Quantification of antibiotic resistance genes and mobile genetic elements in manure from dairy farms in California

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Abstract

Antibiotic resistance genes (ARGs) are emerging environmental contaminants of concern to both human and animal health. Dairy manure is considered reservoir of ARGs. This study is focused on investigating prevalence of ARGs in California dairy farm manure under current common 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*). 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 manure management practices in California dairy farms has limited effects on reducing ARGs and MGEs. Improvement of manure management in dairy farms is thus important to mitigate dissemination of ARGs into the environment.

Key Points

1. Manure management in dairy farms had limited effects on ARG/MGE reduction.
2. Pathogens, including *Psychrobacter* and *Pseudomonas*, were potential ARG/MGE hosts.
3. *sulII* was associated with HGT by transposons.

Introduction

Antibiotic resistance is an emerging threat to public health which compromises the success and costs of therapeutic treatments (CDC 2013; Frieri et al. 2017; Pang et al. 2019; Zaman et al. 2017). In the United States, it is estimated that more than 2.8 million people are infected by antibiotic-resistant bacteria each year, and more than 35,000 died as a direct result of infection (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). Use of antibiotics in animal husbandry is one of the leading factors causing the widespread antibiotic resistance (CDC 2019). Every year, over 13 million kilograms, or approximately 80% of all antimicrobial drugs are applied in animal farming to treat/prevent infectious diseases and promote animal growth in the United States (FDA 2012; FDA 2013). Out of this, approximately 70% is used for non-therapeutic purposes (UCS 2001).

Manure fertilizer is commonly used in cropland, and possible impacts of manure borne antibiotic resistance genes (ARGs) on 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 cattle are considered potential mediators, reservoirs, and disseminators of resistant bacteria and/or ARGs (Allen et al. 2010; Guardabassi et al. 2004). When dairy manure is applied as fertilizer or directly deposited on land by grazing livestock, antibiotic resistant bacteria, ARGs and antibiotic residues may transfer into soil and ambient waterbodies such as river and lakes during rainfall and runoff events (Bennett 2008; Gogarten and Townsend 2005). Antibiotic resistance in environmental bacteria is selected by antibiotic residues or other stress environmental factors (Baquero et al. 2008; Pruden et al. 2013). Once transported, ARGs are persistent in environment for a prolonged period. They proliferate in the host bacteria, and transfer to other microbes including human pathogens through horizontal gene transfers (HGTs), which are mediated by transposons, integrons, and plasmids (Bennett 2008; Gogarten and Townsend 2005).

More than 369 million tons of manure are produced in the USA annually (USDA 2012), and majority of this quantity is used as fertilizer in cropland. Considering the amount of dairy manure produced, and its subsequent use as fertilizers, improved understanding of ARGs in manure could help in identifying the potential impacts on environment. Currently, California is the top milk producing state in the United States, and produces around 60 million tons of manure annually (USDA 2016). Flushed system is one of the most commonly adopted methods for manure handling and management in dairy farms in California due to many benefits, including low labor, ease of handling and reduced operating cost (CARB 2017; Kaffka et al. 2016; Meyer et al. 2011). In a flushed system (Fig. 1), a dairy barn is flushed with recycled water from a lagoon, and then flushed manure passes through a 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 applied into cropland as fertilizers. Liquid portion of manure is stored in lagoon systems for 3–6 months, and eventually it is used as fertilizers. Despite the intensive management of flushed manure, the understanding of regulating ARG in the process is still unknown.

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). 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. Authors found *tet* and *sul* ARGs in chicken layer lagoons were lowest compared with lagoons of swine and dairy facilities. Hurst et al. (2019) studied the abundance of 13 ARGs in untreated manure blend pits and long-term storage systems in Northeastern U.S dairy farms, and a majority of farms use a scrape system to collect manure. The authors found ARGs abundance varied among farms, and ARG concentrations generally did not correlate to average antimicrobial usage due to complex environmental factors. Most manure conditions were simulated at the bench scale, and only few analyzed commonly used practices in dairy farms (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). Wang et al. (2012) simulated the environmental conditions of swine manure treatment by lab scale thermophilic composting and ambient temperature lagoon storage with modest aeration over a 48-day period. Authors found five *erm* genes and five *tet* genes dramatically declined after composting, while no significant reduction of *erm* or *tet* genes was observed during the lagoon treatment. Selvam et al. (2012) reported that resistance genes for tetracycline, sulfonamide, and fluoroquinolone were undetectable after 28–42 days of swine manure composting in lab scale. An investigation in three pig farm wastewater treatment systems in China showed relative abundance of 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.

A major technology to detect antibiotic resistance is by bacteria culture. However, data from current literature showed a wide range use of culture media, incubation time and antibiotic concentrations. Guidelines of standard culture for antibiotic resistance are lacking (Allen et al. 2010). Besides, many non-culturable species and unexpressed ARGs could not be identified (D'Costa et al. 2006; Ghosh and LaPara 2007), and these ARGs can be activated under suitable environmental conditions. It was reported only less than 1% of bacteria are culturable by standard methods (Allen et al. 2010). Knowledge of species and antibiotic resistance profile of unculturable bacteria is lacking. Therefore, the use of culture-independent methods, such as polymerase chain reaction (PCR) and quantitative polymerase chain reaction (qPCR), has a potential to produce relatively more comprehensive and reproducible knowledge of ARG profiles.

The goal of this study was to investigate the fate of ARGs and MGEs under dairy farm manure management. The specific objectives of this study were: 1) estimate the prevalence of ARGs and MGEs in different manure management conditions, including flushed manure, fresh pile, compost pile, primary lagoon, and secondary lagoon; 2) quantify ARGs and MGEs in dairy manure by qPCR and compare abundance of targeted genes in different manure management conditions; and 3) evaluate the relationships among ARGs, MGEs, and microbial communities by network analysis. To the author's knowledge, this study is the first to quantify ARGs by qPCR in dairy manure from California dairy farms, and authors attempt will help improving existing understanding of ARGs in flushed dairy manure, lagoon system, and piled solid dairy manure.

Materials And Methods

Solid and liquid manure sampling in dairy farms

Liquid and solid dairy manure samples were collected from multiple dairy farms in Central Valley, California. Sample information is described elsewhere and 16S rRNA gene sequencing results were published (Pandey et al. 2018). Briefly, thirty-three solid/liquid manure samples were collected from Tulare, Glenn, and Merced counties in California Central Valley, which has approximately 91 percent of dairy cows and over 80 percent of dairy facilities in California (CARB 2017). Solid samples were collected from fresh/old piles (0 to 6 months old) (n = 14), and liquid samples were collected from flushed manure pits and primary/secondary lagoons (0 to 6 months old storage) (n = 19). The solid 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 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 liter of liquid manure sample from each pond, and 600 g of solid manure from each pile were collected in sterile bottles. Samples were transported on wet ice after collection and stored at -20 ºC before DNA extraction.

DNA extraction for dairy farm 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.), depending on the sample consistency. 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, 10 mL of each sample were centrifuged in 50 mL polypropylene tubes at 13,000 rpm for 10 minutes and 0.25 g 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 samples were stored at -20 °C before PCR amplification.

PCR assays for detection of resistance genes

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 to amplify ARGs (Garder et al. 2014; Hu et al. 2015; Pei et al. 2007) as in Table 1. Subsequently, PCR assays were performed to determine gene detectability in the study 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 of 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. PCR products were verified by gel electrophoresis, purified, and cloned using the TOPO TA Cloning Kit (Invitrogen) according to the manufacturer's instructions. 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.

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	*sulI*	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	(Hardwick et al. 2008)
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				

Real-time quantitative PCR (RT-qPCR)

Targeted genes and 16S rRNA gene qPCR reactions were performed using a StepOnePlus™ System (Life Technology) in a 20 µL reaction mixture (10 µL PowerUp™ SYBR™ Green Master Mix [2x]) (Life Technology), 2 µL 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, 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 by triplicates. The average copy and standard deviation were calculated among triplicates 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

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 as described in previous studies (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 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 ($p < 0.01$).

Table 2
Detection of resistance gene families in dairy manure

	Sample ID	*sulI*	*sulII*	*sulIII*	*sulA*	*tetO*	*tetW*	*ermB*	*ermF*	*tnpA*	*intI1*	Percentage	Average
1	FP1	+	+	+	+	+	+	+	+	+	+	100%	81%
2	FP2	+	+	-	-	-	+	-	-	+	+	50%	
3	FP3	+	+	-	-	+	+	-	+	+	-	60%	
4	FP4	+	+	-	-	-	+	+	+	+	+	70%	
5	FP5	+	+	+	+	+	+	+	+	+	+	100%	
6	FP6	+	+	+	-	+	+	+	+	+	+	90%	
7	FP7	+	+	+	+	+	+	+	+	+	+	100%	
8	CP1	+	+	-	-	-	+	-	+	+	+	60%	47%
9	CP2	+	+	+	-	+	+	-	+	+	-	70%	
10	CP3	-	-	-	-	-	-	-	-	-	+	10%	
11	CP4	+	+	+	-	+	+	+	+	+	+	90%	
12	CP5	-	+	-	-	-	-	-	-	-	+	20%	
13	CP6	+	+	-	-	-	+	-	-	+	-	40%	
14	CP7	-	+	-	-	-	+	-	+	+	-	40%	
15	FM1	+	+	-	-	-	+	+	+	+	+	70%	78%
16	FM2	+	+	-	-	-	+	+	+	+	+	70%	
17	FM3	+	+	+	-	+	+	+	+	+	+	90%	
18	FM4	+	+	-	-	+	+	+	+	+	+	80%	
19	FM5	+	+	-	-	+	+	+	+	+	+	80%	
20	FM6	+	+	-	-	+	+	+	+	+	+	80%	
21	PL1	+	+	-	-	+	+	+	+	+	+	80%	75%
22	PL2	-	-	-	-	-	-	-	-	-	-	0	
23	PL3	+	+	+	-	+	+	+	+	+	+	90%	
24	PL4	+	+	+	-	+	+	+	+	+	+	90%	
25	PL5	+	+	+	-	+	+	+	+	+	+	90%	
26	PL6	+	+	-	-	+	+	+	+	+	+	80%	
27	PL7	+	+	-	-	+	+	+	+	+	+	80%	
28	PL8	+	+	+	-	+	+	+	+	+	+	90%	
39	SL1	+	+	-	-	-	+	+	+	+	+	70%	80%
30	SL2	+	+	+	-	+	+	+	+	+	+	90%	
31	SL3	+	+	+	-	-	+	+	+	+	+	80%	
32	SL4	+	+	+	+	+	+	+	+	+	+	100%	
33	SL5	+	+	-	-	+	+	-	+	+	-	60%	

+: present; -: absent.

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

Sample ID	*sulI*	*sulII*	*sulIII*	*sulA*	*tetO*	*tetW*	*ermB*	*ermF*	*tnpA*	*intI1*	Percentage	Average
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												

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. 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 3.

Table 3

qPCR results for 33 dairy samples. Gene concentrations were normalized to the number of copies of bacterial 16S rRNA gene. Underlined data were beyond lower outer fence: Q1−3 IQ and upper outer fence: Q3 + 3 IQ. They were considered as extreme outliers and removed from the table for average calculation and statistical analysis.

	Sample ID	treatment	*sulII*	*tetW*	*ermF*	*intI1*	*tnpA*
1	FP1	FP	8.40E-04	2.10E-05	7.58E-06	4.81E-04	2.23E-05
2	FP2	FP	4.16E-05	1.07E-06	3.15E-08	2.52E-05	8.12E-06
3	FP3	FP	9.32E-07	3.79E-08	2.18E-05	2.62E-06	4.79E-07
4	FP4	FP	4.71E-05	2.84E-05	1.45E-09	1.98E-04	1.71E-04
5	FP5	FP	2.01E-03	2.11E-04	1.26E-05	1.09E-04	2.08E-04
6	FP6	FP	1.31E-04	3.82E-04	9.67E-06	1.04E-04	1.89E-05
7	FP7	FP	3.08E-04	1.15E-04	6.11E-06	2.13E-04	5.16E-05
8	CP1	CP	4.44E-06	6.49E-08	2.03E-07	8.53E-06	7.90E-06
9	CP2	CP	1.61E-06	1.14E-07	1.13E-05	1.50E-06	1.41E-07
10	CP3	CP	4.18E + 00	6.27E-02	0.00E + 00	6.14E-02	1.88E + 01
11	CP4	CP	2.93E-04	1.61E-05	1.96E-06	2.43E-04	1.37E-05
12	CP5	CP	6.59E-05	2.32E-05	3.45E-09	5.37E-05	4.79E-06
13	CP6	CP	9.21E-05	2.85E-05	5.84E-07	8.33E-05	4.40E-05
14	CP7	CP	6.00E-04	7.01E-06	2.16E-08	3.62E-05	4.34E-04
15	FM1	FM	1.01E-04	6.92E-04	5.36E-06	2.65E-05	2.58E-05
16	FM2	FM	3.11E-05	1.42E-04	1.40E-06	9.02E-06	1.59E-05
17	FM3	FM	3.08E-05	1.33E-04	3.62E-06	2.32E-05	1.65E-05
18	FM4	FM	2.22E-04	2.21E-04	1.23E-06	8.08E-06	1.07E-04
19	FM5	FM	1.27E-04	1.91E-03	4.21E-06	1.40E-05	1.62E-04
20	FM6	FM	1.53E-04	5.20E-04	1.31E-06	2.58E-05	9.91E-05
21	PL1	PL	1.75E-04	1.13E-04	2.03E-05	3.90E-04	4.25E-06
22	PL2	PL	4.56E-04	2.43E-03	0.00E + 00	2.54E-04	1.75E-04
23	PL3	PL	6.71E-05	5.30E-05	1.17E-05	1.58E-05	1.76E-05
24	PL4	PL	5.31E-05	6.81E-05	9.51E-06	1.43E-05	3.12E-05
25	PL5	PL	4.25E-05	1.06E-04	6.58E-06	1.15E-05	1.63E-05
26	PL6	PL	6.93E-05	2.04E-04	1.33E-06	4.92E-06	2.52E-05
27	PL7	PL	1.42E-04	3.25E-04	3.78E-06	2.39E-05	6.81E-05
28	PL8	PL	5.17E-05	5.73E-04	1.54E-06	5.80E-06	4.11E-05
39	SL1	SL	1.75E-04	2.69E-05	7.95E-07	4.70E-04	3.14E-05
30	SL2	SL	4.11E-05	1.08E-04	1.45E-05	1.51E-05	4.71E-06
31	SL3	SL	2.94E-05	3.04E-05	3.50E-05	1.78E-05	2.23E-06
32	SL4	SL	3.78E-05	8.71E-06	2.13E-06	1.51E-05	4.36E-06
33	SL5	SL	3.33E-05	6.62E-03	1.34E-06	6.13E-04	1.44E-03
Average			1.04E-04	1.43E-04	5.98E-06	1.10E-04	4.67E-05

As shown in Fig. 2 (Table 3), 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 4.67×10^{-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 (α = 0.05). One-way ANOVA showed manure management had no significant effect on four of the five genes. Effect of 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 conditions such as anaerobic lagoons and composting. 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).

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

Network analysis indicates *intI1* and *Psychrobacter*, *ermF* and *Pseudomonas*, were significantly correlated ($p < 0.05$). This suggests *Psychrobacter* was potential hosts of *intI1*, and abundance of *ermF* could be attributable to the presence of *Pseudomonas*.

In general, ARGs are persistent in the environment, since they not only proliferate in the host bacteria, but also transfer to other microbes and pathogens through HGT mechanisms by transposons, integrons, and plasmids (Bennett 2008; Gogarten and Townsend 2005). It was reported that integrons and transposons are responsible for the acquisition and dissemination of ARGs by HGT (Han et al. 2016; Sandberg and LaPara 2016). The *sulII* gene was reported on a broad-host plasmid RSF1010 (Rådström and Swedberg 1988; Yau et al. 2010). The plasmid was also found to be integrated into transposons (Cain et al. 2010). Huang et al. (2019) reported abundance of ARGs and transposase genes, which were decreased during anaerobic digestion of swine manure. Most ARGs including *sul* genes were significantly correlated with transposase genes. This study showed that *tnpA* and *sulII* abundance were significantly correlated, which indicates that the *sulII* was possibly related to the HGT by transposons. Correlations between other genes were not significant, and this may 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).

Psychrobacter species have been found in various environmental conditions including extremely low temperatures and highly saline ecosystems. These species are considered as rare opportunistic human pathogens. 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 horizontal gene transfer (Poole 2011).

Although current animal waste treatment systems in dairy farms are not specifically designed to remove ARGs, it is important to understand the potential impacts of existing manure management practices on removal of ARGs. ARGs are considered as an environmental contaminant, which may adversely impact human health. 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. $p = 0.02$). Network analysis showed that *sulII* was significantly correlated with HGT by transposase gene, and certain pathogens (*Psychrobacter* and *Pseudomonas*) were potential ARG and MGE hosts ($p < 0.05$). Results of this study showed that ARGs are widely present in liquid (lagoon samples) and solid dairy farm manure (fresh and compost piles). Manure management such as liquid-solid separation, piling, and lagoon storage may not have significant impacts on ARG and MGE reductions. Current manure management practices need to be improved to mitigate the transmission of ARGs into the environment.

Declarations

Ethics approval and consent to participate

This article does not contain any studies with either human participants or animals. Ethical approval and consent to participate is not required.

Consent for publication

All authors gave their consent for publication.

Availability of data and material

All data supporting the results & discussion, and conclusions of this study are included in the manuscript.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Yi Wang, Pramod Pandey, and Richard Jeannotte formed the idea and were involved in writing and analysis. Yi Wang and Colleen Chiu worked on the extraction and processing of samples. Sundaram Kuppu, Ruihong Zhang, Richard Pereira, Bart Weimer, Nitin Nitin, and Sharif Aly assisted in analysis and writing of manuscript.

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References

Alexander TW, Yanke JL, Reuter T, Topp E, Read RR, Selinger BL, McAllister TA (2011) Longitudinal characterization of antimicrobial resistance genes in feces shed from cattle fed different subtherapeutic antibiotics. BMC Microbiol 11:19. doi:10.1186/1471-2180-11-19

Allen HK, Donato J, Wang HH, Cloud-Hansen KA, Davies J, Handelsman J (2010) Call of the wild: antibiotic resistance genes in natural environments. Nat Rev Microbiol 8:251-259. doi:10.1038/nrmicro2312

Aminov R, Garrigues-Jeanjean N, Mackie R (2001) Molecular ecology of tetracycline resistance: development and validation of primers for detection of tetracycline resistance genes encoding ribosomal protection proteins. Appl Environ Microbiol 67:22-32. doi:10.1128/AEM.67.1.22-32.2001

Andersson DI, Hughes D (2010) Antibiotic resistance and its cost: is it possible to reverse resistance? Nat Rev Microbiol 8:260. doi:10.1038/nrmicro2319

Baquero F, Martínez JL, Cantón R (2008) Antibiotics and antibiotic resistance in water environments. Curr Opin Biotechnol 19:260-265. doi:10.1016/j.copbio.2008.05.006.

Bennett P (2008) Plasmid encoded antibiotic resistance: acquisition and transfer of antibiotic resistance genes in bacteria. Br J Pharmacol 153:S347-S357. doi:10.1038/sj.bjp.0707607

Burch TR, Sadowsky MJ, LaPara TM (2016) Modeling the fate of antibiotic resistance genes and class 1 integrons during thermophilic anaerobic digestion of municipal wastewater solids. Appl Microbiol Biotechnol 100:1437-1444. doi:10.1007/s00253-015-7043-x

Cain AK, Liu X, Djordjevic SP, Hall RM (2010) Transposons related to Tn 1696 in IncHI2 plasmids in multiply antibiotic resistant Salmonella enterica serovar Typhimurium from Australian animals. Microb. Drug Resist 16:197-202. doi:10.1089/mdr.2010.0042

CARB (2017) California Dairy 101: Overview of dairy farming and manure methane reduction opportunities. California Air Resources Board. https://ww3.arb.ca.gov/cc/dairy/documents/08-21-17/dsg1-dairy-101-presentation.pdf Accessed 1 March 2019

CDC (2013) Antibiotic resistance threats in the United States. Centers Dis. Control Prevention. https://www.cdc.gov/drugresistance/threat-report-2013/pdf/ar-threats-2013-508.pdf Accessed 1 March 2019

CDC (2019) Antibiotic resistance threats in the United States. Centers Dis. Control Prevention. https://www.cdc.gov/drugresistance/pdf/threats-report/2019-ar-threats-report-508.pdf Accessed 1 March 2019

Chen J, Yu Z, Michel FC, Wittum T, Morrison M (2007) Development and application of real-time PCR assays for quantification of *erm* genes conferring resistance to macrolides-lincosamides-streptogramin B in livestock manure and manure management systems. Appl Environ Microbiol 73:4407-4416. doi:10.1128/AEM.02799-06

Cheng W, Chen H, Su C, Yan S (2013) Abundance and persistence of antibiotic resistance genes in livestock farms: A comprehensive investigation in eastern China. Environ Int 61:1-7. doi:10.1016/j.envint.2013.08.023

D'Costa VM, McGrann KM, Hughes DW, Wright GD (2006) Sampling the antibiotic resistome. Science 311:374-377. doi:10.1126/science.1120800

Di Cesare A, Eckert EM, D'Urso S, Bertoni R, Gillan DC, Wattiez R, Corno G (2016) Co-occurrence of integrase 1, antibiotic and heavy metal resistance genes in municipal wastewater treatment plants. Water Res 94:208-214. doi:10.1016/j.watres.2016.02.049

Dungan RS, McKinney CW, Leytem AB (2018) Tracking antibiotic resistance genes in soil irrigated with dairy wastewater. Sci Total Environ 635:1477-1483. doi:10.1016/j.scitotenv.2018.04.020

FDA (2012) Drug Use Review. U.S. Food and Drug Administration. https://www.fda.gov/media/84216/download Accessed 1 March 2019

FDA (2013) Summary Report on Antimicrobials Sold or Distributed for Use in Food-Producing Animals. U.S. Food and Drug Administration. https://www.fda.gov/media/91700/download Accessed 1 March 2019

Flores-Orozco D, Patidar R, Levin DB, Sparling R, Kumar A, Çiçek N (2020) Effect of ceftiofur on mesophilic anaerobic digestion of dairy manure and the reduction of the cephalosporin-resistance gene *cmy-2*. Bioresour Technol 301:122729. doi:10.1016/j.biortech.2019.122729

Frieri M, Kumar K, Boutin A (2017) Antibiotic resistance. J Infect Public Health 10:369-378. doi:10.1016/j.jiph.2016.08.007

Garder JL, Moorman TB, Soupir ML (2014) Transport and Persistence of Tylosin-Resistant Enterococci, Genes, and Tylosin in Soil and Drainage Water from Fields Receiving Swine Manure. J Environ Qual 43:1484-1493. doi:10.2134/jeq2013.09.0379

Ghosh S, LaPara TM (2007) The effects of subtherapeutic antibiotic use in farm animals on the proliferation and persistence of antibiotic resistance among soil bacteria. ISME J 1:191-203. doi:10.1038/ismej.2007.31

Gogarten JP, Townsend JP (2005) Horizontal gene transfer, genome innovation and evolution. Nat Rev Microbiol 3:679-687. doi:10.1038/nrmicro1204

Gou M, Hu HW, Zhang YJ, Wang JT, Hayden H, Tang YQ, He JZ (2018) Aerobic composting reduces antibiotic resistance genes in cattle manure and the resistome dissemination in agricultural soils. Sci Total Environ 612:1300-1310. doi:10.1016/j.scitotenv.2017.09.028

Guardabassi L, Schwarz S, Lloyd DH (2004) Pet animals as reservoirs of antimicrobial-resistant bacteria. J Antimicrob Chemother 54:321-332. doi:10.1093/jac/dkh332

Han XM, Hu HW, Chen QL, Yang LY, Li HL, Zhu YG, Li XZ, Ma YB (2018) Antibiotic resistance genes and associated bacterial communities in agricultural soils amended with different sources of animal manures. Soil Biol Biochem 126:91-102. doi:10.1016/j.soilbio.2018.08.018

Han XM, Hu HW, Shi XZ, Wang JT, Han LL, Chen D, He JZ (2016) mpacts of reclaimed water irrigation on soil antibiotic resistome in urban parks of Victoria, Australia. Environ Pollut 211:48-57. doi:10.1016/j.envpol.2015.12.033

Hardwick SA, Stokes H, Findlay S, Taylor M, Gillings MR (2008) Quantification of class 1 integron abundance in natural environments using real-time quantitative PCR. FEMS Microbiol Lett 278:207-212. doi:10.1111/j.1574-6968.2007.00992.x

He Y, Yuan Q, Mathieu J, Stadler L, Senehi N, Sun R, Alvarez PJJ (2020) Antibiotic resistance genes from livestock waste: occurrence, dissemination, and treatment. NPJ Clean Water 3:4. doi:10.1038/s41545-020-0051-0

Howes SA (2017) The Effect of Thermophilic Anaerobic Digestion on Ceftiofur and Antibiotic Resistant Gene Concentrations in Dairy Manure. Dissertation, Virginia Tech

Hu HW, Han XM, Shi XZ, Wang JT, Han LL, Chen D, He JZ (2015) Temporal changes of antibiotic resistance genes and bacterial communities in two contrasting soils treated with cattle manure. FEMS Microbiol Ecol 92 (2). doi:10.1093/femsec/fiv169

Huang X, Zheng J, Tian S, Liu C, Liu L, Wei L, Fan H, Zhang T, Wang L, Zhu G, Xu K (2019) Higher Temperatures Do Not Always Achieve Better Antibiotic Resistance Gene Removal in Anaerobic Digestion of Swine Manure. Appl Environ Microbiol 85:e02878-02818. doi:10.1128/aem.02878-18

Hurst JJ, Oliver JP, Schueler J, Gooch C, Lansing S, Crossette E, Wigginton K, Raskin L, Aga DS, Sassoubre LM (2019) Trends in Antimicrobial Resistance Genes in Manure Blend Pits and Long-Term Storage Across Dairy Farms with Comparisons to Antimicrobial Usage and Residual Concentrations. Environ Sci Technol 53:2405-2415. doi:10.1021/acs.est.8b05702

Kaffka S, Barzee T, El-Mashad H, Williams R, Zicari S, Zhang R (2016) Evaluation of Dairy Manure Management Practices for Greenhouse Gas Emissions Mitigation in California. Contract 14:456. https://biomass.ucdavis.edu/wp-content/uploads/ARB-Report-Final-Draft-Transmittal-Feb-26-2016.pdf Accessed 1 March 2019

Klindworth A, Pruesse E, Schweer T, Peplies J, Quast C, Horn M, Glöckner FO (2012) Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. Nucleic Acids Res 41(1):e1-e1. doi:10.1093/nar/gks808

Koike S, Aminov RI, Yannarell AC, Gans HD, Krapac IG, Chee-Sanford JC, Mackie RI (2010) Molecular Ecology Of Macrolide–Lincosamide–Streptogramin B Methylases in Waste Lagoons and Subsurface Waters Associated with Swine Production. Microb Ecol 59:487-498. doi:10.1007/s00248-009-9610-0

Kumar K, Gupta SC, Baidoo S, Chander Y, Rosen CJ (2005) Antibiotic uptake by plants from soil fertilized with animal manure. J Environ Qual 34:2082-2085. doi:10.2134/jeq2005.0026

Ma Z, Wu H, Zhang K, Xu X, Wang C, Zhu W, Wu W (2018) Long-term low dissolved oxygen accelerates the removal of antibiotics and antibiotic resistance genes in swine wastewater treatment. Chem Eng J 334:630-637. doi:10.1016/j.cej.2017.10.051

Marti E, Jofre J, Balcazar JL (2013) Prevalence of antibiotic resistance genes and bacterial community composition in a river influenced by a wastewater treatment plant. PLoS One 8:e78906. doi:10.1021/es504157v

McKinney CW, Loftin KA, Meyer MT, Davis JG, Pruden A (2010) *Tet* and *sul* antibiotic resistance genes in livestock lagoons of various operation type, configuration, and antibiotic occurrence. Environ Sci Technol 44:6102-6109. doi:10.1021/es9038165

Meyer D, Price PL, Rossow HA, Silva-del-Rio N, Karle BM, Robinson PH, DePeters EJ, Fadel JG (2011) Survey of dairy housing and manure management practices in California. J Dairy Sci 94:4744-4750. doi:10.3168/jds.2010-3761

Muyzer G, De Waal EC, Uitterlinden AGJA (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl Environ Microbiol 59:695-700.

Pandey P, Chiu C, Miao M, Wang Y, Settles M, del Rio NS, Castillo A, Souza A, Pereira R, Jeannotte R (2018) 16S rRNA analysis of diversity of manure microbial community in dairy farm environment. PLoS One 13:e0190126. doi:10.1371/journal.pone.0190126

Pang Z, Raudonis R, Glick BR, Lin TJ, Cheng Z (2019) Antibiotic resistance in Pseudomonas aeruginosa: mechanisms and alternative therapeutic strategies. Biotechnol Adv 37:177-192. doi:10.1016/j.biotechadv.2018.11.013

Parashar D, Chauhan D, Sharma V, Katoch V (2006) Applications of real-time PCR technology to mycobacterial research. Indian J Med Res 124:385.

Pei R, Cha J, Carlson KH, Pruden A (2007) Response of antibiotic resistance genes (ARG) to biological treatment in dairy lagoon water. Environ Sci Technol 41:5108-5113. doi:10.1021/es070051x

Pei R, Kim SC, Carlson KH, Pruden A (2006) Effect of river landscape on the sediment concentrations of antibiotics and corresponding antibiotic resistance genes (ARG). Water Res 40:2427-2435. doi:10.1016/j.watres.2006.04.017

Poole K (2011) *Pseudomonas aeruginosa*: resistance to the max. Front Microbiol 2:65-65. doi:10.3389/fmicb.2011.00065

Pruden A, Larsson DJ, Amézquita A, Collignon P, Brandt KK, Graham DW, Lazorchak JM, Suzuki S, Silley P, Snape JR (2013) Management options for reducing the release of antibiotics and antibiotic resistance genes to the environment. Environ Health Perspect 121:878. doi:10.1289/ehp.1206446

Rådström P, Swedberg G (1988) RSF1010 and a conjugative plasmid contain *sulII*, one of two known genes for plasmid-borne sulfonamide resistance dihydropteroate synthase. Antimicrob Agents Chemother 32:1684-1692. doi:10.1128/aac.32.11.1684

Roberts RR, Hota B, Ahmad I, Scott RD, Foster SD, Abbasi F, Schabowski S, Kampe LM, Ciavarella GG, Supino M (2009) Hospital and societal costs of antimicrobial-resistant infections in a Chicago teaching hospital: implications for antibiotic stewardship. Clin Infect Dis 49:1175-1184. doi:10.1086/605630

Sandberg KD, LaPara TM (2016) The fate of antibiotic resistance genes and class 1 integrons following the application of swine and dairy manure to soils. FEMS Microbiol Ecol 92(2). doi:10.1093/femsec/fiw001

Selvam A, Xu D, Zhao Z, Wong JW (2012) ate of tetracycline, sulfonamide and fluoroquinolone resistance genes and the changes in bacterial diversity during composting of swine manure. Bioresour Technol 126:383-390. doi:10.1016/j.biortech.2012.03.045

Storteboom HN, Kim SC, Doesken KC, Carlson KH, Davis JG, Pruden A (2007) Response of antibiotics and resistance genes to high-intensity and low-intensity manure management. J Environ Qual 36:1695-1703. doi:10.2134/jeq2007.0006

Sun W, Qian X, Gu J, Wang XJ, Duan ML (2016) Mechanism and effect of temperature on variations in antibiotic resistance genes during anaerobic digestion of dairy manure. Sci Rep 6:1-9. doi:10.1038/srep30237

UCS (2001) Hogging it!: Estimates of antimicrobial abuse in livestock. Union of Concerned Scientists. https://www.ucsusa.org/resources/hogging-it-estimates-antimicrobial-abuse-livestock#.W77eXi_Mz2Q Accessed 1 March 2019

USDA (2012) Census of Agriculture. USDA National Agricultural Statistics Service. https://www.nass.usda.gov/Publications/AgCensus/2012/ Accessed 1 March 2019

USDA (2016) National Agricultural Statistics Service. https://www.nass.usda.gov/Charts_and_Maps/Cattle/ Accessed 1 March 2019

Wang L, Oda Y, Grewal S, Morrison M, Michel FC, Yu Z (2012) Persistence of Resistance to Erythromycin and Tetracycline in Swine Manure During Simulated Composting and Lagoon Treatments. Microb Ecol 63:32-40. doi:10.1007/s00248-011-9921-9

Wind L, Krometis LA, Hession WC, Chen C, Du P, Jacobs K, Xia K, Pruden A (2018) Fate of pirlimycin and antibiotic-resistant fecal coliforms in field plots amended with dairy manure or compost during vegetable cultivation. J Environ Qual 47:436-444. doi:10.2134/jeq2017.12.0491

Xia J, Sinelnikov IV, Han B, Wishart DS (2015) MetaboAnalyst 3.0—making metabolomics more meaningful. Nucleic Acids Res 43:W251-W257. doi:10.1093/nar/gkv380

Yau S, Liu X, Djordjevic SP, Hall RM (2010) RSF1010-like plasmids in Australian Salmonella enterica serovar Typhimurium and origin of their sul2-strA-strB antibiotic resistance gene cluster. Microb Drug Resist 16:249-252. doi:10.1089/mdr.2010.0033

Zaman SB, Hussain MA, Nye R, Mehta V, Mamun KT, Hossain N (2017) A review on antibiotic resistance: alarm bells are ringing. Cureus 9(6). doi:10.7759/cureus.1403

Zhang R, Wang X, Gu J, Zhang Y (2017) Influence of zinc on biogas production and antibiotic resistance gene profiles during anaerobic digestion of swine manure. Bioresour Technol 244:63-70. doi:10.1016/j.biortech.2017.07.032

Zhao X, Wang J, Zhu L, Ge W, Wang J (2017) Environmental analysis of typical antibiotic-resistant bacteria and ARGs in farmland soil chronically fertilized with chicken manure. Sci Total Environ 593:10-17. doi:10.1016/j.scitotenv.2017.03.062

Zhu YG, Johnson TA, Su JQ, Qiao M, Guo GX, Stedtfeld RD, Hashsham SA, Tiedje JM (2013) Diverse and abundant antibiotic resistance genes in Chinese swine farms. Proc Natl Acad Sci USA 110:3435-3440. doi:10.1073/pnas.1222743110

Figures

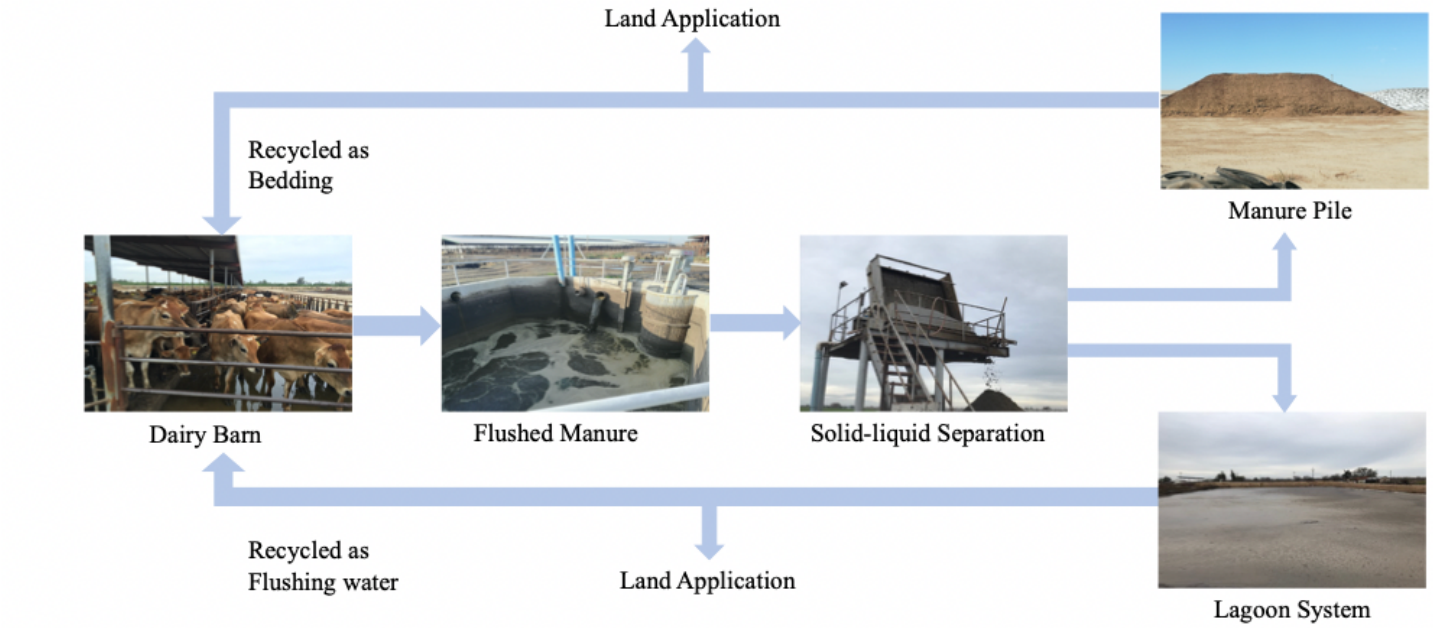

Figure 1

Typical processing of flushed manure in dairy farms in Central Valley California, USA

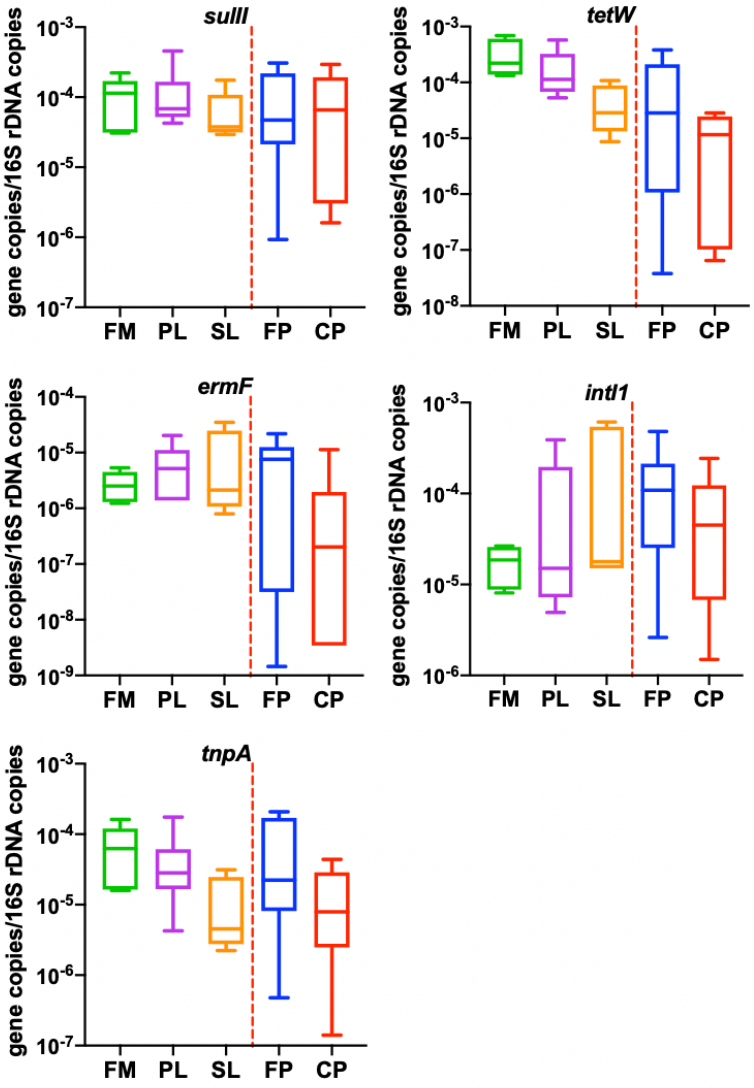

Figure 2

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.

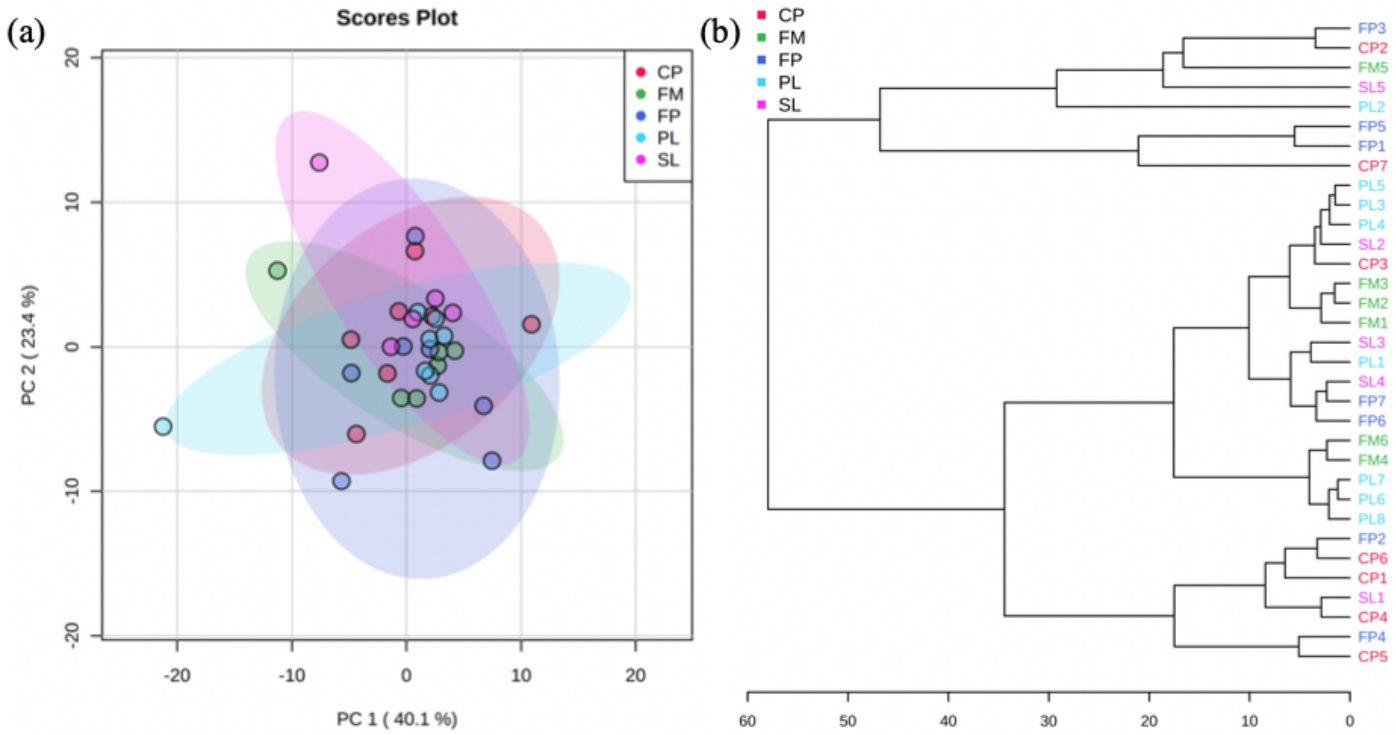

Figure 3

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

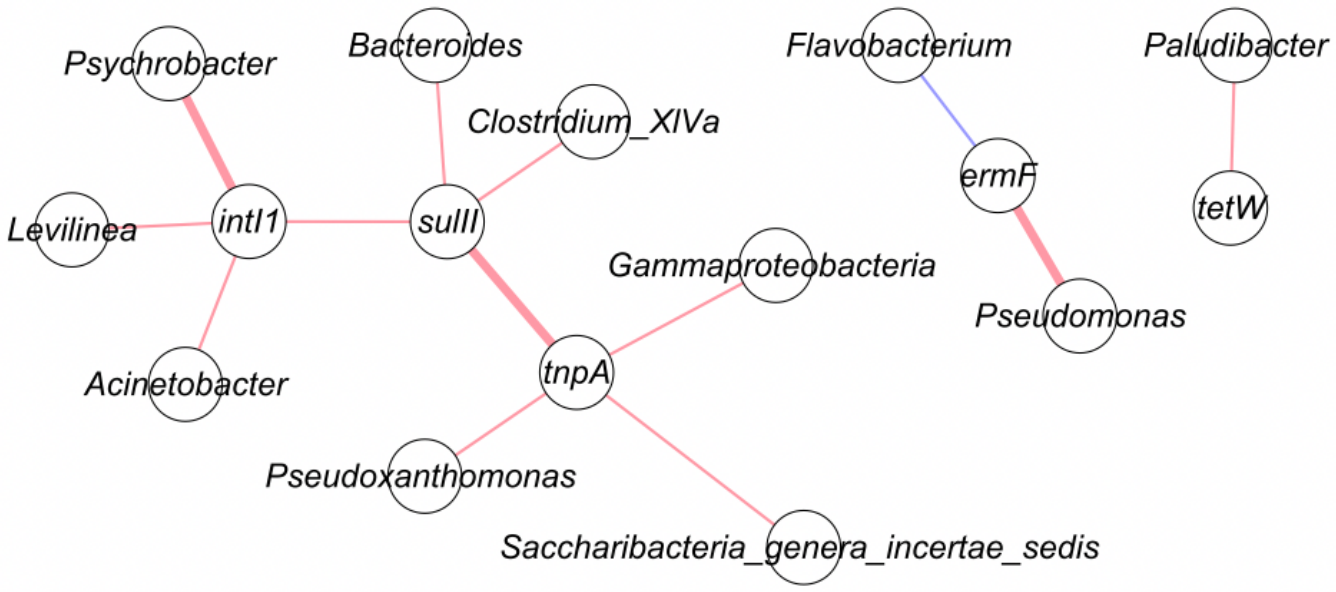

Figure 4

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

Supplementary Files

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