoside resistance genes were dominant in all the livestock manure. Interestingly, fewer aminoglycoside resistance genes were found in wastewater samples collected during the second summer of the sampling scheme. It should be noted that aminoglycosides 2024_branch gene, *aad*A1, *aad*A, *aad*A24, *aad*E, *aph*6, and *aad*A5 were the most prevalent genes in all the samples (Fig. 4A).

The ARGs conferring resistance to tetracycline (a total of 30 ARG types) were most prevalent in swine compared to other microbiomes, as noted above for aminoglycosides. >60 % of *tet* genes were associated directly with animal manure. The most prevalent *tet* genes were *tet*40, *tet*44, *tet*Q, *tet*W, *tet*O, *tet*M, and *tet*X (Fig. 4B). On the contrary, the least detected *tet* genes were *tet* 2043, *tet*32, *tet*39, *tet*A, *tet*B, *tet*K, and *tet*L. Most of the *tet* genes were detected in animal manure compared to very low prevalence in the HRAP fall and DLE winter samples. For the Beta-lactam resistance genes identified in the samples, bla_{CTX}, bla_{OXA}, and bla_{TEM} were the most prevalent (Fig. 4C). In fact, bla_{OXA} was present in almost all the samples. Beta-lactam resistance genes were more evenly distributed in most of the samples compared to tetracycline and aminoglycosides, which were more prevalent in animal manure. Still, the highest frequencies of beta-lactam resistances genes were found in the DLE fall sample and swine manure. Macrolide has about 24 resistance gene types in all the microbiome, and *inu*C was the most frequent gene type, followed by *inu*B, *inu*A, *erm*F, and *mef*A (Fig. 4D). Most of the macrolides detected were associated with swine manure. MDR and efflux pump were prevalent primarily in swine and cattle manure (Fig. 4E). Other additional ARGs detected were *sul*1, *sul*2, and *flo*R genes that were also more prevalent in animal manure (Fig. 4F).

Redundancy analysis (RDA) was performed to explore the correlation between bacterial communities in 24 metagenome samples with (a) ARGs abundance (Fig. 5A), and (b) virulence factor genes (Fig. 5B). Tables S2A and S3A summarizes the weight of the variable that makes the canonical



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axis of the RDA in Fig. 5A and B. The results indicate that 28.7 % (corrected to variables in ARGs) of the variance in bacterial diversity can be explained by ARGs. Among the ARGs only the Bacitracin resistance *bac*A gene (permutations 999, *p*-value 0.005) has a significant correlation with the bacterial community across the different samples (Table S2B). The RDA1 and RDA2 explained 52.36 % and 22.17 % of the total variance, respectively. The bacterial community and VFGs have a significant correlation (permutations 999, p-value 0.0036). After correction to the variable in VFGs, 94.87 % of the variance in the bacterial community can be explained by VFGs. A total of 16 VFGs have a significant correlation with the bacterial community across 24 metagenome samples. Based on p-value the VFGs were categorized into three groups, (a) *bmh*A, *exc*, IS942 (p-value 0.001), (b) *mob*A, *rte*A, *tet*Q, *kor*B, *tni*C (*P*-value < 0.01), and (c) int, *mob*A, *mob*C, *kle*E, *klu*A, *qac*E, *tni*A, *tni*B (*P*-value < 0.05). Table S3B summarizes the variance and p-value of VFGs. The RDA1 and RDA2 explained 40.9 % and 25.07 % of the total variance in the bacterial community.

3.3. Co-occurrence network analysis

Several properties for network analysis were calculated to describe the complicated patterns of the inter-relationship among ARGs subtypes, VFGs, and microbial taxa. Spearman's rank correlation coefficients were calculated among 29 ARG mechanisms and 38 VFGs for both manure samples (Fig. 6A) and dairy lagoon- HRAPS treatment samples (Fig. 6B). After the filtration process (Rho > 0.6 and *P*-value < 0.05), significant correlations were identified with network visualization which consists of 24 nodes (10 ARGs and 14 VFGs) and 69 edges for the manure (Fig. 7A) and 13 nodes (7 ARGs and 6 VFGs) and 18 edges for the dairy lagoon- HRAPS treatment (Fig. 7B). In the network analysis, nodes represent the objects of interest which are ARGs and VFGs. The nodes are colored based on ARGs and VFGs (green for ARGs, orange for VFGs). The edges- the connection between nodes represents strong Spearman correlation (absolute value of rank coefficient Rho > 0.6 and significant ($P < 0.05$)). Node size is weighted based on

the number of connections. Edges are weighted according to the correlation coefficient. Color of edge indicates a positive (blue) and negative (red) correlation. The network connectivity of ARGs shows strong correlation among several genes such as transporter-gene and regulator, efflux pump and Involved-in -polymyxin- resistance, aminoglycoside and beta-lactam, aminoglycoside *and* macrolide with VFGs (Figs. 6&7). A possible reason for these co-occurrences of ARG subtypes with VFGs may be that they are from the same microbial taxa or share the same environment. The strongest correlations among VFGs and ARGs were associated with aminoglycoside, Beta-lactam resistance, and Macrolide.

In addition, the network analysis was conducted to determine the correlation between ARGs and microbial taxa (Phyla) in manure (Fig. 7A) and dairy lagoon- HRAPS treatment (Fig. 7B). Twenty-nine ARGs and 47 phyla were employed to generate Spearman's correlation ranks. The correlations obtained after filtration (Rho > 0.6 and *P*-value < 0.05) were used as inputs for Fig. 7A&B. The network (Fig. 7A) consisted of 49 nodes (15 ARGs and 34 phyla) and 73 edges, while Fig. 7B comprised 19 nodes (6 ARGs and 13 phyla) and 14 edges. For the correlation between ARGs and VFGs, the average number of edges per node was 5.75, 2.77 for Fig. 6 A&B and 2.98, 1.47 for Fig. 7 A&B. The average path lengths were 1.60, 3.00, 3.35, and 2.04 for the same network above. The clustering coefficients were 0.67, 0.43, 0.0, and 0.0 while the modularity indexes were 0.358, 0.415, −0.403, −627.862 for these figures. These results indicated that Fig. 6A&B have modular structures of highly interconnected subgroup (ARGs and VFGs), while Fig. 7A&B have more clusters of ARGs, and bacterial phyla connected to multiple non-interconnected nodes.

3.4. Metagenome assembled genome (MAGs) analysis

A total of 2665 bins were extracted from 24 metagenomes. Quality assessment of the bins with CheckM v1.0.18 yielded each bin's taxonomic placement, completeness, and contamination. Data provided in the link (https://github.com/asbhattacharjee/Animal_Microbiome/blob/main/Bins_Extracted_From_All_Metagenomes.xlsx). One thousand and five bins with contamination below 5 % and completeness >30 % were used to assess taxonomic affiliation, gene prediction, and annotation. Five hundred and forty-four out of 1005 bins had >70 % completeness. These 544 bins were classified as metagenome-assembled genomes (MAGs). Metabolic reconstruction by prediction and functional annotation of genes for each MAG revealed their role in each microbiome. Three hundred and forty-four MAGs had genes that confer resistance to antibiotics, and KEGG classified some of these genes with drug resistance mechanisms. The most prevalent antibiotic resistance mechanism is presented in Fig. 8. KEGG Metagenome assembled genome (MAGs) analysis showed that the most prevalent drug resistance mechanisms were associated with carbapenem resistance, MDR, and efflux pump AdeABC, which was present in almost all the samples. This was followed by the bla resistance system in 13 samples present in the secondary treated wastewater effluent. Putting together all the mechanisms, carbapenem, MDR, and efflux pump were the most

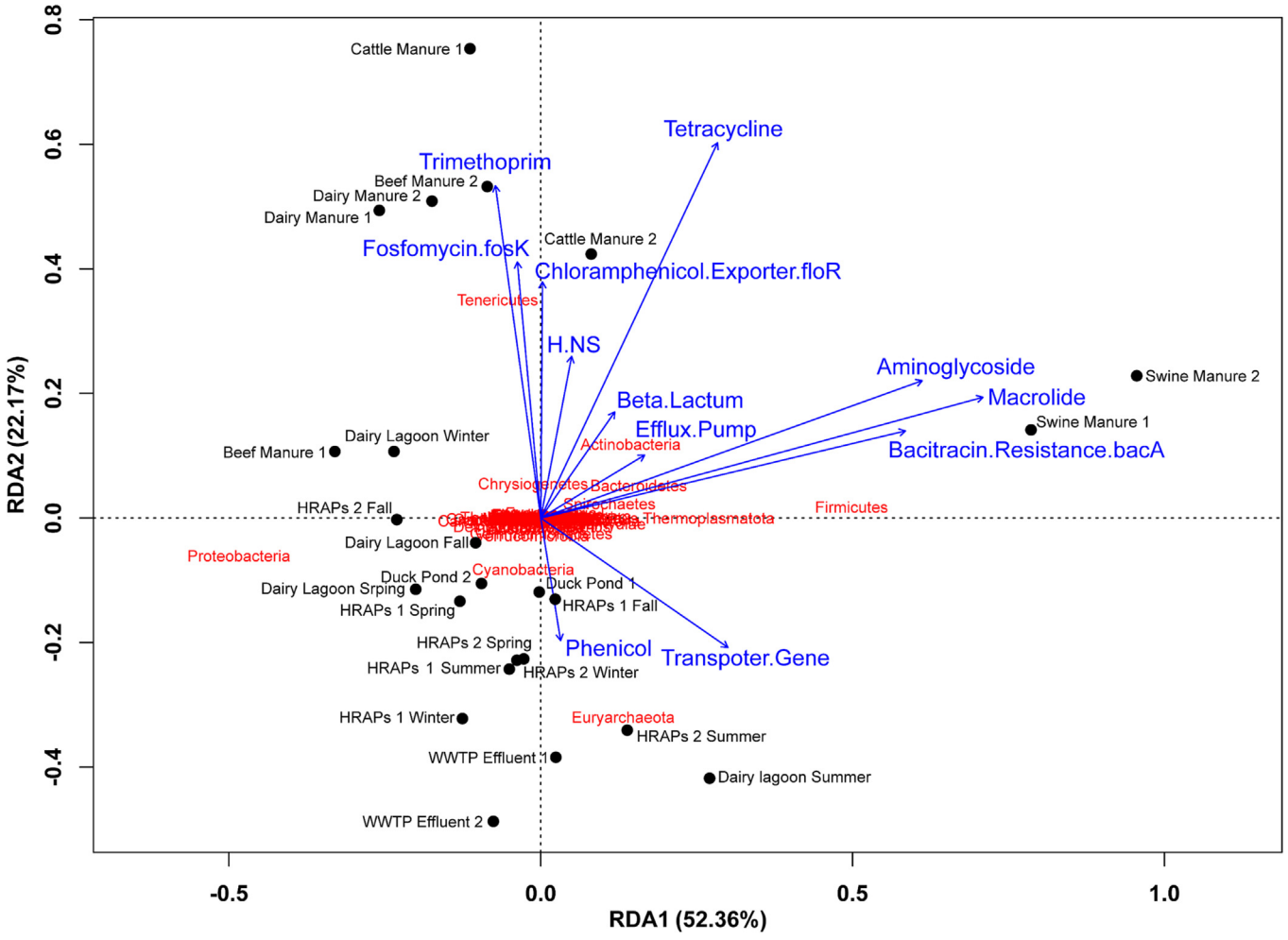
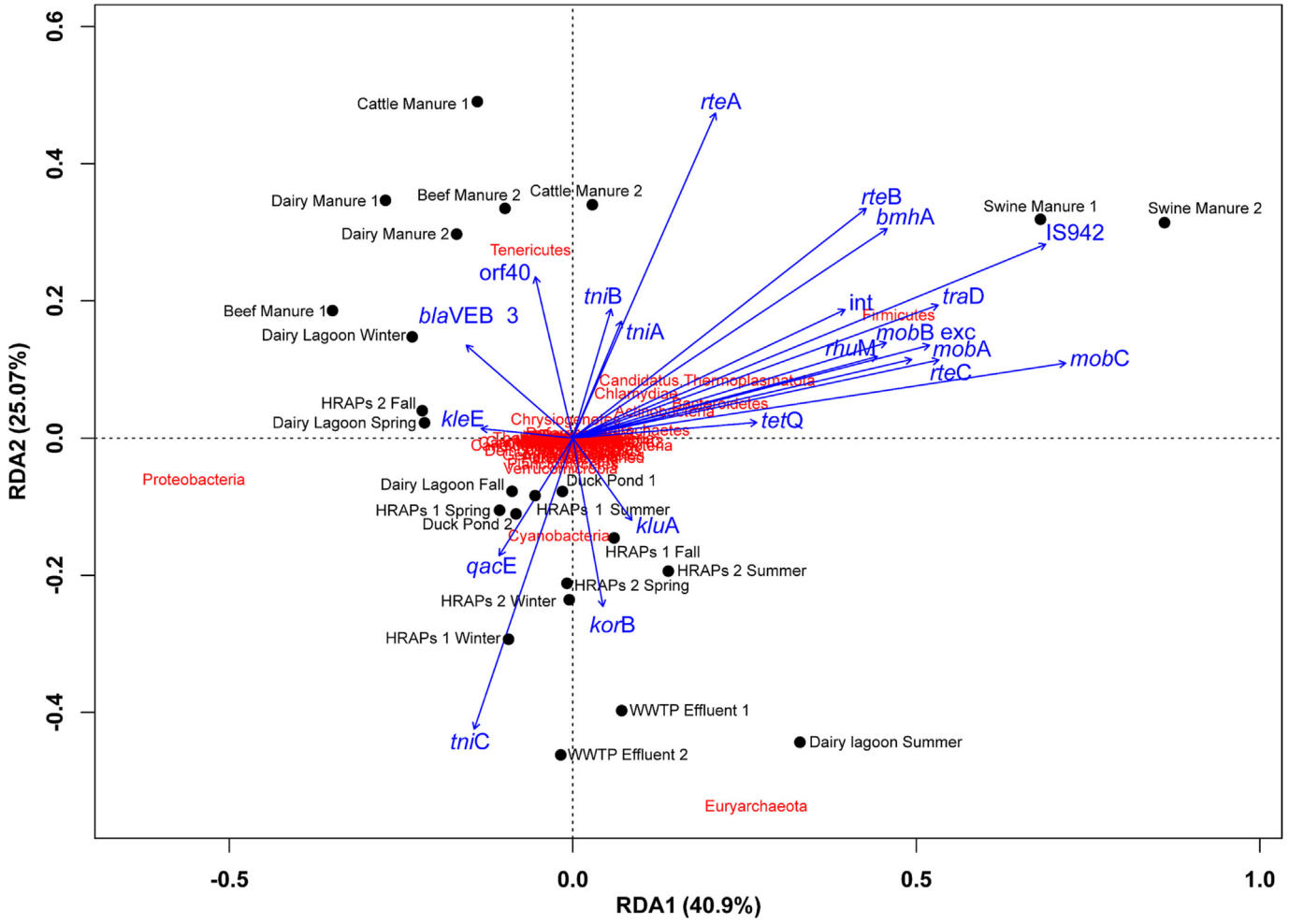


Fig. 5. Redundancy analysis of 24 microbiome. The redundancy analysis (RDA) antibiotic resistance genes (ARGs), and bacterial communities in 24 metagenome samples (A). The redundancy analysis (RDA) virulence factor genes (VF), and bacterial communities in 24 metagenome samples (B).



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dominant mechanisms observed from these samples. The 344 MAGs have genes that impact partial (minimum 60 % of the mechanism pathway) and complete resistance to antibiotics. Some of these MAGs are probable antibiotic-resistant bacteria (ARBs) in the 24 metagenomes.

3.5. Metabolic potential of high-quality MAGs in HRAP in N cycle

The metagenome-assembled genomes of bacterial species of the HRAP microbiome were metabolically reconstructed for delineating their role in nitrogen cycling. A total of 40 MAGs (>70 % completeness) and three bins (>30 % completeness) with genes for nitrogen cycling were recovered from ten metagenomes in the four HRAPs fed with DLE (Fig. 9). The N uptake rate in the HRAP varied across seasons and peaked during summer (Schwartz et al., 2021). During summer, *Bacteroidales* was involved in dissimilatory nitrate reduction (DNRA) in the dairy lagoon. DNRA is the reduction of nitrate to ammonia by bacteria. With *Bacteroidales* as the only MAG extracted from dairy lagoon metagenome during summer suggests the lagoon might have had high ammonia concentrations (Schwartz et al., 2021). Nitrification bacteria *Flavobacterium* and *Pseudomonas fluorescens* were recovered from HRAP summer metagenomes. These bacteria have the potential to oxidize ammonia to nitrite and to nitrate in the HRAP during the process of nitrification. During fall, the dairy lagoon hosts bacteria, *Aeromonas Salmonicidia*, *Rubrivivax*, and *Pararheinheimera* (Fig. 8) that are involved in assimilatory nitrate reduction (ANR). These bacteria reduce nitrate to ammonia. The ammonia-rich dairy lagoon effluents are treated in HRAPs. In fall the HRAP also harbors bacteria that are assimilatory nitrate reducers, *Saprospiraceae OLB9*, *Sediminibacterium*, *UBA2357 sp016790005*, JAGLBJ01, *Tabrizicola*, and *Paludibacter*. Both ANR and denitrification lead to production of ammonia in HRAPs that can be used by algae. For the winter and spring seasons, a similar trend was recorded where bacteria involved in ANR and denitrification were identified in the dairy lagoon and HRAP microbiome. These bacteria provide ammonia to algae in the HRAPs for growth, N uptake, and efficient treatment. In addition, a denitrifier *Algoriphagus* resides in the HRAP microbiome. A total of 19 MAGs in Fig. 9 are antibiotic-resistant bacteria that are involved in nitrogen cycling. However, RDA analysis of nitrogen cycle and antibiotic-resistant bacteria showed no statistically significant effects of ARGs on nitrogen cycle in this study, except some ARBs were involved in nitrogen cycling (Fig. S4A&B).

4. Discussion

Some bacterial species in manure and wastewater carry ARGs and VFGs that are disseminated through horizontal gene transfer to other bacteria (He et al., 2020). In this study, *Proteobacteria* was the dominant phyla in all the livestock and wastewater samples but one, swine manure. Firmicutes were dominant in swine manure. This agrees with Wan et al., 2021, which showed *Proteobacteria* and *Chloroflexi* as the dominant phyla in sheep and dairy manure, while *Firmicutes* dominated in swine and chicken manure. *Proteobacteria, Bacteroidetes, Firmicutes*, and *Actinobacteria,* represented the possible bacterial hosts carrying ARGs in most manure and manure-amended soils (Forsberg et al., 2014; Su et al., 2015). These authors

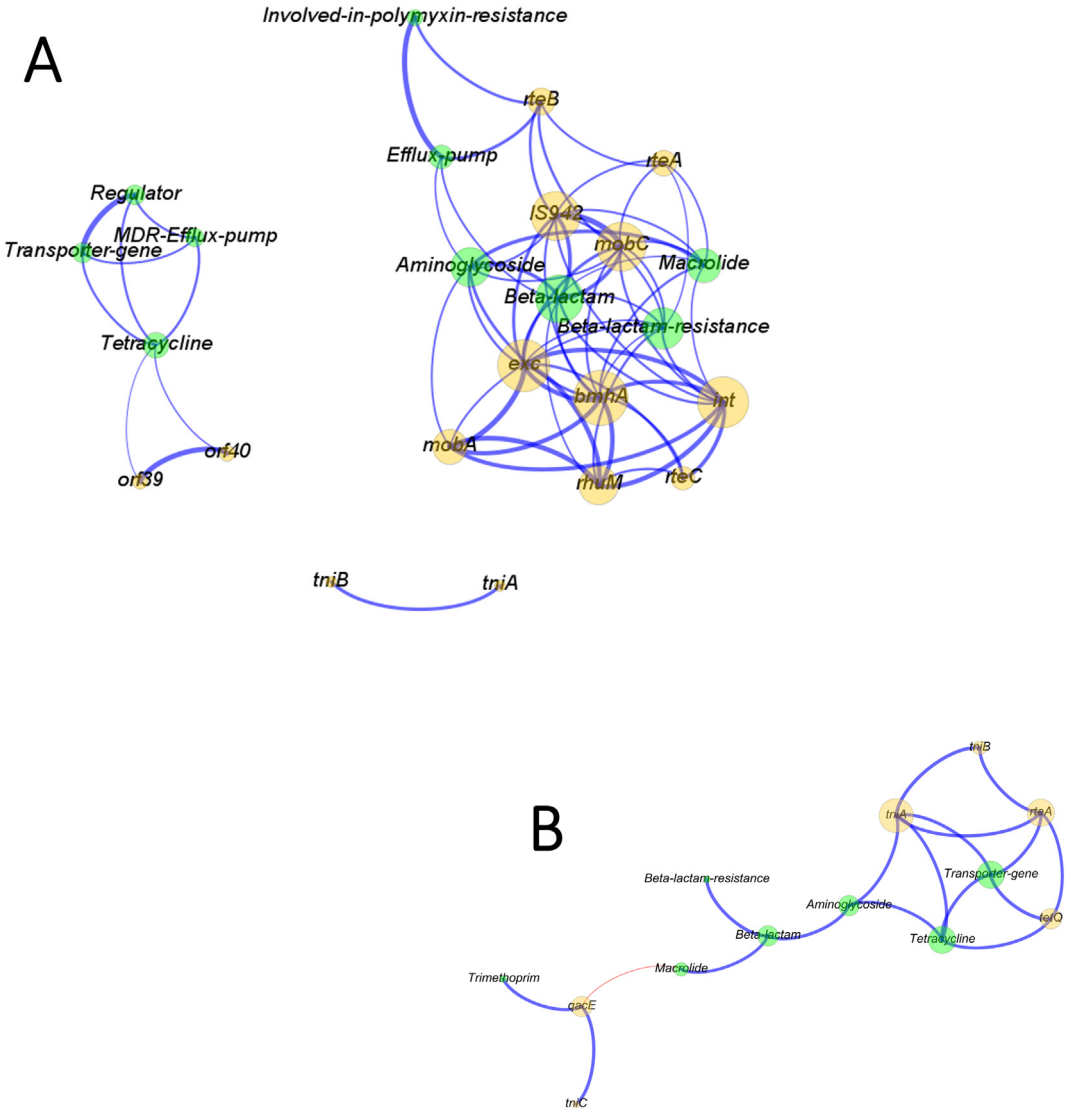

Fig. 6. Network analysis revealing the co-occurrence patterns between antibiotic resistance genes (ARGs) and virulence factor genes (VFGs) in (A) Manure samples and (B) Dairy Lagoon-HRAP treatments. The nodes are colored based on ARGs and VFGs (green for ARGs, orange for VFGs). A connection between nodes represents strong Spearman correlation (absolute value of rank coefficient Rho > 0.6 and significant ($P < 0.05$)). Node size is weighted based on the number of connections. Edges are weighted according to the correlation coefficient. Color of edge indicates a positive (blue) and negative (red) correlation.

suggested that feeding methods can determine both chemical and biological properties of livestock manure. However, diversity index based on InvSimpson and Chao1 test suggest that bacterial diversity was not significantly different from each other ($P = 0.05$) but varied among different microbiome sources (Fig. 1). However, VFGs from swine manure was significantly ($P = 0.03$) higher in diversity than HRAPs and dairy lagoon effluent (Fig. 2). Similarly, ARGs from swine manure was significantly different from that of WWTP, HRAPs, and DLE (Fig. 3). The spread of ARB and ARGs from livestock production and WWTP is a major threat to human and soil health by facilitating the dissemination of ARGs to arable soil and edible crops (Zalewska et al., 2021). However, both liquid and solid manure from livestock production are major sources of nutrients to agricultural production. Therefore, there is a need to find the balance of when and how to use these materials for agricultural production and to determine drivers of contaminations from these materials to the environment. Application of manure and treated wastewater can stimulate the proliferation of other soil bacterial that are not native to the soil before manure application resulting in changes in soil resistome (Forsberg et al., 2014; Su et al., 2015). This can affect the diversity and prevalence of ARGs (Chen et al., 2016). Also, nutrient input from manure can stimulate the growth of the soil bacterial community resulting in a bloom of some native antibiotic-resistant bacteria (ARB) (Udikovic-Kolic et al., 2014; Hu et al., 2016).

Despite some of the negative impacts, manures continue to be regarded as valuable agricultural resources, because they are important sources of plant nutrients and are well known to improve soil physical and biological properties through the addition of organic matter. That value may result from improvements in soil quality, increases in yield, and replacement of commercial nutrient required for crop production. With today's technology, manure can be used more efficiently and, in more ways, than ever, which should mitigate many of the environmental impacts that result when manure is treated as a waste. For instance, the bioconversion of animal manure to animal feed, fertilizer, or soil amendments, composting and vermicomposting are some of the ways manure may became a useful product. Furthermore, converting manure to biochar may be a feasible way to

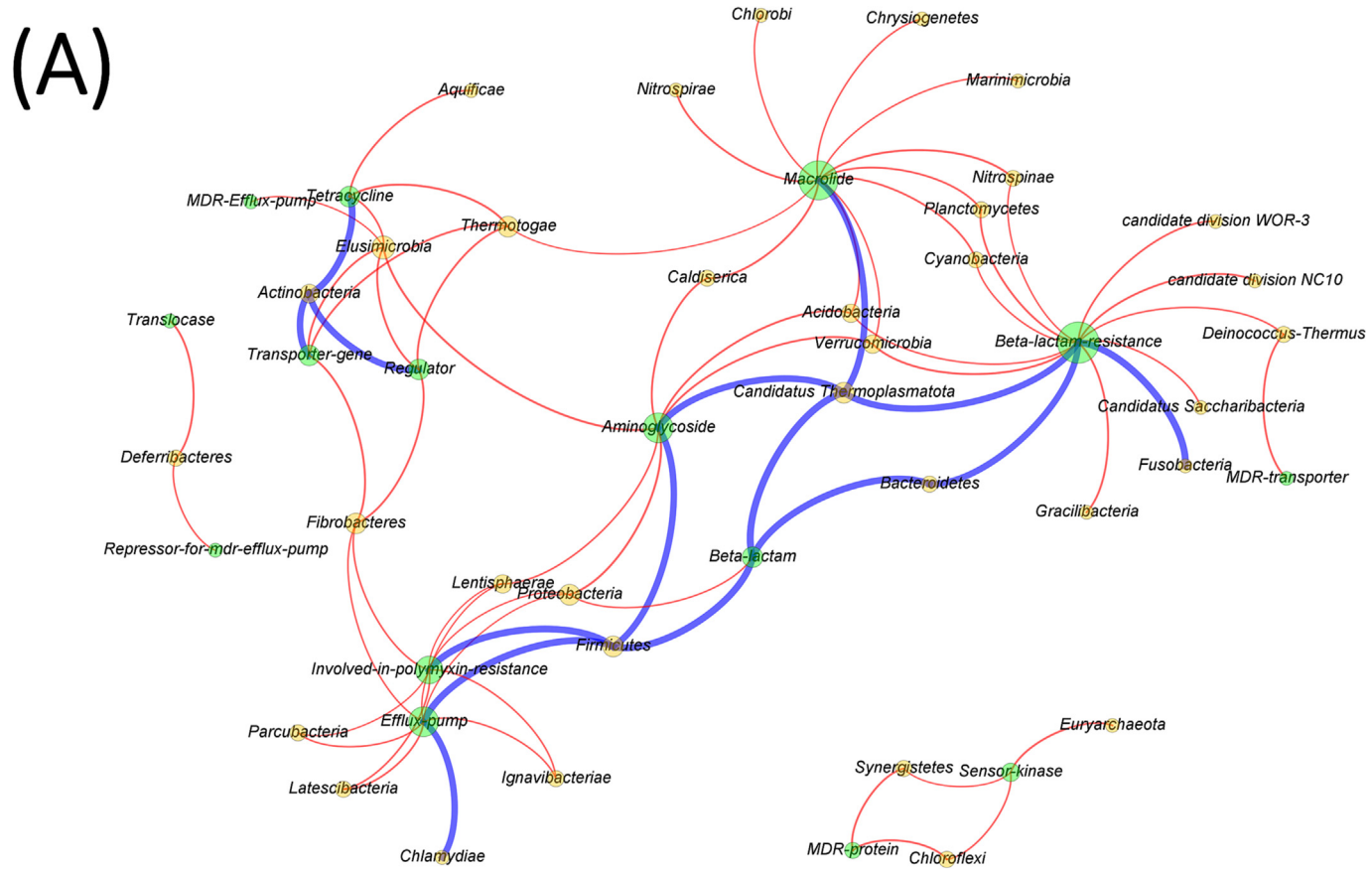

Fig. 7. A: Network analysis revealing the co-occurrence patterns between antibiotic resistance gene (ARG) subtype and microbial taxa (Phylum) in (A) Manure samples and (B) Dairy Lagoon-HRAP treatments. The nodes are colored based on ARGs and microbial taxa (green for ARGs, orange for Phylum). A connection between nodes represents strong Spearman correlation (absolute value of rank coefficient Rho > 0.6 and significant ($P < 0.05$)). Node size is weighted based on the number of connections. Edges are weighted according to the correlation coefficient. Color of edge indicates a positive (blue) and negative (red) correlation. **B:** Network analysis revealing the co-occurrence patterns between ARG subtype and microbial taxa (Phylum) in (A) Manure samples and (B) Dairy Lagoon-HRAP treatments. The nodes are colored based on ARGs and microbial taxa (green for ARGs, orange for Phylum). A connection between nodes represents strong Spearman correlation (absolute value of rank coefficient Rho > 0.6 and significant ($P < 0.05$)). Node size is weighted based on the number of connections. Edges are weighted according to the correlation coefficient. Color of edge indicates a positive (blue) and negative (red) correlation.

control ARG pollution caused by manure but still hold partial nutrients of manure that contribute to soil quality improvement.

4.1. Co-occurrence between ARG subtypes, VFGs, and bacterial taxa

The most representative co-occurrent patterns between ARGs and VFGs were among aminoglycoside, beta-lactam resistance, and macrolide and five VFGs *IS942, mobC, exc, bmhA,* and *int* suggesting that these ARGs and VFGs might be carried by the same bacterial species, and these were positively correlated. The strong positive correlation may suggest the presence of MGEs such as plasmids that can aid in the transfer of ARGs and VFGs (Uhlemann et al., 2014; Wright et al., 2015; Cloeckaert et al., 2017). MGEs are known to co-select for ARGs and VFs in bacteria (Forsberg et al., 2014; Prieto et al., 2016), as well as in the dissemination when equipped on the proper transfer machinery (Kaito et al., 2013; Penades et al., 2015). Since the recovery of ARGs and VFGs from the dairy lagoon- HRAPs treatments were much lower than from the manure samples, a smaller network between ARGs and VFGs was observed (Fig. 6B). From this network, we observed that the most connected nodes for ARGs were tetracycline, transporter-gene, aminoglycoside, and Beta-lactam while the most connected node for VFGs were *tnA* following by *rteA* and *tetQ*. Interestingly, one negative correlation was observed between macrolide and *qaE* in this network. The general observation was the overall reduction in ARGs and VFGs in the algal based HRAPs system. This may be due to the low pH and high temperature in the ponds and the effect of sunlight coupled with high dissolved oxygen in the HRAPs that shifted dominant bacteria species to non-pathogenic thermoacidophiles (Schwartz et al., 2021; Ibekwe et al., 2016).

Network correlation between ARGs and bacterial taxa (Fig. 7A) shows that *Firmicutes*, *Candidatus Themoplasmatota*, *Actinobacteria*, and *Bacteroidetes* were strongly correlated with most of the ARG subtypes. For example, *Firmicutes* was highly correlated with multiple ARGs including Aminoglycoside, Beta-lactam, Efflux-pump, *and* Involved-in-polymycin- resistance. This is consistent with our observation of the high abundance of *Firmicutes* and antibiotic resistances in swine manures (Fig. 6A&B). *Actinobacteria* have strong correlation with transporter-gene, regulator, and tetracycline. While other phyla such as *Proteobacteria*, *Elusimicrobia*, *Acidobacteria* have negative correlations with multiple ARG subtypes. For instance, *Proteobacteria* negatively correlated with Efflux-pump, Involved- in -polymycin-resistance, aminoglycoside, and beta-lactam. Several ARGs such as beta-lactam resistance and aminoglycoside had strong negative correlation with multiple phyla. However, in the DLE treatment train (Fig. 7.B), the

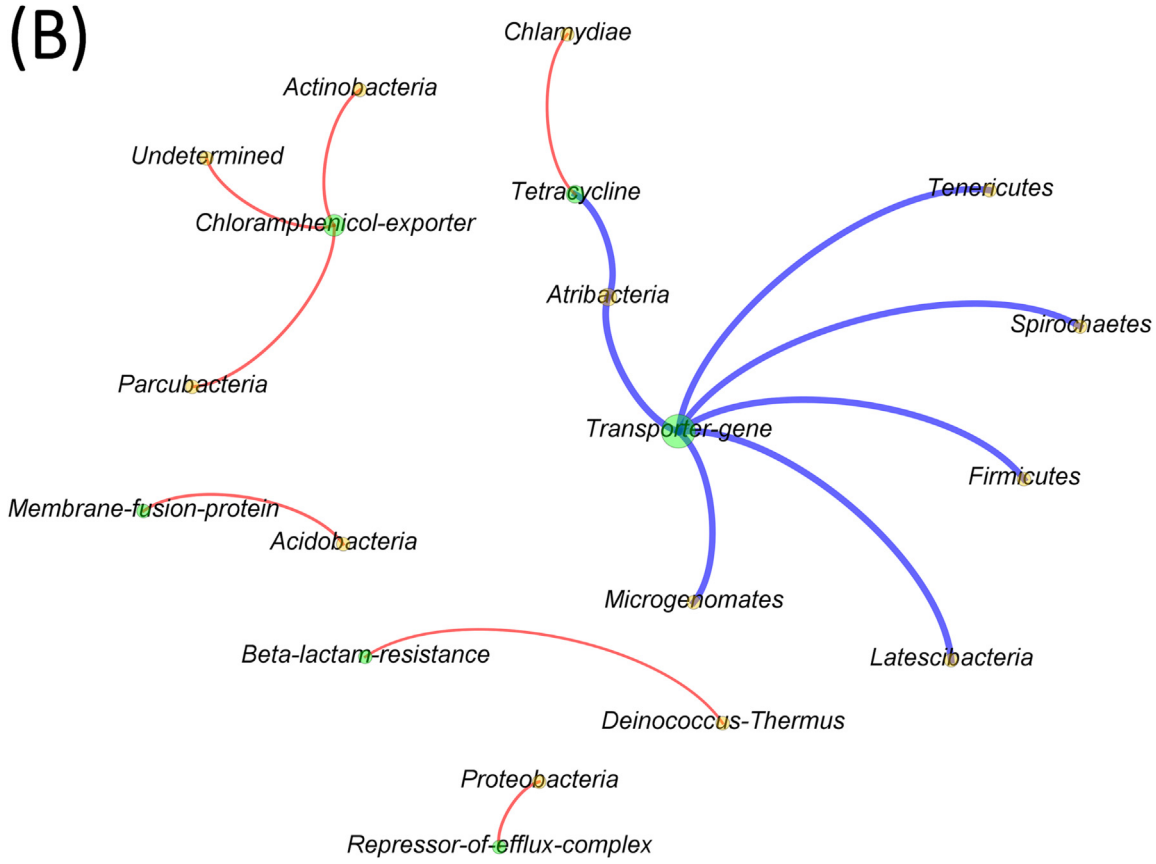

Fig. 7 (*continued*).

transporter-gene has a strong positive correlation for multiple taxa such as *Atribacteria, Tenericutes*, *Spirochaetes*, *Firmicutes*, *Latescibateria*, and *Microgenomates*. While chloramphenicol-exporter had a negative correlation with variety of taxa.

4.2. Nitrogen cycling in Dairy Lagoon and HRAP

Manure from livestock is major sources of nitrogen and phosphorous to agricultural land. On land application of manure, excess N and P leach from soil into freshwater reservoirs and or percolate to groundwaters. Excess N causes algal blooms in water reservoirs. High-rate algal ponds (HRAP) can treat manure-derived nutrients (N and P). In this study, bacterial metabolism dominated the nutrient removal process in HRAP. The normalized abundance of *nrfA*, the marker gene for dissimilatory nitrate reduction to ammonium (DNRA), was approximately four times higher in HRAPs than in the DLE. Regarding denitrification, which competes with DNRA for dissimilatory nitrate/nitrite reduction, the trend was less clear. The HRAP is a unique system for N removal from DLE, and our data suggest that this process may involve different nitrogen related enzymes and bacterial species. During our study, *Bacteroidales* was involved in dissimilatory nitrate reduction (DNRA) in the HRAP because DNRA was higher. It has been reported that denitrification rates may be inhibited by antibiotics treatments, and synergistic inhibition effect has been observed for multiple antibiotics exposure (Yin et al., 2017). These authors suggested that different classes of antibiotics may affect N_2O release rates differently. They concluded that multiple antibiotics exposure may lead to stimulatory effect, and that the abundances of denitrifying functional genes were inhibited by multiple antibiotics exposure due to the antimicrobial properties, and different inhibition on denitrifiers were the major mechanism for the variations of N_2O release rates.

In conclusion, this study used shotgun metagenomics to analyze the resistome of different livestock manure and secondary treated wastewater to determine the reservoirs of ARB, ARGs, and VFGs as well as to track their dissemination from DLE through four high-rate algae ponds (HRAPs) to quantify the effect of HRAPS on bacterial diversity and ARGs. Significantly higher diversity and abundance of ARGs were observed in swine manure, including those resistances to clinically critical important antibiotics. *Bacteroidetes fragilis* and *Enterobacter aerogenes* were the dominant hosts of VFGs, and co-occurrence patterns were between several genes such as transporter-gene and regulator, efflux pump and involved-in-polymyxin- resistance, aminoglycoside, beta-lactam, and macrolide with VFGs and bacterial taxa such as *Firmicutes*, *Candidatus* Themoplasmatota, *Actinobacteria*, and *Bacteroidetes*. These four phyla were the dominant hosts of most of the ARGs. Metagenome-assembled genome (MAGs) analysis showed that the most prevalent drug resistance mechanisms were associated with Carbapenem resistance, MDR, and efflux pump. This study showed the abundance of ARGs and VFGs in livestock manure which may serve as an important reservoir for the dissemination of ARGs to the surrounding farmlands and the environments.

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CRediT authorship contribution statement

Abasiofiok M. Ibekwe, conceive and write.
Ananda S. Bhattacharjee, data analysis.
Duc Phan, data analysis.
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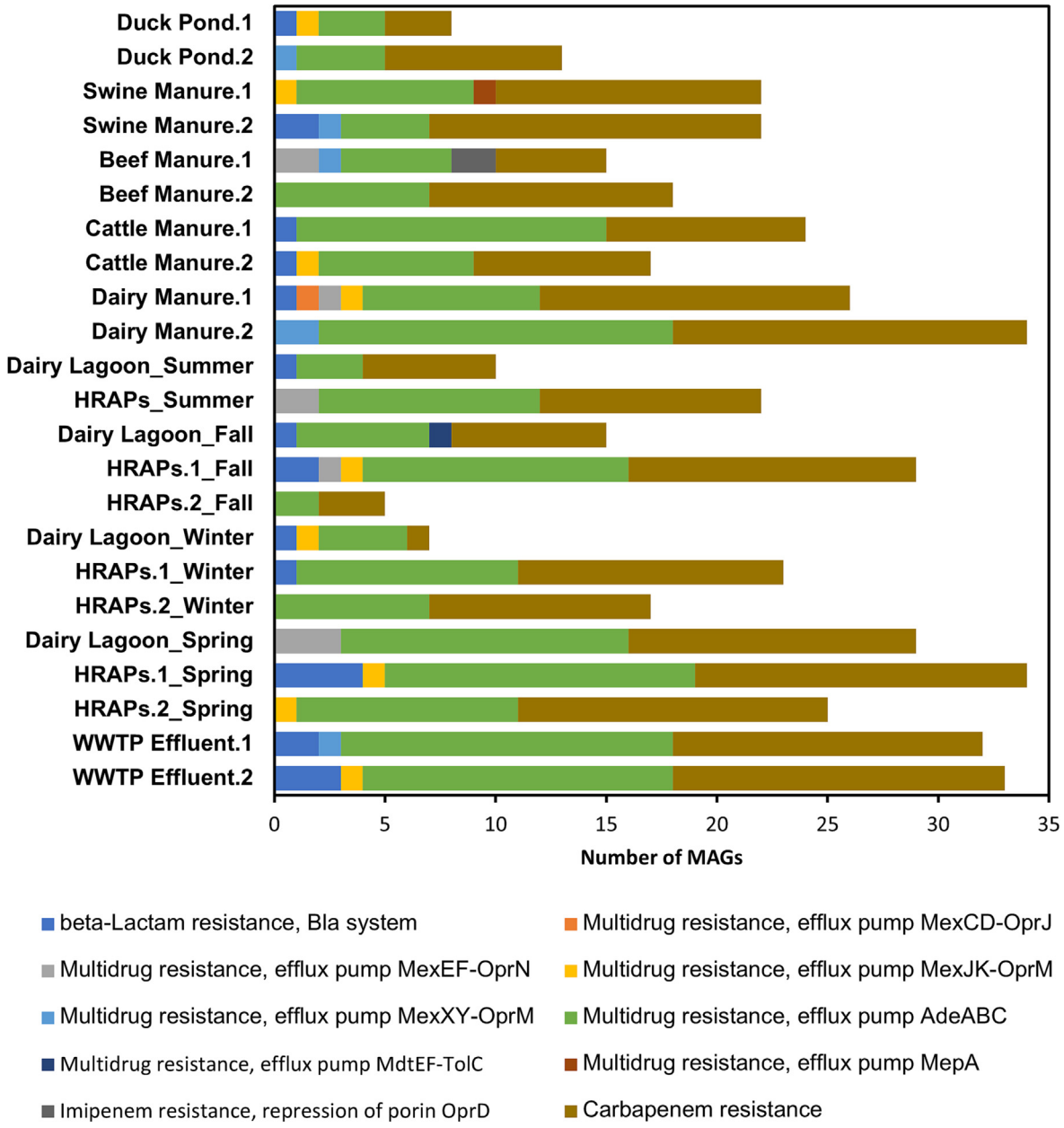

Fig. 8. Antibiotic resistance mechanism of metagenome-assembled genomes (MAGs) of all samples. The figure only represents the number of antibiotic resistance mechanism pathways that are >60 % complete with antibiotic resistance genes. MAGs are >70 % completion and <5 % contamination.

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Data availability

There is a link to data, stastistics, and MAG. We can also provide the link upon request.

Declaration of competing interest

All authors declare no conflict of interest.

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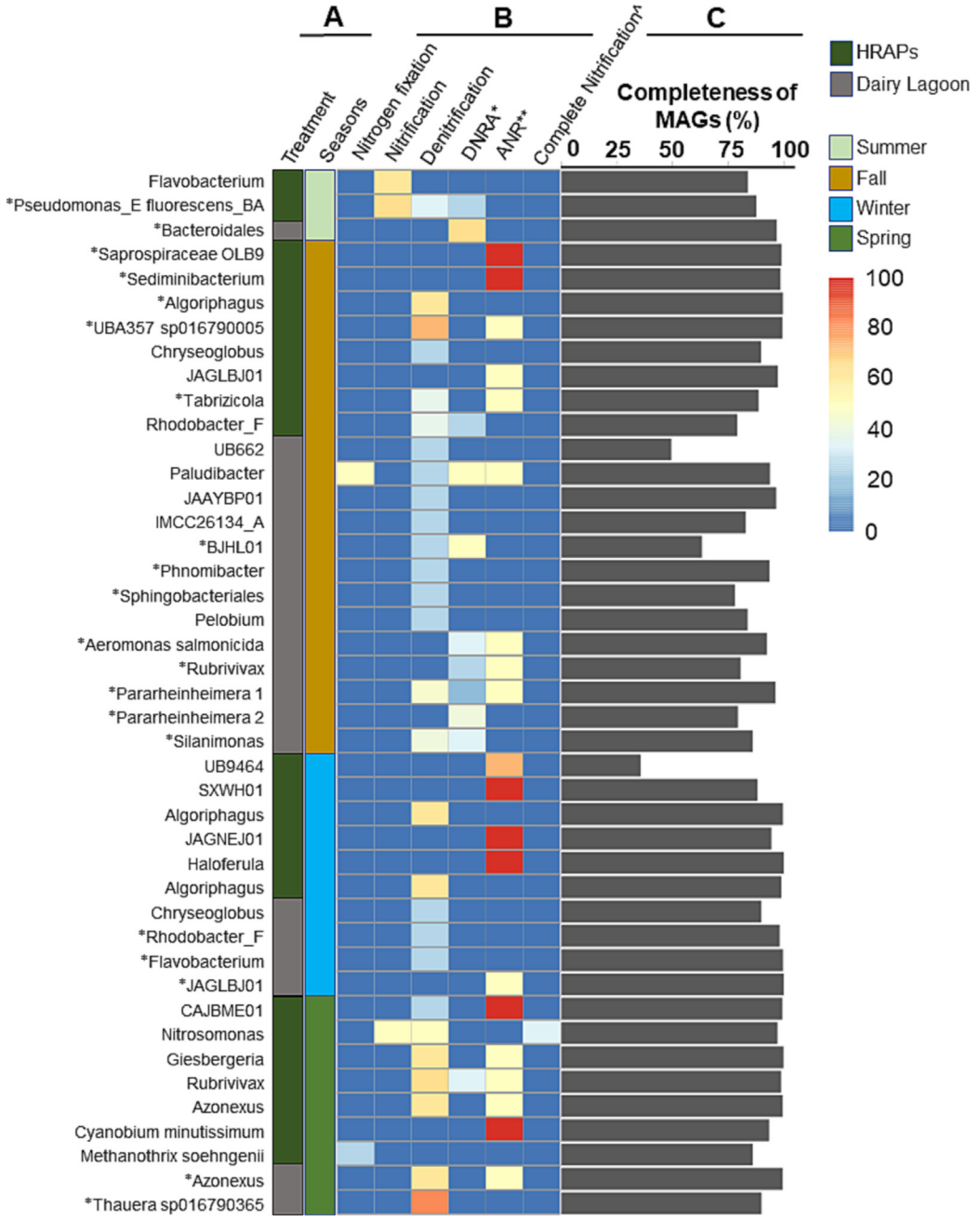

Fig. 9. Nitrogen fixation genes in HRAPs and DLE. A total of 40 MAGs (>70 % completeness) and three bins (>30 % completeness) with genes for nitrogen cycling were recovered from ten metagenomes in the four HRAPs fed with DLE. A, nutrient removal treatments. B, MAGs extracted during different seasons. C, Nitrogen cycling by MAGs and completeness of MAGs involved in nitrogen cycling. MAGs highlighted by (*) are antibiotic-resistant bacteria that are involved in Nitrogen cycling.

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