

UC Davis

UC Davis Previously Published Works

Title

Degradation of antibiotic resistance genes and mobile gene elements in dairy manure anaerobic digestion.

Permalink

<https://escholarship.org/uc/item/2rs9b1xb>

Journal

PloS one, 16(8)

ISSN

1932-6203

Authors

Wang, Yi

Pandey, Pramod K

Kuppu, Sundaram

et al.

Publication Date

2021

DOI

10.1371/journal.pone.0254836

Copyright Information

This work is made available under the terms of a Creative Commons Attribution License, available at <https://creativecommons.org/licenses/by/4.0/>

Peer reviewed

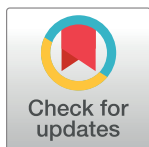
RESEARCH ARTICLE

Degradation of antibiotic resistance genes and mobile gene elements in dairy manure anaerobic digestion

Yi Wang^{1,2}, Pramod K. Pandey^{1*}, Sundaram Kuppu¹, Richard Pereira¹, Sharif Aly¹, Ruihong Zhang²

1 Department of Population Health and Reproduction, School of Veterinary Medicine, University of California-Davis, Davis, California, United States of America, **2** Department of Biological and Agricultural Engineering, University of California-Davis, Davis, California, United States of America

* pkpandey@ucdavis.edu



Abstract

Antibiotic resistance genes (ARGs) are emerging contaminants causing serious global health concern. Interventions to address this concern include improving our understanding of methods for treating waste material of human and animal origin that are known to harbor ARGs. Anaerobic digestion is a commonly used process for treating dairy manure, and although effective in reducing ARGs, its mechanism of action is not clear. In this study, we used three ARGs to conduct a longitudinal bench scale anaerobic digestion experiment with various temperatures (28, 36, 44, and 52°C) in triplicate using fresh dairy manure for 30 days to evaluate the reduction of gene abundance. Three ARGs and two mobile genetic elements (MGEs) were studied: sulfonamide resistance gene (*sullI*), tetracycline resistance genes (*tetW*), macrolide-lincosamide-streptogramin B (MLS_B) superfamily resistance genes (*ermF*), class 1 integrase gene (*intl1*), and transposase gene (*tnpA*). Genes were quantified by real-time quantitative PCR. Results show that the thermophilic anaerobic digestion (52°C) significantly reduced ($p < 0.05$) the absolute abundance of *sullI* (95%), *intl1* (95%), *tnpA* (77%) and 16S rRNA gene (76%) after 30 days of digestion. A modified Collins–Selleck model was used to fit the decay curve, and results suggest that the gene reduction during the startup phase of anaerobic digestion (first 5 days) was faster than the later stage, and reductions in the first five days were more than 50% for most genes.

OPEN ACCESS

Citation: Wang Y, Pandey PK, Kuppu S, Pereira R, Aly S, Zhang R (2021) Degradation of antibiotic resistance genes and mobile gene elements in dairy manure anaerobic digestion. PLoS ONE 16(8): e0254836. <https://doi.org/10.1371/journal.pone.0254836>

Editor: Abasiofiok M. Ibekwe, USDA-ARS Salinity Laboratory, UNITED STATES

Received: January 30, 2021

Accepted: July 3, 2021

Published: August 25, 2021

Copyright: © 2021 Wang et al. This is an open access article distributed under the terms of the [Creative Commons Attribution License](https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Data Availability Statement: All relevant data are within the paper and its [Supporting Information](#) files.

Funding: No special funding was received for this study.

Competing interests: The authors have declared that no competing interests exist.

Introduction

Antibiotic resistance is an emerging issue causing serious health concerns. In the United States, at least 2.8 million antibiotic-resistant infections are reported annually, and more than 35,000 human deaths occur as a result of antibiotic-resistant infections [1]. It has been reported that the deaths caused by methicillin-resistant *Staphylococcus aureus* (MRSA) in the US could be higher than that caused by HIV [2,3]. Antimicrobial drugs are known to be

important for the selection of antimicrobial resistance in addition to being a shared resource between human and animal populations [4–7].

Each year, more than 11 million kilograms of antimicrobial drugs are used in food-producing animals, within which 52% are medically important drugs [8]. In 2016, 87.5% of US cattle feedlots gave antibiotics in feed, water, or by injection to treat, prevent, and control diseases [9]. More than half of feedlots (55.6%) used medically important antibiotics (i.e., important for treating human disease) in feed. To prevent, control, or treat respiratory disease, 41.8% of feedlots treated cattle with antibiotics in feed. In addition, 80% of feedlots treated sick animals individually with antibiotics by injection.

Up to 90% of used antibiotics are excreted via urine and feces in the unchanged form as well as the active metabolites, including tylosin, tetracycline, and sulfonamides [10,11]. When animal excretion is used as fertilizers on agricultural lands and/or directly deposited on grazing land by livestock, antibiotics and ARGs may be transferred to croplands [12–14]. Excessive use of manure in the field increases the chances of manure borne ARGs in contaminating soil and water resources [12,15]. Some antibiotic resistant bacteria in manure are human pathogens such as *S. Typhimurium* and Shiga-toxigenic *E. coli* [16,17], and other bacteria in the environment including pathogens may gain ARGs by horizontal gene transfer (HGT) mechanisms mediated by transposons, integrons, and plasmids [18,19] via transformation, transduction, and conjugation [20,21]. To that end, several national and state regulations to limit the use of antibiotics have been made. For example, the U.S. Drug and Food Administration (FDA)'s directive (Veterinary Feed Directive (VFD) prohibits subtherapeutic doses in animal feed and/or animal drinking water to promote growth and improve feed efficiency. In California, Senate Bill No. 27 (2015) requires a prescription from a California-licensed veterinarian to purchase and use medically important antimicrobial drugs in livestock.

Although policies that limit the uses of antibiotics in animal-agriculture system have the potential to control excessive use of antibiotics, the duration of survival and threat of ARGs being effectively transferred to bacteria in the environment is not understood. Furthermore, relying on natural degradation of ARGs in the environment over time may not be realistic given that studies have shown that the rate of resistance reversibility at the community level may be slow due to evolution, mutations, and genetic co-selection [22,23].

One potential solution for the degradation of ARGs in the environment is anaerobic digestion, which can serve as a step for manure treatment prior to application on crop fields. Previous studies showed anaerobic digestion process could help in reducing ARGs in animal manure [24–27]. Primarily, anaerobic digestion is used to treat manure and produce biogas, a source of renewable energy [28,29]. In addition, anaerobic digestion process produces beneficial organic fertilizer, control odor, and reduces pathogen/bacterial levels in animal waste [30–33]. Compared to mesophilic and ambient-temperature anaerobic process, thermophilic anaerobic process is known to accelerate the digestion process, pathogen reduction, and biogas production [26,34]. Both mesophilic and thermophilic digesters are used to treat organic waste material, however, how an increase in temperature influences ARG removal in dairy manure is not well understood. In general, the impacts of temperature on ARG removal is uncertain [25,35–37].

Hence, the goal of this study was to investigate the effectiveness of anaerobic digestion on neutralizing ARGs in dairy manure. The specific objectives of this research were to: 1) understand the impacts of anaerobic digestion on the reduction of ARGs and MGEs in dairy manure; 2) determine the impacts of mesophilic and thermophilic temperatures on the reduction of ARGs and MGEs; and 3) fit a model to predict gene reductions in anaerobic digestion process over time.

Materials and methods

Batch anaerobic digestion experiment of liquid manure

Fresh manure was collected from the University of California, Davis research dairy facility. Manure was diluted with DI water (0.5 L water/kg of manure) and filtered with an 850 μm standard sieve (Fisher Scientific, Hampton, NH). Subsequently, manure was mixed with granules-inocula, as an inoculum to start anaerobic process inside the reactors. The granules were obtained from a mesophilic UASB (i.e., upflow anaerobic sludge blanket) reactor treating potato starch waste (Ingredion Incorporated, Richland, WA).

Total solid content (TS) and volatile solid content (VS) of original manure, filtered manure, granules-inocula, and mixed feedstock in this experiment were measured according to the American Public Health Association Methods [38]. Mixed feedstock refers to the mixture of granules-inocula and filtered liquid manure that was fed directly to the flask digester. To set up the batch anaerobic digestion experiment (Fig 1), twelve 1000 mL volume Erlenmeyer flasks (Corning Life Sciences, Tewksbury, MA) were filled with 430 mL of liquid manure and 70 g of inocula. Liquid manure and inocula were stirred well before feeding to each flask so that there was no difference between each batch. All the bottles were purged with nitrogen for 2 min and then tightly plugged by a rubber stopper in order to maintain an anaerobic environment. The rubber stopper was inserted by two 8-gauge dispensing needles. One was inserted into the liquid portion for sampling. The other needle in the headspace was connected to a Tedlar bag as a biogas outlet. Connections were sealed by liquid nails (i.e., adhesive) and the seal integrity was tested for each reactor.

The anaerobic digestion batch experiments were performed in four water baths at temperatures 28°C (moderate condition), 36°C (mesophilic condition), 44°C (high mesophilic condition), and 52°C (thermophilic condition). Each temperature had three digesters. Filtered manure, granules-inocula, and mixed feedstock were sampled for DNA extraction and gene quantification. Samples were taken on 0, 1, 2, 3, 5, 7, 10, 14, 18, 22, 26, 30 days, with a total of 12 time points. By connecting a syringe to the liquid inlet, 10 mL digestate at each time point was sampled. After collection, samples were stored at -20°C until use.

Quantitative reverse transcription PCR (RT-qPCR)

Total DNA was extracted by DNeasy PowerSoil Kit (Qiagen, Valencia, CA). Two mL of each sample was centrifuged at 13,000 rpm for 10 min and the pellet was used for DNA extraction.

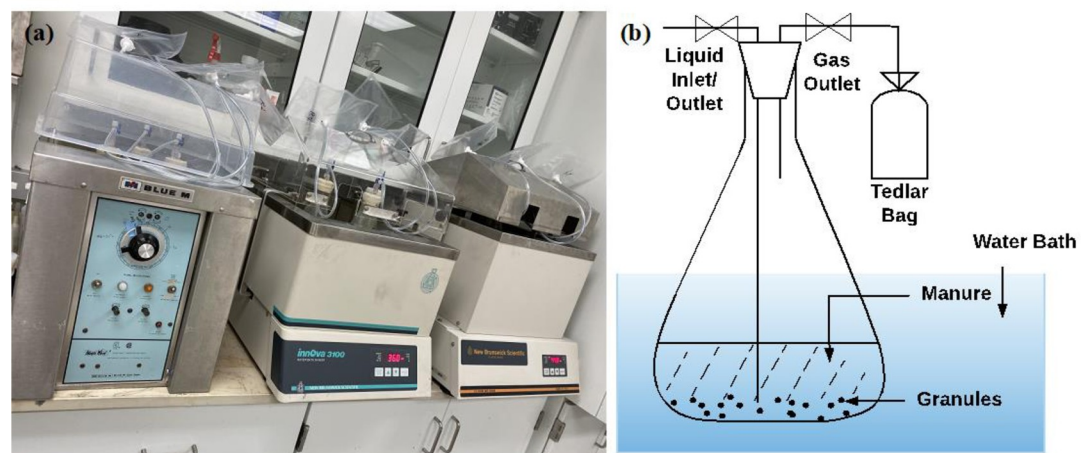


Fig 1. (a) Anaerobic digester setup. (b) Schematic graph of the anaerobic digester.

<https://doi.org/10.1371/journal.pone.0254836.g001>

The quality and concentration of the DNA were assessed by NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE). All extracted DNA samples were stored at -20°C before qPCR amplification.

Five types of genes were under specific concern to be investigated in this study: [sulfonamide resistance genes (*sulII*), tetracycline resistance genes (*tetW*), macrolide-lincosamide-streptogramin B (MLS_B) superfamily (erythromycin is a member) resistance genes (*ermF*), and MGEs (*intI1*, *tnpA*)], which are important for assessing the persistence and dispersal of ARGs. Tetracycline, macrolide, and sulfonamide are three of the most commonly used antibiotics for animal treatment. In 2014, 15.5% of US dairy operations fed tetracycline as medicated milk replacer to preweaned heifers, specifically chlortetracycline (1.6% of operations), Oxytetracycline (4.9% of operations), or combination of neomycin and oxytetracycline (9% of operations) [39]. Furthermore, macrolides were the primary antibiotics to treat respiratory disease in preweaned heifers (18.2% of operations) and weaned heifers (14.1% of operations), and about 66% of weaned heifers were treated by sulfonamides as their primary antibiotics for diarrhea.

Primers designed in previous work were used to amplify targeted ARGs as in Table 1. PCR products were purified, verified by gel electrophoresis, and ligated into a pCR2.1-TOPO vector by TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Ligations were transformed into DH5 α competent cells. Plasmids carrying the targeted ARGs were extracted from cell cultures using QIAprep Spin Miniprep kit (Qiagen, Valencia, CA) and sequenced to verify the insert of the targeted ARGs. The quality and concentration of plasmids were assessed by NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE). The copy number of genes per μL plasmid DNA was calculated by [40]:

$$\text{copy of genes}/\mu\text{LDNA} = \frac{b \times c}{L \times a \times 10^{12}} \quad (1)$$

where a is the weight of kb DNA per pmol (1 kb DNA = 0.66 $\mu\text{g}/\text{pmol}$), b the Avogadro's constant ($6.022 \times 10^{23}/\text{mol}$), L the length of template containing the target gene, c the concentration of template measured in $\mu\text{g}/\mu\text{L}$.

Standard curves of DNA standards were constructed for each 96-well qPCR assay, and each standard curve was generated by 12-fold serial dilutions of plasmid DNA. DNA dilutions that fell out of the linear region of the standard curve were removed from the analysis. Generally, the quantitative limit of qPCR was 10^3 copies/mL digestate. Efficiencies of PCR standards were

Table 1. Synthetic oligonucleotides and temperature regimes used for qPCR reactions.

Primer	Target gene	Sequences (direction 5'-3')	qPCR annealing temp ($^{\circ}\text{C}$)	Amplicon size (bp)	Reference
sulII-FW	<i>sulII</i>	TCCGGTGGAGGCCGGTATCTGG	60	191	[40]
sulII-RV		CGGGAATGCCATCTGCCTTGAG			
tetW-FW	<i>tetW</i>	GAGAGCCTGCTATATGCCAGC	55	168	[41]
tetW-RV		GGGCGTATCCACAATGTTAAC			
ermF-189f	<i>ermF</i>	CGACACAGCTTTGGTTGAAC	55	309	[42]
ermF-497r		GGACCTACCTCATAGACAAG			
HS463a	<i>intI1</i>	CTGGATTTTCGATCACGGCAGC	57	473	[43]
HS464		ACATGCGTGTAAATCATCGTTCG			
tnpA-04F	<i>tnpA-04</i>	CCGATCACGGAAAGCTCAAG	56	101	[44]
tnpA-04R		GGCTCGCATGACTTCGAATC			
357F	16S rRNA gene	CCTACGGGAGGCAGCAG	56	193	[45]
518R		ATTACCGCGGCTGCTGG			

<https://doi.org/10.1371/journal.pone.0254836.t001>

calculated by: Efficiency = $10^{-(1/\text{slope})} - 1$, which were all between 90% and 110% with $r^2 \geq 0.99$ for a minimum of six points.

Targeted ARGs and 16S rRNA gene qPCR reactions were performed using the AriaMx Real-time PCR System (Agilent Technologies, Santa Clara, CA) in a 10 μL reaction mixture (5 μL PowerUp SYBR Green Master Mix [2 \times] (Life Technology, Carlsbad, CA), 0.5 μL 10 mM each primer, and 1 μL of template) with a thermal cycling 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 55°C-60°C; 1 min at 72°C. Each reaction was repeated three times. The average copy and DNA standard deviation were calculated from three dataset for each reaction.

The absolute copy number of genes, which is called absolute abundance (AA), was quantified by referring to the corresponding DNA standard curve obtained by plotting threshold cycles versus log-copy number of genes, which represents gene copy numbers per milliliter of digestate sample. The AA of 16S rRNA gene also represents the total bacterial biomass [46–49]. Levels of targeted ARGs 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 (RA) in manure samples [50–52]. Relative abundance (RA) of genes represents gene absolute abundance normalized to the total number of 16S rRNA genes present in the sample. This normalization provides the proportion of bacterial populations carrying the ARGs/MGEs of interest [53].

Statistical analysis

Statistical analysis showed gene abundance data followed a log-normal distribution, which was also reported in other studies [47,49]. Therefore, data were log-transformed before statistical analysis. Gene AAs of a total of 144 samples were analyzed, representing 12 reactors at 12 time points. The *ermF* was excluded for further analysis because the gene concentration in samples was below the detection limit (10^3 copies/mL). Two-factor mixed-design ANOVA analysis was conducted to evaluate the impacts of digestion time and temperature on gene reductions as measured using AA. Each reactor was treated as a block. Time was regarded as a within-subject factor, whereas temperature was a between-subject factor. Residuals passed the Brown-Forsythe test for heteroscedasticity and the Anderson-Darling test for normality ($p < 0.05$). Tukey test was used for multiple comparisons between Day 0, 5, and 30 for each gene ($\alpha = 0.05$). Multiplicity adjusted p -value was reported for each comparison.

Pearson's correlation matrix between gene relative abundance was generated by pooling of gene abundance data together (144 samples with 12 time points and 12 digesters). The correlation coefficient values range from "0" meaning no correlations to "1" which is a monotone increasing relationship, and "-1" which is a monotone decreasing relationship.

The decay rate of ARGs was determined by a Collins-Selleck model (Eq 2). Collins-Selleck model was initially developed to describe the inactivation of coliform microbes in domestic wastewater by chlorine [54,55]. This model is also used in alternative disinfection methodologies [54]. Previous studies have shown that the decay of ARGs in wastewater solids-amended soil microcosms [49] and anaerobic digestion of municipal wastewater solids [47] can be described by a modified Collins-Selleck model [54,55]. The model (Eq 2) is a power function.

$$\log_{10} \left(\frac{N}{N_0} \right) = \Lambda_{CS} [\log_{10} t - \log_{10} b] \quad (2)$$

In this equation, N is the number of ARG copies at time t , N_0 is the initial quantity of ARGs, Λ_{CS} is the specific lethality coefficient and b is a lag coefficient. Nonlinear regression curves based on gene concentrations versus time was established. The average of three

replicates (samples from three digesters) of each treatment (temperature) was used for curve fitting. Least squares regression was chosen as the fitting method. Residuals analysis passed the normality test (Anderson-Darling) and homoscedasticity test checked by the Diagnostics tab. A p -value of less than 0.05 was considered significant. All statistical analyses were conducted using GraphPad Prism 8.

Results and discussion

Absolute abundance and relative abundance of ARGs and MGEs in anaerobic digestion

The analysis of gene AA in granules-inocula showed *sulIII*, *tetW*, *ermF*, *intI1*, and *tnpA* levels were below the detection limit in inoculum, which indicates mixing granules-inocula did not add external ARGs and MGEs of concern into the dairy manure. Total solid content (TS) and volatile solid (VS) mixed feedstocks (filtered liquid manure mixed with granules) were 6.64% and 5.37%, respectively (S1 Table).

Gene absolute abundance at Day 0, 5, 30 were compared as shown in Fig 2. *sulIII*, *intI1* and 16S rRNA gene were significantly reduced ($p < 0.001$) in anaerobic digestion at all temperatures by Day 30. *tnpA* was significantly reduced under 52°C ($p < 0.05$) by 77% on Day 30. The reduction in *tetW* abundance was not significant, and *tetW* was increased by 12–13% on Day 5 at 44°C and on Day 30 at 52°C (S2 Table). Results of ANOVA analysis (S3 Table) showed that the impacts of time on gene absolute abundance was significant ($p \leq 0.0001$). Impacts of temperature and interaction effects of time and temperature on gene *sulIII*, *tetW*, *intI1* and 16S rRNA gene were significant ($p \leq 0.05$). Among all the sources of variation, effect of time explained most of the total variation for each gene, ranging from 43% (*sulIII*) to 75% (16S rRNA gene). These results suggest that the impacts of digestion time on reduction of gene AA was considerable during anaerobic digestion process.

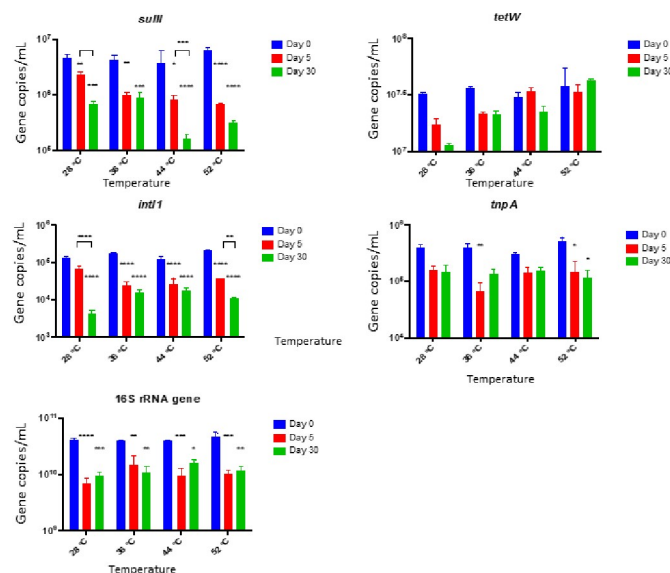


Fig 2. Absolute abundance of ARGs and MGEs at Day 0, 5, 30 in anaerobic digestion. Values represent the means based on three replicates. Stars on bars of Day 5 and Day 30 represent their p value when gene AAs were compared with the corresponded initial value (Day 0). Stars on square brackets showed p value of comparison between Day 5 and Day 30. Error bars represent standard errors. Symbol and meaning: $p \leq 0.05$ (*); $p \leq 0.01$ (**); $p \leq 0.001$ (***); $p \leq 0.0001$ (****).

<https://doi.org/10.1371/journal.pone.0254836.g002>

At Day 0, *tetW* was the most abundant among targeted ARGs with AA > 10⁷ copies/mL followed by *sulIII* (>10⁶ copies/mL). The AAs of both *intI1* and *tnpA* were >10⁵ copies/mL. The AA of 16S rRNA gene were >10¹⁰ copies/mL. After a digestion of 30 days, the reduction in *sulIII* level was significantly higher at 44°C and 52°C compared to 28°C and 36°C ($p < 0.05$). At 44°C and 52°C, *sulIII* level was reduced by 95% and 96%, respectively. The *intI1* level at 52°C was reduced by 95%. The highest reduction in *tnpA* was at 52°C (reduced by 77%). However, at low temperature, *tetW* level was reduced by 64% at 28°C.

The reduction in 16S rRNA gene is shown in Fig 2. After 30 days of anaerobic digestion, 16S rRNA gene was reduced by 76–77%. In general, *sulIII*, *intI1*, *tnpA*, and 16S rRNA gene reductions were overall highest at thermophilic temperature (i.e., 52°C). Similar level of reductions were obtained at 44°C for *sulIII* and at 28°C for *intI1* and 16S rRNA. The reduction on gene AA at 52°C was in the following order: *sulIII* = *intI1* > *tnpA* = 16S rRNA gene > *tetW*.

While analyzing the impacts of digestion time, results showed that the levels of *sulIII* and *intI1* AAs on Day 30 were lower than that of Day 5 under all temperature conditions. This indicates that the longer digestion period will likely to be more effective in reducing genes compared to less than a week of digestion period. The gene reduction percentages from Day 5 to Day 30 was significant in some cases (*sulIII* at 28°C and 44°C, *intI1* at 2°C and 52°C). In few genes, for example, the *tnpA* gene level remained at the same level post 5 days of digestion.

Relative abundance (i.e., RA) of *sulIII*, *intI1* were significantly reduced during anaerobic process (Fig 3). Both 44°C and 52°C resulted in reduction of *sulIII* significantly on Day 30 ($p < 0.01$) (S4 Table). Similarly, RA of *intI1* was significantly reduced at 52°C on Day 30 ($p < 0.01$), with reductions of log 0.80 and 0.65, respectively. There were no significant declines in the RAs of *tnpA*. In few cases, for example, ARG *tetW* showed relatively higher level at high mesophilic (44°C) and thermophilic temperature conditions (52°C). In general, RAs of *sulIII* and *intI1* were reduced under all temperature conditions at the end of 30 days of anaerobic digestion. In a previous study, a similar anomaly with regards to *tetW* and *tetO* was reported, where Wallace et al. (2018) [56] investigated the abundance of sulfonamides and tetracyclines resistant ARGs (log (ARG Copies/16S rRNA)) in a full-scale anaerobic digester, and authors found that *sul1* and *sul2* copies were reduced significantly ($P < 0.001$) by 5% and 10% respectively, however, tetracyclines-related genes (*tetW* and *tetO*) concentrations were unchanged.

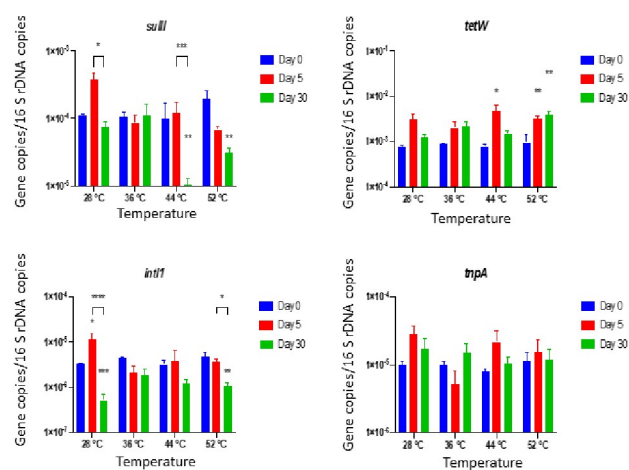


Fig 3. Relative abundance of ARGs and MGEs detected at Day 0, 5, 30 during anaerobic digestion. Values represent the means based on three replicates. Bars represent standard errors. Symbol and meaning: $P \leq 0.05$ (*); $P \leq 0.01$ (**); $P \leq 0.001$ (***) ; $P \leq 0.0001$ (****).

<https://doi.org/10.1371/journal.pone.0254836.g003>

Considering the existing information with regards to ARGs reductions in anaerobic digestion process, previous research suggested that anaerobic digestion process may have an impact on reducing ARGs. As an example, Flores Orozco [57] demonstrated that anaerobic digestions removed more than 90% of cephalosporin-resistance gene *cmv-2* in dairy manures in a semi-continuous lab-scale anaerobic digester under mesophilic temperature conditions, with a hydraulic retention time of 30 days. Zhang, Yang [27] found that among 35 studied ARG subtypes, only 8 subtypes were reduced by > 90.0% under thermophilic temperature conditions. A range of other studies, however, suggested that the impact of anaerobic process on gene reduction could be gene specific [25,58–60]. Howes [58] examined changes of antibiotic resistant genes in lab scale anaerobic digesters at thermophilic temperatures, and found that *tetQ* and *cfxA* concentrations reduced significantly by 70%, whereas *mefA* concentrations was increased during 10 days of anaerobic digestion. Similarly, Gao, Gu [61] investigated tetracycline resistance genes and the integrase gene *intI1* in thermophilic anaerobic co-digestion study and reported that all tetracycline resistance genes were reduced except *tetW*. Authors reported that *tetW* encodes ribosomal protection protein, which is relatively more persistent to thermophilic conditions. Besides, the persistence of *tetW* in anaerobic digestion may be explained by a wide variety of microbial hosts. Roberts [62] reported the host range of the *tetW* gene includes Gram-positive, Gram-negative, aerobic and anaerobic bacteria, and *tetW* gene is also associated with conjugative elements which may contribute to its spread. Many factors such as the type of ARGs, bacteria community, digestion parameters, and associated metabolic pathways in the anaerobic digestion process could be attributed to the inconsistent effect of anaerobic digestion on ARG removal, and it also underscores the need for additional research [63].

Correlations between ARGs and MGEs

Correlation plot of the four genes in Fig 4 showed that *intI1* and *tnpA* were significantly positive correlated with *sulII* and *tetW* ($P < 0.01$). *sulII* and *tetW* were significantly correlated with *intI1*, which means if a sample was found to have high level of *intI1* and *tnpA* abundance, the abundance of *sulII* and *tetW* was higher. The degrees of correlations of *tetW* with *intI1* and *tnpA* were higher than *sulII*, indicating *tetW* abundance was more likely to be positively influenced by *intI1* and *tnpA* abundance than *sulII*. The correlation coefficient of *intI1* and *tnpA* was also significant that indicates that *intI1* tended to co-exist with *tnpA*. The correlation coefficient of *sulII* and *tetW* was found to be low (-0.01) and not significant ($p = 0.89$), which indicates that the abundance of these genes was not correlated. Interspecies gene transfer may have played a role up to an extent to establish linkages between genes. Sørensen, Bailey [64] suggested horizontal gene transfer (due to gene transfer via MGEs between different bacterial hosts) and vertical gene transfer (due to the proliferation of bacterial hosts) are two main factors for ARGs abundance. Mobile genetic elements (MGEs), including transposons, plasmids, and integrons facilitates horizontal gene transfer (HGT) and induce a higher abundance of ARGs [44]. Mobile integrons usually carry ARGs and mobility is supported by transposons and plasmids [65]. Huang, Zheng [35] investigated transposase genes and ARGs during anaerobic digestion of swine manure, and transposase genes showed significant correlations with most ARG types including sulfonamide and tetracycline resistance genes.

Gene reduction curves fit using Collins-Selleck model

Results showed that the modified Collins–Selleck model provided a good fit for estimating the reductions of ARGs, MGEs, and 16S rRNA genes over time during the anaerobic digestion

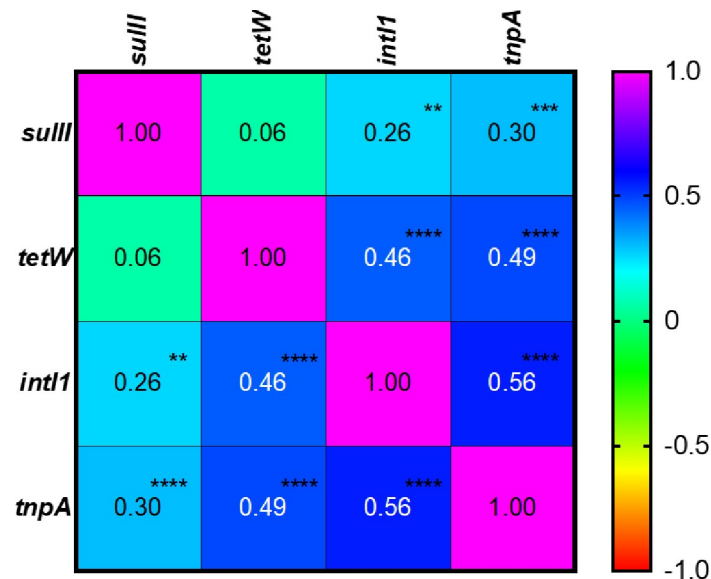


Fig 4. Pearson correlation matrix of relative abundance of ARGs/ MGEs. **significant at $P \leq 0.01$; *** significant at $P \leq 0.001$; **** significant at $P \leq 0.0001$.

<https://doi.org/10.1371/journal.pone.0254836.g004>

(Figs 5 and 6) except *tetW* and *tnpA*, where r^2 value was low. The low r^2 value was caused by increase of the gene abundance over time. The coefficients of model fit are shown in Table 2.

Λ_{CS} was the slope of the transformed log X and Y curve, which described the rate of reduction for each gene. At 36°C, *sullI* gene reduction rate was 1–2 times greater than that at 28°C and 52°C. *tnpA* reduction rate was also higher at temperature 36°C. For *tetW* and *int11*, however, the reduction rate was found to be greater at 28°C and 44°C. 16S rRNA gene reduction rate was highest at 52°C.

It is important to note that this study uses SYBR green approach for evaluating ARGs. Other alternative methods such as TaqMan probe provides improved specificity. There are advantages and disadvantages in both methods (TaqMan and SYBR). While SYBR is easy and cost effective, specificity is better in TaqMan, however, TaqMan requires specific probe for each target. Considering the important issues such as ARGs, it is important to adapt multiple approaches and testing methods for robust dataset in order to make the informed decision and reduce the biasness caused by testing methods. When grouping coefficients by temperatures, there was no significant difference among the specific lethality coefficient Λ_{CS} under different temperature conditions, which indicates that the decay rate of each gene might vary with temperature. The lethality coefficient could be gene specific. The *int11*, *tnpA*, and 16S rRNA genes had higher reduction rates (i.e., specific lethality coefficient Λ_{CS} compared to *sullI* and *tetW* ($P < 0.05$)). In most cases, ARGs/MGEs declined quickly in the beginning but then the reduction slowed down. Reductions by Day 5 were generally above 50% for all the genes except *tetW* (Table 2). The quick reduction of targeted ARGs in the beginning phase of anaerobic digestion could be explained by the significant reduction of aerobic bacteria such as Actinomycetales and Bacilli [66]. The calculations of reductions rates using the modeling tools such as Collins–Selleck can provide insights while comparing the impacts of various treatment on gene reductions, and help in decision making in terms of temperature and retention time needed for controlling various genes in digestate.

The use of Collins-Selleck model for understanding the decay of ARGs in landfill leachate in aerobic and anaerobic conditions [67], land application of sludge bio-drying products [68], and thermophilic aerobic digestion of sewage sludge [69] are reported and results indicate that

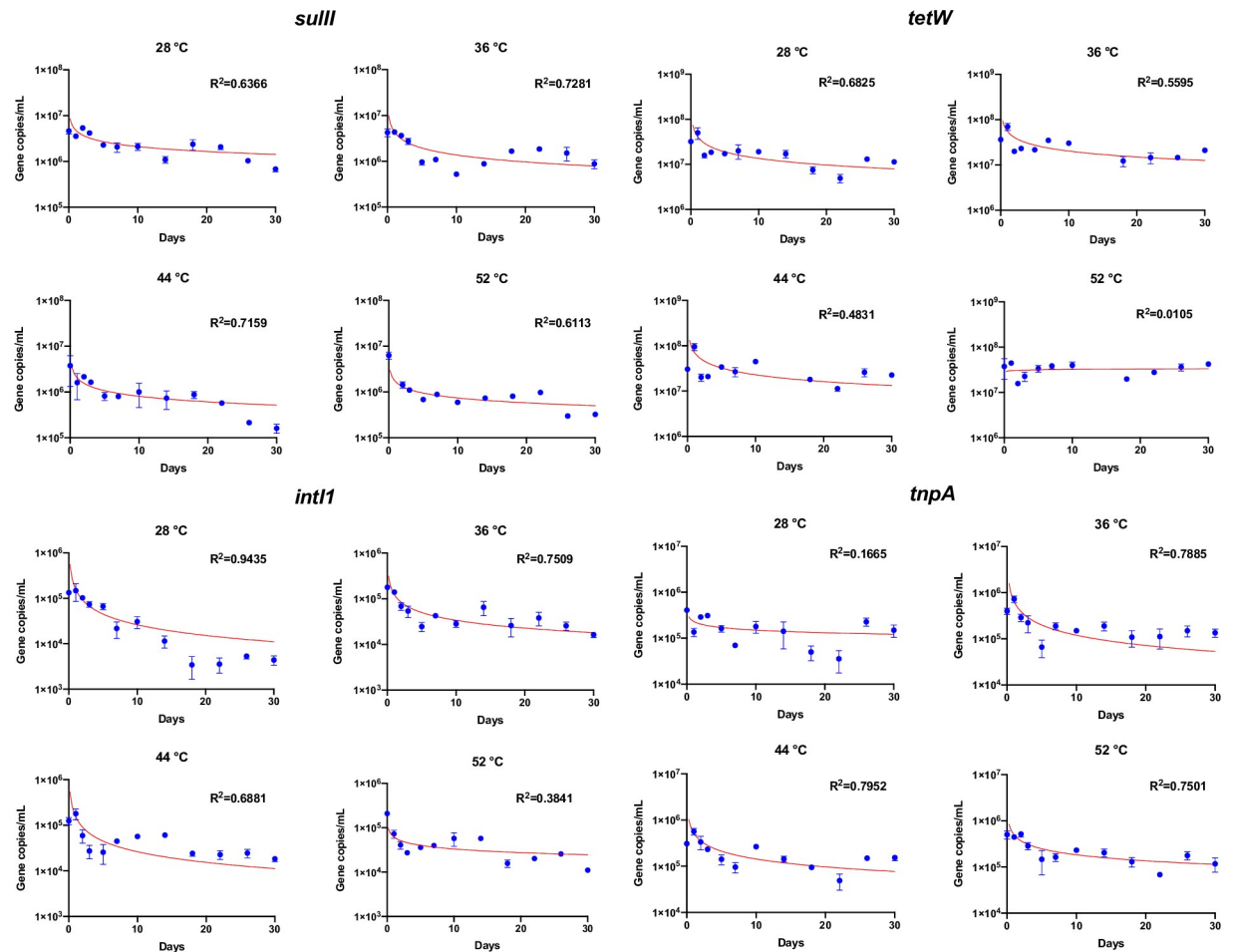


Fig 5. Modelling fit of *sullI*, *tetW*, *intI1*, and *tnpA*. Bars represent standard errors. Blue dots represent the mean value of triplicate reactors under each temperature. Solid red lines represent the best fit of the data to a modified Collins-Selleck model. The model coefficients are shown in Table 2.

<https://doi.org/10.1371/journal.pone.0254836.g005>

this model can assist in understanding the reduction rates and determining the impacts of environmental conditions on ARGs reductions. In a previous study, Burch, Sadowsky [70] investigated the fate of six ARGs (*sulI*, *tetA*, *tetW*, *tetX*, *ermB*, *qnrA*) and one integrase gene (*intI1*) during various treatments conditions (air drying, aerobic digestion, mesophilic/ thermophilic anaerobic digestion, pasteurization, and alkaline stabilization) of wastewater solids-amended soil microcosms. The authors reported that in most cases, ARGs declined quickly in the initial phase of treatment, and subsequently the reduction rate was reduced. The model was able to capture the reduction rates under various conditions. Similarly, another study by Burch, Sadowsky [47] conducted laboratory-scale thermophilic anaerobic digestion experiments at 40, 56, 60, and 63°C. The authors reported decrease in ARGs (*tetW*, *tetX*, *qnrA*) and *intI1* abundance under all temperature conditions, and reduction rates of genes could be understood better by Collins-Selleck model than first-order and second-order kinetic models. Sandberg and LaPara [49] investigated the fate of ARGs (*ermB*, *tetA*, *tetW*, *tetX*) and class 1 integron (*intI1*) in swine and dairy manure amended soil and found that Collins-Selleck model described the decay of ARGs in the soil microcosms well. Other methods such as linear model fit may underestimate the length of time needed for ARG reduction.

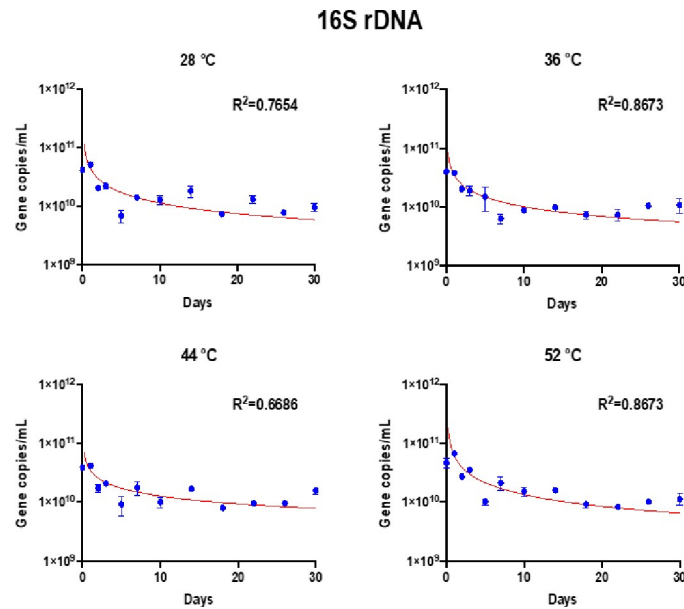


Fig 6. 16S rRNA gene in anaerobic digestion over time. Bars represent standard errors. Blue dots represent the mean value of triplicate reactors under each temperature.

<https://doi.org/10.1371/journal.pone.0254836.g006>

While there are previous studies elaborating the impacts of anaerobic digestion of ARG reductions [71–75] in a range of feedstocks such as waste water, sludge and dairy manure, the

Table 2. Coefficients for modified Collins–Selleck model by least squares nonlinear regression.

Targeted gene	Temperature	N_0	A_{CS}	B	R^2	Reduction at Day 5
<i>sullI</i>	28°C	4.62E+06	-0.36	1.12	0.64	41%
	36°C	4.26E+06	-0.50	1.07	0.73	54%
	44°C	3.77E+06	-0.41	0.23	0.72	72%
	52°C	6.30E+06	-0.35	0.02	0.61	85%
<i>tetW</i>	28°C	3.21E+07	-0.49	1.70	0.68	41%
	36°C	3.63E+07	-0.43	2.62	0.56	24%
	44°C	3.04E+07	-0.50	5.67	0.48	-6%
	52°C	3.75E+07	0.03	1716.11	0.01	15%
<i>intI1</i>	28°C	1.34E+05	-0.78	1.24	0.94	66%
	36°C	1.78E+05	-0.57	0.53	0.75	72%
	44°C	1.25E+05	-0.77	1.33	0.69	64%
	52°C	2.09E+05	-0.27	0.01	0.38	81%
<i>tnpA</i>	28°C	4.09E+05	-0.19	0.05	0.17	58%
	36°C	4.00E+05	-0.74	1.94	0.79	50%
	44°C	3.07E+05	-0.57	2.59	0.80	31%
	52°C	5.03E+05	-0.43	0.98	0.75	51%
16S rRNA gene	28°C	4.13E+10	-0.60	1.14	0.77	59%
	36°C	4.00E+10	-0.54	0.80	0.87	63%
	44°C	3.91E+10	-0.44	0.80	0.67	55%
	52°C	4.70E+10	-0.67	1.55	0.87	54%

N_0 is the initial quantity of genes (copies/mL) at Day 0. A_{CS} is the specific lethality coefficient (unitless) and b is a lag coefficient (copies*day/mL). R^2 indicates goodness of fit. Normality of Residuals were checked by Anderson-Darling test. Model also passed homoscedasticity test.

<https://doi.org/10.1371/journal.pone.0254836.t002>

performance of anaerobic digesters substantially depends on the feedstock, therefore, it is important to consider existing manure management in animal-agriculture system and the type of feedstock fed to digesters in order to understand the possible impacts of anaerobic digestion on ARGs reductions. For example, study by Zhang et al. (2011) [71] is focused on studying activated sludge, and sludge characteristics differ than flushed manure. Similarly, research by Ma et al. (2011) [72] studied waste water treatment process of sludge in thermophilic temperature. While the principles of anaerobic digestions process are similar but feedstock characteristics have substantial impacts on anaerobic process, which may be one possible reason for differences in results among studies. Antunes [75] reported that sulfonamide resistance genes are likely to be correlated with class 1 integron. Article by Sun et al (2016) [73] studied scrapped dairy manure in moderate, mesophilic, and thermophilic temperature to understand the dynamics and bacterial communities by q PCR and 16S rRNA and results indicate that anaerobic digestion has a potential to influence ARGs. Temperature can cause selective selection of bacteria carrying a lower number of ARG on their genome plasmids, which can help in ARGs removal. This study was focused on evaluating the manure of flush system, which is often used for flushing the manure from confined dairy operations, and the results of this study may not be transferable to sludge digester systems. Additional studies are needed to improve the existing knowledge for various types of organic matter fed to anaerobic digesters. As antibiotic concern is relatively a new issue, existing set of knowledge corresponding to various waste treatment processes and feedstock characteristics yet to be developed robustly. Future studies, and existing knowledge likely to help in developing improved treatment methods, which can likely to reduce the possible ARGs load from waste material to environment.

Conclusions

This study was focused on evaluating anaerobic digesters impacts on ARGs removal in dairy manure management system, where flush system was used. Findings of this study based on three ARGs, and 16S rRNA gene indicates that anaerobic digesters can help reducing ARGs in dairy manure, however, additional studies are needed to improve the findings, which elaborates further on the effects of feedstock, and temperature. Results showed that after 30-day thermophilic digestion (52°C), the absolute abundance of *sulII*, *intII*, *tnpA*, 16S rRNA gene was reduced by 76%-95%, however, absolute abundance of *tetW* was increased by 13%. Reduction of *intII* and 16S rRNA was comparable at moderate and thermophilic conditions, indicating that the impact of thermophilic anaerobic digestion on gene reduction may not necessarily be greater than lower temperatures. Results of decay curves fitted with modified Collins–Sellack model indicates that reduction of antibiotic resistance genes during startup phase was greater than the later stages, when dairy manure inside digester was relatively acclimatized.

Supporting information

S1 Table. TS, VS and VS/TS of original manure, filtered manure, granules-inocula, and mixed feedstock.

(DOCX)

S2 Table. Gene AA reductions on Day 5 and Day 30 compared with Day 0. Numbers are in percentage.

(DOCX)

S3 Table. Two-factor mixed-design ANOVA for effects of time and temperature on ARGs.

(DOCX)

S4 Table. Gene RA reductions on Day 5 and Day 30 compared with Day 0. Numbers are in log scale.
(DOCX)

Acknowledgments

The authors thank the Division of Agriculture and Natural Resources (ANR) and School of Veterinary Medicine Extension, University of California, Davis.

Author Contributions

Conceptualization: Pramod K. Pandey.

Investigation: Pramod K. Pandey.

Methodology: Sharif Aly.

Supervision: Pramod K. Pandey, Ruihong Zhang.

Visualization: Richard Pereira.

Writing – original draft: Yi Wang.

Writing – review & editing: Pramod K. Pandey, Sundaram Kuppu, Richard Pereira, Sharif Aly, Ruihong Zhang.

References

1. CDC. Antibiotic resistance threats in the United States. 2019. [Cited 2021, January 20] <https://www.cdc.gov/drugresistance/pdf/threats-report/2019-ar-threats-report-508.pdf>.
2. Liu B, Pop M. ARDB—antibiotic resistance genes database. *Nucleic Acids Res.* 2008; 37(suppl_1): D443–D7. <https://doi.org/10.1093/nar/gkn656> PMID: 18832362
3. Ventola CL. The antibiotic resistance crisis: part 1: causes and threats. *P T.* 2015; 40(4): 277–83. PMID: 25859123.
4. Sieber RN, Skov RL, Nielsen J, Schulz J, Price LB, Aarestrup FM, et al. Drivers and Dynamics of Methicillin-Resistant Livestock-Associated *Staphylococcus aureus* CC398 in Pigs and Humans in Denmark. *mBio.* 2018; 9(6): e02142–18. <https://doi.org/10.1128/mBio.02142-18> PMID: 30425152
5. Davis GS, Price LB. Recent research examining links among *Klebsiella pneumoniae* from food, food animals, and human extraintestinal infections. *Current environmental health reports.* 2016; 3(2): 128–35. <https://doi.org/10.1007/s40572-016-0089-9> PMID: 27022987
6. Berge ACB, Atwill ER, Sischo WM. Animal and farm influences on the dynamics of antibiotic resistance in faecal *Escherichia coli* in young dairy calves. *Preventive Veterinary Medicine.* 2005; 69(1): 25–38. <https://doi.org/10.1016/j.prevetmed.2005.01.013> PMID: 15899294
7. Pereira R, Lima S, Siler JD, Foditsch C, Warnick LD, Bicalho RC. Ingestion of milk containing very low concentration of antimicrobials: longitudinal effect on fecal microbiota composition in preweaned calves. *PLoS One.* 2016; 11(1): e0147525. <https://doi.org/10.1371/journal.pone.0147525> PMID: 26808865
8. FDA. Summary Report on Antimicrobials Sold or Distributed for Use in Food-Producing Animals. 2019. [Cited 2021, January 20] <https://www.fda.gov/media/133411/download>.
9. NAHMS. Antimicrobial use and stewardship on US feedlots, 2017. National Animal Health Monitoring System, United States Department of Agriculture. 2019. [Cited 2021, January 20] https://www.aphis.usda.gov/animal_health/naahms/amr/downloads/amu-feedlots_1.pdf.
10. Sarmah AK, Meyer MT, Boxall AB. A global perspective on the use, sales, exposure pathways, occurrence, fate and effects of veterinary antibiotics (VAs) in the environment. *Chemosphere.* 2006; 65(5): 725–59. <https://doi.org/10.1016/j.chemosphere.2006.03.026> PMID: 16677683
11. Massé DI, Saady NMC, Gilbert Y. Potential of biological processes to eliminate antibiotics in livestock manure: an overview. *Animals.* 2014; 4(2): 146–63. <https://doi.org/10.3390/ani4020146> PMID: 26480034.

12. Manyi-Loh C, Mamphweli S, Meyer E, Okoh A. Antibiotic use in agriculture and its consequential resistance in environmental sources: potential public health implications. *Molecules*. 2018; 23(4): 795. <https://doi.org/10.3390/molecules23040795> PMID: 29601469.
13. Udikovic-Kolic N, Wichmann F, Broderick NA, Handelsman J. Bloom of resident antibiotic-resistant bacteria in soil following manure fertilization. *Proceedings of the National Academy of Sciences*. 2014; 111(42): 15202–7. <https://doi.org/10.1073/pnas.1409836111> PMID: 25288759
14. Kyselková M, Kotrbová L, Bhumibhamon G, Chroňáková A, Jirout J, Vrchotová N, et al. Tetracycline resistance genes persist in soil amended with cattle feces independently from chlortetracycline selection pressure. *Soil Biol Biochem*. 2015; 81: 259–65. <https://doi.org/10.1016/j.soilbio.2014.11.018>.
15. Chee-Sanford JC, Mackie RI, Koike S, Krapac IG, Lin Y-F, Yannarell AC, et al. Fate and transport of antibiotic residues and antibiotic resistance genes following land application of manure waste. *Journal of Environmental Quality*. 2009; 38(3): 1086–108. <https://doi.org/10.2134/jeq2008.0128> PMID: 19398507
16. Walczak JJ, Xu S. Manure as a Source of Antibiotic-Resistant *Escherichia coli* and Enterococci: a Case Study of a Wisconsin, USA Family Dairy Farm. *Water, Air, Soil Pollut*. 2011; 219(1): 579–89. <https://doi.org/10.1007/s11270-010-0729-x>.
17. Omojowo F, Omojasola F. Antibiotic resistance pattern of bacterial pathogens isolated from poultry manure used to fertilize fish ponds in new Bussa, Nigeria. *Albanian Journal of Agricultural Sciences*. 2013; 12(1): 81. <https://doi.org/10.15835/nsb518998>.
18. Gogarten JP, Townsend JP. Horizontal gene transfer, genome innovation and evolution. *Nat Rev Microbiol*. 2005; 3(9): 679–87. <https://doi.org/10.1038/nrmicro1204> PMID: 16138096
19. Bennett P. Plasmid encoded antibiotic resistance: acquisition and transfer of antibiotic resistance genes in bacteria. *Br J Pharmacol*. 2008; 153(S1): S347–S57. <https://doi.org/10.1038/sj.bjp.0707607>.
20. Alanis AJ. Resistance to antibiotics: are we in the post-antibiotic era? *Arch Med Res*. 2005; 36(6): 697–705. <https://doi.org/10.1016/j.arcmed.2005.06.009> PMID: 16216651.
21. Von Wintersdorff CJ, Penders J, Van Niekerk JM, Mills ND, Majumder S, Van Alphen LB, et al. Dissemination of antimicrobial resistance in microbial ecosystems through horizontal gene transfer. *Front Microbiol*. 2016; 7: 173. <https://doi.org/10.3389/fmicb.2016.00173> PMID: 26925045
22. Andersson DI, Hughes D. Antibiotic resistance and its cost: is it possible to reverse resistance? *Nat Rev Microbiol*. 2010; 8: 260. <https://doi.org/10.1038/nrmicro2319> PMID: 20208551
23. Melnyk AH, Wong A, Kassen R. The fitness costs of antibiotic resistance mutations. *Evolutionary applications*. 2015; 8(3): 273–83. Epub 2014/08/27. <https://doi.org/10.1111/eva.12196> PMID: 25861385
24. Liao J, Chen Y. Removal of int1 and associated antibiotics resistant genes in water, sewage sludge and livestock manure treatments. *Rev Environ Sci Biotechnol*. 2018; 17(3): 471–500. <https://doi.org/10.1007/s11157-018-9469-y>.
25. He Y, Yuan Q, Mathieu J, Stadler L, Senehi N, Sun R, et al. Antibiotic resistance genes from livestock waste: occurrence, dissemination, and treatment. *npj Clean Water*. 2020; 3(1): 4. <https://doi.org/10.1038/s41545-020-0051-0>.
26. Youngquist CP, Mitchell SM, Cogger CG. Fate of antibiotics and antibiotic resistance during digestion and composting: a review. *J Environ Qual*. 2016; 45(2): 537–45. <https://doi.org/10.2134/jeq2015.05.0256> PMID: 27065401
27. Zhang T, Yang Y, Pruden A. Effect of temperature on removal of antibiotic resistance genes by anaerobic digestion of activated sludge revealed by metagenomic approach. *Appl Environ Microbiol*. 2015; 99(18): 7771–9. <https://doi.org/10.1007/s00253-015-6688-9> PMID: 25994259
28. Pandey PK, Soupir ML. Impacts of temperatures on biogas production in dairy manure anaerobic digestion. *International Journal of Engineering and Technology*. 2012; 4(5): 629. <https://doi.org/10.7763/IJET.2012.V4.448>.
29. Biswas S, Niu M, Pandey P, Appuhamy J, Leytem AB, Kebreab E, et al. Effect of Dairy Manure Storage Conditions on the Survival of *E. coli* O157:H7 and *Listeria*. *J Environ Qual*. 2018; 47(1): 185–9. <https://doi.org/10.2134/jeq2017.06.0224> PMID: 29415095.
30. Holm-Nielsen JB, Al Seadi T, Oleskowicz-Popiel P. The future of anaerobic digestion and biogas utilization. *Bioresour Technol*. 2009; 100(22): 5478–84. <https://doi.org/10.1016/j.biortech.2008.12.046> PMID: 19217772
31. Pandey PK, Soupir ML. *Escherichia coli* inactivation kinetics in anaerobic digestion of dairy manure under moderate, mesophilic and thermophilic temperatures. *AMB express*. 2011; 1(1): 18. <https://doi.org/10.1186/2191-0855-1-18> PMID: 21906374.
32. Pandey PK, Cao W, Wang Y, Vaddella V, Castillo AR, Souza A, et al. Simulating the effects of mesophilic anaerobic and aerobic digestions, lagoon system, and composting on pathogen inactivation. *Ecological Engineering*. 2016; 97: 633–41. <https://doi.org/10.1016/j.ecoleng.2016.10.047>.

33. Biswas S, Pandey PK, Farver TB. Assessing the impacts of temperature and storage on *Escherichia coli*, Salmonella, and *L. monocytogenes* decay in dairy manure. *Bioprocess and Biosystems Engineering*. 2016; 39(6): 901–13. <https://doi.org/10.1007/s00449-016-1569-x> PMID: 26922419.
34. Pandey PK, Biswas S, Vaddella VK, Soupir ML. *Escherichia coli* persistence kinetics in dairy manure at moderate, mesophilic, and thermophilic temperatures under aerobic and anaerobic environments. *Bioprocess and biosystems engineering*. 2015; 38(3): 457–67. <https://doi.org/10.1007/s00449-014-1285-3> PMID: 25248871.
35. Huang X, Zheng J, Tian S, Liu C, Liu L, Wei L, et al. Higher temperatures do not always achieve better antibiotic resistance gene removal in anaerobic digestion of swine manure. *Appl Environ Microbiol*. 2019; 85(7): e02878–18. <https://doi.org/10.1128/aem.02878-18> PMID: 30683745
36. Ma Y, Wilson CA, Novak JT, Riffat R, Aynur S, Murthy S, et al. Effect of various sludge digestion conditions on sulfonamide, macrolide, and tetracycline resistance genes and class I integrons. *Environ Sci Technol*. 2011; 45(18): 7855–61. <https://doi.org/10.1021/es200827t> PMID: 21815642.
37. Sun W, Gu J, Wang X, Qian X, Peng H. Solid-state anaerobic digestion facilitates the removal of antibiotic resistance genes and mobile genetic elements from cattle manure. *Bioresour Technol*. 2018. <https://doi.org/10.1016/j.biortech.2018.09.013> PMID: 30529334
38. APHA. Standard methods for the examination of water and wastewater. American Public Health Association. 2005. [Cited 2021, January 20] http://www.just.edu.jo/CoursesAndLabs/ENVIRONMENTAL%20ANALYTICAL%20CHEMISTRY_CHEM734/chem%20734.doc.
39. NAHMS. Dairy 2014 health and management practices in US Dairy operation, 2014. USDA, Animal and Plant Health Inspection Service, Veterinary Services, Center for Epidemiology and Animal Health, National Animal Health Monitoring System. 2018. [Cited 2021, January 20] https://www.aphis.usda.gov/animal_health/nahms/dairy/downloads/dairy14/Dairy14_dr_PartIII.pdf.
40. Pei R, Kim SC, Carlson KH, Pruden A. Effect of river landscape on the sediment concentrations of antibiotics and corresponding antibiotic resistance genes (ARG). *Water Res*. 2006; 40(12): 2427–35. <https://doi.org/10.1016/j.watres.2006.04.017> PMID: 16753197
41. Aminov R, Garrigues-Jeanjean N, Mackie R. Molecular ecology of tetracycline resistance: development and validation of primers for detection of tetracycline resistance genes encoding ribosomal protection proteins. *Appl Environ Microbiol*. 2001; 67(1): 22–32. <https://doi.org/10.1128/AEM.67.1.22-32.2001> PMID: 11133424
42. Chen J, Yu Z, Michel FC, Wittum T, Morrison M. 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*. 2007; 73(14): 4407–16. <https://doi.org/10.1128/AEM.02799-06> PMID: 17496134
43. Hardwick SA, Stokes H, Findlay S, Taylor M, Gillings MR. Quantification of class 1 integron abundance in natural environments using real-time quantitative PCR. *FEMS Microbiol Lett*. 2008; 278(2): 207–12. <https://doi.org/10.1111/j.1574-6968.2007.00992.x> PMID: 18042230
44. Zhu YG, Johnson TA, Su JQ, Qiao M, Guo GX, Stedtfeld RD, et al. Diverse and abundant antibiotic resistance genes in Chinese swine farms. *Proc Natl Acad Sci USA*. 2013; 110(9): 3435–40. <https://doi.org/10.1073/pnas.1222743110> PMID: 23401528
45. Muyzer G, De Waal EC, Uitterlinden AG. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl Environ Microbiol*. 1993; 59(3): 695–700 <https://doi.org/10.1128/aem.59.3.695-700.1993> PMID: 7683183.
46. Ghosh S, Ramsden SJ, LaPara TM. The role of anaerobic digestion in controlling the release of tetracycline resistance genes and class 1 integrons from municipal wastewater treatment plants. *Appl Environ Microbiol*. 2009; 75(4): 791–6. <https://doi.org/10.1007/s00253-009-2125-2> PMID: 19597810
47. Burch TR, Sadowsky MJ, LaPara TM. Modeling the fate of antibiotic resistance genes and class 1 integrons during thermophilic anaerobic digestion of municipal wastewater solids. *Appl Environ Microbiol*. 2016; 82(3): 1437–44. <https://doi.org/10.1007/s00253-015-7043-x> PMID: 26481624
48. Diehl DL, LaPara TM. Effect of temperature on the fate of genes encoding tetracycline resistance and the integrase of class 1 integrons within anaerobic and aerobic digesters treating municipal wastewater solids. *Environ Sci Technol*. 2010; 44(23): 9128–33. <https://doi.org/10.1021/es102765a> PMID: 21058743.
49. Sandberg KD, LaPara TM. The fate of antibiotic resistance genes and class 1 integrons following the application of swine and dairy manure to soils. *FEMS Microbiol Ecol*. 2016; 92(2). <https://doi.org/10.1093/femsec/fiw001> PMID: 26738555
50. Marti E, Jofre J, Balcazar JL. Prevalence of antibiotic resistance genes and bacterial community composition in a river influenced by a wastewater treatment plant. *PLoS One*. 2013; 8(10): e78906. <https://doi.org/10.1021/es504157v> PMID: 24205347

51. Alexander TW, Yanke JL, Reuter T, Topp E, Read RR, Selinger BL, et al. Longitudinal characterization of antimicrobial resistance genes in feces shed from cattle fed different subtherapeutic antibiotics. *BMC Microbiol.* 2011; 11(1): 19. <https://doi.org/10.1186/1471-2180-11-19> PMID: 21261985
52. Selvam A, Xu D, Zhao Z, Wong JW. Fate of tetracycline, sulfonamide and fluoroquinolone resistance genes and the changes in bacterial diversity during composting of swine manure. *Bioresour Technol.* 2012; 126: 383–90. <https://doi.org/10.1016/j.biortech.2012.03.045> PMID: 22537403
53. Gupta S, Arango-Argoty G, Zhang L, Pruden A, Vikesland P. Identification of discriminatory antibiotic resistance genes among environmental resistomes using extremely randomized tree algorithm. *Microbiome.* 2019; 7(1): 123. <https://doi.org/10.1186/s40168-019-0735-1> PMID: 31466530
54. Crittenden JC, Trussell RR, Hand DW, Howe KJ, Tchobanoglous G. *MWH's water treatment: principles and design*: John Wiley & Sons, Hoboken; 2012.
55. Selleck RE, Collins HF, Saunier BM. Kinetics of bacterial deactivation with chlorine. *J Environ Eng.* 1978; 104(6): 1197–212.
56. Wallace JS, Garner E, Pruden A, Aga DS. Occurrence and transformation of veterinary antibiotics and antibiotic resistance genes in dairy manure treated by advanced anaerobic digestion and conventional treatment methods. *Environ Pollut.* 2018; 236:764–772. <https://doi.org/10.1016/j.envpol.2018.02.024> PMID: 29455089.
57. Flores-Orozco D, Patidar R, Levin DB, Sparling R, Kumar A, Çiçek N. Effect of ceftiofur on mesophilic anaerobic digestion of dairy manure and the reduction of the cephalosporin-resistance gene *cmy-2*. *Bioresour Technol.* 2020; 301: 122729. <https://doi.org/10.1016/j.biortech.2019.122729> PMID: 31931335.
58. Howes, SA. The Effect of Thermophilic Anaerobic Digestion on Ceftiofur and Antibiotic Resistant Gene Concentrations in Dairy Manure. Virginia Polytechnic Institute and State University. Masters Thesis 2017; <http://hdl.handle.net/10919/86532>.
59. Pu C, Liu H, Ding G, Sun Y, Yu X, Chen J, et al. Impact of direct application of biogas slurry and residue in fields: In situ analysis of antibiotic resistance genes from pig manure to fields. *J Hazard Mater.* 2018; 344: 441–9. <https://doi.org/10.1016/j.jhazmat.2017.10.031> PMID: 29096257
60. Zhang R, Wang X, Gu J, Zhang Y. Influence of zinc on biogas production and antibiotic resistance gene profiles during anaerobic digestion of swine manure. *Bioresour Technol.* 2017; 244: 63–70. <https://doi.org/10.1016/j.biortech.2017.07.032> PMID: 28779677
61. Gao P, Gu C, Wei X, Li X, Chen H, Jia H, et al. The role of zero valent iron on the fate of tetracycline resistance genes and class 1 integrons during thermophilic anaerobic co-digestion of waste sludge and kitchen waste. *Water Res.* 2017; 111: 92–9. <https://doi.org/10.1016/j.watres.2016.12.047> PMID: 28061387.
62. Roberts MC. Update on acquired tetracycline resistance genes. *FEMS Microbiol Lett.* 2005; 245(2): 195–203. <https://doi.org/10.1016/j.femsle.2005.02.034> PMID: 15837373
63. Xu R, Yang ZH, Wang QP, Bai Y, Liu JB, Zheng Y, et al. Rapid startup of thermophilic anaerobic digester to remove tetracycline and sulfonamides resistance genes from sewage sludge. *Sci Total Environ.* 2018; 612: 788–98. <https://doi.org/10.1016/j.scitotenv.2017.08.295> PMID: 28866406
64. Sørensen SJ, Bailey M, Hansen LH, Kroer N, Wuertz S. Studying plasmid horizontal transfer in situ: a critical review. *Nat Rev Microbiol.* 2005; 3(9): 700–10. <https://doi.org/10.1038/nrmicro1232> PMID: 16138098
65. Naas T, Mikami Y, Imai T, Poirel L, Nordmann P. Characterization of In53, a class 1 plasmid-and composite transposon-located integron of *Escherichia coli* which carries an unusual array of gene cassettes. *J Bacteriol Res.* 2001; 183(1): 235–49. <https://doi.org/10.1128/JB.183.1.235-249.2001> PMID: 11114922.
66. Sun W, Qian X, Gu J, Wang X-J, Duan M-L. Mechanism and effect of temperature on variations in antibiotic resistance genes during anaerobic digestion of dairy manure. *Sci Rep.* 2016; 6(1): 1–9. <https://doi.org/10.1038/srep30237> PMID: 28442746
67. Su Y, Wang J, Xia H, Xie B, Li X. Anaerobic/aerobic conditions determine antibiotic resistance genes removal patterns from leachate by affecting bacteria taxa-genes co-occurrence modules. *Chemosphere.* 2019; 223: 28–38. <https://doi.org/10.1016/j.chemosphere.2019.02.007> PMID: 30763913
68. Zhang J, Sui Q, Lu T, Zhong H, Shen P, Wei Y. Sludge bio-drying followed by land application could control the spread of antibiotic resistance genes. *Environ Int.* 2019; 130: 104906. <https://doi.org/10.1016/j.envint.2019.104906> PMID: 31203029
69. Min Jang H, Choi S, Shin J, Kan E, Mo Kim Y. Additional reduction of antibiotic resistance genes and human bacterial pathogens via thermophilic aerobic digestion of anaerobically digested sludge. *Bioreour Technol.* 2019; 273: 259–68. <https://doi.org/10.1016/j.biortech.2018.11.027> PMID: 30448677
70. Burch TR, Sadowsky MJ, LaPara TM. Effect of different treatment technologies on the fate of antibiotic resistance genes and class 1 integrons when residual municipal wastewater solids are applied to soil. *Environ Sci Technol.* 2017; 51(24): 14225–32. <https://doi.org/10.1021/acs.est.7b04760> PMID: 29148730.

71. Zhang T., Yang Y. & Pruden A. Effect of temperature on removal of antibiotic resistance genes by anaerobic digestion of activated sludge revealed by metagenomic approach. *Appl Microbiol Biotechnol* 99, 7771–7779 (2015). <https://doi.org/10.1007/s00253-015-6688-9> PMID: 25994259
72. Ma Yanjun, Wilson Christopher A., Novak John T., Riffat Rumana, Aynur Sebnem, Murthy Sudhir, et al *Environmental Science & Technology* 2011 45 (18), 7855–7861 <https://doi.org/10.1021/es200827t> PMID: 21815642
73. Sun W., Qian X., Gu J. et al. Mechanism and Effect of Temperature on Variations in Antibiotic Resistance Genes during Anaerobic Digestion of Dairy Manure. *Sci Rep* 6, 30237 (2016). <https://doi.org/10.1038/srep30237> PMID: 27444518
74. Miller et al., 2016 Miller J.H., Novak J.T., Knocke W.R., Pruden A. Survival of antibiotic resistant bacteria and horizontal gene transfer control antibiotic resistance gene content in anaerobic digesters *Front. Microbiol.*, 7 (2016), p. 263. <https://doi.org/10.3389/fmicb.2016.00263> PMID: 27014196
75. Antunes P, Machado J, Sousa JC, Peixe L. Dissemination of sulfonamide resistance genes (sul1, sul2, and sul3) in Portuguese *Salmonella enterica* strains and relation with integrons. *Antimicrob Agents Chemother.* 2005 Feb; 49(2):836–9. <https://doi.org/10.1128/AAC.49.2.836-839.2005> PMID: 15673783; PMCID: PMC547296.