

BIOAg Project Final Report

Report Type: FINAL

Title:

Impact of manure-derived fertilizers on bacterial community and antibiotic resistance genes in washington red raspberry fields

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Abstract:

Washington State is the number one producer of processed red raspberries in the nation. Raspberry growers in Whatcom County rely on dairy manure as a ready source to improve soil health before planting. Soil is a dynamic entity harboring billions of microorganisms and plays a crucial role in plant production. The microbial quality of soil is also directly associated with food safety, which is impacted by fertilizer practices. Antibiotic resistant bacteria and antibiotic resistance genes (ARGs) have emerged as important contaminants in soil and produce, posing a serious human health risk. Unfortunately, manure fertilizer application could directly or indirectly alter bacterial community structure by enriching antibiotic resistant bacteria and increasing the risk of spread of ARGs among soil bacteria. However, up to now, knowledge about impacts of manure application on soil microbial ecology and the abundance of ARGs of raspberry fields or other production system is very limited, which leaves a critical knowledge gap. The objective of this study was to investigate the impacts of manure-derived fertilizers use on soil bacterial ecology and ARGs abundance using soil samples from the Raspberry beds. The abundance of soil bacterial community and the major categories of ARGs were analyzed using Illumina MiSeq sequencing and quantitative PCR amplification, respectively. This study provides information about soil microbial ecology and ARGs profiles related to manure fertilizer application, which provides critical preliminary data for accessing manure fertilizer applications on microbiological safety, soil microbial ecology and ARGs profiles of red raspberry and other produces.

Project Description:

Washington is a major state of dairy and beef cow production. Dairy cows and feedlot beef cattle are commonly housed in a confined area, where prodigious quantities of manure accumulate. Cow manures are a valuable resource of nutrients and organic matter for soil enrichment and crop production. Raspberry growers in Whatcom County rely on dairy manure as a ready source to improve soil health prior to planting. However, manures applied to farmland could directly or indirectly alter the bacterial community by enriching antibiotic-resistant bacteria. With the wide application/interests of manure-derived fertilizers in raspberry fruit production, it is imperative to understand the microbial ecology, the ARGs prevalence, and persistence in the raspberry field. The overall objective of this study was to investigate the impacts of manure-derived fertilizer application on soil bacteria ecology and ARGs abundance in soil samples from the same Raspberry beds in Whatcom County using metagenomics sequencing and quantitative PCR approach. We have been pursuing two specific objectives. 1) Evaluate impacts of manure-derived fertilizer

application on soil bacteria ecology/structure using a metagenomics sequencing, which enables us to detect bacteria at phylum, family, and genus levels that are not able to culture and recovery using the conventional culture method (Heuer and Smalla, 2007). 2) Examine the prevalence of major ARG groups in soils with or without manure-derived fertilizer application.

Outputs:

1. Methods

Soil sampling: Soil samples were collected from the raspberry field study from a commercial red raspberry field at Honcoop Farm (Whatcom County, WA). Two manure derived fertilizers, standard aerobically composted dairy manure (COM), a raw manure straight lagoon (SL) were compared to control (CON), a standard fertilizer.

Extraction of soil bacterial DNA: Bacterial DNA was extracted from soil samples using the DNASy PowerSoil Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol.

Amplification of bacterial 16S ribosomal DNA and MiSeq sequencing: Soil bacterial abundance was analyzed at Initiative for Bioinformatics and Evolutionary Studies (IBEST) Genomics Resources Core at University of Idaho using Illumina MiSeq dual-barcoded two-step PCR amplicon sequencing per our established method (Kang et al, 2019).

Bioinformatic analysis: Bioinformatic analysis of sequencing data was conducted using the QIIME 1.5.0 software suite by a bioinformatics data scientist at IBEST. Reading merging, quality filtering, denoising, and chimera checking were conducted per protocol and criteria used at IBEST. The representative sequence of each Operational Taxonomic Units (OTU) was clustered and taxonomically classified using the first Ribosomal Database Project (RDP) (Michigan State University), from which we obtained the soil bacterial community structure at different taxonomic levels (from phylum to genus).

Analyses of antibiotic resistance genes (ARGs): The antibiotic resistance genes in the respective soil samples were analyzed by PCR method using published primer sets (Stedtfeld et al., 2018). A total of 161 ARGs regarding to the most frequently used antibiotics in cattle and had clinically importance in human was selected and used for this study (Stedtfeld et al., 2018).

Statistical analysis: Data were analyzed with one-way Analysis of Variance (ANOVA) using IBM SPSS Statistics, version 19.0 software (Chicago, IL). Mean difference was determined by Least Significant Difference (LSD) multiple comparison, where $P \leq 0.05$ was considered statistically significant.

2. Results

Bacterial community changes in soils amended with manure-derived fertilizers: The bacterial community comprised up to 35 phyla in all soil samples and only 20 different phyla represented at least 89.6% of the profile for most bacterial communities analyzed (Fig. 1A). The most abundant four bacterial phyla were *Proteobacteria*, *Acidobacteria*, *Actinobacteria*, and *Bacteroidetes* in all soil samples (Fig. 2A). The application of SL significantly increased the abundance of *Chlamydiae*

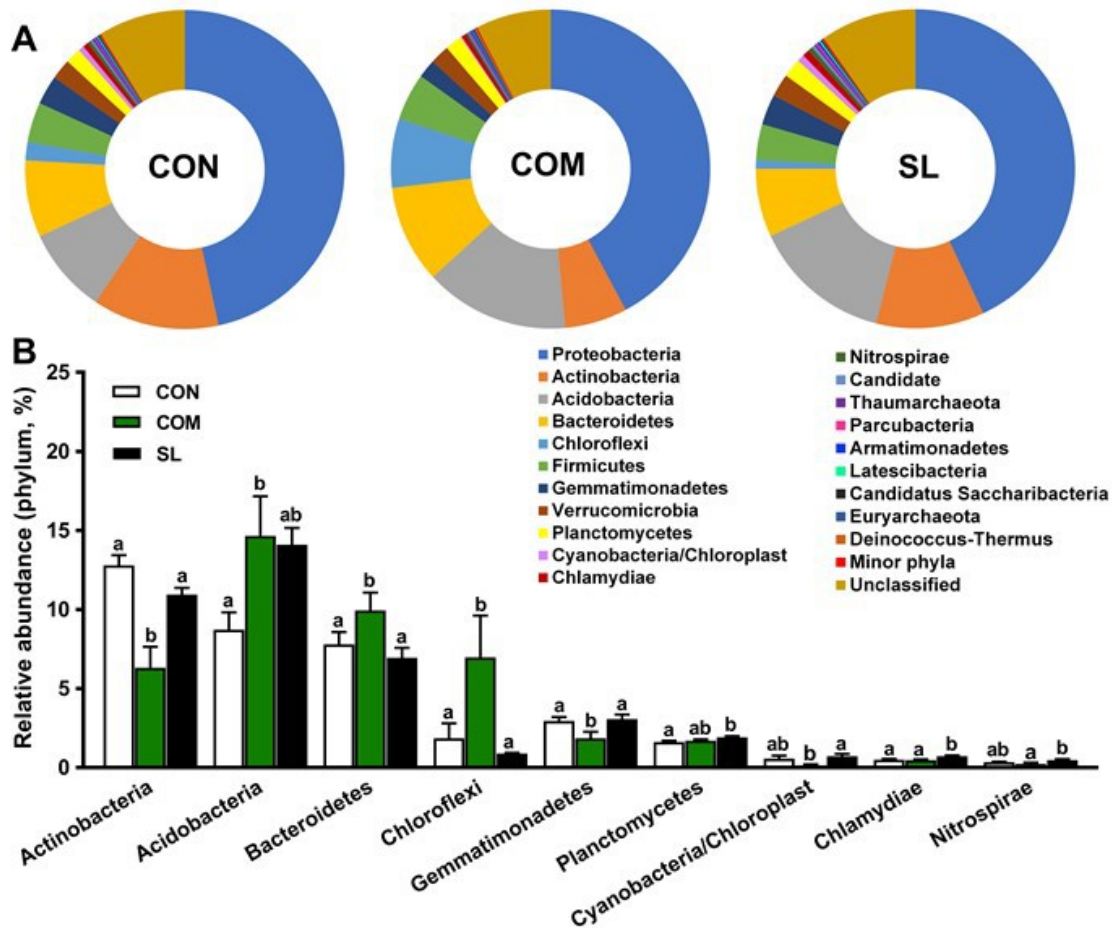


Fig. 1. The microbiota composition and abundance in soil samples post different fertilizers application at phyla level. A. Overall comparison of soil microbiota at phylum level; B. Selected bacterial phylum. Mean \pm SEM, n=4. ^{a-b} Histogram bars in a bacterial phylum without common letter differ significantly ($p < 0.05$). CON: standard fertilization; COM: compost; SL: straight lagoon. *Candidate*: *Candidate* division WPS-1.

compared to the application of CON in soils (Fig. 1B). The application of COM significantly increased the abundance of *Acidobacteria*, *Bacteroidetes* and *Chloroflexi* compared to the application of CON in soils (Fig. 1B). However, the abundance of *Actinobacteria* and *Gemmatimonadetes* were significantly decreased in soil samples amended with COM compared to that with CON and SL (Fig. 1B).

There were about 300 bacterial families identified in all soil samples (Fig. 2). The major and minor bacteria families varied among soil samples amended with manure-derived fertilizers compared to that with CON (Fig. 2). The application of SL in soils significantly increased the abundance of *Acidobacteria* Gp3 family, *Acidobacteria* Gp6 family, *Planctomycetaceae*, *Comamonadaceae* and *Reyraneliaceae* compared with the application of CON in soils (Fig. 2). The abundance of *Microbacteriaceae*, *Flavobacteriaceae* and *Anaerolineaceae* were significantly increased in soil samples amended with COM compared to that with CON (Fig. 2). Other bacterial families including *Hyphomicrobiaceae*, *Planococcaceae*, *Polyangiaceae*, *Oxalobacteraceae*, *Erythrobacteraceae*, *Rhizobiales_incertae_sedis*, *Caulobacteraceae* *Phyllobacteriaceae*, and

Ohtaekwangia were significantly increased in soil samples amended with COM compared to that with CON (Fig. 2).

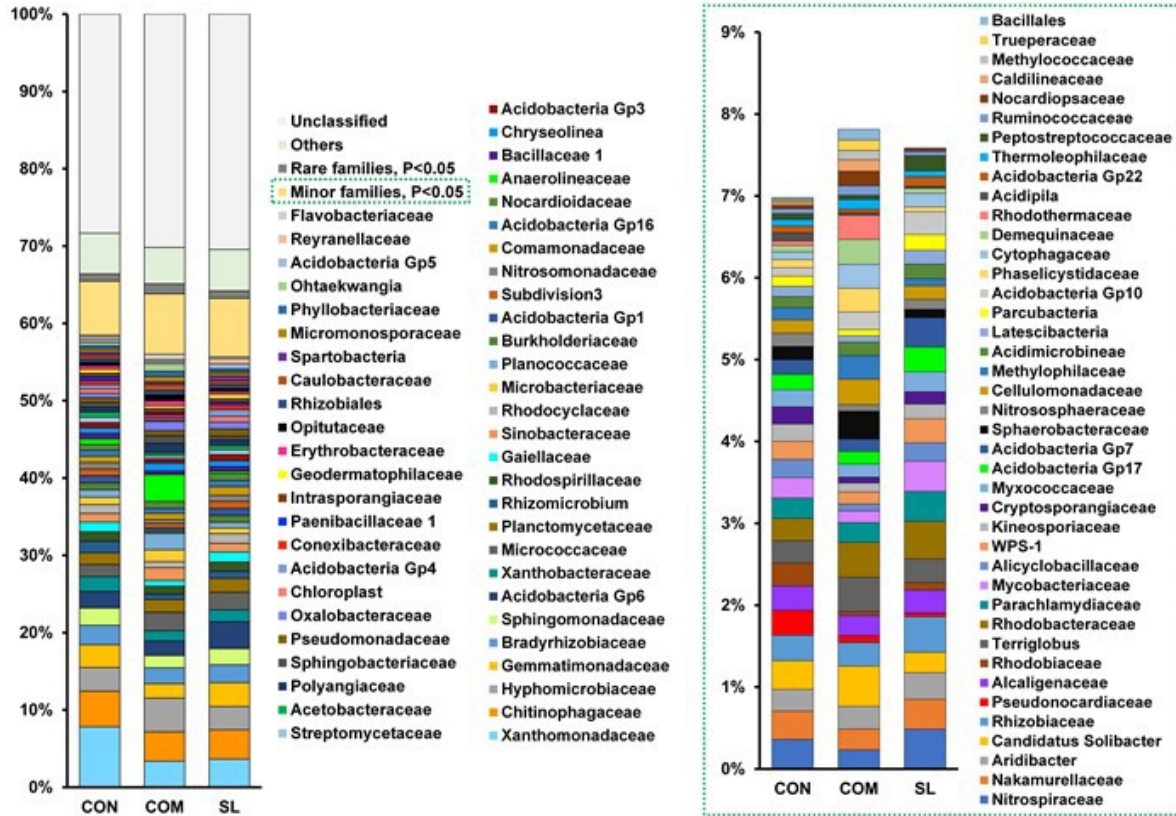


Fig. 2. The microbiota composition and abundance in soil samples with different fertilizers at family level. Left figure showed 51 most abundant families (mean abundance > 0.5% in either of three samples) Right figure showed bacterial minor families. Mean \pm SEM, n=4. CON: standard fertilization; COM: compost; SL: straight lagoon. *Rhizobiales*: *Rhizobiales_incertae_sedis*; *Spartobacteria*: *Spartobacteria_family_incertae_sedis*; *Subdivision3*: *Subdivision3_family_incertae_sedis*; *WPS-1*: *WPS-1_family_incertae_sedis*; *Acidimicrobinae*: *Acidimicrobinae_incertae_sedis*; *Latescibacteria*: *Latescibacteria_family_incertae_sedis*; *Parcubacteria*: *Parcubacteria_family_incertae_sedis*; *Bacillales*: *Bacillales_incertae_sedis XII*.

There were about 900 bacterial genera identified in soil samples supplemented with different fertilizers. The application of SL in soils significantly increased the abundance of *Acidobacteria* genus Gp6 and *Nakamurella* compared with the CON group. The abundance of *Ohtaekwangia* genus, *Pedobacter*, *Planomicrobium*, *Hyphomicrobium*, *Devosia*, and *Massilia* were significantly increased in soils amended with COM compared to that with CON. The abundance of *Gaiella* *Gemmatimonas*, *Pseudolabrys*, *Rhizomicrobium*, *Bradyrhizobium*, *Burkholderia*, and *Subdivision3_genera_incertae_sedis* were significantly decreased in soils amended with COM compared to that with CON.

Antibiotic resistance genes in soils amended with manure derived fertilizers: Overall, we detected 52 ARGs from 161 ARGs that selected from the published primer sets (Stedtfeld et al., 2018) according to the most frequently used antibiotics in cattle and had clinically importance in

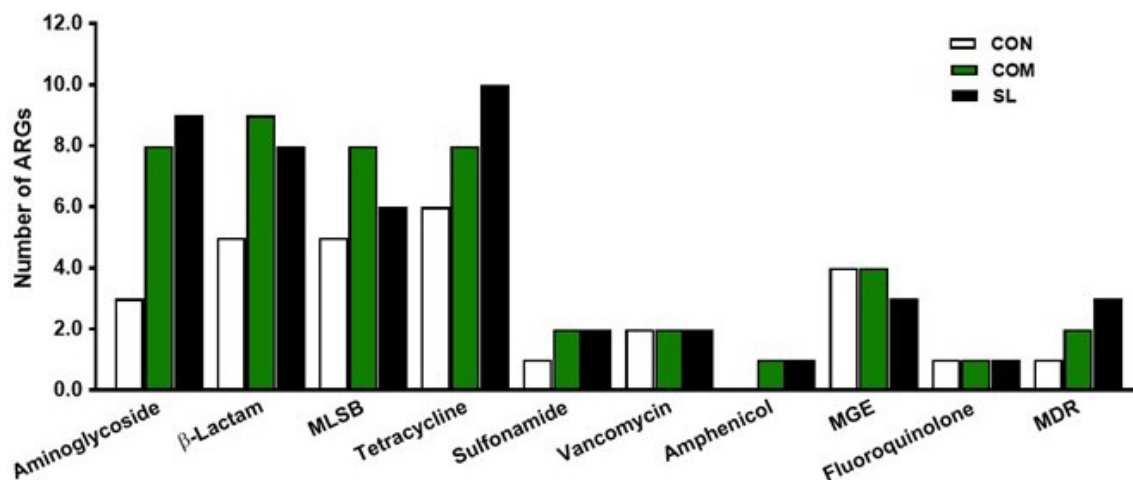


Fig. 3. Overall categories of antibiotic resistance gene detected in soil samples amended with different type of fertilizers. Data are present as the number of antibiotic resistance genes (ARGs) detected in soil samples amended with individual fertilizer with 4 replications per treatment and if the same ARG is detected in all four replications, this was only counted as detection of one ARG. CON: standard fertilization; COM: compost; SL: straight lagoon. MLSB: macrolide–lincosamide–streptogramin B; MGE: mobile genetic element; MDR: multidrug resistance.

human. There were 28, 45, and 45 type of ARGs were detected in soils amended with CON, COM, and SL, respectively (Fig. 3). The ARGs detected in soils confer resistance to the antibiotics including aminoglycoside, β-lactam, macrolide-lincosamide-streptogramin B, tetracycline, sulfonamide, vancomycin, amphenicol, fluoroquinolone (Fig. 3). ARGs relevant to multidrug resistance and mobile genetic elements were also detected (Fig. 3). Soils contain certain type of ARGs regardless of fertilizer application including *aadA2*, *aphA1*, *aac6-aph2*, *blaMOX*, *bla1*, *cphA*, *blaCMY*, *blaTEM*, *lmrA*, *ermT*, *ermX*, *lnuA*, *lnuB*, *tetK*, *tetW*, *tetO*, *tetX*, *tetPB*, *tetG*, *sul2*, *vanD*, *vanSA*, *oqxA*, *tnpA_1*, *tnpA_2*, *tnpA_3*, *intl3*, *tigB*. The ARGs including *ant4-ib*, *tetQ*, *tetC*, *qac_new_356*, and *mexB* were only detected in soils amended with SL; while *sugE* was only detected in soils amended with COM. ARGs including *aacA/aphD*, *aadA5*, *aadA9*, *aph6ia*, *ant6-ia*, *blaROB*, *blaDHA*, *pbp5*, *ampC*, *ermF*, *msrC*, *ermB*, *tetA*, *tetL*, *folA*, and *floR* were detected in soils amended with COM and SL but was not detected in soils amended with CON.

3. Discussion

We observed a significantly higher richness and evenness of bacterial communities in soils amended with COM compared to that with CON and SL. A similar trend was also found in soils amended with manure-based compost compare to that with no fertilizer treatment (Tian et al., 2015a). *Proteobacteria* were the most abundant phyla in all soil samples regardless of fertilizer application. Similarly, *Proteobacteria* is the most abundant phyla in soils post-horse manure application (Ding et al., 2016). *Acidobacteria*, *Actinobacteria*, *Verrucomicrobia*, *Bacteroidetes*, *Chloroflexi* were also observed as major phyla in all soil samples and were similar to the previous observation in soils (Janssen, 2006). The abundance of several bacteria phyla including *Acidobacteria*, *Bacteroidetes*, and *Chloroflexi* were increased in soil samples amended with COM compared to that with CON, which is consistent with the observation found in soils amended with pig manure-based compost (Tian et al., 2015b). Genera belonging to the phylum *Chlamydiae* can

cause diseases in animals and humans (Taylor-Brown et al., 2015). In our study, we found both *Parachlamydia* and *Neochlamydia* belong to phylum *Chlamydiae* significantly increased in SL amended soils compared with COM or CON amended soils and indicated the risks of using raw manure as a soil amendment. *Anaerolineaceae* and *Micromonosporaceae* were the major bacterial families during composting (Meng et al., 2020), and we observed the increased abundance of these families in soil samples amended with COM compared to that with CON. We also observed the increased abundance of *Magnetospirillum* and *Pseudomonas* in soils amended with COM or SL and these bacteria secrete siderophores to promote plant growth (Grobelač and Hiller, 2017). Soilborne pathogens *Phytophthora rubi* and *Pratylenchus penetrans* that caused damage to the raspberry plant (Gigot et al., 2013) were not detected in the tested raspberry field regardless of fertilizer application. Data collectively indicate that compost application to the raspberry field has beneficial effects on soil microbiome.

The application of COM and SL in soils brought ARGs conferring resistance to aminoglycoside, β -lactam, MLSB, tetracycline, sulfonamide, and vancomycin. These antibiotics are frequently used for cattle (FDA, 2017) and might induce antibiotic resistance in cattle gut microbiome during the treatment of infections (Chee-Sanford et al., 2009). Similarly, ARGs conferring resistance to β -lactam were increased in soils amended with cow manure (Udiković-Kolić et al., 2014). ARGs conferring resistance to aminoglycoside, β -lactam, MLSB, tetracycline, sulfonamide, vancomycin, fluoroquinolone was detected in soil samples regardless of fertilizer applications. To support our findings, ARGs conferring resistance to vancomycin, tetracycline, β -lactam, aminoglycoside, and sulfonamides are most common type of ARGs found in environmental metagenomes (Nesme et al., 2014). However, the exact source of these ARGs is not known. ARGs are also disseminated via mobile genetic elements like transposons, integrons, and insertion elements (Szekeres et al., 2018), which were detected in soil samples with or without the application of manure-derived fertilizers, further supporting the prevalence of ARGs even in soils without manure-derived fertilizer amendments.

4. Publications:

One meeting abstract has been submitted to 2021 International Association of Food Protection meeting and a manuscript is in preparation.

Impacts

- Short-Term and Intermediate-Term:

This study showed impacts of soil amendments on the soil ecology and the frequency of antimicrobial resistance genes in soils in Washington raspberry field. Data provide useful information to identify a potential manure derived fertilizer for berry production to maximize the diversity of soil microbial community and health condition of soil and minimize the abundance of ARGs.

- Long-Term:

Development of an effective manure derived fertilizer will increase the profitability of raspberry farm and food production, facilitating further expansion of other berries and organic food production; 2) Successful using manure derived organic fertilizer will reduce abundance, prevalence and spreading of ARGs while increase soil microbial diversity and render a health

soil microbial ecology, improving the production/ wholesomeness of raspberry and other food products.

Additional funding applied for/secured:

Using the data generated we are planning to submit proposals to USDA-AFRI foundation and USDA-OREI program.

Graduate students funded:

This project has supported a doctoral student Xiaoye Shen and a visiting PhD student Jingyi Wang.

Recommendations for future research: It is valuable to investigate long-term fertilization besides the transient fertilization on bacterial communities and antimicrobial resistance bacteria for comprehensively understanding the influence of manure-derived fertilizers.

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