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Ryan R. Busby, David B. Ringelberg, and Carina M. Jung

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Cover Photo: Closeup of *Lespedeza cuneata*; online image source: Invasive Species and Ecosystem Health (https://www.invasive.org/browse/subinfo.cfm?sub=3033).

Flavonoids Differentially Influence Rhizosphere Bacterial Communities from Native and Introduced Lespedeza Roots

Ryan R. Busby

Construction Engineering Research Laboratory
U.S. Army Engineer Research and Development Center
2902 Newmark Drive
Champaign, IL 61822

David B. Ringelberg

Cold Regions Research and Engineering Laboratory
U.S. Army Engineer Research and Development Center
72 Lyme Road
Hanover, NH 03755

Carina M. Jung

Environmental Laboratory
U.S. Army Engineer Research and Development Center
3909 Halls Ferry Road
Vicksburg, MS 39180

Final report

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103 Army Pentagon
Washington, DC 20314-1000

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Abstract

Military training can create disturbances that facilitate invasive plant establishment. Introduced plant species' interactions with soil microbial communities through root exudates often aid plants in colonizing new locales. This study tested the hypothesis that rhizosphere bacterial communities associated with the native legume, *Lespedeza virginica*, and the non-native legume, *Lespedeza cuneata*, respond differently to plant-exuded molecules. Bacterial communities collected from coexisting populations of the two species were grown in the presence of four separate flavonoids at four concentrations. Following 96 hours of incubation, DNA was recovered from the enrichment cultures and analyzed using next-generation sequencing. In cultures receiving a flavonoid, *L. virginica* enrichments were characterized by a greater operational taxonomic unit (OTU) richness and exhibited a dose-response relationship to one of the flavonoids. The *L. cuneata* enrichments were characterized by a decreased OTU richness. Bacterial genera containing known pathogenic taxa occurred at a significantly greater relative frequency in *L. cuneata* enrichments than in the *L. virginica* enrichments. However, calculation of a species diversity index indicated greater OTU diversity in the *L. cuneata* enrichments across all four flavonoid treatments. These results indicate the rhizosphere microbial communities of co-existing *L. cuneata* and *L. virginica* legumes exhibit different responses when exposed to plant communication molecules.

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Preface

This work was conducted for the Assistant Secretary of the Army (Acquisition, Logistics and Technology (ASA(ALT)) under the U.S. Army Basic Research Program of PE 61102, BT25, Task 01, "Environmental Science Basic Research." The specific project covered by this report is Project 14-05, "Molecular Impact of Invasive Species Introduction on Military Lands." The technical monitor was Mr. Alan Anderson, CEERD-CZT, Technical Director for Sustainable Ranges and Lands.

The work was directed by the Ecological Processes Branch of the Installations Division (CEERD-CNN) at the U.S. Army Engineer Research and Development Center, Construction Engineering Research Laboratory (ERDC-CERL). At the time of publication, Dr. Chris Rewerts was Chief, CEERD-CNN, and Mr. Donald J. Hicks was Chief, CEERD-CN. The Deputy Director of ERDC-CERL was Dr. Kirankumar Topudurti, and the Director was Dr. Lance D. Hansen.

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1 Introduction

1.1 Background

Introduced plant species' interactions with soil microbial communities often aid in the plants' ability to effectively colonize new locales (Callaway et al. 2004; Reinhart and Callaway 2006). These interactions include suppression of native mutualists (Vogelsang and Bever 2009), advantageous associations with native mutualists (Callaway et al. 2011; Hu et al. 2014), pathogen avoidance (Callaway et al. 2011), and facilitation of native plant pathogens (Eppinga et al. 2006). These interactions have been shown to rely on chemical signals exuded from plant roots (Nolan et al. 2014; Yuan et al. 2014). While most of these signaling chemicals have not been identified, introduced plants appear to produce greater concentrations, numbers, and species-specific secondary metabolites (Kim and Lee 2011; Macel et al. 2014). Some of the compounds identified and attributed to specific microbial responses include: benzyl- and allyl-glucosinolate, root exudates of garlic mustard, Alliaria petiolata, which impact arbuscular mycorrhizal fungi communities; the glucoside alliarinoside, which alters bacterial composition (Lankau 2011); and the flavonoid catechin, a root exudate of Centaurea stoebe (spotted knapweed) which has variable effects on bacterial communities (Pollock et al. 2011).

Plant root exudates contain a variety of chemical constituents that perform a variety of functions for the plant (Uren 2001). Individual exudate chemicals, particularly phenolic compounds, can have significant influences on the soil microbial community by selecting for different bacteria, stimulating and suppressing bacteria, and altering species richness and composition (Badri et al. 2013). Included in these compounds are the secondary metabolite flavonoids. Flavonoids are polyphenolic compounds that are ubiquitous in plants and plant tissues. Because of the variety of modifications possible to the carbon skeleton, approximately 9,000 different flavonoid molecules have been identified from plants (Ferrer et al. 2008). Flavonoids are involved in a number of important plant functions, including roles in providing adaptation to abiotic stresses and pigmentation. In soils specifically, flavonoids function to protect plants against herbivory and bacterial pathogens, aid in competitive interactions with neighboring plants, aid in communication with soil microbes, and aid in increasing nutrient availability (Shaw et al. 2006; Weston and Mathesius 2013). One of

the primary functions of flavonoids in soils is to attract symbiotic microorganisms, including rhizobia by legumes (Aoki et al. 2000), actinomycetes by actinorhizal plants (Benoit and Berry 1997), and arbuscular mycorrhizal fungi (Siqueira et al. 1991).

Four flavonoids that have been isolated from *Lespedeza* spp. include kaempferol, orientin, quercetin, and quercitrin. Kaempferol and quercetin perform a variety of functions in rhizospheres, including induction of Nod genes, antibacterial activity, iron chelation, allelopathy, stimulation of mycorrhizal colonization, and nematode repellency (Ulanowska et al. 2007; Cesco et al. 2012; Weston and Mathesius 2013). Both kaempferol and quercetin are effective at disrupting quorum sensing and biofilm formation in bacterial isolates (Vikram et al. 2010). Quercetin further stimulates hyphal branching in mycorrhizal fungi and exhibits antifungal activity, while kaempferol inhibits germination of pathogenic fungal spores (Hartwig et al. 1991; Cesco et al. 2012). Isolates of *Rhizobium* and *Pseudomonas* have been observed to degrade quercetin and even utilize it as a sole carbon source (Shaw et al. 2006). Kaempferol appears to resist microbial degradation, persisting for days after clover was applied to soil as green manure and long after all other flavonoids (including quercetin) had been degraded (Carlsen et al. 2012). Much less research has been conducted on the functions of orientin and quercitrin in the plant rhizosphere. However, research investigating their roles in medicine indicates that orientin possesses antifungal properties and provides protection against radiation (Morris 2008), while quercitrin exhibits antioxidant activity (Panat et al. 2015).

Lespedeza cuneata is an introduced legume from eastern Asia (Peterson et al. 2003) and its current range in North America overlaps with numerous native *Lespedeza* species, including the widespread *L. virginica* (Busby et al. 2016). *L. cuneata* has a high polyphenol content and is associated with lower nematode densities in soils (Kardol et al. 2010). *L. cuneata* forms extensive stands (Yannarell et al. 2011) and benefits from greater rhizobial associations than does the native species *L. virginica* (Hu et al. 2014). Further, nodule-associating bacteria differ significantly in taxonomy between *L. cuneata* and *L. virginica* when coexisting in a single stand (Busby et al. 2016). The different interaction with the soil microbiological community favors establishment and growth of *L. cuneata*, which can lead to its dominance in only a few growing seasons (Coykendall and Houseman 2014).

1.2 Objective

The goal of this research was to test the hypothesis that rhizosphere bacterial communities associating with the native *Lespedeza virginica* and non-native *Lespedeza cuneata* respond differently to plant secondary metabolite communication molecules.

1.3 Approach

Lespedeza virginica and *Lespedeza cuneata* rhizosphere samples were collected from coexisting populations of the two species. Bacteria were isolated and stored for flavonoid exposure. Four flavonoids (kaempferol, orientin, quercetin, and quercitrin) were selected to represent both common and unique constituents in *Lespedeza* spp. Each of the four flavonoids were added separately to culture wells at concentrations of 0, 50, 100 and 200 μM, replicated 3 times each. Each well then received 2 μL of *L. cuneata* or *L. virginica* rhizosphere bacterial culture. Well plates were incubated at 30°C for 96 h. Following incubation, 100 μL of culture was recovered from each well. In addition, 100 μL aliquots of the original, time zero, non-incubated bacterial cultures were recovered in a similar fashion. Recovered bacteria were sequenced using next generation sequencing with the Illumina MiSeq platform. Effects of plant, flavonoid, and flavonoid concentrations on OTU richness and diversity were analyzed, along with relative abundances of commonly occurring bacterial orders. Effects on OTU composition were analyzed using multiple response permutation procedures (MRPP). Details of the materials and methods used are in Chapter 2.

2 Materials and Methods

2.1 Sample collection

Lespedeza virginica and *Lespedeza cuneata* rhizosphere samples were collected from coexisting populations of the two species in an open forest site at Fort Leonard Wood, Missouri (Busby et al. 2016). Root balls, approximately 20 x 20 cm centered on the stem, were excavated in June and transported intact to the laboratory. Bulk soil was carefully removed from the roots by hand shaking. Roots were then suspended in 50 ml of filter-sterilized 0.1% sodium pyrophosphate in a 50 ml screw-cap Falcon tube and shaken for 60 min at 150 rpm and 4°C. Tubes were allowed to stand for 2 min to allow large soil particles to settle out. The supernatant was then recovered using a sterile pipet, transferred to sterile 50 mL Falcon tubes, and centrifuged at 6,000 x g for 7 min to pellet the recovered cells. The pelleted cells were washed once with DPBS (Dulbecco's phosphate-buffered saline), re-pelleted at 6,000 x g for 7 min, then resuspended in 2.5 ml of PBS (phosphate-buffered saline) containing 5% dimethyl sulfoxide. Each cell suspension was divided into 0.5 mL aliquots in cryogenic vials, and frozen at -80°C.

2.2 Experimental design

Growth media consisted of 5 g each of glucose, peptone, tryptone, and yeast extract, and 0.6 g $MgSO4{\cdot}7H_2O$, and 0.06 g $CaCl_2$ in 1 L deionized water. The pH of the media was adjusted to between 6.8 and 7.0, and then autoclaved. Autoclaved growth media (100 µL) was added to each utilized well of a 96-well plate. Four flavonoids were selected to represent both common and unique constituents in *Lespedeza* spp. Because *L. virginica* phytochemistry has not been elucidated, a closely related species (*L. capitata*) with overlapping geographical occurrence (including occurrences near the sampling location) was used as a surrogate in terms of flavonoid production. Flavonoids occurring in *L. capitata* include kaempferol, quercetin, and orientin (Wagner et al. 1972; Bisby et al. 1994), while flavonoids occurring in *L. cuneata* include kaempferol, quercetin, and quercitrin (Bisby et al. 1994; Yoo et al. 2015; Min and Shim 2016). Quercitrin is believed to be unique to *L. cuneata*, orientin to be unique to *L. capitata,* and kaempferol and quercetin are believed to be common to both species.

Each of the four flavonoids were added separately to the respective culture wells at concentrations of 0, 50, 100, and 200 µM, replicated 3 times each. Each well then received 2 µL of *L. cuneata* or *L. virginica* rhizosphere bacterial culture. Unused wells in each plate were filled with 100 µL of ultrapure water to increase humidity and prevent culture drying. Well plates were incubated at 30°C for 96 hr. Following incubation, 100 µL of culture was recovered from each well by repeated agitation with a sterile pipette tip to dislodge any biofilm that formed on well sides. In addition, 100 µL aliquots of the original, time zero, non-incubated bacterial cultures were recovered in a similar fashion. All recovered aliquots were centrifuged at 10,000 rpm for 5 min, the supernatant was decanted, and remaining cell pellets were frozen at -80◦C for sequencing.

2.3 Next-generation sequencing

A semi-quantitative survey method 16S rDNA community analysis via next-generation sequencing with the Illumina MiSeq platform (Illumina Inc., San Diego, CA) was performed. DNA was extracted with Qiagen's Power Soil DNA Isolation Kit (Valencia, CA). Resultant DNA was amplified with uniquely barcoded primers specifically designed for 16S rRNA sequencing with the Illumina MiSeq from the 515-806 base pair (bp) region of the 16S gene (*E. coli* numbering) (Caporaso et al. 2012). Amplicons were normalized and combined to 15 pmol and further combined with 7.5% PhiX control, according to Illumina MiSeq instructions. The sample was added to a 300-cycle MiSeq kit for sequencing. Relative abundances of microbial community composition between samples were compared using QIIME software (Caporaso et al. 2011, 2012). Reported operational taxonomic units (OTUs) comprised at least 0.5% of the total relative abundance (0%–100%), based on the total number of reads for a given sample.

2.4 Data analysis

Effects of plant, flavonoid, and flavonoid concentration on OTU richness were analyzed using PROC GLM in SAS software (SAS Version 9.2, SAS Institute, Cary, North Carolina). Relative abundances of individual bacterial orders with relative frequencies greater than 80% were also compared using PROC GLM in SAS. Significant differences were analyzed using Tukey's Honest Significant Difference (HSD) test at an alpha of 0.05. Simpson's Index of Diversity was calculated for each experimental unit by QIIME, using the following equation:

$$1 - \Sigma(n/N)2 \qquad \text{(Eq. 1)}$$

where:

n = the total number of individuals for a species
N = the total number of individuals across all species.

Composition changes in OTUs were analyzed using MRPP in PC ORD Version 5 software (MjM Software Design, Gleneden Beach, Oregon) at the order level. Because MRPP only allows one categorical variable to be compared, concentrations were combined to observe relationships between flavonoids independent of concentration, and individual concentrations were analyzed separately to compare concentration effects within flavonoids. OTU composition was evaluated at the order level, using orders that comprised at least 5% of cells, to minimize the influence of rare species.

3 Results

A total of 356 OTUs (355 bacteria and 1 archaea), 266 in the *L. cuneata* samples and 304 in the *L. virginica* samples, were identified in the initial rhizosphere extracts and in the post-experimental cultures. A total of 87 OTUs were found to be unique to *L. cuneata* and 51 to be unique to *L. virginica*. The initial, non-incubated, time zero rhizosphere extracts were taken from frozen stocks the day the flavonoid incubations were begun. The derived Simpson diversity index, expressed as a mean value, was found to be greater in these initial rhizosphere extracts (0.806 in *L. virginica* and 0.906 in *L. cuneata*) when compared to the 96-hour post-experimental cultures (0.56 in *L. virginica* and 0.707 in *L. cuneata*). The initial time zero extracts were also high in OTU richness, with mean richness values calculated at 217.5 in the *L. cuneata* isolate and 146.0 in the *L. virginica* isolate. These OTU richness values declined sharply over the time course of the experiment. The 96-hour control cultures exhibited mean OTU richness values of only 30.0 and 27.0 for *L. cuneata* and *L. virginica*, respectively. A number of bacteria orders in both plant species were observed to fall below detection limits during the laboratory incubation period (Table 1).

The three most abundant bacterial orders detected—Pseudomonadales, Enterobacteriales, and Xanthomonadales (all in the Gammaproteobacteria class)—comprised 96.3% of *L. cuneata* and 92.8% of *L. virginica* sequence reads when expressed as mean relative abundances (Figure 1). The relative abundance of these Gammaproteobacteria increased from a total of 54.9% in the initial *L. cuneata* rhizosphere extract to 99.8% in the post-experimental cultures and from 40.5% in the initial *L. virginica* extracts to 96.5% in the post-experimental cultures (Table 1).

Table 1. Mean relative abundance of bacterial taxa identified in initial cultures of both Lespedeza cuneata and L. virginica and in post-experiment 96 hr controls.

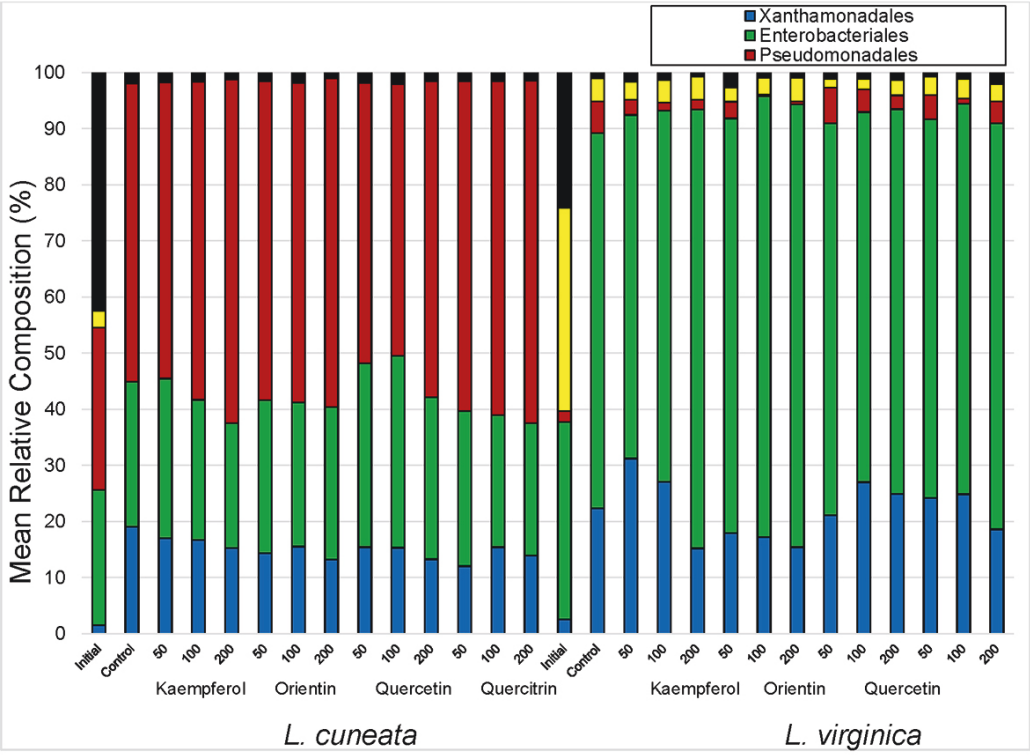
	Rhizosphere Source Plant			
	Lespedeza cuneata		*Lespedeza virginica*	
	Relative Abundances (%)		Relative Abundances (%)	
Order	Initial	Control	Initial	Control
Acidobacteriales	1.857	0	0.5718	0
Actinomycetales	0.9777	0	3.06	0
Alteromonadales	0.0903	0.662	0.0903	0.0135

	Rhizosphere Source Plant			
	Lespedeza cuneata		*Lespedeza virginica*	
	Relative Abundances (%)		Relative Abundances (%)	
Order	Initial	Control	Initial	Control
Chloroflexi TK10	0.6856	0	0.0012	0
Bacillales	5.905	0.0007	2.25	0.1157
Unclassified Betaproteobacteria	0.055	0	0.0255	0.0025
Burkholderiales	3.055	0.0027	36.2	4.083
Caulobacterales	1.312	0	0.64	0
Chromatiales	0.0005	0.002	0	0.002
Chthoniobacterales	1.896	0	0.3052	0
Enterobacteriales	24.14	25.79	35.16	66.92
Unclassified Gammaproteobacteria	0.0633	1.013	0.0109	0.9148
Gemmatales	1.037	0	0.365	0
Methylophilales	0.0019	0	0.102	0.0132
Nostocales	3.005	0.0002	4.471	0
Oceanospirillales	0.0925	0.0074	0.6625	0.0008
Pseudomonadales	28.9	53.18	1.97	5.608
Rhizobiales	7.384	0.0461	2.362	0
Rhodospirillales	2.278	0	0.46	0
Solibacterales	4.863	0.0003	0.3853	0
Solirubrobacterales	0.996	0	0.089	0
Sphingomonadales	1.279	0	1.32	0
Thermogemmatisporales	0.921	0	0.0072	0
Candidate Division WPS-2	1.316	0.0003	0.033	0
Xanthomonadales	1.48	19.08	2.545	22.325

The mean relative abundance of Burkholderiales (class Betaproteobacteria) was found to be more than an order of magnitude greater in the initial *L. virginica* cultures compared to *L. cuneata* cultures (Figure 1), but this mean relative abundance dropped significantly in all cultures post-experimental incubation. Mean relative abundances of Enterobacteriales and Xanthomonadales were also found to be greater in the *L. virginica* cultures (Figure 1). The mean relative abundance of Enterobacteriales nearly doubled in the *L. virginica* cultures, post-experimental incubation, whereas Enterobacteriales abundance remained almost unchanged in the *L. cuneata* cultures. In contrast, the mean relative abundance of Xan-

thomonadales increased significantly in both the *L. virginica* and *L. cuneata* cultures post-experimental incubation. The mean relative abundance of Pseudomonadales was detected at a greater percentage in the *L. cuneata* time zero initial extracts (Figure 1). The relative abundance of the Pseudomonadales more than doubled in the *L. cuneata* cultures post-experimental incubation and remained relatively unchanged in the *L. virginica* cultures.

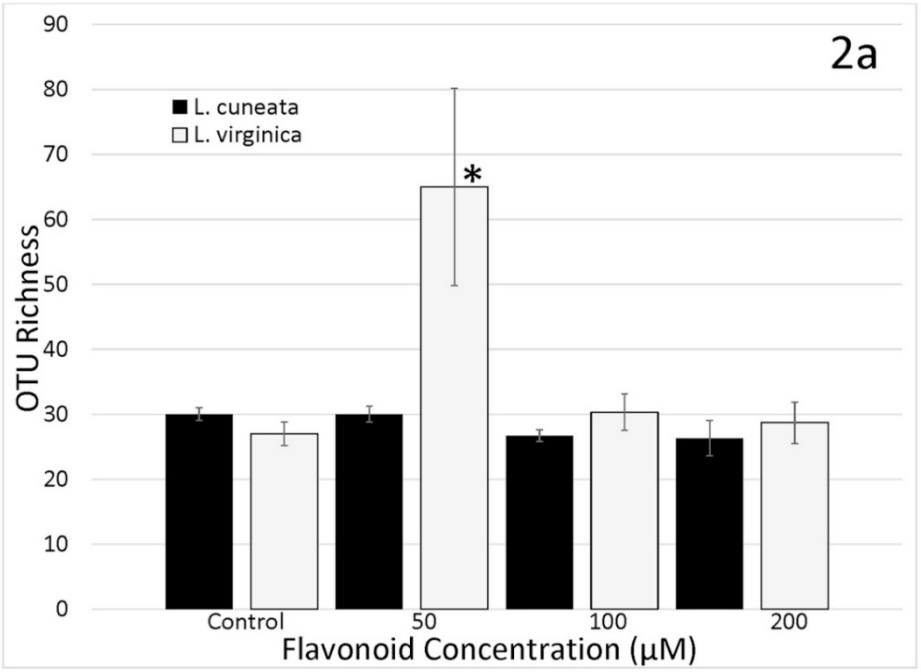
Figure 1. Mean relative abundance of the most prevalent bacterial orders by rhizosphere source, initial rhizosphere extra, flavonoid, and concentration.

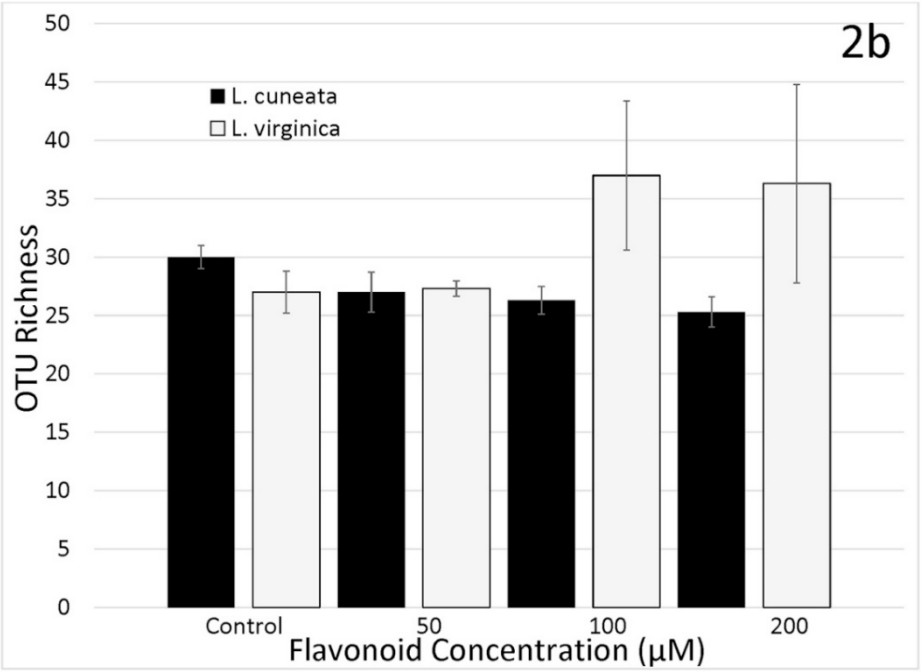


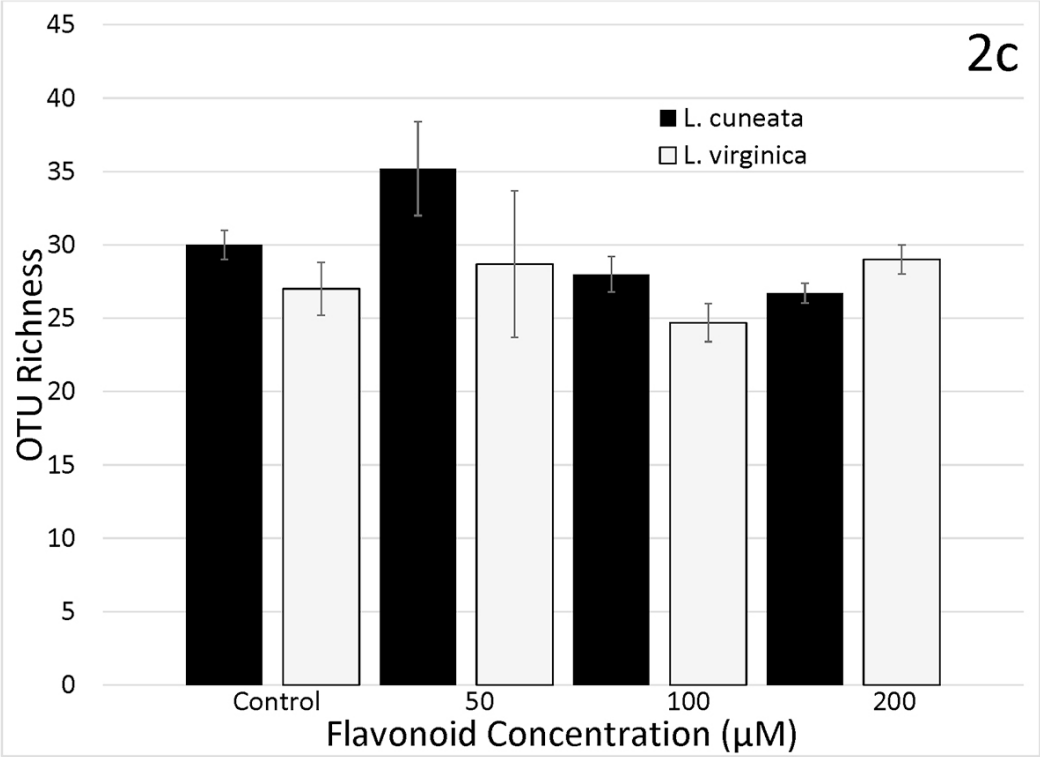
Flavonoids had a significant influence on OTU richness in the different rhizosphere bacterial communities, exhibiting a plant x flavonoid x concentration interaction ($F = 10.17$, $p < 0.001$). Mean OTU richness in the *L. cuneata* cultures decreased 5% relative to the control, but increased 41% in the *L. virginica* cultures. OTU richness in the *L. cuneata*-associated bacterial communities responded negatively to kaempferol and orientin (Figure 2a and Figure 2b), exhibited a dose-specific response to quercetin (Figure 2c), and exhibited no response to quercitrin (Figure 2d). Likewise, OTU richness in the *L. virginica*-associated bacterial community exhibited dose-specific responses to kaempferol and orientin (Figure 2a and Figure

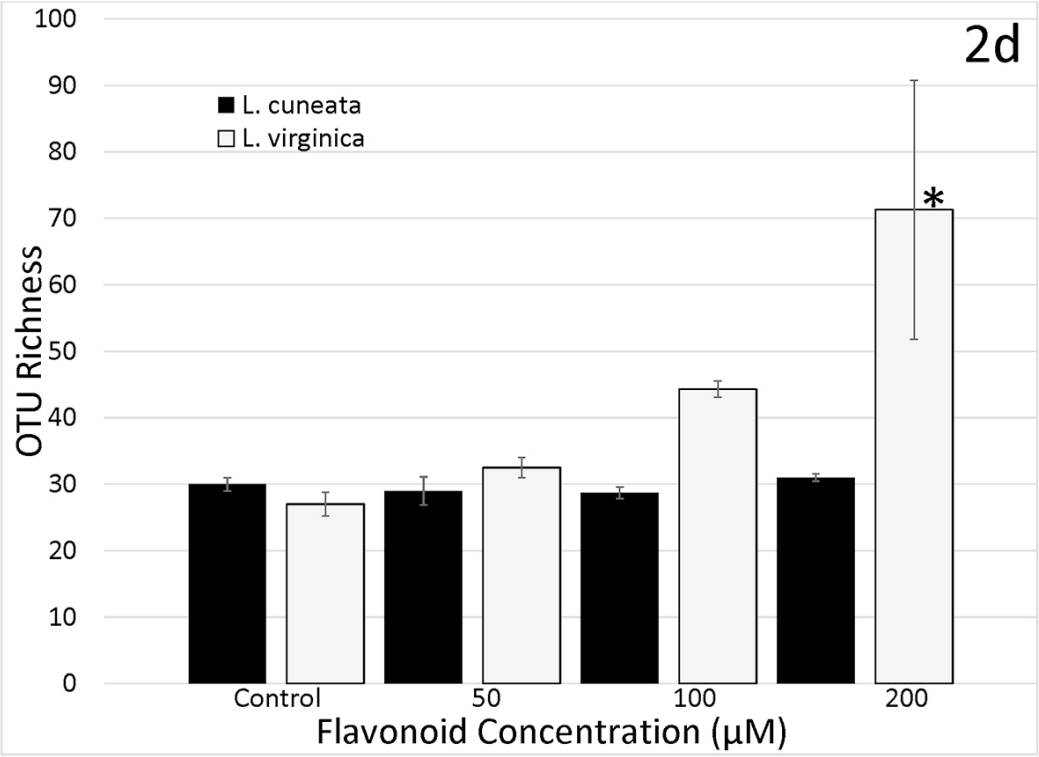
2b), exhibited no response to quercetin (Figure 2c), and exhibited a strong positive response to quercitrin (Figure 2d).

Figure 2. Effects of flavonoids (a) kaempferol, (b) orientin, (c) quercetin, (d) quercitrin at varying concentrations on mean bacterial OTU richness in rhizosphere bacterial communities of *L. cuneata* and *L. virginica*. Bars represent standard errors.






2c
L. cuneata
L. virginica
OTU Richness
0
5
10
15
20
25
30
35
40
45
Control
50
100
200
Flavonoid Concentration (µM)


2d
L. cuneata
L. virginica
OTU Richness
0
10
20
30
40
50
60
70
80
90
100
*
Control
50
100
200
Flavonoid Concentration (µM)

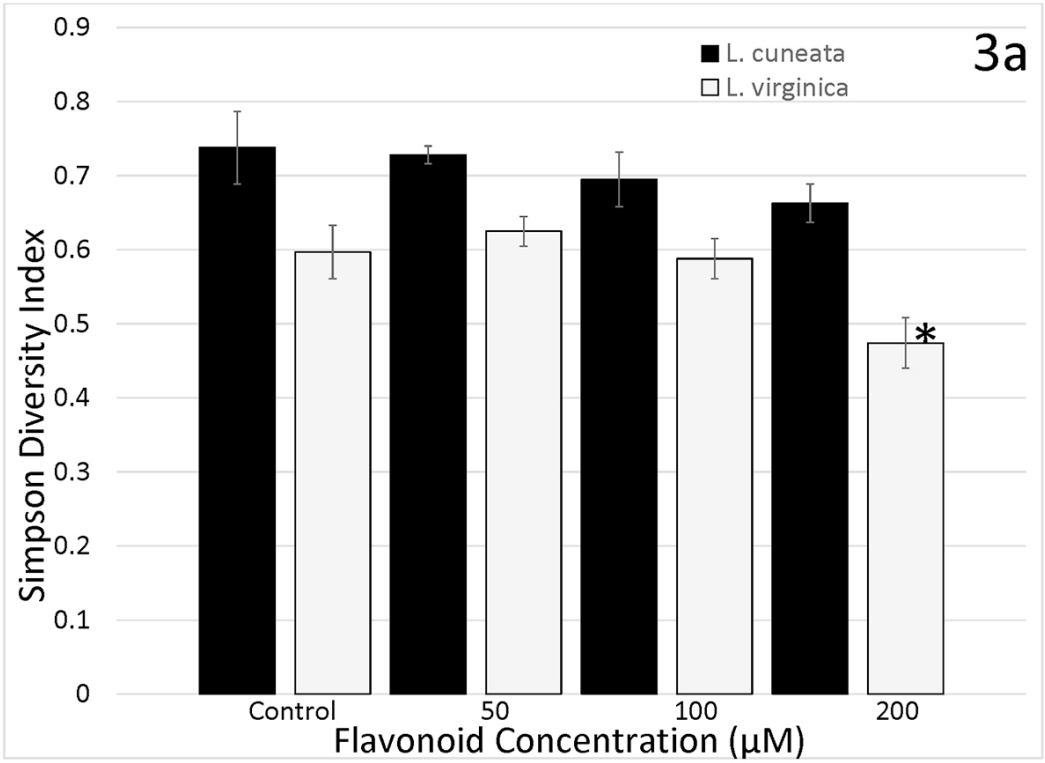
Plant ($F = 132.6$, $p < 0.001$) and flavonoid type ($F = 4.57$, $p < 0.01$) had a significant effect on Simpson diversity in the post-incubation cultures (Figure 3a–d). Diversity decreased with increasing kaempferol (Figure 3a), orientin (Figure 3b), and quercitrin (Figure 3c) concentrations for both plant rhizosphere sources. Quercetin concentrations had little effect on diversity from either rhizosphere source (Figure 3d). Analyses revealed that both plant ($F = 31.82$, $p < 0.0001$) and flavonoid type ($F = 3.17$, $p = 0.03$) affected the relative frequency of Xanthomonadales (Table 2). Within L. cuneata treatments, Burkholderiales exhibited a flavonoid x concentration effect ($F = 3.71$, $p < 0.01$). Flavonoid type alone was found to have a significant effect on Enterobacteriales ($F = 4.72$, $p < 0.01$) and Burkholderiales ($F = 4.66$, $p < 0.01$), but only in the *L. virginica* cultures (Table 2).

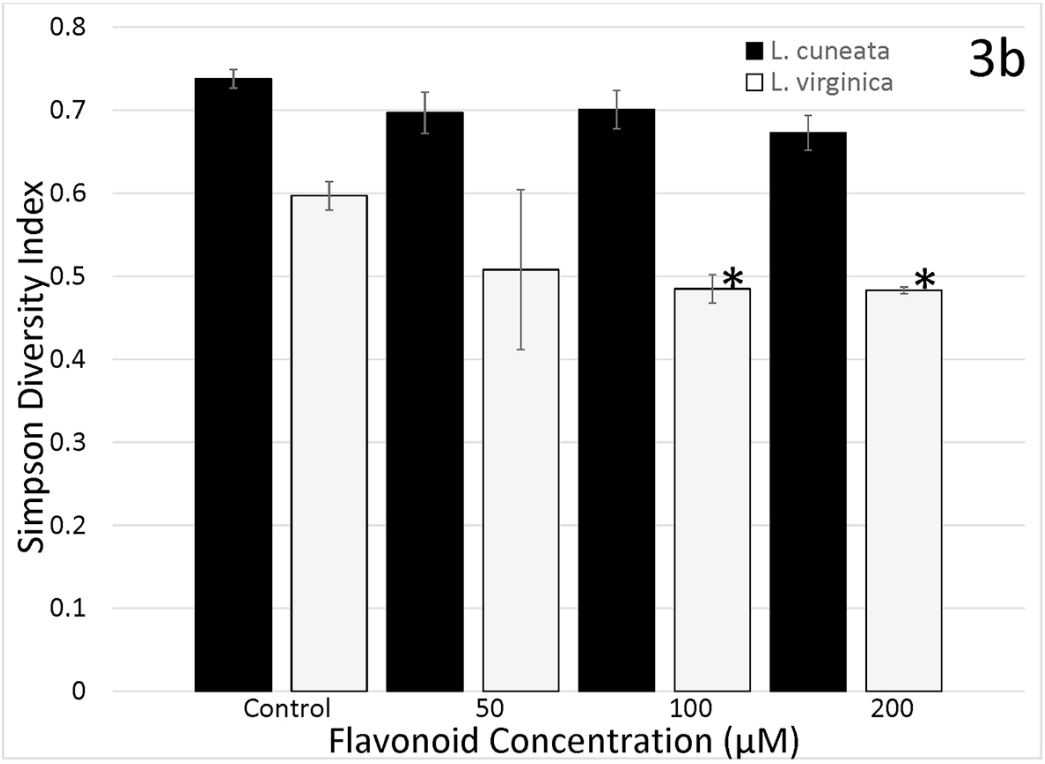
Table 2. Effects of flavonoids on mean relative frequencies of common bacterial orders. Standard errors are in parentheses.

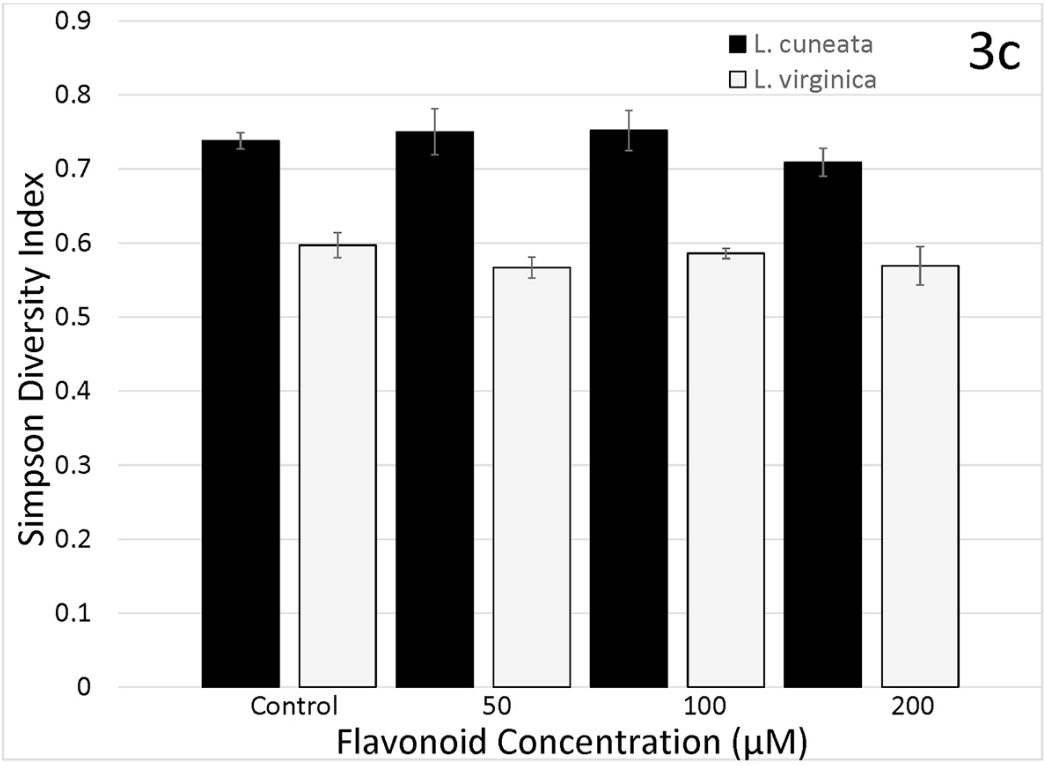
	Rhizosphere Source		
	L. virginica		*Lespedeza* combined
Flavonoid	Enterobacteriales	Burkholderiales	Xanthomonadales
None	66.92 (1.16)	4.08 (1.87)	20.7 (1.37)
Kaempferol	68.55 (2.98)	3.78 (0.28)	20.39 (1.77)
Orientin	77.13 (2.61)	3.24 (0.34)	15.61 (1.31)
Quercetin	68.15 (1.32)	2.05 (0.23)	19.49 (1.57)
Quercitrin	70.1 (1.6)	3.32 (0.23)	17.81 (1.51)

Flavonoids also had a significant effect on community composition (Table 3). Quercitrin treatments with *L. cuneata* cultures resulted in a unique bacterial community that differed from those associated with all the other flavonoids ($A = 0.09$, $p = 0.03$ vs. kaempferol; $A = 0.08$, $p < 0.05$ vs. orientin; $A = 0.14$, $p = 0.01$ vs. quercitrin). Within the *L. virginica* cultures, the presence of orientin resulted in a unique bacterial community that differed from all other flavonoids ($A = 0.09$, $p <= 0.05$ vs. kaempferol; $A = 0.19$, $p < 0.01$ vs. quercetin; $A = 0.09$, $p = 0.04$ vs. quercitrin).

Figure 3. Effects of flavonoids: (a) kaempferol, (b) orientin, (c) quercetin, (d) quercitrin at varying concentrations on mean Simpson diversity indices in rhizosphere bacterial communities of *L.cuneata* and *L. virginica*. Bars represent standard errors.






3c
L. cuneata
L. virginica
Simpson Diversity Index
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Control
50
100
200
Flavonoid Concentration (µM)

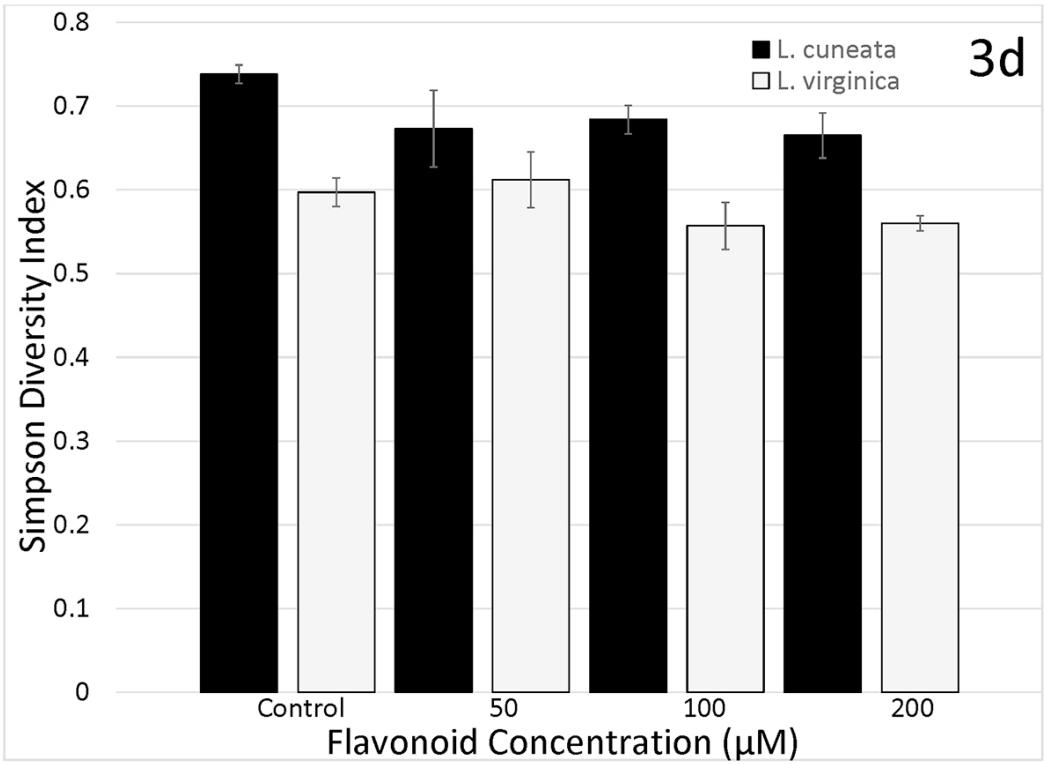

3d
L. cuneata
L. virginica
Simpson Diversity Index
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Control
50
100
200
Flavonoid Concentration (µM)

Table 3. MRPP comparisons of bacterial OTU composition of rhizosphere bacteria associated with two plant species between flavonoids across and between different concentrations. Different letters in each column correspond to significantly different composition at the order level at an uncorrected p level < 0.05.

	All Concentrations		50 µM		100 µM		200 µM	
Flavonoid	*L. cuneata*	*L. virginica*	*L. cuneata*	*L. virginica*	*L. cuneata*	*L. virginica*	*L. cuneata*	*L. virginica*
Kaempferol	B	B	A	A	AB	B	A	AB
Orientin	B	A	A	AB	AB	A	A	B
Quercetin	A	B	A	B	A	B	A	A
Quercitrin	B	B	A	B	B	AB	A	A

4 Discussion

We failed to reject our hypothesis that rhizosphere bacterial communities associating with the native *Lespedeza virginica* and non-native *L. cuneata* legumes would respond differently to plant communication molecules. Our results identified a decline in mean OTU richness in *L. cuneata* rhizosphere cultures following exposure to increasing concentrations of kaempferol, orientin, and quercetin, but no response to quercitrin. Quercetin was associated with a unique *L. cuneata*-derived bacterial community that differed in composition from all other flavonoids. Mean OTU richness in *L. virginica* cultures was found to be more variable than observed in *L. cuneata*; the variation increased significantly with increasing quercitrin concentration, showed specific positive dose responses to kaempferol and orientin, and had no response to quercetin. Orientin was associated with a unique *L. virginica*-derived bacterial community that differed in composition from all of the other flavonoids. The dose-specific responses observed in *L. virginica* cultures indicate a Goldilocks effect for these flavonoids on the studied bacterial community, where too little has no measurable effect and too much has a suppressive effect. This effect has been observed in previous research, where some flavonoids increased biological activity with increasing concentrations, while others peaked and then declined (Begum et al. 2001; Ulanowska et al. 2007). These previous studies also found high variability between bacterial responses to different flavonoids at the individual bacterial isolate level.

Invasive plant species have been shown to alter the bacterial community in invaded soils compared to native plants (Batten et al. 2006; Swedo et al. 2008; Bray et al. 2017). A previous study observed that *L. cuneata* benefits from greater rhizobial associations than does the native species, *L. virginica* (Hu et al. 2014); this observation supports the advantageous associations with native mutualists' hypothesis of plant invasion (Callaway et al. 2011). Other previous research observed that nodule-associating bacteria differ significantly in taxonomy between invasive and native species when they were coexisting in a single stand. Burkholderiales has been observed in *Lespedeza* nodules; however, the family Burkholderiaceae occurred exclusively in native Lespedeza nodules, while the family Comamonadaceae, which includes the pathogenic bacteria *Acidovorax*, occurred exclusively

in *L. cuneata* (Busby et al. 2016). These prior findings support the "facilitation of native plant pathogens hypothesis" of plant invasion (Eppinga et al. 2006).

In this study, the four bacterial orders found to differ significantly between the two rhizosphere sources were further broken up into pathogenic genera, based on Agrios (2005) and compared to OTU alignments in the cultures. Richness of these pathogenic genera were virtually identical between the two plant rhizospheres; however, the relative abundance of these pathogenic genera was much higher in the *L. cuneata* initial extracts and cultures, when compared to *L. virginica*. This finding provides further support for the facilitation of native plant pathogens hypothesis of plant invasion.

Within the Enterobacteriales, *Serratia* (a genus of common plant pathogens) was predominant in *L. cuneata* cultures, while *Citrobacter* (a non-pathogenic genus) was predominant in *L. virginica* cultures. A similar result was observed with respect to the Pseudomonadales, where *Pseudomonas* (a genus of common plant pathogens) was predominant in *L. cuneata* cultures, while *Acinetobacter* (a genus of non-pathogens) was predominant in *L. virginica* cultures. The Xanthomonadales (an order containing many common bacterial pathogens) was comprised primarily of OTUs aligned most closely with the genus *Stenotrophomonas*, a group of bacterial species not known to be pathogenic but rather to function as plant growth promoters (Ryan et al. 2009). This genus was much more prevalent, in terms of relative OTU abundance, in association with *L. virginica* than *L. cuneata*. While both *Pseudomonas* and *Serratia* contain pathogens, they also contain plant growth-promoting bacteria (Preston 2004; Zaheer et al. 2016). These observations both lend support to the facilitation of the native plant pathogens hypothesis and suppression of the native mutualists' hypotheses of plant invasion (Vogelsang and Bever 2009).

Unfortunately, at the level of resolution for our data and with only two initial rhizosphere extracts compared, it cannot be stated that pathogens were higher in association with *L. cuneata* compared to *L. virginica* or that native mutualists were suppressed in the presence of the invasive species. Results from this study did identify that genera with no known pathogenic species were higher in relative abundance in association with *L. virginica* compared to *L. cuneata* within the most common bacterial orders, while genera with known pathogenic species were much higher in

relative abundance in association with *L. cuneata* compared to *L. virginica* within the most common bacterial orders. Thus, it is unknown if the mutualists associating with the native *Lespedeza* were suppressed by the invader, outcompeted by more aggressive pathogens, or absent for other reasons. Additional research using a greater number of rhizosphere samples and higher sequencing resolution to identify bacterial species would determine whether these observed trends could potentially support a prevailing hypothesis for plant invasion using two (and possibly more) congeners with overlapping ranges.

Because no data was available regarding *L. virginica* flavonoid exudates, *L. capitata* exudates were used instead. While it is possible that the exudates used are not produced by *L. virginica*, it is equally possible that all four flavonoids are produced by both *Lespedeza* species, but these species have not been fully characterized yet. In legume species that have been more fully characterized, all four of these flavonoids do occur together, for instance, in *Onobrychis vicifolia* (Regos et al. 2009) and in *Desmodim canadense* (Puodziunene et al. 2009), a common native legume that overlaps both with *L. virginica*'s native range and the invaded range of *L. cuneata*.

Results of this study demonstrate that rhizosphere bacteria communities isolated from native and introduced congeners from a common location differ in their composition and responses to flavonoid exposure. Flavonoid type influenced bacterial community responses, as a function of the unique plant rhizosphere. Further, the native plant rhizosphere maintained higher bacterial richness when flavonoids were present, while the introduced plant rhizosphere lost richness and was observed to have increased abundances of genera containing known pathogens. However, the introduced plant rhizosphere maintained greater bacterial diversity in the presence of flavonoids compared to the native plant rhizosphere. These findings indicate that the studied rhizosphere communities are highly variable and differentially responsive to added plant communication molecules, and that the invader associates with a more diverse rhizosphere bacterial community that is less responsive to flavonoids.

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14. ABSTRACT

Military training can create disturbances that facilitate invasive plant establishment. Introduced plant species' interactions with soil microbial communities through root exudates often aid plants in colonizing new locales. This study tested the hypothesis that rhizosphere bacterial communities associated with the native legume, *Lespedeza virginica*, and the non-native legume, *Lespedeza cuneata*, respond differently to plant-exuded molecules. Bacterial communities collected from coexisting populations of the two plant species were grown in the presence of four separate flavonoids at four concentrations. Following 96 hours of incubation, DNA was recovered from the enrichment cultures and analyzed using next-generation sequencing. In cultures receiving a flavonoid, *L. virginica* enrichments were characterized by a greater OTU richness and exhibited a dose-response relationship to one of the flavonoids. The *L. cuneata* enrichments were characterized by a decreased OTU richness. Bacterial genera containing known pathogenic taxa occurred at a significantly greater relative frequency in *L. cuneata* enrichments than in the *L. virginica* enrichments. However, calculation of a species diversity index indicated greater OTU diversity in the *L. cuneata* enrichments across all four flavonoid treatments. These results indicate that the rhizosphere microbial communities of co-existing *L. cuneata* and *L. virginica* legumes exhibit different responses when exposed to plant communication molecules.

15. SUBJECT TERMS
Military training, Invasive plants, Plant-soil relationships, Microbial ecology, Lespedeza cuneata, Flavonoids

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