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The Slug Microbiome: Elucidating the Influences of an Invasive Slug on Soil Bacterial Communities

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DEDICATION

I dedicate this dissertation

to my son

Avery Camargo.

I love you.

ABSTRACT OF THE DISSERTATION

The Slug Microbiome: Elucidating the Influences of an Invasive Slug on Soil Bacterial Communities

by

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Doctor of Philosophy, Graduate Program in Microbiology
University of California, Riverside, September 2020
Dr. Emma Aronson, Chairperson

The invasive terrestrial gastropod, the slug *Lehmannia valentiana,* is distributed throughout California. It originates from Europe and is a serious pest of gardens, plant nurseries, and greenhouses. Terrestrial slugs frequently ingest soil and plant bacteria from dead and living plant material while also picking up bacteria on their bodies and in their mucus. These bacteria can then be dispersed as they travel which could possibly cause disturbances to native soil microbial community functioning, as well as cause concern for human health or agricultural crops. Altering soil microbial community composition by slugs, such as interrupting the action of free-living microbes, altering rates of nutrient supply and the portioning of resources may be detrimental to plant communities.

Horticulturalists, agronomists, and land managers are recognizing the need to control slug populations in an ecologically sound and effective manner.

Prior to this research, little was known about slug bacterial dispersal. Additionally, information regarding whole slug bacterial microbiomes is limited. This knowledge could provide valuable information for biocontrol studies. Moreover, this research provides evidence for the potential of slugs to disperse bacteria. With the advances of next-generation sequencing, focus on host-microbiome systems have expanded and now include a wider range of plants and animals, notably invertebrates. Bacteria associated with invertebrates may be ecologically significant as these microbes potentially perform many functions, including supporting overall fitness and health. Although invertebrates make excellent study models, minimal work has been conducted on characterizing bacterial associations with terrestrial slugs.

This novel research investigated an invasive California slug, *L. valentiana*, and documented the ability of this slug to disperse bacteria while providing critical information about its bacterial microbiome and effect on soil bacterial communities. This research may raise awareness to the adverse contributing factors slugs have in environmental and agricultural settings. Additionally, these findings fill a gap in current malacological research, provide material on new methods of slug DNA extraction, and assist in slug bacterial microbiome discovery, which could offer valuable information for future biocontrol studies.

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INTRODUCTION

One of the most challenging pests of crops farmers face that are often overlooked, are members of the class Gastropoda- the terrestrial slugs [1,3]. Primitively, Gastropoda are marine animals, but several groups in this molluscan class have made the adaptive shift from aquatic to terrestrial existence [4] including the terrestrial slug. There are thousands of species of terrestrial slugs living in a variety of habitats from temperate to tropical regions [4,5]. Slugs are soft-bodied, produce mucus, have a rasping mouthpart called a radula, two pairs of tentacles and breathe through an opening in their mantle called a pneumostome [6]. Slugs have the ability to thrive in a range of conditions and their success is attributed to mucus production that deters predators, high reproduction rates and having an adaptable appetite [5]. They are known to digest even unpalatable material when choice of food is limited, and when a palatable food source is found, they repeatedly return to that location to feed [7]. Slugs consume by scraping the surface of their food with their radula, which can include seeds, roots, stems and leaves [8].

Slugs are becoming an increasing concern due to their ability to cause significant destruction to a variety of crops and nursery plants and for their capacity to act as a vector for bacterial transport. Their herbivory behavior implicates them as a threat to plant populations, consuming large amounts of crop and nursery plant material, and, owing to their voracious appetites, populating habitats that provide a variety of food sources. Slugs cause damage to plants above ground affecting morphology and below the

ground targeting roots and tubers [5] making crops and nursery plants a great resource for slug nourishment. These pests cause a considerable amount of economic damage in California arable and horticultural crops, commercial nurseries and home gardens [1,5], both as a direct result of plant destruction and indirectly through attempts at pest control.

In these habitats, slugs are also frequently ingesting soil microbes from dead and living plant material [9], as well as picking up soil microbes on their body and in their mucus. They can therefore become contaminated with bacteria both internally and externally [10]. Their bodies, feces, and mucus can serve as a vector, harboring, and dispersing bacteria wherever they travel. Slugs have been shown to carry E. coli internally and excrete it in their feces [5,10,11] and have the ability to carry coliform bacteria (often used as indicators of contamination, commonly found in fecal waste) both on their surface and in their gastrointestinal tract which could be dispersed in their trail of mucus as well as their feces to various locations [10,12]. Slugs are impacting habitats directly as well as indirectly by consuming, spreading disease, and disturbing the soil microbial community. Soil microbes play key roles in ecosystems and influence a large number of important ecosystem processes and have a big impact on plant productivity [13]. Altering soil microbial community composition by slugs, such as interrupting the action of free-living microbes, altering rates of nutrient supply and the portioning of resources [13] may be detrimental to plant communities.

Most slug species found in California crop systems and garden nurseries are invasive, and many are transported long distances. Known invasive slug species in California include members of the genera *Deroceras* and *Arion*, as well as the species *Milax gagates*, and *Lehmannia valentiana*, all originating from Europe [5,11]. At various garden nurseries in Riverside County, we have collected the slug species *Deroceras*, *Arion* and *Lehmannia valentiana*. *Lehmannia* have been collected in high numbers at Louie's nursery in Riverside, CA. We have also found *Lehmannia* at Mockingbird Nursery, a nursery that specializes in California natives, indicating that invasive slug species are not only localized to imported plants.

Due to their status as serious pests, several slug control options are in place. Present slug control options include chemical methods such as treatment with methiocarb pellets, cultural methods such as repeated seed bed cultivations, and biological approaches such as treatment with a natural slug predator [5]. Slug management with a pesticide is difficult because of the slug's biology. Also, due to the lack of effective chemicals, and because of the slug's ability to produce mucus, most contact poisons are sloughed off and never harm the slug [14]. In addition, pesticides used for slug control often times harm non-targeted small animals and other invertebrates [2]. One biological approach involves nematodes, and sometimes co-occurring bacteria. Nematodes are natural enemies of slugs and elsewhere have evolved an association with pathogenic bacteria that can be lethal to slugs [15], however it is currently only available in Europe (marketed as Nemaslug®) and studies in the United States for this particular biological control are currently underway [5,16,17].

The aim of this novel research was to characterize the bacterial microbiome of the invasive slug *Lehmannia valentiana*, while establishing its core bacterial microbiome. We also sought to verify any changes to the microbiome due to environment and diet, noting dispersal capabilities, using microcosm experiments. These findings herein suggest that the establishment of the slug bacterial microbiome varies individually and that it can be manipulated by dietary and environmental changes. Additionally, these findings demonstrate the possibility of ecosystem changes from soil bacterial invasions mediated by invasive slugs. This research contributes to the field of microbial ecology and plant pathology by demonstrating the devastation slugs are capable of when dispersing bacteria within their environment, disturbing native soil bacterial communities and spreading bacteria that could lead to the infection of various plant or contamination of crops.

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CHAPTER 1

Environment and diet influence the bacterial microbiome of *Lehmannia valentiana*, an invasive slug in California

Abstract

Lehmannia valentiana is an invasive terrestrial gastropod distributed throughout California. It originates from Europe and is a serious pest of gardens, plant nurseries, and greenhouses. Here, we have evaluated the bacterial microbiome of whole slugs to capture a more detailed picture of bacterial diversity and composition in this host. We concentrated on the influences of diet and environment on the core bacterial microbiome of *Lehmannia valentiana* as a starting point for obtaining valuable information that will aid in future slug microbiome studies. *Lehmannia valentiana* were collected from two environments (garden reared and reared from eggs in a laboratory). DNA from whole slugs were extracted and next-generation 16S rRNA sequencing was performed. Microbiomes differed between slug environmental sources (garden nursery vs. lab reared) and the bacterial community structure was influenced by sterile diet and the environment. The data also indicated that there is a core microbiome shared across all treatments. This is consistent with our hypothesis that a core microbiome is present and will not change due to these treatments. Findings from this study will help elucidate the impacts of slug-assisted bacterial dispersal on soils and plants, while providing valuable information about the slug microbiome for potential integrated pest research applications.

Introduction

All invertebrates associate with bacterial communities, which form a component of their microbiome. Notably, invertebrate microbiomes are often overlooked. Bacteria associated with invertebrates play many roles in association with their hosts – including protection or supporting overall health and fitness – or have negative implications for the host, and may therefore be ecologically important [1,2]. Invertebrate systems have been shown to be excellent models for the study of host-bacterial associations, partially due to their smaller size and rather uncomplicated gut communities [3]; yet, to date, little work has been conducted on malacological (the study of mollusks) research focused on bacteria associated with whole terrestrial slugs [4]. With the advances of next-generation sequencing, research focused on host-microbiome systems has expanded and now includes a wider range of plants and animals.

Slugs may serve as vectors for transporting microbes from place to place. Therefore, many slugs that are considered invasive could harbor and translocate a variety of exotic or pathogenic microorganisms within their microbiome. Horticulturalists, agronomists, and land managers recognize the need to effectively control slug populations in an ecologically sound manner. The interactions between slugs, bacteria and their environment can vary; characterizing the bacterial community within – and among – slugs is an important step for elucidating the nature of these various interactions [5]. Given their fundamental role, slugs provide an exemplary system for addressing

questions concerning composition, function and diversity of this malacological microbiome [3,6].

Slugs have the capacity to thrive in a range of conditions. Indeed, their success is attributed to mucus production that deters predators, as well as high reproduction rates and adaptable appetites [4]. They are major pests of plant nurseries and certain agricultural crops, such as corn, soybean, wheat, brassicas, leafy vegetables and strawberry crop systems [7]. Slugs target a variety of plants and grasses, often by killing seedlings outright, causing considerable amounts of economic damage in California arable and horticultural crops, commercial nurseries and home gardens [4,8].

Most slug species found in California nurseries are invasive, with many having been transported long distances. Known invasive slug species in California include members of genera *Deroceras* and *Arion*, as well as the species *Milax gagates*, and *Lehmannia valentiana*, all originating from Europe [4,7]. Although individual slugs do not move rapidly *per se*, trade in horticultural commodities has facilitated their spread. As a result, additional exotic slug species, or species not considered to be endemic to California, are likely to appear there in the near future [4,7].

Malacological studies have yet to determine the bacterial microbiome of whole slugs, and more importantly, address the context wherein diversity of the slug bacterial microbiomes is being shaped. To gain an understanding into the slug's bacterial ecological

relationships, we investigated the bacterial microbiome of *Lehmannia valentiana*, a slug invasive to California, using sterile microcosm experiments. Moreover, to address some factors shaping slug bacterial microbiomes, we evaluated whether the *L. valentiana's* bacterial microbiome can be influenced by changing their diet and environment. To elucidate the impacts of slug-assisted bacterial dispersal on soils and plants, we hypothesized that (1) *L. valentiana* overall bacterial communities can be manipulated via their environment and diet, while (2) a core bacterial microbiome creates the basis for future studies of slug microbiomes on host physiology, but also will provide useful information for Integrated Pest Management applications.

Methods

Study species

Lehmannia valentiana (d'Audebard de Férussac, 1823) evolved in Europe and is invasive in California, geographically distributed throughout at least 29 counties in California [7]. One distinguishing morphological characteristic of *L. valentiana* is the presence of two distinct lines extending down their back along the entire length of their body (Fig. 1.1a). These slugs are wide-scale pests, feeding on plants and decomposing wood [7].

Sample collection

Slugs were collected with sterile gloves from shaded areas beneath flats and pots of various plants from Louie's Nursery, a market garden located in Riverside, California (Fig.1.1b). Upon collection, slugs were placed in sterile 15ml conical tubes and

subsequently returned to the laboratory at the University of California, Riverside, for downstream analyses. For characterizing the initial slug bacterial microbiome, five of these slugs were frozen immediately. The remaining 15 were divided into three sterile microcosms, consisting of five slugs each.

In addition to slugs collected from the garden, slugs were also reared in the laboratory. The eggs from previously collected *L. valentiana* were gathered and kept in a dish with moist paper towels at room temperature. The juvenile slugs that emerged from the eggs were transferred to new dishes, kept moist and fed with carrots and wet dog food. After approximately four-six months of growth, 10 randomly selected laboratory-reared slugs were divided; five were frozen for initial bacterial microbiome analyses and the remaining five were placed into a sterile microcosm.

Sample processing and experimental design

Each microcosm was composed of sterile, autoclaved paper towel, initially moistened with ~5ml of sterile water; thereafter, small amounts of sterile water were added to each microcosm to maintain consistent moisture. Slugs were rinsed gently with sterile water prior to placement into the microcosms. All slugs were fed *ad libitum* with a sterile artificial diet based on a method by Walker (1997). The sterile diet was composed of a mixture of autoclaved carrots, bran, and nutrient agar. To maintain sterility, each microcosm was only opened in a biosafety cabinet; sterile forceps were used for feeding, as well as slug placement or removal.

Laboratory reared slugs were initially fed non-sterile dry and wet canned dog food and carrots. Five of those slugs were placed into a sterile microcosm, as described above. Slugs were housed in sterile microcosms for two weeks before DNA analyses.

Whole slug tissues were prepared for DNA extraction in 15ml conical tubes by blending each slug with sterile water using 14G, 16G, and 18G needles (in sequential order) to create a slug mixture. The amount of sterile water added was determined by slug weight. DNA extraction of the slug mixtures was performed using the MoBio PowerSoil® DNA extraction kit. An aliquot of 250µl of each slug mixture was used in lieu of the 0.25g of soil called for in the kit protocol. Slug DNA extracts were amplified by PCR to capture the full variety of the 16S rRNA genes within each sample. These PCR extracts were sequenced using the Illumina MiSeq system allowing for the sequencing of a ~450bp section of the 16S V3 and V4 region of the 16S rRNA gene The sequences were multiplexed using barcoded indexes and primers from the Illumina Nextera XT kit [9].

Data analysis and bioinformatics

To examine the core bacterial microbiome, as well as the relationship between the core and communities from environment and diet, sequences were processed with Quantitative Insights Into Microbial Ecology (QIIME). This approach was used to determine the relationship between bacterial microbiome communities and host diet, rearing, and sterility variables. We removed low quality and chimeric sequences and computed core

microbiomes in QIIME; we define the core bacterial microbiome of slugs as the bacteria commonly detected among all sampled slugs [10].

To determine if bacterial beta diversity was different between samples, Unifrac distance matrices were created and used to compare community samples. To visualize and explain differences among bacterial communities, we used Non-metric MultiDimensional Scaling (NMDS) plots of the Unifrac distances. Unifrac distances were also used for PERMANOVA analyses of microbial community data using the adonis function in the vegan package of R [11,12]. PERMANOVA was used to compare bacterial community structures across all treatment groups based on the OTU composition and examine the relationship between relative abundances of the most abundant phyla or classes, as well as diet, rearing, and sterility variables.

Indicator Species Analysis

We applied an indicator species analysis to detect bacterial families significantly associated ($p<0.05$) with the two groups sterile/non-sterile, as well as between garden/lab-reared. We calculated the indicator values using the 'multiplatt' function with 9999 permutations in the 'indicspecies' R package [13]. Indicator value indices were used for assessing the predictive values of taxa as indicators of conditions present within the different groups [13].

Results:

The structure of the slug bacterial communities was significantly different between dietary treatments, as well as between environments ($p<0.001$, $p<0.001$; Fig. 1.2). Both lab-reared and the garden slug's bacterial communities adapted similarly after sterile diet and sterile environmental exposure. Prior to sterile diet and sterile environmental exposure, the lab-reared and garden slug microbiomes mostly did not overlap, and the community composition was significantly different. These results support our first hypothesis that the bacterial microbiome of *L. valentiana* can be manipulated via changes in their environment and diet.

We detected a likely core microbiome for *L. valentiana* slugs. There were several slug bacterial OTUs conserved across all slug samples (Table 1.1), which is consistent with our second hypothesis, that a core bacterial microbiome is present and not changed due to these perturbations. While some bacterial taxa were shared across all treatments, both sterility ($p<0.015$) and environment ($p<0.007$) explained the variation among the total slug bacterial microbiomes.

The indicator species analysis yielded a total of 54 significant taxa (Table S1) across all groups. The sterile slug group produced four significant bacterial species ($p<0.01$) accompanied with seven significant bacterial species ($p<0.01$) for the non-sterile slug group. *Propionibacterium acnes* was the most significant ($p<0.01$) indicator for the garden slug group. Highly significant ($p<0.001$) indicators for the lab-reared slug group

included the bacterial genera *Pigmentiphaga*, *Ochrobactrum*, *Leucobacter*, *Candidatus Solibacter*, and *Luteolibacter* and the bacterial families *Rhodobacteraceae* and *Rhodocyclaceae*.

Discussion and Conclusions

In this present study, we investigated the impact of diet and environment on the composition and diversity of *L. valentiana's* bacterial microbiome. We found that bacterial communities of slugs differed, ostensibly resulting from both diet and environment. Sterility treatments in diet and environment subsequently lead to similar shifts in the slug bacterial microbiome. However, we did detect some overlap in bacterial communities across these treatments, indicative of the core microbiome.

Previous studies have shown that diet and environment play a role in determining the bacterial microbiome of a variety of invertebrates. A study from Cavalcante et al. (2012) showed that a diet of only sugarcane produced a shift on the gut microbial communities of *Achatina fulica*, a land snail. Landry et al. (2015) presented evidence that environment, including diet, has a significant effect on the microbial species diversity in the midgut of *Choristoneura fumiferana*, the spruce budworm. Our findings were comparable to the results reported in these and other studies of invertebrate microbiomes. *Lehmannia valentiana* bacterial microbiomes from groups reared in either the garden or laboratory environments differed initially. Moreover, the microbial community of these slugs raised in these diverse environments shifted similarly after exposure to sterile food and

atmospheric conditions. Findings from our study provides evidence that slug bacterial microbiomes are malleable and may depend substantially on both diet and environment. Additionally, the shift in the microbial community from these conditions supports our first hypothesis that diet and environment may have an impact on the bacterial microbiome of *L. valentiana*

Diet and environment of invertebrates directly or indirectly play substantial roles in shaping their microbiome, such as environmental pressures on resident microbiota and overall survival in the invertebrate gut [1,2]. Currently, little is known about mechanisms for slug dispersal of bacteria. Future research aimed at determining whether microorganisms acquired by slugs can influence their microbiome or affect their capacity to disperse microbes to novel environments – as well as examining if the core bacterial microbiome of slugs develop via vertical transmission – would be especially valuable.

Although there was a core microbiome of shared bacterial taxa isolated from *L. valentiana* slugs across all sequenced slug microbiome samples, all but one of the core bacterial taxa found in *L. valentiana*: *Rhodococcus fascians,* were not identified at the species level. We discovered eight bacterial families in the core bacterial microbiome of *L. valentiana* which included bacterial species found in previous studies of gastropod microbiomes. One genus detected across all of our sequenced samples, *Citrobacter,* has not only been found in fecal samples of the slug *Geomalacus maculosus*, but also in the gut of another slug species *Arion ater* [3,4]. *Geomalacus maculosus* also consisted of

similar core bacteria, including such genera as *Aeromonas*, *Buttiauxella*, *Citrobacter*, *Kluyvera* and *Pseudomonas* [4]. We have previously found *Pseudomonas* in the microbiome of *L. valentiana* and *Arion* sp. slugs. These findings support our hypotheses that a core microbiome is present in *L. valentiana*.

Similar to our study, Joynson et al. (2017) also determined that the majority of the gut microbial community of *Arion ater* corresponded to members of the Enterobacteriaceae and Pseudomonadaceae families [14]. Additionally, the bacterial family *Comamonadacea* was detected within the core bacterial microbiome of *L. valentiana*; previous research illustrated that taxa from *Comamonadacea* can be recovered from the gut of the giant African land snail *Achatina fulica* [16]. Other studies also detected genes linked to lignocellulose degradation within the microbiota associated with crops afflicted with the giant African land snail [16,17]. Some genera in the family *Comamonadaceae* have been directly linked to the degradation of lignocellulose [5], which could have implications for host nutritional status or for amplifying economic crop losses. Determining the extent of bacterial genes associated with lignocellulose degradation in *L. valentiana* could be economically significant and relevant for food security. The diversity of the core bacterial microbiome of *L. valentiana* slugs in this study is slightly more diverse than similar previously conducted slug microbiome studies. This could be due to our use of whole slugs in this study, whereas other studies limited their microbiome analyses to specific regions of the slug's anatomy.

Some of the bacterial taxa found across the *L. valentiana* core bacterial microbiome are putative plant pathogens. For instance, *Erwinia* is a genus containing mostly plant pathogenic species [19,20], and *Rhodococcus fascians* is a plant pathogen which causes leafy gall disease in a variety of plants. Additionally, a subset of other plant pathogen genera such as *Pseudomonas viridiflava,* were found in some of our slug samples, but were otherwise absent in others [6].

The indicator species analyses revealed families, genera and species of bacteria, characteristic of each treatment group individually. In fact, according to our indicator species analyses, the most significant taxon in the group raised in sterile conditions included the families *Aeromonadaceae* and *Cerasicoccaceae,* as well as genera *Flavobacterium* and *Mycobacterium*; this could indicate that these taxa may be poor competitors with other members of the non-sterile group. The most highly significant bacterial species found in the non-sterile slug group: *Paracoccus marcusii,* is known to produce astaxanthin, a carotenoid that produces a red/orange pigment which not only provides a variety of plants and animals with their red/orange color but has also been linked to having beneficial (photoprotective, antioxidant, and anti-inflammatory) effects on the skin [22,24]. Additionally, *Paracoccus marcusii* was isolated from the white grub, a serious pest of potatoes, in a study that attempted to find entomopathogenic bacteria associated the grub [25]. In the garden slug group, six bacterial families were identified as indicators. Overall, *Propionibacterium acnes* was the most significant bacterial indicator of the garden group. Although this taxon has been reported as a member of the

skin microbiome and is associated with acne pathogenesis [26], previous studies have not identified *Propionibacterium acnes* as a common garden taxon associated with slugs' microbiomes. Many bacterial taxa, across seven bacterial families, were indicative of the lab-reared slug group. Across these seven bacterial families, many taxa, which were previously isolated from a variety of organisms, have been found to be linked to either gut health or gut microbiome. Of these taxa, *Pigmentiphaga* has been isolated from nematodes [7], *Ochrobactrum* from bees, spiders, nematodes and sand flies [8-11], and *Rhodocyclaceae* from termites and beetle larvae [12,13]. Often detected within woody plant parts; *Leucobacter* has been found to exhibit mutualistic relationships with keystone soil invertebrates, ostensibly due to its ability to degrade lignocellulose into more labile components and bioavailable nutrient sources [14]. Likewise, *Candidatus Solibacter* has also been associated with decomposing dead wood and peat moss [15,16]. *Luteolibacter*, in the family *Verrucomicrobiaceae*, was identified as part of the core microbiome within fecal samples of *Geomalacus maculosus*, a European protected slug [15]. Thus, based on their ecological roles, interactions with plant or animal hosts, or their physiological adaptations to particular environments or diets, the occurrence of the bacterial indicator taxa characteristic of each group may be indicative of changes in either diet (sterile vs non-sterile) and environment (garden and lab-reared).

Our study provides evidence that bacteria associated with slugs are not only ecologically significant but may also be manipulated by both dietary and environmental changes. Several microbes found within our slug bacterial microbiomes have been detected in the

guts or feces of other slugs, which could have functional implications for host processes and dietary parameters. Given that invasive slugs could harbor a variety of plant pathogenic microorganisms within their microbiome, their dispersal could have environmental and agricultural implications for both crop health and plant science. Findings from our study suggest that although a small core microbiome remains consistent, the establishment of the slug bacterial microbiome not only varies among individuals but may also be manipulated by dietary and environmental changes. Nevertheless, a better understanding of the slug bacterial microbiome may provide valuable information regarding biotic threats posed by invasive slugs, as well as insight into potential techniques for holistically managing slug populations.

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Figure 1.1 (a) Study species ***Lehmannia valentiana***: Length- up to 75 mm; mantle with two distinct lateral bands and a less distinct median band; slime copious, watery; keel poorly marked, rounded; lateral bands may run full length of the body (b) Map of study site. Louie's Nursery, 16310 Porter Ave., Riverside, CA 92504

a

b

Figure 1.2

Non-metric multidimensional scaling (NMDS) illustrating how bacterial communities of *Lehmannia valentiana* vary by environmental and dietary treatments. Samples of slugs raised under sterile conditions are represented by red, while slugs in their natural conditions are symbolized by blue; slugs reared in the lab are represented by triangles, and slugs from the garden outside of the laboratory are indicated by circles

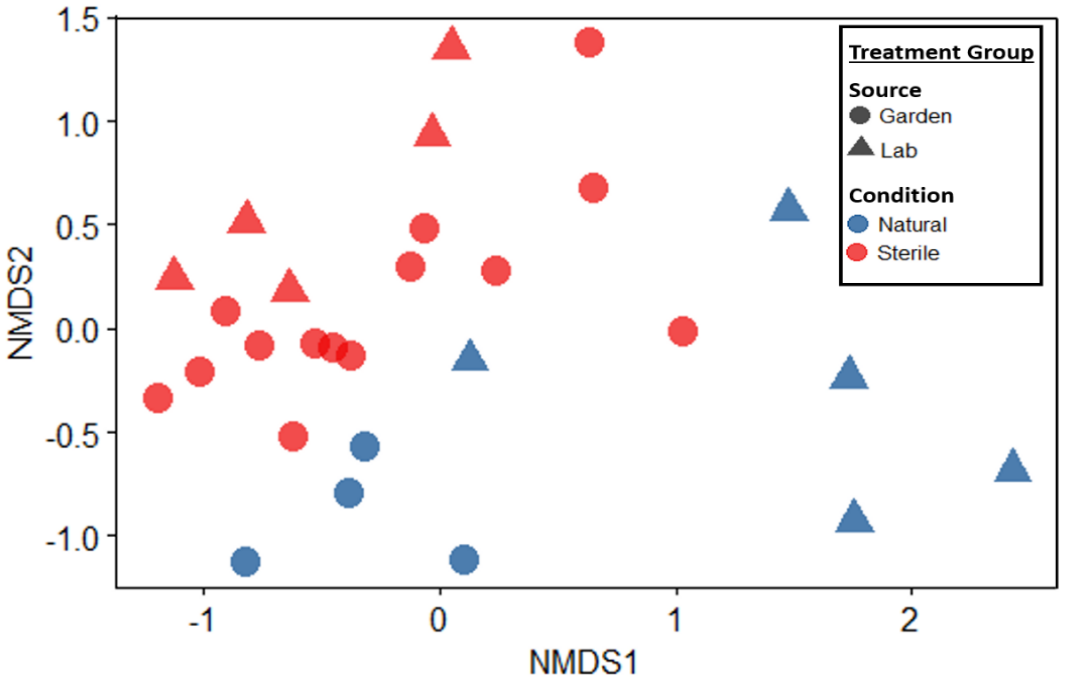

Table 1.1 Bacterial taxa present in all *Lehmannia valentiana* samples

Bacterial genus	Bacterial family
Citrobacter	*Enterobacteriaceae*
Delftia	*Comamonadaceae*
Erwinia	*Enterobacteriaceae*
Arthrobacter	*Micrococcaceae*
Stenotrophomonas	*Xanthomonadaceae*
Pseudomonas	*Pseudomonadaceae*
Rhodococcus	*Nocardiaceae*
Bacillus	*Bacillaceae*

CHAPTER 2

Soil Bacterial Community Response to an Invasive Terrestrial Slug: A Microcosm Study

Abstract

The introduction and spread of invasive bacterial species can disrupt the structure and function of indigenous soil bacterial communities. These non-native species can compete against the native community, possibly reducing their abundance or displacing them entirely. The invasive potential of bacteria can depend on their ability to disperse, and although unintentionally introduced, invasive slugs can provide that mechanism. This novel study is one of the first to demonstrate that the presence of an invasive slug influences the biodiversity of soil bacterial communities in soil microcosms. This study analyzed the species richness and diversity of bacterial communities associated with slugs and soils, prior to and subsequently to the residency of sterile-reared invasive slugs. We used soils from two distinct environments, the San Joaquin Experimental Reserve located in the Sierras (SJER) and Catalina-Jimenez Critical Zone Observatory (CZO) located in Arizona, in soil microcosms to determine if the invasive slug species, *Lehmannia valentiana*, may perhaps disrupt soil bacterial communities. We found that this invasive slug significantly altered the biodiversity of the soil bacterial communities after two weeks of residency in the soil microcosms. These results indicate that in a soil microcosm, an invasive slug species can disturb native soil bacterial communities,

suggesting that slug dispersed invasive bacteria may be devastating to indigenous soil bacterial communities.

Introduction

Within natural habitats, soil bacteria interact more with neighboring, endemic bacteria than they do with non-cosmopolitan bacteria from distant habitats [1]; however, soil bacterial communities can be disrupted by dispersed invasive bacteria [2]. These invasive species can transform communities in which they are introduced and may gain an advantage by outcompeting the native species of that community [20,21]. Microorganisms are not traditionally considered when studying invasive species, and many microbial ecologists do not consider bacteria as invasive species. However, an increasing amount of research is showing that climatic, environmental, and biological drivers, in particular, co-invasion with plants and animals, can introduce bacteria to new environments.

Invasive species are often considered to include a variety of destructive or highly competitive plants and animals; however, microorganisms may also be considered invasive. There are approximately 50,000 species of plants, animals and microbes (such as viruses and fungi) invasive to the U.S. and the quantities of invasive species are increasing [5]. Economic damages and control costs due to invasive species has been estimated at $137 billion per year in the United States and research efforts to understand the ecology of invasive species has been growing over the last 20 years [2,3]. A range of

ecological factors may allow invasive species to become abundant in their new habitats. However, once an invasive species is dominant in a novel location, it competes with native species for nutrients, food or space, triggering changes in ecosystem structure and function, and sometimes spreading pathogens and/or becoming serious ecological threats in their new habitat [2,4,5].

Newly dispersed bacteria are known to invade existing bacterial communities, which can alter community structure and ecosystem functioning in terrestrial environments [2]. However, there are limitations on the ability of these often-non-motile organisms to disperse on their own. One possible mechanism for bacterial dispersal is co-invasion with above-ground consumers, such as terrestrial slugs, that feed upon bacteria that have key ecological roles [10]. Bacterial community structure and composition can be connected to ecosystem function [11] demonstrating that deviations in soil microbial communities may well cause environmental change. These first-principle mechanisms are determined, partly, by native microbial communities [12] and disturbance by invasive slug bacteria could disrupt these native microbial populations that are already established.

Slugs are notable members of the soil fauna, inhabiting soils both above and below ground, allowing them continual contact with the soil bacterial community [5,6]. Slugs are mostly active during the evening and prefer cool, moist soils; however, they can burrow deep into soil during periods of unfavorable conditions and to prevent drying out, generally spending more time below ground and within the soil [15]. Invasive slugs, such

as *Lehmannia valentiana*, continuously and inadvertently ingest bacteria from the soil, their environment [16] and from plants and in addition, bacteria adhere to their mucus, making invasive slugs candidates for bacterial dispersal into indigenous soil bacterial communities. Introduction of invasive species, such as slugs and their associated bacteria, into new regions endangers regional and native biodiversity worldwide [9–11].

In order to uncover the possibility of ecosystem changes from soil bacterial invasions mediated by slugs, and to determine if slugs disperse bacteria, which species of bacteria are dispersed, and what occurs in the soil once the bacteria are deposited, we performed a series of soil microcosm experiments, exposing soils from two distinct environments to a sterile-reared invasive slug. The aim of this study was to investigate the impact of invasive bacteria, dispersed by slugs, on soil bacterial communities. We hypothesized that (1) after soil residency, the soil and slug bacterial microbiome would be altered and (2) *L. valentiana* slugs disperse invasive bacteria into soil environments. The goal of this research is to understand the extent that slugs act as a dispersal mechanism for both beneficial soil bacteria and plant pathogens and how their microbiome is incorporated or changed in relation to the soil microbiome. Additionally, we aim to expose a potential pathway through which slugs mediate bacterial-driven ecosystem services through their effects on the soil bacterial community. Our main objective was to establish whether there were changes in the indigenous soil bacterial community due to the presence of an invasive slug.

Methods

Study species

Lehmannia valentiana (d'Audebard de Férussac, 1823) evolved in Europe and is invasive in California, geographically distributed throughout at least 29 counties in California [19]. One distinguishing morphological characteristic of *L. valentiana* is the presence of two distinct lines extending down their back along the entire length of their body. These slugs are wide-scale pests, feeding on plants and decomposing wood [19].

Sample collection

Sterile-reared slugs

To effectively evaluate slug bacterial dispersal and to track affects between the soil and slug bacterial communities, sterile-reared slugs were used in soil microcosms. 150 slugs were collected from Louie's Nursery, as described in chapter one. For characterizing the initial slug bacterial microbiome, eight of these slugs were frozen immediately (**garden slugs**). The remaining 142 slugs were divided into sterile microcosms.

Sample processing and experimental design

Sterile microcosms

Each microcosm (Fig. 2.1) was composed of sterile, autoclaved paper towel, initially moistened with ~5ml of sterile water; thereafter, small amounts of sterile water were added to each microcosm to maintain consistent moisture. Slugs were rinsed gently with sterile water prior to placement into the microcosms. All slugs were fed *ad libitum* with a

sterile artificial diet based on a method by Walker (1997). The sterile diet was composed of a mixture of autoclaved carrots, bran, and nutrient agar. To maintain sterility, each microcosm was only opened in a biosafety cabinet; sterile forceps were used for feeding, as well as slug placement or removal. To reduce the slug's bacterial biodiversity and ensure slug health, slugs remained in the sterile microcosms for 4-5 to months until placement into soil microcosms. Eight of the slugs were frozen after sterile treatment (**sterile slugs**). The remaining viable sterile slugs were placed into soil microcosms.

Soil microcosms

To determine the bacterial community responses between soils and slugs, two different types of soil were used. These soils were previously analyzed in another study and made available for use in the project. **Soil A** was collected from SJER (San Joaquin Experimental Reserve) in the Sierras, and **Soil B** was collected from the Catalina Jimenez Critical Zone Observatory in Arizona (CTNA). There were five microcosms for each soil type: two replicates, one control and two that received no treatments. Soil microcosms (Fig. 2.2) were composed of an autoclaved glass petri dish, six inches in diameter. To prevent slugs from escaping, copper wire mesh was placed under the lids of each microcosm. Copper causes a chemical reaction to slug skin and mucus, therefore slugs avoid moving over copper [23–25]. The microcosms were autoclaved before the addition of soil and slugs. ~45g of soil was added to each microcosm and each were kept damp with sterile water. 16 of the sterile-reared slugs were placed into each replicate soil microcosm while the control microcosms received only water and sterile food. The no

treatment microcosms received no water or sterile food. Samples of soils were collected prior to (**pre-slug**) and subsequently after (**post-slug**) slug placement. In addition, soils were sampled two weeks after slugs were removed. For comparison, all pre-slug soil samples were grouped together per soil type. All post-slug soil samples were grouped together per soil type.

All slugs were fed and handled as previously described. Slugs remained in the soil replicate microcosms for two weeks. After two weeks, 14 slugs from the Soil A microcosms (**Slugs (Soil A)**) and 16 slugs from the soil B microcosms (**Slugs (Soil B)**) were frozen, while the remaining slugs were moved into a new experiment, outlined in chapter three.

Whole slug tissues were prepared for DNA extraction in 15ml conical tubes by blending each slug with sterile water using 14G, 16G, and 18G needles (in sequential order) to create a slug mixture. The amount of sterile water added was determined by slug weight. DNA extraction of the slug mixtures and soil samples were performed using the MoBio PowerSoil® DNA extraction kit. An aliquot of 250µl of each slug mixture was used in lieu of the 0.25g of soil called for in the kit protocol. Slug and soil DNA extracts were amplified by PCR to capture the full variety of the 16S rRNA genes within each sample. These PCR extracts were sequenced using the Illumina MiSeq system allowing for the sequencing of a ~450bp section of the 16S V3 and V4 region of the 16S rRNA gene The

sequences were multiplexed using barcoded indexes and primers from the Illumina Nextera XT kit [23].

Data analysis and bioinformatics

To examine the relationships between the soil and slug bacterial communities, sequences were processed with Quantitative Insights Into Microbial Ecology 2 (QIIME2). We removed low quality and chimeric sequences in QIIME2. Core microbiomes were determined with original code in R for each of the groupings discussed in this work, defined as the set of taxa matching at least one read in >95% of samples for that group.

Alpha diversity (within-sample) was calculated using Simpson's reciprocal index (1/D) as recommended by Jost (2006). Comparisons of alpha diversity between samples used the Wilcoxon rank-sum test due to unequal variances between groups. Bray-Curtis dissimilarities were calculated on the whole dataset as well as separately on the non-sterile samples (negative controls and autoclaved clay (Ch. 3)) using the vegdist function in the R package (R Core Team, 2013), vegan (Oksanen et. al, 2019). Principal co-ordinates analyses (PCoAs) were conducted with the ape package (Paradis and Schliep, 2018). The extremely low diversity of the sterilized samples masked differences in community composition between the non-sterile samples, so the former are excluded from community analyses in the present work. Visualizations of data were produced using the ggpubr package in R (Kassambara, 2020).

Alpha diversity (between groups) was calculated to provide a further assessment of richness and diversity. We pooled the data of all treatment groups into single samples. This created uniform profiles of each treatment group, allowing expression of the data as a percentage of the total diversity of each group [25,26]. The following α diversity indices were used:

Simpson's reciprocal index (1/D) gives more importance to species abundance (dominance) and considers both species richness and evenness. A higher index value is indicative of a greater degree of biodiversity within the community. The Simpson's reciprocal index (1/D) was calculated as:

$$DI = \frac{N(N-1)}{\sum n(n-1)}$$

Shannon diversity index (H') is weighted towards rare species, independent of sample size and combines both species abundance and richness. The index value increases as both the richness and the evenness of the community increase. The Shannon diversity was calculated as:

$$H' = -\sum_{i=1}^{S} p_i \ln (p_i)$$

Pielou's evenness index (J') is a measure of relative evenness, whereas the evenness (equitability) of a sample implies equality in the number of individuals of species. Values

range from near zero to one with higher values representing more even communities. The Pielou's evenness index was calculated as:

$$J' = \frac{H'}{\ln(S)}$$

Richness was calculated using the Margalef and Menhinick equations and assume that a relationship between S (total no. of species) and n (total no. of individuals) exists. Each were calculated as:

Margalef $R_2 = \frac{S-1}{\ln(n)}$ Menhinick $R_1 = \frac{S}{\sqrt{n}}$

Lastly, we evaluated the Relative species abundance for each group. Relative species abundance refers to how common or rare a species is relative to other species in a defined location or community. These values are reported in percent composition relative to the total number of that taxa in a sample group. Rank abundance curves display relative species abundance, measured on a log scale.

Where:

N = the total number of individuals collected

n = the number of individuals of a species

DI = Simpson Diversity Index

H = the Shannon index value

p_i = the proportion of individuals found in the *i*th species

ln = the natural logarithm

S = the number of species in the community

Results

Beta diversity analyses using the Bray-Curtis distance algorithm illustrated the main separation of communities was by soils pre-slug and soils post-slug for both soil types (Fig. 2.3a). There was also clustering within slug communities by treatment (Fig. 2.3a). The Wilcoxon rank-sum test indicated that there was significant difference between Soil type A and Soil type B pre-slug ($p=0.007$), however, the soils post-slug showed no significant difference. Differences between Soil type A pre-slug and Soil type A post-slug were highly significant ($p<0.0001$, Fig. 2.3b) however not significant between Soil type B pre-slug and Soil type B post-slug ($p=0.056$, Fig. 2.3b).

Although the garden and sterile slug groups were not significantly different, the sterile conditions altered the biodiversity and richness of the slug microbiome. The sterile slug group was composed of few dominant taxa, the richness of the group showed a -2.5-fold change from the garden slug group. Clustering was evaluated by principal coordinate analysis (PCoA).

Alpha diversity indices between groups represented that the Soil A pre-slug group had higher biodiversity and richness than the Soil A post-slug group, whereas the Soil B pre-

slug and post-slug groups had relatively even biodiversity and richness (Fig. 2.4a, Fig. 2.4b, Fig. 2.5a, Fig 2.5b). The biodiversity, richness and evenness decreased in the Garden slug groups after exposure to a sterile environment whereas the biodiversity, richness and evenness increased after the sterile slug group was exposed to either soil types (Fig. 2.4a, Fig. 2.4b, Fig. 2.5a, Fig. 2.5b).

The predominant genera of the Soil A pre-slug group (Fig. 2.4) are of the order *Solirubrobacterales* (~4%), phylum *Saccharibacteria*:TM7 (~3%), genus *Tepidisphaera*:WD2101 (~3%), family *Bradyrhizobiaceae* (2%) and genus *Sporosarcina* (~2%). The predominant genera of Soil A post-slug group (Fig. 2.4) are of the genus *Luteolibacter* (~10%) and *Pedobacter* (~10%), species *Flavobacterium succinicans* (5%) and *Brevundimonas diminuta* (~4%). The predominant genera of the Slugs (Soil A) group (Fig. 2.4) are of the genus *Luteolibacter* (~12%) and *Devosia* (~7%), family *Enterobacteriaceae* (~7%), species *Brevundimonas diminuta* (~5%) and genus *Mycoplasma* (~4%).

The predominant genera of the Soil B pre-slug group (Fig. 2.4) are of the order *Solirubrobacterales* (~4%), families *Bradyrhizobiaceae* (~4%) and *Chitinophagaceae* (~3%), genus *Rhodoplanes* (~3%) and class *Bacilli*:ZB2 (~3%). The predominant genera of the Soil B post-slug group (Fig. 2.4) are of the genus *Luteolibacter* (~6%) and *Pedobacter* (~6%), order *Solirubrobacterales* (~3%), and species *Flavobacterium succinicans* (~3%). The predominant genera of the Slugs (Soil B) group (Fig. 2.4) are

from the Genus Luteolibacter (~15%), Devosia (~7%) and Mycoplasma (~7%), species *Brevundimonas diminuta* (~6%) and family Enterobacteriaceae (~5%).

The predominant genera of the Sterile slug group (Fig. 2.4) are of the family Enterobacteriaceae (~30%), species Sphingobacterium faecium (~12%), genus Agrobacterium (~7%), species Brevundimonas diminuta (~5%) and family Comamonadaceae (~4%).

Taxa not present (or rare) in the pre-slug soils but present in the post-slug soils and slugs (Fig. 2.5) include the species *Brevundimonas diminuta* and *Flavobacterium succinicans*, and the genus *Agrobacterium*, *Devosia*, *Dyadobacter*, *Luteolibacter*, and *Pedobacter*.

These results together support our first and second hypotheses, that the soil and slug bacterial communities can be altered when *L. valentiana* is in a soil microcosm, and that *L. valentiana* slugs disperse bacteria into soil environments.

Discussion and Conclusions

We analyzed the bacterial microbiomes of soils and slugs prior to and subsequently to the residency of the sterile-reared invasive slug species *L. valentiana* in soil microcosms. We found that the soil bacterial communities of two separate soil types, changed significantly in response to the presence of the invasive slug, *L. valentiana*. In addition, the structure of the slug bacterial microbiome differed between Garden, Sterile and Soil slug groups

with some overlap in bacterial communities. Some bacterial taxa identified in this study were rare or not present in pre-slug soil groups but present in post-slug soils, both soil types, and in all slug groups. These include taxa such as *Brevundimonas diminuta* and *Flavobacterium succinicans*. *Brevundimonas* spp. was identified in a soil microbiome responding to chemical contamination [26]. Another study found that the invasion of the bacterium *Limnohabitans planktonicus* in a bacterial community increased previously rare *Flavobacterium* sp. [27].

One notable taxon identified in this study that was also rare or not present in pre-slug soil groups, but present in post-slug soils, both soil types, and in all slug groups is the genus *Luteolibacter*. *Luteolibacter* was the most abundant taxa in the slug groups from both soil types and was also identified in chapter one as a highly significant indicator ($p=<0.001$) for the lab-reared (sterile) group. It has been identified in as part of the core microbiome within fecal samples of *Geomalacus maculosus*, a European protected slug [15] and identified as a competitor towards other bacteria in the rhizosphere. In addition, *Luteolibacter* was found as an indicator of older, decomposing, coarse woody debris [35,36]. It is also associated in late root-galls of nematode infected roots, indicating a strong association with nematode infection [30]. Another important role of *Luteolibacter* is providing protection from reactive oxygen species (ROS) which are known kill bacteria directly and indirectly [30–32].

The taxon *Devosia* was identified as the second most abundant taxon in the post-soil slug groups from both soil types. *Devosia* has been identified in close association with multiple organisms including species of snail, ants, moths, beetles, ticks and ladybugs [33–39]. It has also been recognized as an endosymbiont having various roles within its ciliate host [40–44].

In addition to the potential for slug-dispersed bacteria to interrupt native soil bacterial communities, the dispersed bacteria may also be a health concern for humans and the crops we rely on. Slugs have been shown to carry E. coli internally and excrete it in their feces [13,26], [35]. For another example, in an experiment exposing slugs to *Clostridium botulinum*, known to cause botulism, the bacteria were detected and found viable in slug carcasses, slug feces, and live slugs [46]. It was also revealed that slugs have the ability to carry coliform bacteria (often used as indicators of contamination, commonly found in fecal waste) both on their surface and in their gastrointestinal tract which could be dispersed in their trail of mucus as well as their feces to various locations [13,44]. Slugs have also been shown to transmit plant pathogens such as clover rot and soft rot of potatoes [48,49] and may be linked to the contamination of crops by *E. coli* that were recalled in California [19,50].

Our data indicate that slug presence in soil alter the bacterial communities of both slug and soil. Since soil bacterial diversity and community structure depends on bacterial interactions, it is important to understand the exchanges between soil bacterial

communities and slug bacteria. Similar studies have shown that soil invertebrates can transport both free living and plant symbiotic microorganisms in soils, and in addition, the structure of the rhizosphere microbial community is altered in the presence of macroinvertebrates [26,27]. There has been very little work done on the microbes associated with, or pathogenic to, terrestrial slugs [45] and even less regarding the role of slug bacteria in soils. Studies on the direct impacts of the transformation of the soil bacterial communities by invasive slugs are lacking [10] demonstrating the novelty of this research. Slugs play an important, indirect role in promoting bacterial growth within the soil matrix [53]. Invasive slugs impact habitats directly as well as indirectly by consuming, spreading disease, and disturbing the soil bacterial community. Disturbance of existing soil bacterial communities by slug dispersed soil bacteria could disrupt native bacterial communities and interfere in the functions of that community. Our study indicates that consequences of changes to the soil bacterial diversity by invasive slugs is likely and therefore should be studied more in depth.

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Figure 2.1 Sterile slug microcosm experimental design

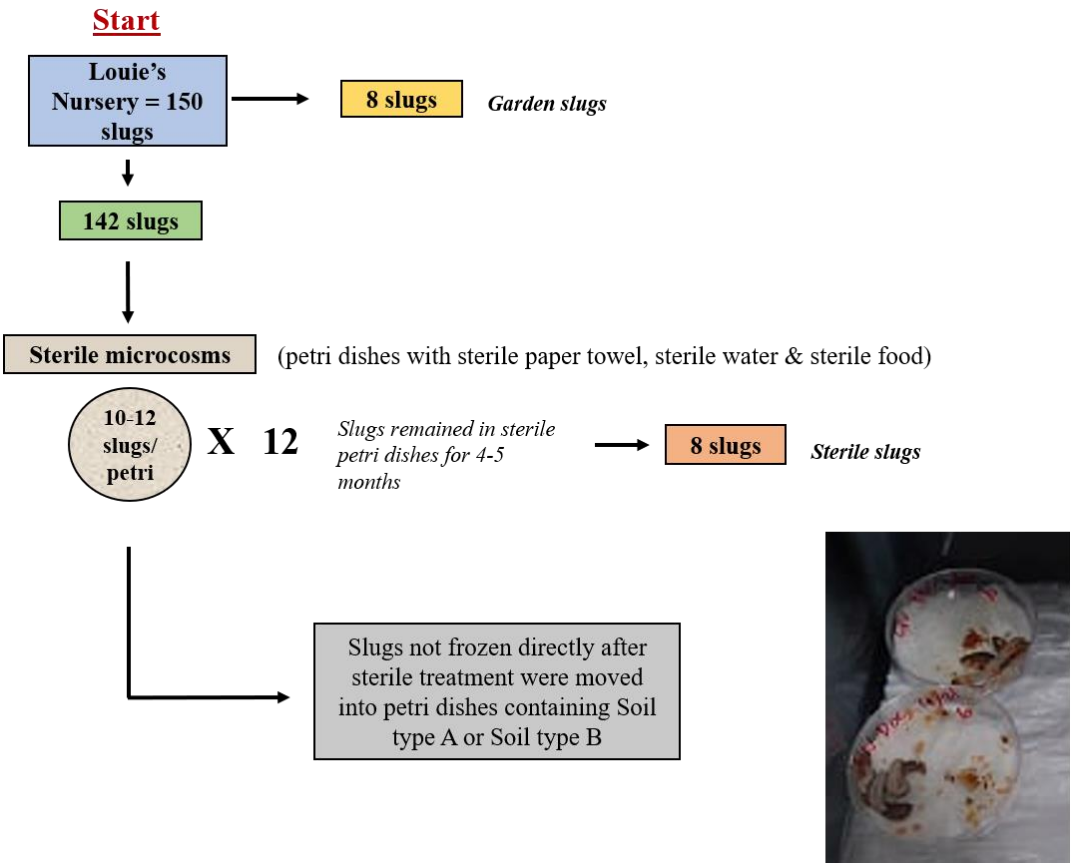


Figure 2.2 Soil type microcosm experimental design

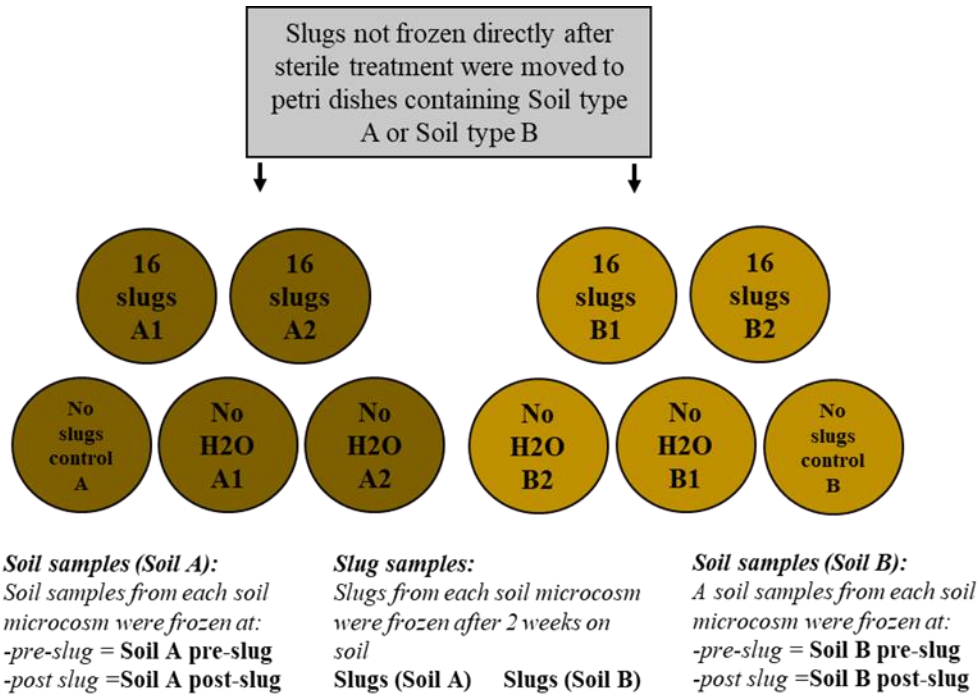
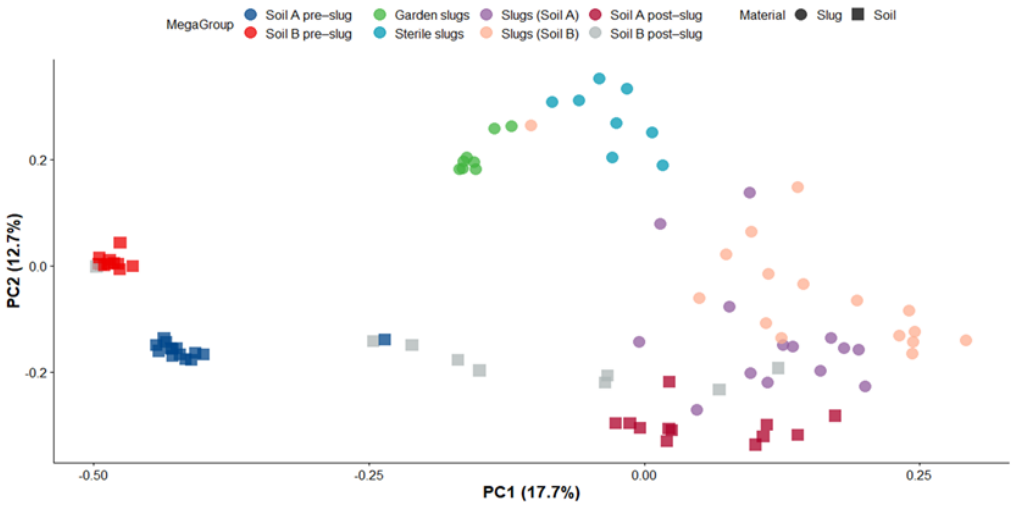


Figure 2.3 Beta and Alpha diversity of the Soil and Slug group samples

(a) Principal Coordinate Analysis (PCoA) plot with Bray-Curtis dissimilarity representing soil and slug microbiomes. Each data point represents an individual sample. The shape indicates sample community type, circle for slugs and square for soils. Samples are colored according to treatment. (b) Boxplot of Simpson's reciprocal diversity (1/D) between soils and slug treatment groups. ****= P<.0001. ***= P<.001, **= P<.01

a


MegaGroup
Soil A pre–slug
Soil B pre–slug
Garden slugs
Sterile slugs
Slugs (Soil A)
Slugs (Soil B)
Soil A post–slug
Soil B post–slug
Material
Slug
Soil
PC2 (12.7%)
PC1 (17.7%)
0.2
0.0
-0.2
-0.50
-0.25
0.00
0.25

b

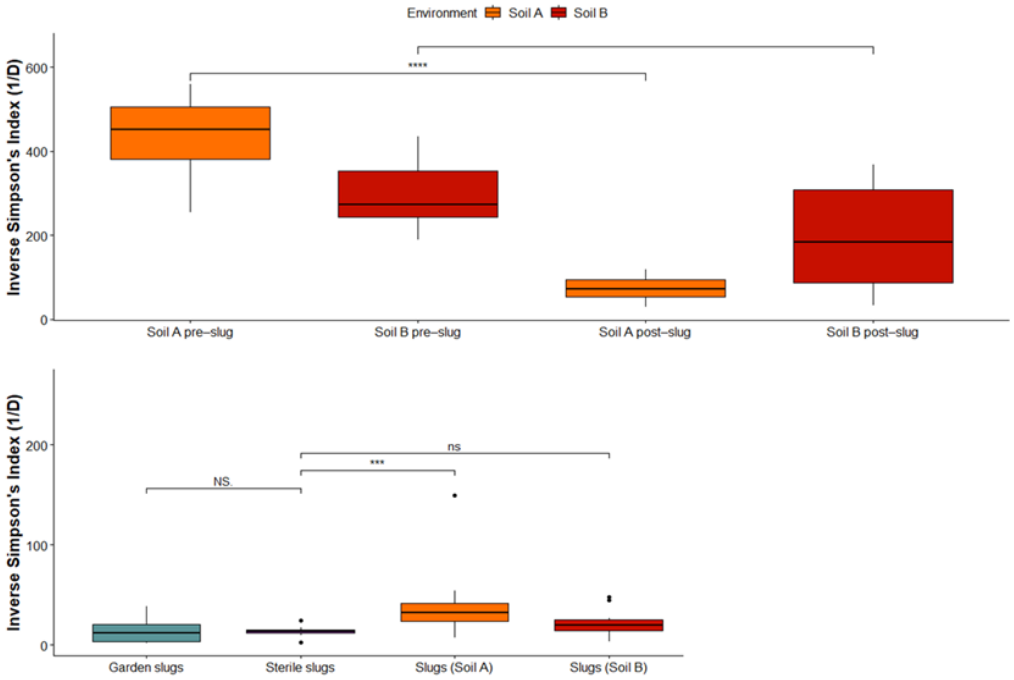
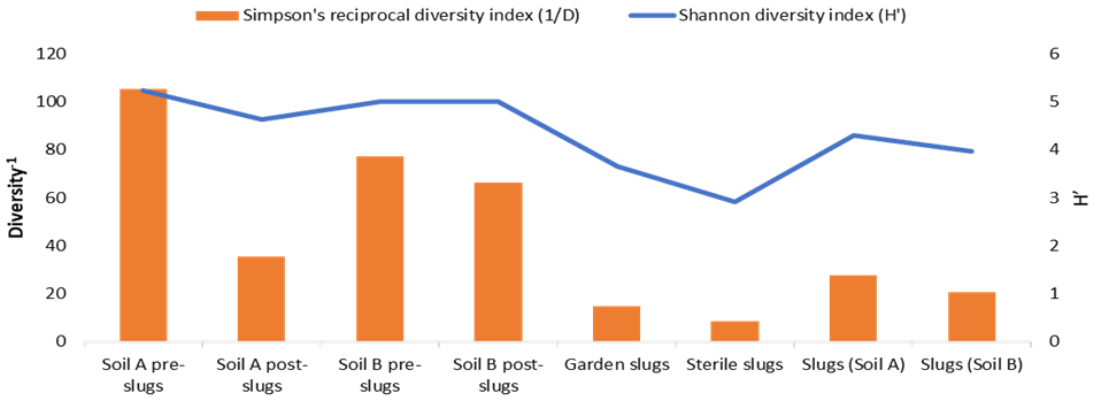

Environment
Soil A
Soil B
Inverse Simpson's Index (1/D)
****
600
400
200
0
Soil A pre–slug
Soil B pre–slug
Soil A post–slug
Soil B post–slug
Inverse Simpson's Index (1/D)
ns
***
NS.
200
100
0
Garden slugs
Sterile slugs
Slugs (Soil A)
Slugs (Soil B)

Figure 2.4 Alpha Diversity of Soil and Slug groups

(a) Simpson's reciprocal index (in orange) where a higher index value indicates a greater degree of biodiversity, gives more importance to species abundance. Shannon diversity index (in blue) is weighted towards rare species where a higher index value indicates an increase in both richness and evenness. Biodiversity decreased in both soil types after exposure to slugs. Sterile environment reduced slug biodiversity. Slug biodiversity increased after exposure to both soil types. (b) Richness and evenness between treatment groups for both soil types and slugs. Slugs reduced richness and evenness in Soil type A. Sterile treatment reduced richness and evenness in slugs. Soil environments increased slug richness for both soil types.

a


Simpson's reciprocal diversity index (1/D)
Shannon diversity index (H')
Diversity-1
H'
120
100
80
60
40
20
0
6
5
4
3
2
1
0
Soil A pre-slugs
Soil A post-slugs
Soil B pre-slugs
Soil B post-slugs
Garden slugs
Sterile slugs
Slugs (Soil A)
Slugs (Soil B)

b

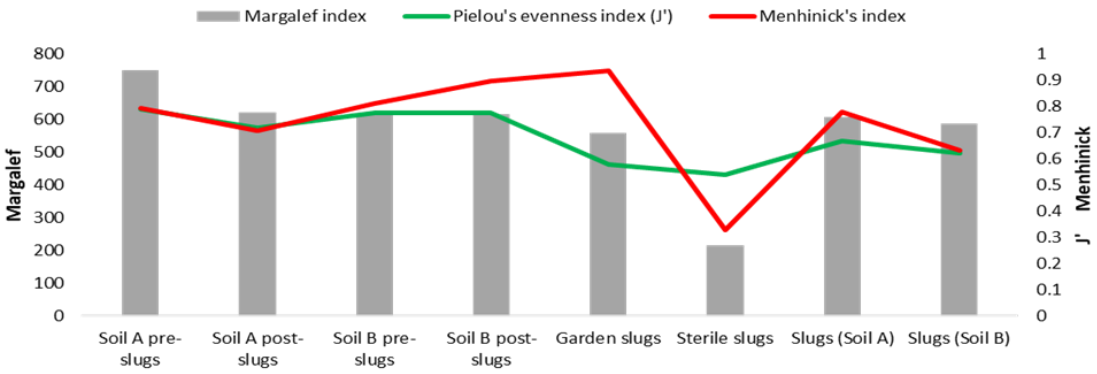

Margalef index
Pielou's evenness index (J')
Menhinick's index
Margalef
J'
Menhinick
800
700
600
500
400
300
200
100
0
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Soil A pre-slugs
Soil A post-slugs
Soil B pre-slugs
Soil B post-slugs
Garden slugs
Sterile slugs
Slugs (Soil A)
Slugs (Soil B)

Figure 2.5 Predominant Relative Species Abundances %: Soil A and B groups

Most dominant taxa among Soil A sample groups (slugs and soil). Slug presence altered community structure in both soil types. Rare and unassigned taxa are not included. The x-axis represents the percent abundance for each taxon in each group.

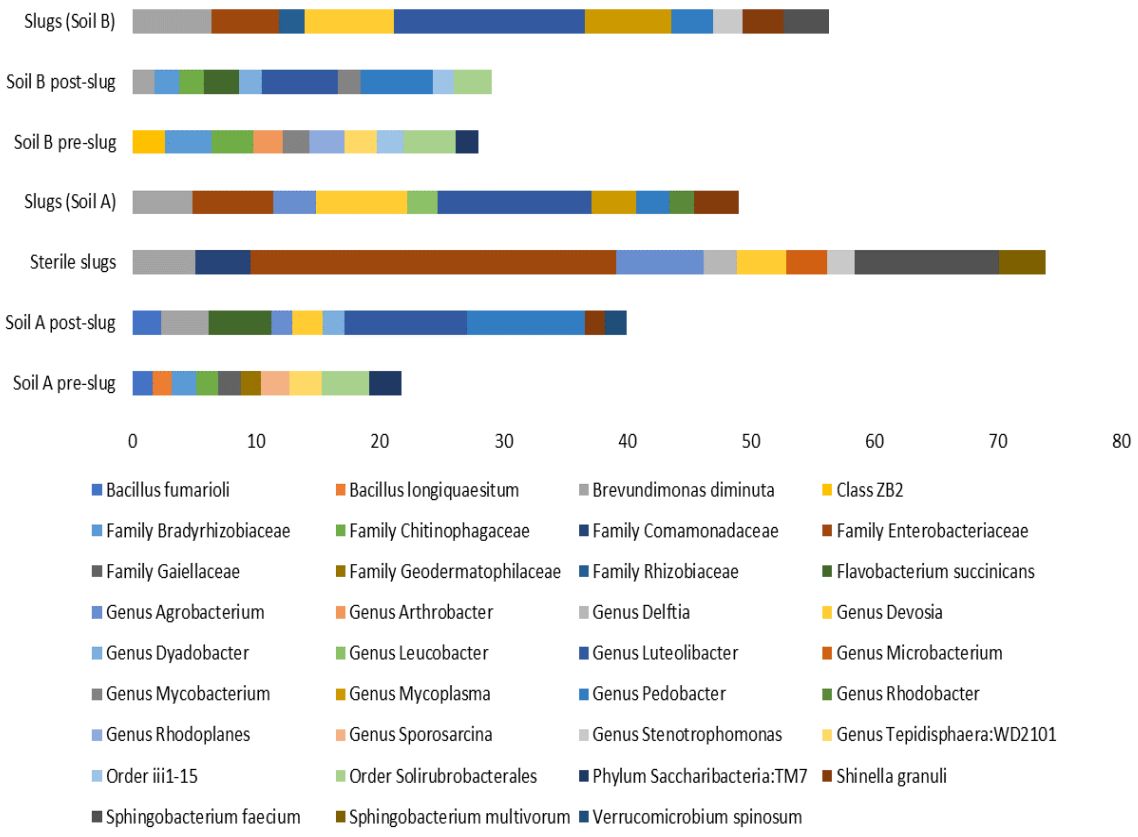
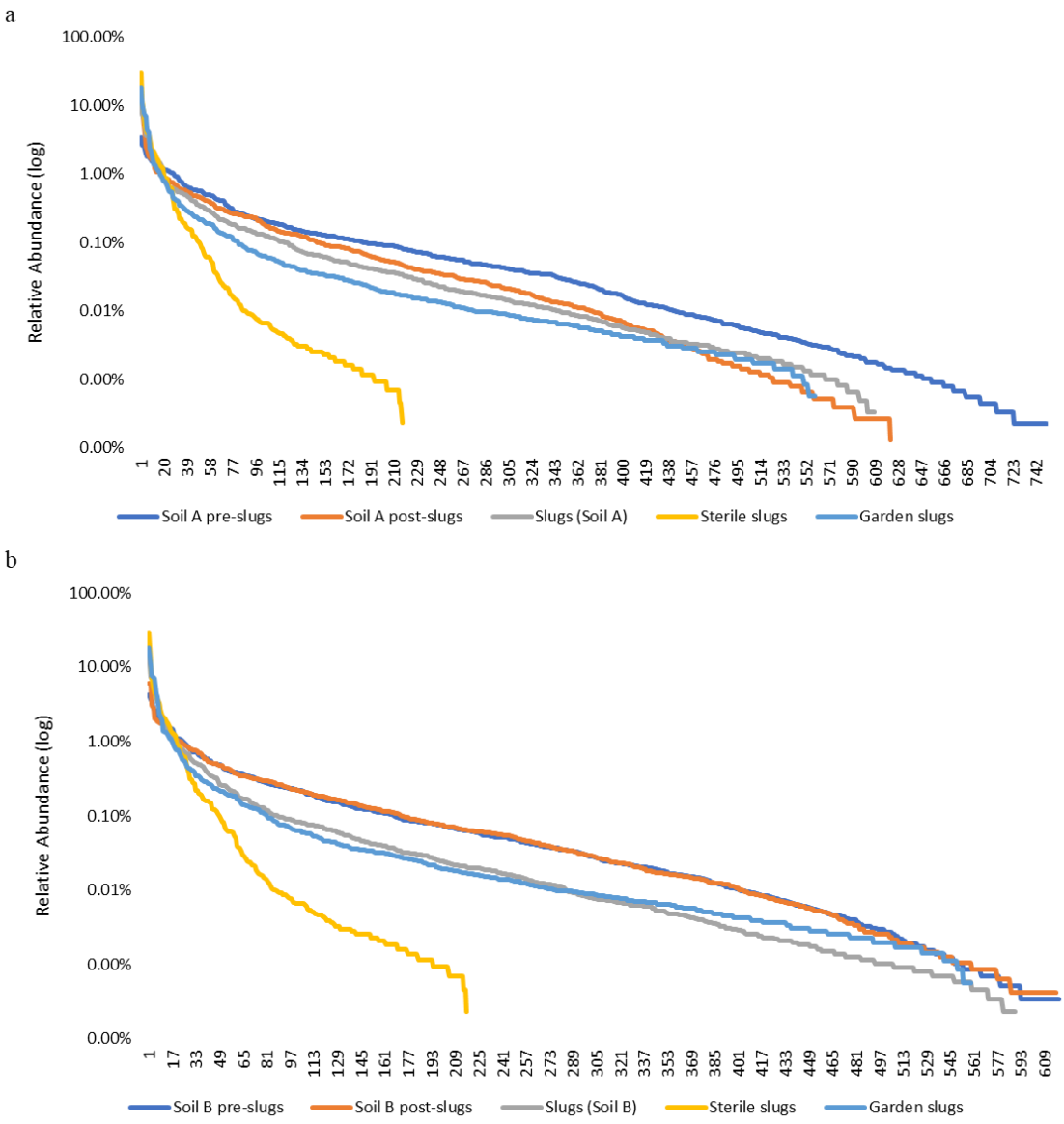


Figure 2.6 Relative Species Abundance Rank: Soil and Slug groups



CHAPTER 3

Bacterial Colonization in an Artificial Substrate in the Presence of Invasive Terrestrial Slug: A Microcosm Study

Abstract

Although invasive slugs are recognized in agricultural related studies as significant pests, almost no attention has been given to their ecological role within the soil environment. Slugs may serve as vectors for transporting microbes from place to place and many slugs considered invasive could be harboring or translocating a variety of exotic or pathogenic microorganisms within their microbiome. Evidence for how invasive bacteria, dispersed by invasive slugs, impact the indigenous soil microbial community is rare. This study is one of first to investigate the colonization of slug acquired soil bacteria dispersed into a new environment. We placed slugs that were previously exposed to soil environments into microcosms consisting of an artificial substrate. These microcosms functioned as a tool that assisted in determining the possibility for bacterial dispersal and bacterial colonization by slugs to occur. In addition, they provided valuable information regarding the transfer of soil bacteria by slugs to a novel soil environment, both from the body wall and the digestive system. In the new environment, we identified specific bacterial taxa deposited by the slugs from their previous soil environment. Adding that the slugs also significantly altered the bacterial diversity of the artificial substrate.

Introduction

The soil bacterial community contains a significant proportion of the world's biodiversity, with just one gram of soil harboring between 100 million and 1 billion bacteria. These bacteria are critical to soil health, contributing to important ecosystem processes such as soil formation, biogeochemical cycling, and perform various symbiotic roles within the environment [12–15]. Soil bacterial communities have a primary role in regulating ecosystem properties such as plant community productivity [5] and have large impacts on plant-plant interactions and consequently on plant diversity and composition [6]. The overall functioning of the soil environment is directly and indirectly impacted by soil bacterial communities [7].

In chapter 2, we focused on bacterial dispersal and influence on a natural soil bacterial community by slugs and found that slug presence significantly alters the biodiversity of the soil bacterial community. What remains to be determined is the fate of the dispersed bacteria into the soil after slug departure. Slugs are commonly found on and within soils and in agricultural environments, dispersing bacteria as they travel. Once bacteria are shed from the slug, into the environment, possible colonization and spread of the bacteria may occur (Fig. 3.1).

Bacterial dispersal promotes bacterial colonization, interfering with previously formed biodiversity, in which the newly established colonizers become dominant within the community [8]. Once an invasive species is dominant in a novel location, it competes

with native species for nutrients, food or space, triggering changes in ecosystem structure and function, and sometimes spreading pathogens and/or becoming serious ecological threats in their new habitat [2–5]. Further, introduced bacteria may be able to perform important ecosystem function previously lacking in that community, allowing them to establish in the novel location and benefit the ecosystem. Invasive bacterial species can create higher colonization pressure by interacting with each other, which, in turn, promotes establishment into the recipient community [12].

Knowledge considering the consequences of invasive slugs within their new environments, and the fate of their shed bacteria, is scarce. The colonization of invasive bacteria dispersed by slugs can cause increased competition between native species. Early detection of vectors (invasive slugs) and pathways can contribute to the reduction of colonization pressure by creating awareness, increasing targeted (slug) management [9]. The purpose of this study was to exam slugs as potential sources of colonizing exogenous bacteria into a new environment. Our main experimental goals were (1) to determine whether slugs are mediators of bacterial dispersal to a new environment, and (2) to evaluate evidence of bacterial colonization associated with an invasive slug.

Methods

Study species

Lehmannia valentiana (d'Audebard de Férussac, 1823) evolved in Europe and is invasive in California, geographically distributed throughout at least 29 counties in California

[13]. One distinguishing morphological characteristic of *L. valentiana* is the presence of two distinct lines extending down their back along the entire length of their body. These slugs are wide-scale pests, feeding on plants and decomposing wood [13].

Sample collection

Soil exposed slugs

150 slugs were collected from Louie's Nursery, as described in chapter one. Initially, slugs were raised in sterile conditions, then transferred into two separate soil bacterial communities as described in chapter two. Finally, the slugs were removed from the soil microcosms and placed into autoclaved calcined clay microcosms.

Sample processing and experimental design

Calcined clay microcosms

For assessment of bacterial dispersal and colonization into a new environment, we used autoclaved calcined clay, as described in Lebeis et al. (2014). The autoclaved calcined clay is used as surrogates for soil, due to the difficulties of removing trace microbial DNA from soil, even after intense sterilization procedures. Autoclaving, rather than irradiating, prevents bacterial colonization that occurs between competing bacteria [15]. Although complete sterility in the calcined clay was not achieved, autoclaving reduces biological activity, bacterial growth and increases time to produce growth [8–10].

There were five calcined clay microcosms, two for Soil A slugs, two for Soil B slugs and one control (Fig. 3.2). To prevent slugs from escaping, copper wire mesh was placed under lids of each microcosm. Copper causes a chemical reaction to slug skin and mucus, therefore slugs avoid moving over copper [11–13]. ~45g of calcined clay was added to each microcosm and each were kept damp with sterile water. The complete microcosms (clay and copper wire) were autoclaved prior to the addition of slugs. 14 Soil A type slugs from chapter two were divided into two calcined clay microcosms. 14 Soil type B slugs from chapter 2 were divided into two calcined clay microcosms. The control received sterile food and water but was not exposed to slugs. Samples of calcined clay were collected prior to (**Calcined pre-slug**) and subsequently after (**Calcined post-slug (Soil A)** and **Calcined post-slug (Soil B)**). In addition, calcined clays were sampled two weeks after slugs were removed. For comparison, all pre-slug calcined clay samples were grouped together per soil type. All post-slug calcined clay samples were grouped together per soil type.

All slugs were fed *ad libitum* with a sterile artificial diet based on a method by Walker (1997). The sterile diet was composed of a mixture of autoclaved carrots, bran, and nutrient agar. To maintain sterility, each microcosm was only opened in a biosafety cabinet; sterile forceps were used for feeding, as well as slug placement or removal. Slugs remained in the calcined clay replicate microcosms for two weeks. After two weeks all slugs (**Slugs (Calcined, Soil A)** and **Slugs (Calcined, Soil B)**) were removed and frozen.

Whole slug tissues were prepared for DNA extraction in 15ml conical tubes by blending each slug with sterile water using 14G, 16G, and 18G needles (in sequential order) to create a slug mixture. The amount of sterile water added was determined by slug weight. DNA extraction of the slug mixtures and calcined clay samples were performed using the MoBio PowerSoil® DNA extraction kit. An aliquot of 250µl of each slug mixture was used in lieu of the 0.25g of soil called for in the kit protocol. Slug and calcined clay DNA extracts were amplified by PCR to capture the full variety of the 16S rRNA genes within each sample. These PCR extracts were sequenced using the Illumina MiSeq system allowing for the sequencing of a ~450bp section of the 16S V3 and V4 region of the 16S rRNA gene The sequences were multiplexed using barcoded indexes and primers from the Illumina Nextera XT kit [21].

Data analysis and bioinformatics

To examine the relationships between the soil and slug bacterial communities as well as the core bacterial microbiome, sequences were processed with Quantitative Insights Into Microbial Ecology 2 (QIIME2). We removed low quality and chimeric sequences in QIIME2. Core microbiomes were determined with original code in R for each of the groupings discussed in this work, defined as the set of taxa matching at least one read in >95% of samples for that group.

Alpha diversity (within-sample) was calculated using Simpson's reciprocal index (1/D) as recommended by Jost (2006). Comparisons of alpha diversity between samples used

the Wilcoxon rank-sum test due to unequal variances between groups. Bray-Curtis dissimilarities were calculated on the whole dataset as well as separately on the non-sterile samples (negative controls and autoclaved clay (Ch. 3)) using the vegdist function in the R package (R Core Team, 2013), vegan (Oksanen et. al, 2019). Principal co-ordinates analyses (PCoAs) were conducted with the ape package (Paradis and Schliep, 2018). The extremely low diversity of the sterilized samples masked differences in community composition between the non-sterile samples, so the former are excluded from community analyses in the present work. Visualizations of data were produced using the ggpubr package in R (Kassambara, 2020).

Alpha diversity indices (between groups) were calculated to provide a further assessment of richness and diversity. We pooled the data of all treatment groups into single samples. This created uniform profiles of each treatment group, allowing expression of the data as a percentage of the total diversity of each group [15,16]. The following α diversity indices were used:

Simpson's reciprocal index (1/D) gives more importance to species abundance (dominance) and takes into account both species richness and evenness. A higher index value is indicative of a greater degree of biodiversity within the community. The Simpson's reciprocal index (1/D) was calculated as:

$$DI = \frac{N(N-1)}{\sum n(n-1)}$$

Shannon diversity index (H') is weighted towards rare species, independent of sample size and combines both species abundance and richness. The index value increases as both the richness and the evenness of the community increase. The Shannon diversity was calculated as:

$$H' = -\sum_{i=1}^{S} p_i \ln (p_i)$$

Pielou's evenness index (J') is a measure of relative evenness, whereas the evenness (equitability) of a sample implies equality in the number of individuals of species. Values range from near zero to one with higher values representing more even communities. The Pielou's evenness index was calculated as:

$$J' = \frac{H'}{\ln (S)}$$

Richness was calculated using the Margalef and Menhinick equations and assume that a relationship between S (total no. of species) and n (total no. of individuals) exists. Each were calculated as:

Margalef $R_2 = \frac{S-1}{\ln (n)}$ Menhinick $R_1 = \frac{S}{\sqrt{n}}$

Lastly, we evaluated the Relative species abundance for each group. Relative species abundance refers to how common or rare a species is relative to other species in a defined location or community. These values are reported in percent composition relative to the total number of that taxa in a sample group. Rank abundance curves display relative species abundance, measured on a log scale.

Where:

N = Total number of individuals collected

n = Number of individuals of a species

DI = Simpson Diversity Index

H = the Shannon index value

p_i = the proportion of individuals found in the *i*th species

ln = the natural logarithm

S = the number of species in the community

Results

Even after sterilization, some taxa were found in the calcined clay pre-slugs, however, this artificial substrate was dominated by few taxa, with just eight species making up 65% of the total community, and had significantly lower diversity than the post-slug calcined clay (Fig. 3.3-3.5). Beta diversity analyses using the Bray-Curtis distance algorithm illustrated the main separation of communities was by calcined clay pre-slug (not present on plot due to extremely low diversity) and calcined clay post-slug for both

soil types (Fig. 3.3a). Wilcoxon rank-sum test indicated that these differences were significant between Calcined pre-slug and Calcined post-slug (Soil A) ($p=0.0003$, Fig. 3.3b) and Calcined pre-slug and Calcined post-slug (Soil B) ($p<0.0001$, Fig. 3.3b). Differences were also significant between Slugs (Soil A) and Slugs (Calcined, Soil A) ($p=0.0049$, Fig.3.3b), however not significant between Slugs (Soil B) and Slugs (Calcined, Soil B) ($p=0.215$, Fig. 3.3b).

Alpha diversity indices between groups represented that the Calcined clay pre-slug group had lower biodiversity and evenness and higher richness than the Calcined clay post-slug groups of either soil type (Fig. 3.4a, Fig. 3.4b, Fig. 3.6). The slugs removed from the calcined clay had lower biodiversity, richness, and evenness than the calcined clay post-slugs for both soil types (Fig. 3.4a, Fig. 3.4b, Fig. 3.6).

The predominant genera of the Calcined pre-slug group (Fig. 3.5) are of the genus *Pseudomonas* (~31%), species *Bacillus flexus* (~9%), and genus *Gluconacetobacter* (~7%) and *Stenotrophomonas* (~5%). The predominant genera of the Calcined post-slug (Soil A) group (Fig. 3.5) are of the genus *Luteolibacter* (~8%), species *Flavobacterium succinicans* (~7%), genus *Dyadobacter* (~5), *Fluviicola* (~5%) and *Pedobacter* (~4%). The predominant genera of the Calcined post-slug (Soil B) group (Fig. 3.5) are of the genus Luteolibacter (~12%), species Flavobacterium succinicans (~9%), genus Pedobacter (~5%) and Dyadobacter (~4%).

Some of the most predominant genera, shared by slugs removed from the calcined clay for both soil types, include the families *Enterobacteriaceae* and *Cytophagaceae*, genus *Luteolibacter* and *Mycoplasma* and the species *Flavobacterium succinicans*.

Also reported here are the core taxa from the slugs of all treatment groups from chapters two and three (Fig. 3.7). This is a likely core microbiome for *L. valentiana*, and includes genera previously found in our chapter one core microbiome results, such as the families *Enterobacteriaceae* and *Bacillaceae*, and genus *Pseudomonas*, *Delftia*, and *Stenotrophmonas*.

Discussion and conclusions

This study investigated the bacterial community recently deposited and recolonized into in an artificial substrate by an invasive slug species *L. valentiana*. Here, in this present study, we observed invasive bacteria, shed into a novel environment by the invasive slug species *L. valentiana*, from two separate soil communities. We also found that slugs from both soil types were significantly different than the slugs removed from the calcined clay microcosms. Furthermore, we analyzed 73 invasive *L. valentiana* slugs from a variety of environments (Garden, Sterile, Soils, and Calcined clay) and uncovered a core microbiome within all slugs (Fig. 3.7), comparable to our previous study. Interestingly, most of the taxa (~70-80%) within the calcined clay slug groups (Fig. 3.5) are also found in their core microbiome (Fig. 3.7). It appears that slugs removed from the calcined clay

retained most of their core microbiome while shedding previous soil bacterial into the calcined clay (Fig. 3.3, Fig. 3.5).

Some of the taxa dispersed into the calcined clay have critical functions among other organisms. *Agrobacterium*, dispersed from both soil type slugs, has been isolated as a rhizome-associated bacterial endophyte of the invasive grass *Sorghum halepense*, which enhance its competition within soil microbial communities [24]. *Agrobacterium* are phytopathogens with a wide host range, using horizontal gene transfer to insert part of their DNA into the plant's tissue resulting in tumor production and have been isolated as the causal agent of aerial galls in rose plants [6,25–27]. In addition to being dispersed into the calcined clay, this taxon has also been identified in this study in the *L. valentiana* core microbiome as well as in the gut microbiome of another slug species, *Arion ater* [28].

One of the most abundant taxa found to colonize into the clay, and identified on the slugs removed from the clay, is *Luteolibacter*. *Luteolibacter* was also identified in chapter 1, as an indicator species for the lab-reared (sterile) slugs and in chapter 2, as the most abundant taxa in the slug groups from both soil types, as well as identified in the microcosms of both soil types. Furthermore, it is the second most abundant taxon in this invasive slug's microbiome (Fig. 3.7). *Luteolibacter* has been identified as a strong indicator in the mucus of coral under stress, abundantly on the skin of an amphibian infected with a trematode and in the presence of the antimicrobial Sulfadimethoxine, and

showed resistance to the antimicrobial triclosan [28–30]. The mucus of slugs has been verified to contain antimicrobial activity [32,33] and considering the antibiotic resistance of *Luteolibacter* and its importance in other organisms, as well as in this study, more research on *Luteolibacter* as a potential candidate of slug immune response should be completed.

The results presented here contribute to the evidence that invasive slugs disperse bacteria and those deposited bacteria successfully recolonize. In a similar soil biodiversity study, invasive earthworms exhibited an affect soil microbial biomass and diversity and created favorable soil conditions for bacterial growth and colonization [34]. Nutrients and physical conditions of soil are major determinants of bacterial colonization [15]. Successful colonization by invasive species is dependent on their ability to survive on the little resources left unconsumed by native species [35]. Limiting resources within these artificial microcosms could reflect the successfulness of each species type to establish and multiply within a new environment. Our artificial microcosms represent a simple model that is illustrative of a novel soil environment in which invasive slug bacteria can establish and colonize. From this study, we have established *L. valentiana* as a mechanism for bacterial dispersal and colonization into a new environment, expanding their position as significant pests.

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Figure 3.1 Slug bacterial dispersal mechanism

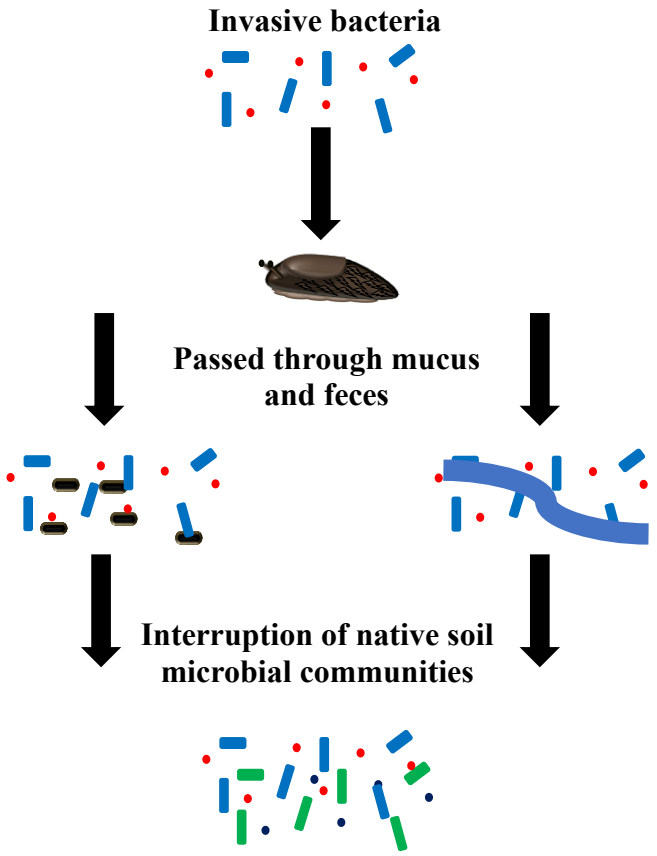
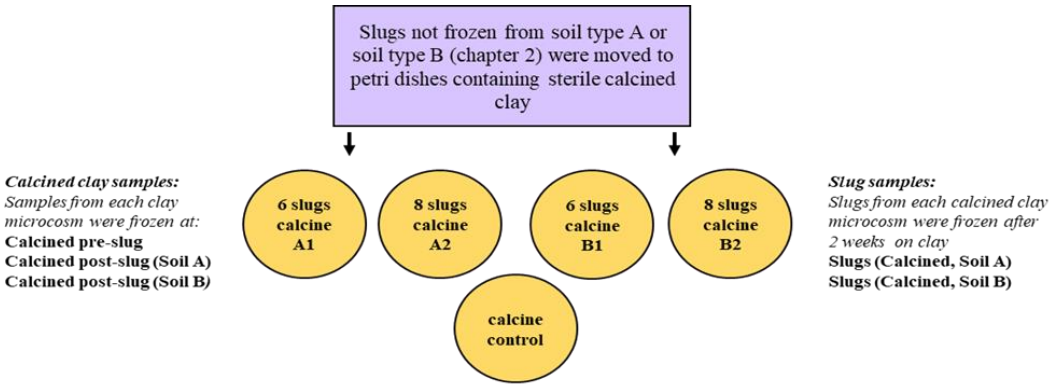


Figure 3.2 Calcined clay microcosm experimental design



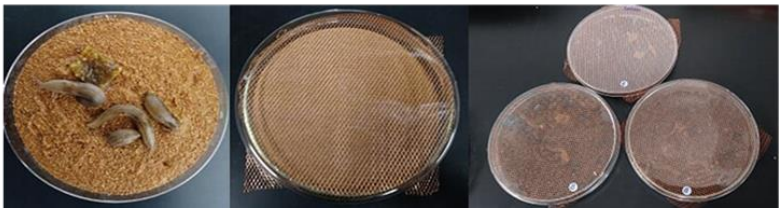
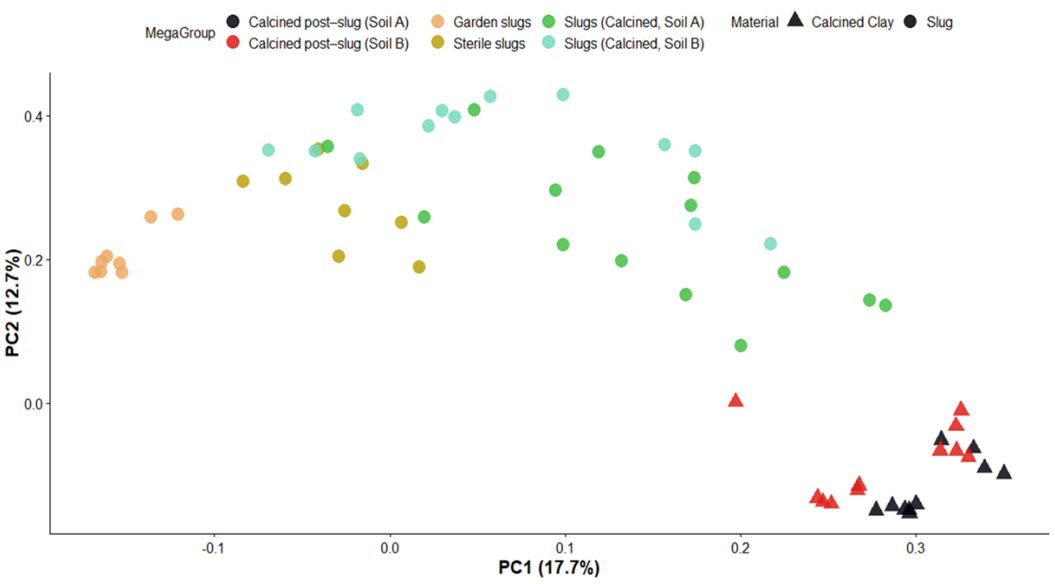


Figure 3.3 Beta and Alpha diversity of the Calcined clay and Slug group samples

(a) Principal Coordinate Analysis (PCoA) plot with Bray-Curtis dissimilarity representing calcined clay and slug microbiomes. Each data point represents and individual sample. The shape indicates sample community type, circle for slugs and triangle for calcined clay. Samples are colored according to treatment. (b) Boxplot of Simpson's reciprocal diversity (1/D) between calcined clay and slug treatment groups. ****= P<.0001. ***= P<.001, **= P<.01

a


MegaGroup
Calcined post−slug (Soil A)
Calcined post−slug (Soil B)
Garden slugs
Sterile slugs
Slugs (Calcined, Soil A)
Slugs (Calcined, Soil B)
Material
Calcined Clay
Slug
PC2 (12.7%)
PC1 (17.7%)
0.4
0.2
0.0
-0.1
0.0
0.1
0.2
0.3

b

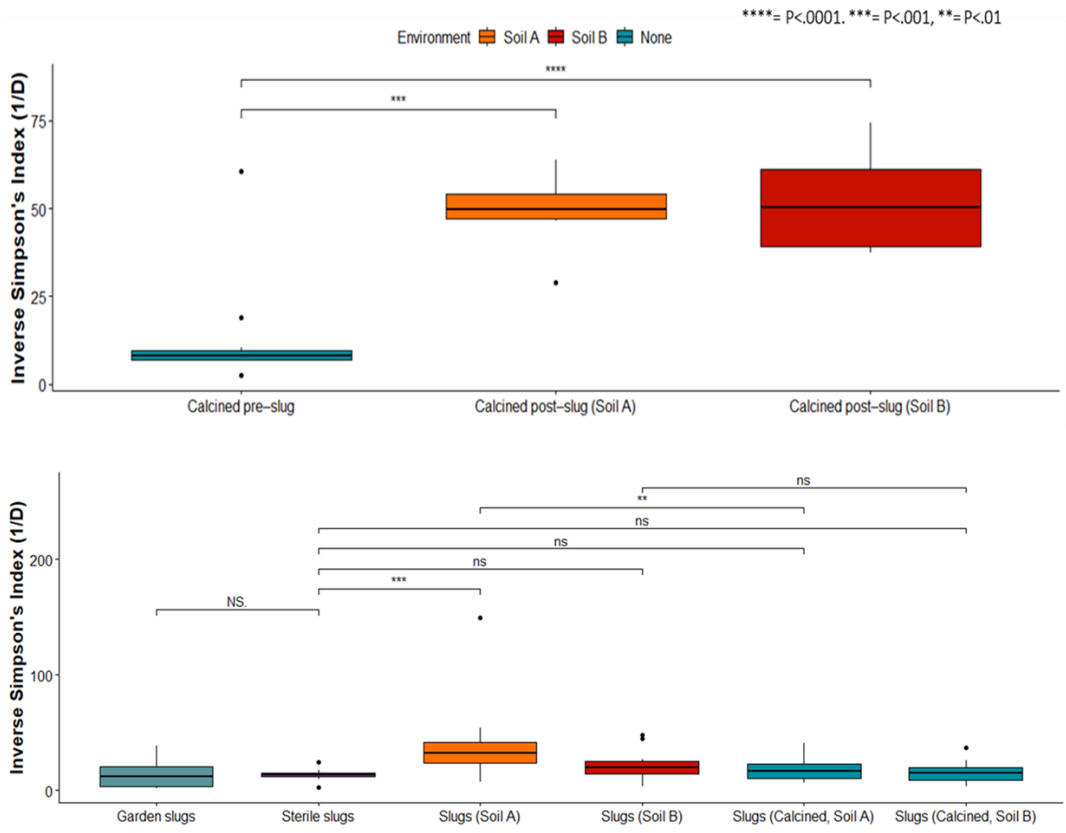

****= P<.0001. ***= P<.001, **= P<.01
Environment
Soil A
Soil B
None
Inverse Simpson's Index (1/D)
****
***
75
50
25
0
Calcined pre−slug
Calcined post−slug (Soil A)
Calcined post−slug (Soil B)
Inverse Simpson's Index (1/D)
ns
**
ns
ns
ns
***
NS.
200
100
0
Garden slugs
Sterile slugs
Slugs (Soil A)
Slugs (Soil B)
Slugs (Calcined, Soil A)
Slugs (Calcined, Soil B)

Figure 3.4 Alpha Diversity of Calcined clay and Slug groups

(a) Simpson's reciprocal index (in orange) where a higher index value indicates a greater degree of biodiversity, gives more importance to species abundance. Shannon diversity index (in blue) is weighted towards rare species where a higher index value indicates an increase in both richness and evenness. Biodiversity increased in in calcined clay microcosms after exposure to slugs from both soil types. (b) Richness and evenness between treatment groups for both Calcined clay and slugs. Calcined clay showed higher evenness and richness prior to slug placement.

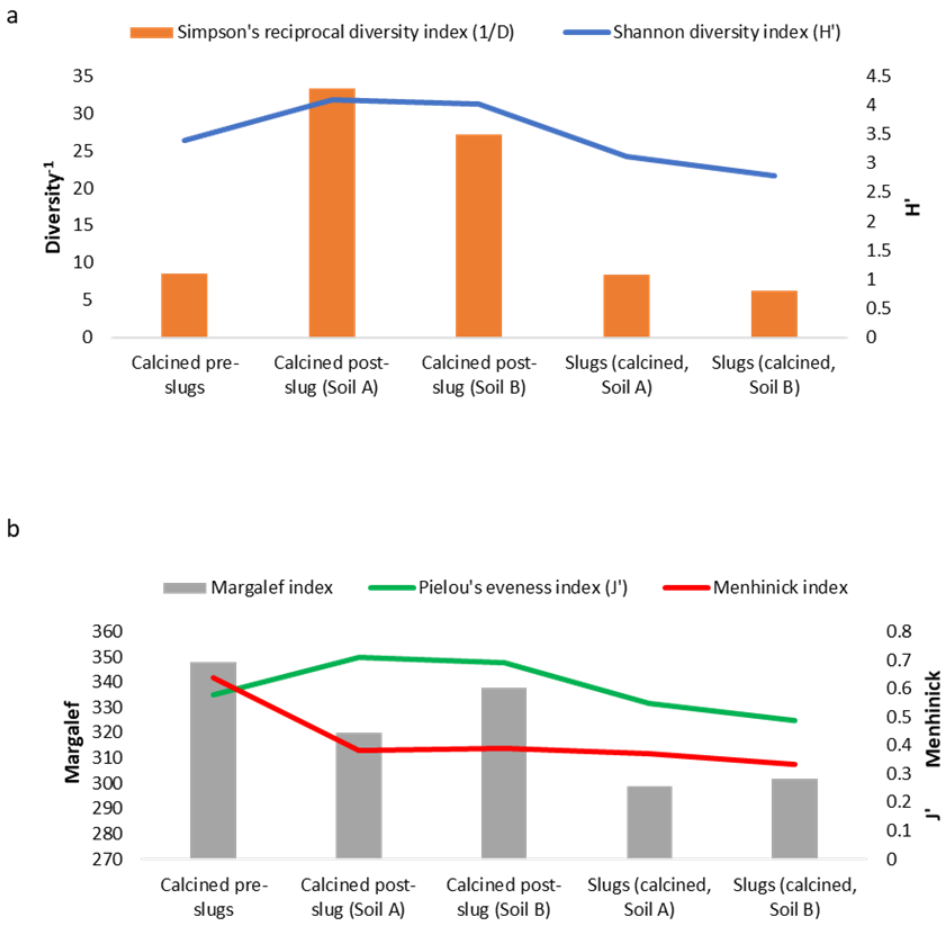


Figure 3.5 Predominant Relative Species Abundances %: Calcined Soil A and B groups

Prior to slug exposure, calcined clay community consisted of few dominant taxa. Community structure in calcined clay was altered after slug presence. Rare and unassigned taxa are not included. The x-axis represents the percent abundance for each taxon in each group.

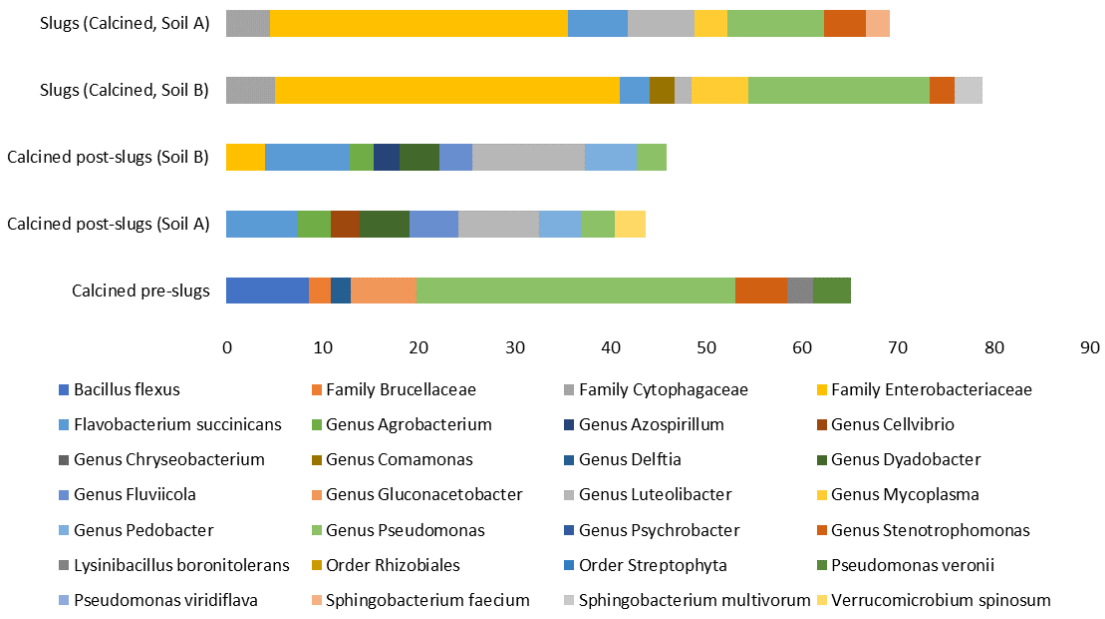


Figure 3.6 Relative Species Abundance Rank: Calcined Clay and Slug groups

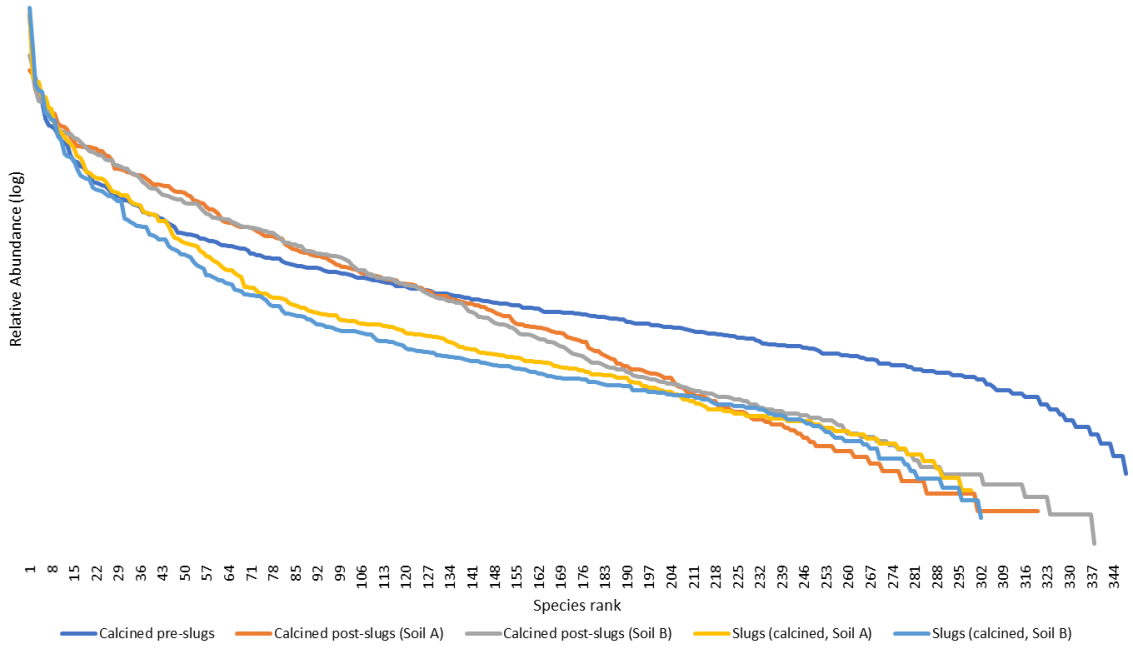


Fig. 3.7 Relative abundance of bacterial taxa present in all *Lehmannia valentiana* samples (chapter two and three).

Rare and unassigned taxa are not included. The x-axis represents the percent abundance for each taxon in each group.

0.000 10.000 20.000 30.000 40.000 50.000 60.000 70.000 80.000 90.000 100.000

- o__Enterobacteriales f__Enterobacteriaceae __ __
- o__Verrucomicrobiales f__Verrucomicrobiaceae g__Luteolibacter s__
- o__Pseudomonadales f__Pseudomonadaceae g__Pseudomonas __
- o__Mycoplasmatales f__Mycoplasmataceae g__Mycoplasma s__
- o__Rhizobiales f__Hyphomicrobiaceae g__Devosia s__
- o__Caulobacterales f__Caulobacteraceae g__Brevundimonas s__diminuta
- o__Pseudomonadales f__Pseudomonadaceae g__Pseudomonas s__
- o__Sphingobacteriales f__Sphingobacteriaceae g__Sphingobacterium s__faecium
- o__Rhizobiales f__Rhizobiaceae g__Agrobacterium s__
- o__Cytophagales f__Cytophagaceae g__ s__
- o__Xanthomonadales f__Xanthomonadaceae g__Stenotrophomonas s__
- o__Flavobacteriales f__Flavobacteriaceae g__Flavobacterium s__succinicans
- o__Rhizobiales f__Rhizobiaceae g__Shinella s__granuli
- o__Sphingobacteriales f__Sphingobacteriaceae g__Sphingobacterium s__multivorum
- o__Sphingobacteriales f__Sphingobacteriaceae g__Pedobacter s__
- o__Burkholderiales f__Comamonadaceae __ __
- o__Rhizobiales __ __ __
- o__Rhizobiales f__Rhizobiaceae __ __
- o__Burkholderiales f__Comamonadaceae g__Comamonas s__
- o__Rhizobiales f__Rhizobiaceae g__Kaistia s__
- o__Pseudomonadales f__Pseudomonadaceae g__Pseudomonas s__viridiflava
- o__Actinomycetales f__Microbacteriaceae g__Leucobacter s__
- o__Rhodobacterales f__Rhodobacteraceae g__Rhodobacter s__
- o__[Saprospirales] f__Chitinophagaceae g__Sediminibacterium s__
- o__Procabacteriales f__Procabacteriaceae g__Procabacter s__
- o__Actinomycetales f__Microbacteriaceae g__Microbacterium __
- o__Xanthomonadales f__Xanthomonadaceae g__Stenotrophomonas __
- o__Enterobacteriales f__Enterobacteriaceae g__Gluconacetobacter s__
- o__Burkholderiales f__Comamonadaceae g__Delftia s__
- o__Flavobacteriales f__[Weeksellaceae] g__Chryseobacterium s__
- o__Pseudomonadales f__Pseudomonadaceae g__Pseudomonas s__veronii
- o__Rhizobiales f__Brucellaceae __ __
- o__Burkholderiales f__Alcaligenaceae g__Achromobacter s__
- o__Actinomycetales f__Microbacteriaceae __ __
- o__[Saprospirales] f__Chitinophagaceae g__ s__
- o__Actinomycetales f__Mycobacteriaceae g__Mycobacterium s__
- o__Burkholderiales f__Oxalobacteraceae g__Ralstonia s__
- o__Bacillales f__Bacillaceae __ __
- o__Burkholderiales f__Oxalobacteraceae __ __
- o__Burkholderiales f__Alcaligenaceae g__Pigmentiphaga s__
- o__Rhizobiales f__Brucellaceae g__Pseudochrobactrum s__
- o__Rhizobiales f__Rhizobiaceae g__Shinella __
- o__Lactobacillales f__Carnobacteriaceae g__Carnobacterium s__
- o__iii1-15 f__ g__ s__
- o__Burkholderiales f__Comamonadaceae g__Curvibacter s__
- o__Bacillales f__Bacillaceae g__Anaerobacillus s__
- o__Alteromonadales f__Alteromonadaceae g__Cellvibrio s__
- o__Rhizobiales f__Rhizobiaceae g__Rhizobium s__
- o__Flavobacteriales f__Cryomorphaceae g__Fluviicola s__
- o__Burkholderiales f__Alcaligenaceae __ __
- o__Burkholderiales f__Alcaligenaceae g__ s__