Quantification of antibiotic resistance genes and mobile genetic in dairy manure

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Background. Antibiotic resistance genes (ARGs) are considered to be emerging environmental contaminants of concern potentially posing risks to human and animal health, and this research studied the prevalence of antimicrobial resistance in dairy manure.

Methods. This study is focused on investigating prevalence of ARGs in California dairy farm manure under current common different manure management. A total of 33 manure samples were collected from multiple manure treatment conditions: 1) flushed manure (FM), 2) fresh pile (FP), 3) compost pile (CP), 4) primary lagoon (PL), and 5) secondary lagoon (SL). After DNA extraction, all fecal samples were screened by PCR for the presence of 8 ARGs: four sulfonamide ARGs (*sulI*, *sulII*, *sulIII*, *sulA*), two tetracycline ARGs (*tetW*, *tetO*), two macrolide-lincosamide-streptogramin B (MLS_B) ARGs (*ermB*, *ermF*). Samples were also screened for two mobile genetic elements (MGEs) (*intI1*, *tnpA*), which are responsible for dissemination of ARGs. Quantitative PCR was then used to screen all samples for five ARGs (*sulII*, *tetW*, *ermF*, *tnpA* and *intI1*).

Results. Prevalence of genes varied among sample types, but all genes were detectable in different manure types. Results showed that liquid-solid separation, piling, and lagoon conditions had limited effects on reducing ARGs and MGEs, and the effect was only found significant on *tetW* ($p = 0.01$). Besides, network analysis indicated that *sulII* was associated with *tnpA* ($p < 0.05$), and *Psychrobacter* and *Pseudomonas* as opportunistic human pathogens, were potential ARG/MGE hosts ($p < 0.05$). This research indicated current different manure management practices in California dairy farms has limited effects on reducing ARGs and MGEs. Improvement of different manure management in dairy farms is thus important to mitigate dissemination of ARGs into the environment.

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Abstract

Background. Antibiotic resistance genes (ARGs) are considered to be emerging environmental contaminants of concern, which poses risks to human and animal health, and this research studied the prevalence of antimicrobial resistance in dairy manure under various manure management practices.

Methods. This study is focused on investigating the prevalence of ARGs in California dairy farm manure under different manure management practices. A total of 33 manure samples were

collected from various farms under multiple manure treatment conditions: 1) flushed manure (FM), 2) fresh pile (FP), 3) compost pile (CP), 4) primary lagoon (PL), and 5) secondary lagoon (SL). Manure samples of these treatments were collected for DNA extraction. After DNA extraction, DNAs of all fecal samples were screened by PCR for the presence of 8 ARGs: four sulfonamide ARGs (*sulI*, *sulII*, *sulIII*, *sulA*), two tetracycline ARGs (*tetW*, *tetO*), two macrolide-lincosamide-streptogramin B (MLS_B) ARGs (*ermB*, *ermF*). DNAs samples were also screened for two mobile genetic elements (MGEs) (*intI1*, *tnpA*), which are considered to be responsible for the dissemination of ARGs in the environment. In addition to PCR, Quantitative PCR (qPCR) was used to screen DNA samples for five ARGs (*sulII*, *tetW*, *ermF*, *tnpA* and *intI1*).

Results. Results of PCR and qPCR showed the prevalence of various genes in dairy manure samples. All tested genes were detectable in different types of manure samples. Liquid-solid separation, piling, and lagoon conditions had limited effects on reducing ARGs and MGEs, and the effect of these treatments was significant in *tetW* ($p = 0.01$) reductions. This research indicated current different manure management practices in California dairy farms have limited effects on reducing ARGs and MGEs. Improvement in different manure management practices in dairy farms is thus important to investigate for mitigating the dissemination of ARGs into the environment.

Introduction

Antibiotic resistance is an emerging concern to public health (CDC 2013; Frieri et al. 2017; Pang et al. 2019; Zaman et al. 2017). In the United States of America, it is estimated that more than 2.8 million people are possibly infected by antibiotic-resistant bacteria each year (CDC 2019). The total economic cost to the U.S. economy is estimated up to $55 billion a year due to lost wages, extended hospital stays, and premature deaths (CDC 2013; Roberts et al. 2009). The use of antibiotics in animal husbandry is one of the leading factors causing widespread antibiotic

resistance (CDC 2019). Every year, approximately 80% of all antimicrobial drugs are applied in animal farming to treat/prevent infectious diseases (FDA 2012; FDA 2013). Out of this, approximately 70% is used for non-therapeutic purposes (UCS 2001). The elevated quantity of antibiotic residue in fecal matter is potentially due to low absorption in the cattle body (Jjemba 2002).

Dairy manure is used as fertilizers in cropland, and potential impacts of manure borne antibiotic resistance genes (ARGs) on the environment are yet to be fully understood (Baquero et al. 2008; Han et al. 2018; Kumar et al. 2005; Wind et al. 2018; Zhao et al. 2017). Dairy manure is considered potential mediator and reservoir for ARGs (Allen et al. 2010; Guardabassi et al. 2004). When dairy manures are applied as fertilizer, manure born ARGs and antibiotic residues may transfer into crop land soil, and subsequently to ambient water bodies (Bennett 2008; Gogarten and Townsend 2005).

Previous research showed that antibiotic resistance in environmental bacteria is selected by antibiotic residues (Baquero et al. 2008; Pruden et al. 2013), and ARGs can proliferate in the host bacteria and transfer to other microbes, including human pathogens, through horizontal gene transfers (HGTs) (Bennett 2008; Gogarten and Townsend 2005).

In terms of quantity, more than 369 million tons of manure are produced in the USA annually (USDA 2012), and the majority of this dairy manure is used as fertilizer in various cropland. Currently, California is the top milk producing state in the United States of America, and dairy farms also produces around 60 million tons of manure annually (USDA 2016).

While managing manure in dairy farms, the flushed system is one of the most commonly adopted methods for manure handling and management in dairy farms in California. Flush system has many benefits, including low labor, ease of handling, and reduced operating cost

(CARB 2017; Kaffka et al. 2016; Meyer et al. 2011), however, it also produces enormous amount of liquid manure. Identifying the suitable treatment methods, which can reduce the contamination levels in manure is important for food safety. Therefore, this study was focused on studying the prevalence of ARGs in the flush manure management system. In a flushed system (Fig. 1), a dairy barn is flushed with recycled water from a lagoon system which holds liquid manure and then flushed manure passes through a liquid-solid separator, where it is separated into solid and liquid waste streams. Solid manure is piled, and in some cases, it is composted in dairy farms before being applied into cropland as fertilizers.

Previous reports emphasize the importance of understanding the fate of ARGs in livestock manure treatments (Flores-Orozco et al. 2020; Gou et al. 2018; Howes 2017; Ma et al. 2018). The abundance of ARGs in livestock waste varies among farm types and locations (He et al. 2020). McKinney et al. (2010) examined the behavior of ARGs in eight livestock lagoon systems. Hurst et al. (2019) studied the abundance of 13 ARGs in untreated manure blend pits. The authors found ARGs abundance varied among farms, and ARG concentrations generally did not correlate to average antimicrobial usage due to complex environmental factors (Flores-Orozco et al. 2020; Huang et al. 2019; Pei et al. 2007; Selvam et al. 2012; Sun et al. 2016; Wang et al. 2012).

Previous findings of an investigation in three pig farm wastewater treatment systems in China showed a relative abundance, and most ARGs were significantly higher in wastewater lagoon than in fresh manures even after treatment (Cheng et al. 2013). Though these studies do provide preliminary understanding, knowledge about the ARGs in California flushed system in dairy farm is yet to be understood.

97 While issues of antibiotics in environment are considered important, standard guidelines
98 and practices capable of reducing antibiotic resistance are yet to be proposed (Allen et al. 2010
99 D'Costa et al. 2006; Ghosh and LaPara 2007). Currently, knowledge of species and antibiotic
100 resistance profile of unculturable bacteria is lacking. The use of culture-independent methods,
101 such as polymerase chain reaction (PCR) and quantitative polymerase chain reaction (qPCR) are
102 preferred due to simplicity and fast results and have the potential to produce relatively more
103 comprehensive and reproducible knowledge of ARG profiles.
104 Considering the importance of animal-agricultural system in food supply, and associated
105 by products, understanding the role of various manure management practices in reducing manure
106 borne contamination is crucial for both public health and environmental health. Human health is
107 closely connected to animal health and their shared environment (One Health), therefore, it is
108 important to explore antimicrobial resistance genes content of manure and the optimal
109 management practices to reduce ARGs content for reducing unwanted potential impacts on
110 reduced efficacy of antimicrobial drugs in clinical practice. The aim of this study was to
111 investigate the fate of ARGs and MGEs in manure under the flushed manure management
112 system. The specific objectives of this research were: 1) estimate the prevalence of antibiotic
113 resistance genes in manure under different manure management practices; 2) quantify ARGs
114 and MGEs in dairy manure; and 3) determine the relationships among ARGs, MGEs, and
115 microbial communities.

116 ## Materials & Methods

117 *Solid and liquid manure sampling in dairy farms*
118 In dairy farms, liquid and solid dairy manure samples were collected in California Central Valley
119 from multiple dairy farms (Pandey et al. 2018). Thirty-three solid/liquid manure samples were

collected from Tulare, Glenn, and Merced counties in California Central Valley. California Central Valley has approximately 91 percent of dairy cows and over 80 percent of dairy facilities in California (CARB 2017). In dairy farms, solid manure samples were collected from fresh/old piles (0 to 6 months old) (n = 14), and liquid manure samples were collected from flushed manure pits and primary/secondary lagoons (0 to 6 months old storage) (n = 19). The solid manure samples collected from fresh piles (less than 2 weeks old pile) were termed as Fresh Pile (FP). The solid samples collected from old piles were termed as Compost Pile (CP). The studied CP here does not necessarily mean that the piles were maintained under thermophilic temperature and mixing conditions of a standard composting process. Similarly, lagoon system in dairy farms were not necessarily under strict anaerobic environments. The liquid manure samples collected from flushed manure pit were termed as Flushed Manure (FM), while the liquid manure samples collected from primary lagoons and secondary lagoons were termed as Primary Lagoon (PL) and Secondary Lagoon (SL), respectively. In each dairy facility, 1 L of liquid manure sample from each pond, and approximately 500-600 g of solid manure from each pile were collected in sterile bottles. Samples were then transported on wet ice after collection and stored at -20 ºC before DNA extraction.

DNA extraction from manure samples

In manure samples, genomic DNA was extracted either using the MO BIO PowerSoil® DNA Isolation Kit or MO BIO PowerWater® DNA Isolation Kit (MO BIO Laboratories Inc.). All solid samples and liquid samples with turbid and sludge-like consistency were processed by the MO BIO PowerSoil® kit. For sludge-like liquid samples (sample with high solid), 10 mL of each sample were centrifuged in 50 mL polypropylene tubes at 13,000 rpm for 10 minutes and 0.25g of the resulting pellet was used for bead beating. Liquid samples with clear-to-low

turbidity were processed by the MO BIO PowerWater® kit, and 10 - 200 mL of each was filtered through a Millipore filter (0.45-µm pore size). The quality and concentration of the DNA were assessed by NanoDrop 1000 spectrophotometer (Thermo Scientific). All extracted DNA from manure samples were stored at -20°C before PCR amplification.

PCR assays for detection of resistance genes in manure

PCR assays for detection of *sul*, *tet* and *erm* were designed. It was reported *sul*, *tet* and *erm* are three of the most frequently detected ARGs classes in livestock waste, which match the major classes of antibiotics used in animal growth promotion and disease control (He et al., 2020). Primers designed in previous work targeting *sul*, *tet* and *erm* genes were used in this study to amplify ARGs (Garder et al. 2014; Hu et al. 2015; Pei et al. 2007), which are shown in Table 1. Subsequently, PCR assays were performed to determine gene detectability in the manure samples. These assays were carried out using the KAPA2G Robust HotStart Ready Mix PCR Kit (KAPA) in a 25 µL volume reaction. The PCR reaction consisted 12.5 µL 2 x KAPA2G Robust Hotstart Ready Mix, 1.25 µL 10 mM each primer, and 2 µL of the template. The temperature program consisted of initial denaturation at 95°C, followed by 40 cycles of 15 s at 95°C; 30 s at the 60°C (55°C for *tetO*, *tetW*, *ermB* and *ermF*); 30 s at 72°C, and a final extension step for 1 min at 72°C. Primer and temperature conditions for *intI* and *tnpA* genes are presented in Table 1**.** PCR products were verified by gel electrophoresis. Purificaiotn, and cloning was done using the TOPO TA Cloning Kit (Invitrogen) according to the manufacturer's instructions. Subsequently, clones were sequenced to verify the insert of the targeted gene (sequencing and verification results are shown in Fig. S1-S6). Plasmids carrying the target genes were extracted and used as positive controls for qPCR to generate standard curves.

Real-time quantitative PCR (RT-qPCR) analysis in manure

Targeted genes and 16S rRNA gene qPCR reactions were performed using a StepOnePlus™ System (Life Technology) in a 20 µL of reaction mixture (10 µL PowerUp™ SYBR™ Green Master Mix [2x]) (Life Technology), 2 µL of 10 mM each primer, and 2 µL of template) with a temperature program of 2 min at 50 °C for UDG activation and 2 min at 95 °C for Dual-Lock™ DNA polymerase activation. This was followed by 40 cycles of 15 s at 95 °C; 15 s at 50 °C-60 °C (60 °C for $T_m > 60$ °C and at T_m for $T_m < 60$ °C); 1 min at 72 °C. Each reaction was conducted in triplicates.

The average copy and standard deviation were calculated using triplicate for each reaction. Melting curve analysis was used to detect nonspecific amplification. Standard curves were included in each qPCR plate by performing serial 10-fold dilutions of the standards. The efficiency of the PCR was calculated by Efficiency = $10^{-(1/\text{slope})} - 1$. All standard curves had a r^2 > 0.99 and an amplification efficiency of 90%–110%. The detection limit for each gene was determined by the highest dilution that produced a consistent C_T value (within 5% deviation). If the standard deviation was more than 5% then two samples with the smallest difference were used for calculation.

The absolute copy number of genes was quantified by referring to the corresponding standard curve obtained by plotting threshold cycles versus log-copy number of genes. Levels of targeted genes were normalized as the percentage of copy number of a gene/copy number of 16S rRNA gene for each sample to emphasize the relative abundance in environmental samples (Alexander et al. 2011; Marti et al. 2013; Selvam et al. 2012).

16S rRNA gene sequencing in manure samples

The high-throughput sequencing for 16S rRNA gene is described elsewhere (Pandey et al. 2018). Sequencing was performed by DNA Technologies Core Facility of the Genome Center at the

University of California-Davis using the Illumina MiSeq platform. The V3 and V4 hypervariable region of the 16S rRNA gene was amplified using the forward primer: (5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3′) and the reverse primer: (5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3′) (Klindworth et al. 2013). For quality control, barcodes and primers were allowed to have 1 and 4 mismatches, respectively. Primer sequence reads were then trimmed, and sequences were merged into a single amplicon sequence using FLASH2. Assignment of sequence to phylotypes was performed in the RDP database using the RDP Bayesian classifier (bootstrap confidence score > 50%). Further, covariates were generated using relative abundance of bacterial taxa in each sample. Stepwise discriminant analysis models built in JMP Pro 13.0 were performed until only variables with a p-value < 0.05 were retained (Pandey et al., 2018).

Data analysis

Statistical analysis on gene abundance data was performed using the published approaches (Burch et al. 2016; Sandberg and LaPara 2016; Sun et al. 2016). Data were log-transformed before statistical analysis. Ordinary one-way ANOVA was used to evaluate the influence of dairy manure conditions on gene reductions by GraphPad Prism 8. Residuals were checked by Brown-Forsythe test of heteroscedasticity and Anderson-Darling test of normality ($\alpha = 0.05$). Tukey's multiple comparison test was used for comparing gene levels under different conditions ($\alpha = 0.05$). Multiplicity adjusted p-value was reported for each comparison. Principal Component Analysis (PCA) Plot and Hierarchical Clustering Plot were conducted by MetaboAnalyst 3.0 to find similarity among samples. Correlation networks were created by MetScape 3.1.3 and Cytoscape 3.4.0. CorrelationCalculator 1.0.1 was used based on Debiased Sparse Partial

Correlation (DSPC) method to calculate partial correlation values and *p*-values for each pair in the network. Range for edges was set to partial correlation values (corr. pcor) of < -0.20 or > +0.20.

Results

PCR for gene presence

Firstly, PCR assays were applied to explore whether the gene was detectable or not in each sample. PCR screening results in all 33 samples are shown in Table 2. Four different manure management groups, FP, FM, PL, and SL, had similar positive percentages of gene types. CP group had a significantly lower percentage ($p = 0.02$), with an average of 47% types of targeted genes. One sample (PL2) was found with no detection. Four samples (FP1, FP5, FP7, and SL4) were found with all ten genes. The most abundant gene was *sulII*, and it was present in a total of 93.9% among all samples, with a percentage of 92.9% in solid samples and 94.7% in liquid samples. The lowest one, *sulA*, was positive in a total of 12.1% among all samples, and was detected in 21.4% solid samples and 5.3% liquid samples. Liquid samples normally had a higher percentage of detectable genes, except for *sulIII* and sul*A*. *sulII*, *tetW*, *ermF*, *tnpA* and *intI1* were selected for further study to quantify the gene concentrations because of their representation of different antibiotic resistance mechanisms and high prevalence among the samples. Fisher's exact test for contingency table analysis showed the overall gene detection rate in CP group was significantly lower than FP, FM, PL, and SL ($p < 0.01$).

Quantification of resistance related genes

Five genes (*sulII*, *tetW*, *ermF*, *tnpA* and *intI1*) were quantified by qPCR in 33 dairy manure samples taken from different manure management conditions. DNA templates for qPCR were the same batch of extractions as PCR (Table 1). The numbers of copies of the five resistance

related genes quantified at each sample were then normalized to the number of copies of bacterial 16S rRNA gene. Data is shown in Table S1.

As shown in Fig. 2 (Table S1), the average gene concentrations for *sulII*, *tetW*, and *intI1* were similar (~1×10^{-4} gene copies/16S rDNA copies). The *tetW* was the highest (1.43×10^{-4} gene copies/16S rDNA copies). The concentrations of *ermF* and *tnpA* were 5.98×10^{-6} and 4.67 $\times 10^{-5}$ (gene copies/16S rDNA copies), respectively (lower by one and two order of magnitudes). In ordinary one-way ANOVA, diagnostic of residuals showed data passed Brown-Forsythe test and Anderson-Darling test ($\alpha = 0.05$). One-way ANOVA showed different manure management had no significant effect on four of the five genes. Effect of different manure management practices was only found significant on *tetW* ($p = 0.01$). Tukey test for multiple comparisons showed *tetW* in Compost Pile were significantly lower than Flushed Manure (adjusted $p = 0.02$) and Primary Lagoon (adjusted $p = 0.02$).

PCA and cluster plots for relative abundance of five genes were drawn by MetaboAnalyst 3.0 (Xia et al. 2015) as shown in Fig. 3. Relative abundance of five genes were log transformed and then normalized by median, followed by mean centering as the data scaling method. Figure 3 (a) shows PC 1 captured 40.1% of the variation between samples, and PC 2 captured 23.4%. These two PCs captured 63.5% of the variation between the samples. The CP, FP, PL, SL, and FM groups were overlapped, which means they were not significantly different from each other. In agglomerative hierarchical cluster analysis shown in Fig. 3 (b), each sample began as a separate cluster and the algorithm proceeded to combine them until all samples belonged to one cluster. Results showed that PL and FM, CP and FP were similar, as they tended to cluster together. However, different manure conditions did not fall into separate clusters, indicating their ARG profiles were not significantly different from each other. As the CP, FP, PL, SL, and FM

groups were overlapped in Fig. 3 (a) and did not fall into separate clusters in Fig. 3 (b), it can be inferred that liquid-solid separation, lagoon system and piling process may have limited to no impacts on ARGs reductions.

Cooccurrence of ARGs, MGEs, and microbial communities

Figure 4 shows the correlation network of five genes with top 50 bacterial taxa in the manure samples. Bacterial community data was used for network analysis. Range for edges was set to partial correlation values (corr. pcor) of < -0.20 or > +0.20. Red lines indicate positive correlation, while blue lines represent negative correlation. A bold line shows a *p*-value less than 0.05. These were three significant correlations: *tnpA – sulII* (corr. pcor = 0.415); *intI1 – Psychrobacter* (corr. pcor = 0.519); *ermF – Pseudomonas* (corr. pcor = 0.466).

Discussion

Prevalence and quantification of resistance related genes

The PCR results showed manure under different conditions possessed variety of ARGs and MGEs. Both traditional PCR and RT-qPCR were able to amplify DNA. RT-qPCR provided both qualitative and quantitative data by measuring the kinetics of the reaction in the exponential phase. Traditional method by agarose gels provided only qualitative results by measuring amplification products at endpoint of the PCR reaction (Parashar et al. 2006). In our study, targeted genes were screened firstly by PCR and selected gene were then quantified by RT-qPCR. It was noticed that some of genes were not detectable in PCR, and the same genes were detectable in qPCR. For example, *sulII*, *tnpA* and *intI1* in PL2 were detectable in qPCR but were not detectable in PCR. This may be due to the limitation of UV visualization because some bands in agarose gels were not visible clearly under UV light. Relative abundance of *intI1* in PL2

and SL5 samples was both above average in qPCR but *intI1* gene in these samples was not detected in PCR.

The results showed that *sulII*, *ermF*, *tnpA*, and *intI1* concentrations were not significantly different among five manure conditions (FP, CP, FM, PL, SL), and only one gene—*tetW*, was found at a significantly lower concentration in CP compared with the FM and PL. Previous studies showed various responses of ARGs to biological treatment methods such as anaerobic lagoons and composting (Zhang et al., 2021). This may be due to different experimental conditions and complex microbial ecologies involved (Pruden et al. 2013). McKinney et al. (2010) observed reductions of *tet* ARGs but increases of *sul* ARGs in anaerobic lagoons. Zhang et al. (2017) found that absolute abundances of 13 out of 14 ARGs and two integrase genes increased after 52 days of anaerobic digestion of swine manure. Sun et al. (2016) stated that 4 out of 10 detected ARGs declined during dairy manure anaerobic digestion under 20 °C. Storteboom et al. (2007) reported reduction of *tet*O but increase of *tetW* during horse manure composting process. Previous studies reported a higher decrease of cultivated antibiotic resistant bacteria in composting process compared to lagoon system (Wang et al. 2012). While IS6, family members of bacterial and archaeal insertion sequences are known to play crucial role in spreading antibiotic-resistance genes, overproduction inhibition phenomenon presents challenges (Munoz-Lopez and Garcia-Perez, 2010; Harmer and Hall, 2019). The overproduction and inhibition phenomenon is the property of some transposases where they display lower activity at higher concentrations; this could affect the detection of transposases as the concentration must be taken into account when evaluating impacts of these genes (Harmer and Hall, 2019; Harmer and Hall, 2020). A pitfall in the current study is that the primer used to screentransposons *tnpA* was specific to those previously observed in the IS6, and may have limited complete identification of

all potential *tnpA* in the samples; a general concern with transposons is the potential role they may play on dissemination of various ARGs, and further studies would need to be conducted to specifically quantify their corroboration towards this effect ,

It was noticed that average *sulII*, *tetW*, and *intI1* concentrations identified in this study were lower than previous findings. As an example, McKinney et al. (2010) reported *sulII* and *tetW* of ~10^{-1} and 10^{-2} copies/16S rRNA respectively in a dairy lagoon samples in Colorado. Dungan et al. (2018) reported *intI1* gene copies of 10^{-2} /16S rRNA gene in the dairy wastewater in Idaho. Differences in ARG levels may be due to site-specific physical/chemical conditions, manure handing methods, and historical intensity of antibiotic use (He et al. 2020). However, *tet* and *sul* were reported to be the most abundant ARGs in livestock waste (He et al. 2020), which is aligned to the findings of this study.

Cooccurrence of ARGs, MGEs, and microbial communities

Integrons and transposons have been reported as responsible for the acquisition and dissemination of ARGs by HGT, which indicates that HGT could be a potential mechanism for the spread and dissemination of specific ARGs (Han et al. 2016; Sandberg and LaPara 2016). In addition, the *sulII* gene was reported on a broad-host-range (BHR) plasmid RSF1010 (Rådström and Swedberg 1988; Yau et al. 2010), and BHR plasmids are of considerable interest because they are reported not only in single bacterial species but also members of different taxonomic group, and play an important role in HGT. Existing knowledge in terms of how anaerobic processes influences ARGs is weak, particularly the effects of anaerobic digesters treating dairy manure on ARGs of manure is not well understood. Preliminary research such as a study by Huang et al. (2019) investigated the abundance of ARGs and transposase genes during anaerobic process, and authors found a reduction in ARG and transposons genes during AD of pig manure.

These findings suggest that transposons genes could be correlated with ARGs. Correlations between other genes were not significant, and this may be due to resistance genes not located in integrons/transposons and non-specific selection agents in the manure (Andersson and Hughes 2010; Di Cesare et al. 2016; McKinney et al. 2010).

While assessing the presence of ARGs, it is crucial to understand the correlation between ARGs and various microbial species. Previous studies indicate that gram-negative bacteria such as *Psychrobacter* and *Pseudomonas*, which are abundant in the environment and able to tolerate both cold and hot environment, showed correlation with ARGs and MGE. As an example, one of the species in *Pseudomonas* genus, *Pseudomonas aeruginosa*, is an opportunistic pathogen that causes infections in humans with a high mortality rate. Presence of *ermF* in *Pseudomonas* could compromise clinical treatment by MLS_B antibiotics. *Pseudomonas* is resistant to a variety of antimicrobials due to multidrug efflux pumps, chromosomal mutations and the acquisition of resistance genes via HGT (Poole, 2011). These findings are important because the presence of ARGs in environmental bacteria have the potential to be received by human and animal pathogens through HGT, which increases the risk to public and animal healths via antimicrobial resistance (AMR) exposure.

While these results indicate the possibility of putative microorganisms hosting ARGs, additional studies are needed for strengthening the findings considering a preliminary nature of this study. A previous study by Flores-Orozco et al. (2020) identified more than 180 unique antibiotic-resistance genes in dairy manure, and changes in ARGs levels were found to be related with the shifts in the microbial community. Detailed bioinformatic analysis was conducted to evaluate the co-occurrence of microbial groups and ARGs results revealed the presence of potential microbial ARG hosts.

Many ARGs such as tetX, sul1, sul2, and tetG were dominant in various farm's soils (Duan et al., 2019). Studies (Duan et al., 2019; Zhang et al., 2021) focused on evaluating the impacts of manure on soil's ARGs, and anaerobic digestion process effects on the reduction of ARGs in manure suggested that cropland's soil receiving manure showed more than 2 times higher ARGs compared to the soil without manure. Manure treatment process such as anaerobic digestion was found to be effective in reducing ARGs levels in manure, and changes in microbial community during anaerobic digestion resulted in reduced ARGs level (Flores-Orozco et al., 2020; Zhang et al., 2021).

One of the major limitations of this study is fewer sample numbers and samples from limited dairy farms. Further, manure management practices change from one farm to another substantially, which pose challenges in comparing the microbial and ARGs data among farms. Understanding the deviation in characteristics of ARGs and microbial community within and between farms with similar management practices can provide greater understanding of temporal changes in ARGs present in liquid manure versus solid manure.

Conclusions

In this research, we studied the prevalence of ARGs and MGEs in flushed manure, primary lagoon manure, secondary lagoon manure, fresh pile manure, and compost pile manure. Manure samples were obtained from multiple dairy farms located in Central Valley, California. Prevalence of genes varied among sample types, but all of the studied genes were detectable in different manure types. Among five genes quantified, only *tetW* was found at significantly lower concentration in compost pile comparing with flushed manure (adj. $p = 0.02$) and primary lagoon samples (adj. $p = 0.02$). Results of this study showed that ARGs are widely present in liquid (lagoon samples) and solid dairy farm manure (fresh and compost piles). Different manure

management such as liquid-solid separation, piling, and lagoon storage may not have significant impacts on ARG and MGE reductions.

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Table 1(on next page)

Synthetic oligonucleotides

Table 1 Synthetic oligonucleotides used in this study

Primer	Target gene	Sequences (direction 5′–3′)	Traditional PCR annealing temp (°C)	qPCR annealing temp (°C)	Amplicon size (bp)	Reference
sulI-FW	*sul*I	CGCACCGGAAACATCGCTGCAC	60	60	163	(Pei et al. 2006)
sulI-RV		TGAAGTTCCGCCGCAAGGCTCG				
sulII-FW	*sulII*	TCCGGTGGAGGCCGGTATCTGG	60	60	191	
sulII-RV		CGGGAATGCCATCTGCCTTGAG				
sulIII-FW	*sulIII*	TCCGTTCAGCGAATTGGTGCAG	60	60	128	
sulIII-RV		TTCGTTCACGCCTTACACCAGC				
sulA-FW	*sulA*	TCTTGAGCAAGCACTCCAGCAG	60	60	299	
sulA-RV		TCCAGCCTTAGCAACCACATGG				
tetW-FW	*tetW*	GAGAGCCTGCTATATGCCAGC	55	53.9	168	(Aminov et al. 2001)
tetW-RV		GGGCGTATCCACAATGTTAAC				
tetO-FW	*tetO*	ACGGARAGTTTATTGTATACC	55	48.5	171	
tetO-RV		TGGCGTATCTATAATGTTGAC				
ermB-FW	*ermB*	GGTTGCTCTTGCACACTCAAG	55	51.2	191	(Koike et al. 2010)
ermB-RV		CAGTTGACGATATTCTCGATTG				
ermF-189f	*ermF*	CGACACAGCTTTGGTTGAAC	55	51.4	309	(Chen et al. 2007)
ermF-497r		GGACCTACCTCATAGACAAG				
HS463a	*intI1*	CTGGATTTCGATCACGGCACG	60	55.7	473	(Barlow et al., 2004)
HS464		ACATGCGTGTAAATCATCGTCG				
tnpA-04F	*tnpA*-04	CCGATCACGGAAAGCTCAAG	60	56	101	(Zhu et al. 2013)
tnpA-04R		GGCTCGCATGACTTCGAATC				
357F	16S rRNA gene	CCTACGGGAGGCAGCAG	60	56	193	(Muyzer et al. 1993)
518R		ATTACCGCGGCTGCTGG				

1

Table 2(on next page)

Detection of resistance gene families

Table 2 Detection of resistance gene families in dairy manure

	Sample ID	*sulI*	*sulII*	*sulIII*	*sulA*	*tetO*	*tetW*	*ermB*	*ermF*	*tnpA*	*intI1*	Percentage
1	FP1	+	+	+	+	+	+	+	+	+	+	100%
2	FP2	+	+	-	-	-	+	-	-	+	+	50%
3	FP3	+	+	-	-	+	+	-	+	+	-	60%
4	FP4	+	+	-	-	-	+	+	+	+	+	70%
5	FP5	+	+	+	+	+	+	+	+	+	+	100%
6	FP6	+	+	+	-	+	+	+	+	+	+	90%
7	FP7	+	+	+	+	+	+	+	+	+	+	100%
8	CP1	+	+	-	-	-	+	-	+	+	+	60%
9	CP2	+	+	+	-	+	+	-	+	+	-	70%
10	CP3	-	-	-	-	-	-	-	-	-	+	10%
11	CP4	+	+	+	-	+	+	+	+	+	+	90%
12	CP5	-	+	-	-	-	-	-	-	-	+	20%
13	CP6	+	+	-	-	-	+	-	-	+	-	40%
14	CP7	-	+	-	-	-	+	-	+	+	-	40%
15	FM1	+	+	-	-	-	+	+	+	+	+	70%
16	FM2	+	+	-	-	-	+	+	+	+	+	70%
17	FM3	+	+	+	-	+	+	+	+	+	+	90%
18	FM4	+	+	-	-	+	+	+	+	+	+	80%
19	FM5	+	+	-	-	+	+	+	+	+	+	80%
20	FM6	+	+	-	-	+	+	+	+	+	+	80%
21	PL1	+	+	-	-	+	+	+	+	+	+	80%
22	PL2	-	-	-	-	-	-	-	-	-	-	0
23	PL3	+	+	+	-	+	+	+	+	+	+	90%
24	PL4	+	+	+	-	+	+	+	+	+	+	90%
25	PL5	+	+	+	-	+	+	+	+	+	+	90%
26	PL6	+	+	-	-	+	+	+	+	+	+	80%
27	PL7	+	+	-	-	+	+	+	+	+	+	80%
28	PL8	+	+	+	-	+	+	+	+	+	+	90%
39	SL1	+	+	-	-	-	+	+	+	+	+	70%
30	SL2	+	+	+	-	+	+	+	+	+	+	90%
31	SL3	+	+	+	-	-	+	+	+	+	+	80%
32	SL4	+	+	+	+	+	+	+	+	+	+	100%
33	SL5	+	+	-	-	+	+	-	+	+	-	60%
Positive percentage		87.9%	93.9%	42.4%	12.1%	63.6%	90.9%	69.7%	84.8%	90.9%	81.8%	

+: present; -: absent.

FP: Fresh Pile; CP: Compost Pile; FM: Flushed manure; PL: Primary Lagoon; SL: Secondary Lagoon

1

Figure 1

Manure flow

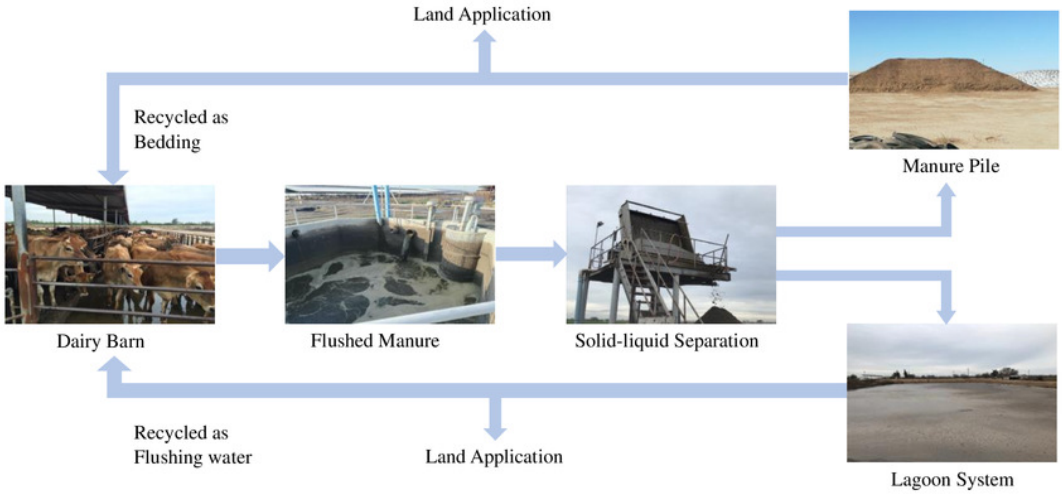

Figure 1 Typical processing of flushed manure in dairy farms in Central Valley California (source: Pandey et al. 2019).

Figure 2

Copies of Resistance genes

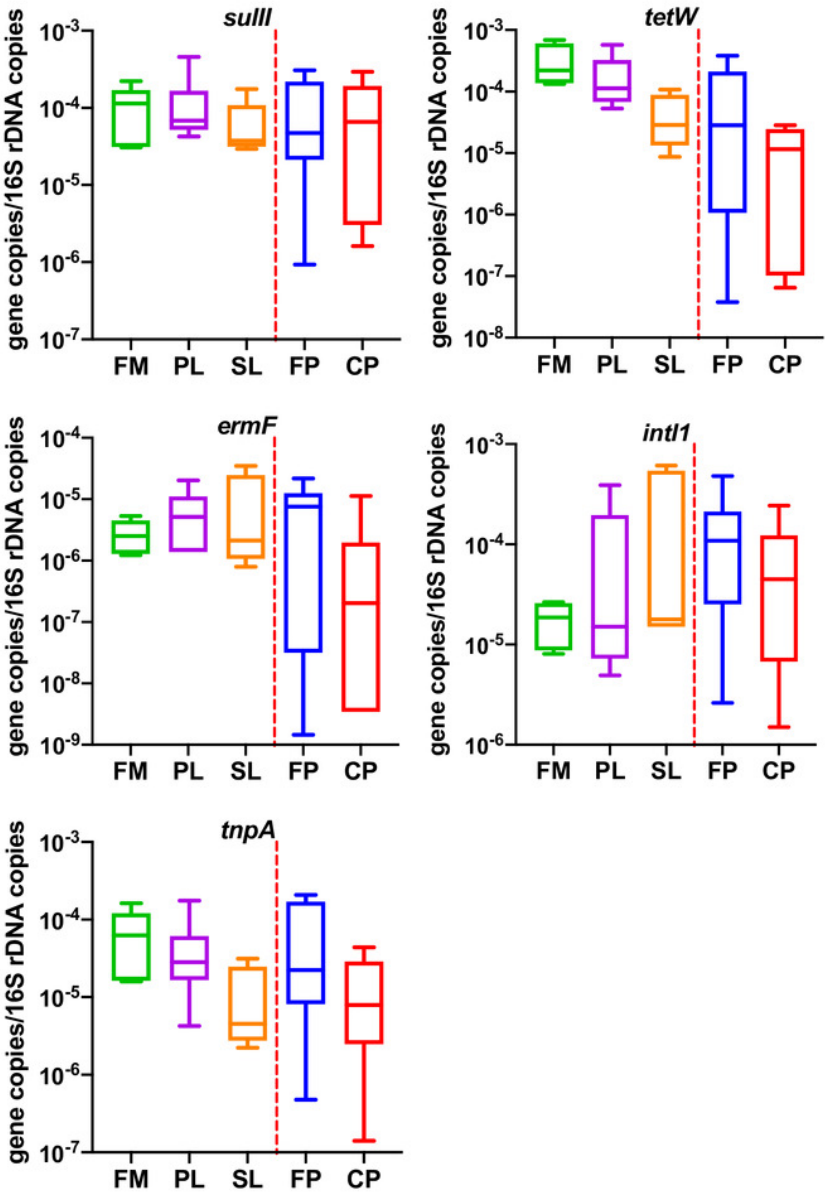

Figure 2 Copies of resistance related genes normalized to the number of bacterial 16S rRNA gene genes in different dairy manure. X-axis labels indicate the type of dairy treatments, rectangular boxes indicate the interquartile range of the data; median value is indicated by the horizontal line inside the box. Whiskers show min to max of data. Extreme outliers (< Q1 – 3 IQ or > Q3 + 3 IQ) were removed and shown as "--" in Table 3. FM: Flushed Manure; PL: Primary Lagoon; SL: Secondary Lagoon; FP: Fresh Pile; CP: Compost Pile. Liquid samples and solid samples are separated by a red vertical line.

Figure 3

PCA analysis

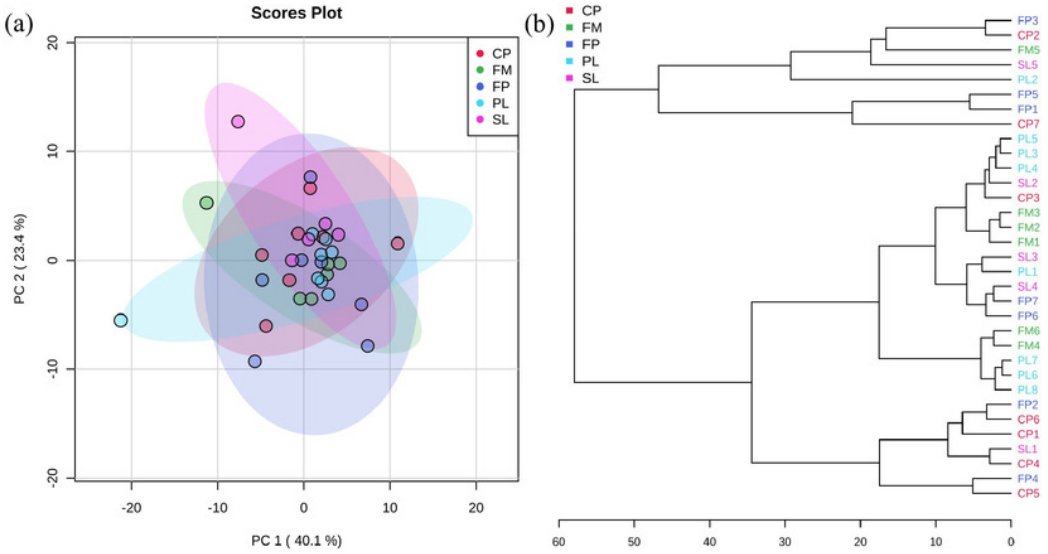

Figure 3 (a) Principal Component Analysis (PCA) Plot (Colors representing 95% confidence regions). **(b) Hierarchical Clustering Plot** (distance measure using Euclidean, and clustering algorithm using Ward).

Figure 4

Network analysis

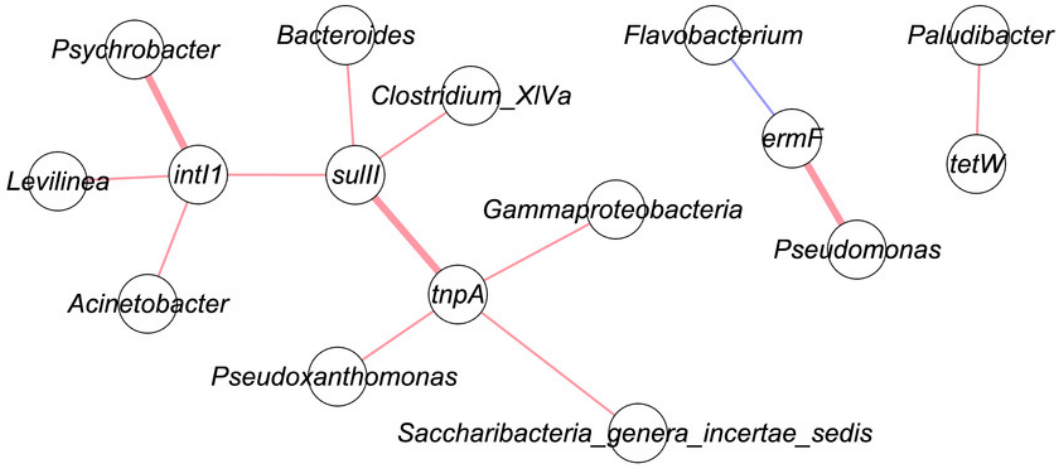

Figure 4 Network Analysis of targeted genes with bacterial communities. Red line: positive correlation; blue line: negative correlation; bold line: p-value < 0.05.

1