COVER SHEET

TITLE: Bacterial strains within the fungus gardens of leaf-cutter ants show evidence of mutual and commensal interactions

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ABSTRACT

Leaf-cutter ants have a symbiotic relationship with a fungal cultivar known as *Lecuoagaricus gongylophorus*. The cultivar functions as an external gut by breaking down plant polysaccharides and supplies the ants with simple sugars. A consistent community of bacteria lives in consortium with this fungus, and the community composition is conserved across ant species. However, the exact role of these bacteria remains uncertain. We carried out several experiments to examine possible roles of the bacterial community. One experiment involved using antibiotic infused-leaf discs to attempt to reduce bacterial abundance within fungus gardens. The other experiment involved growing pure isolates of bacteria from *L. gonglyophorus* alongside the fungal culture. Conflicting results were obtained from both experiments, so genus level bacterial primers were designed to evaluate bacterial abundance within antibiotic treated fungus gardens. These investigations will provide insight into how the bacterial community interplays with the rest of the leaf-cutter ant system.

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Abstract:

Leaf-cutter ants have a symbiotic relationship with a fungal cultivar known as *Lecuoagaricus gongylophorus*. The cultivar functions as an external gut by breaking down plant polysaccharides and supplies the ants with simple sugars. A consistent community of bacteria lives in consortium with this fungus, and the community composition is conserved across ant species. However, the exact role of these bacteria remains uncertain. We carried out several experiments to examine possible roles of the bacterial community. One experiment involved using antibiotic infused-leaf discs to attempt to reduce bacterial abundance within fungus gardens. The other experiment involved growing pure isolates of bacteria from *L. gonglyophorus* alongside the fungal culture. Conflicting results were obtained from both experiments, so genus level bacterial primers were designed to evaluate bacterial abundance within antibiotic treated fungus gardens. These investigations will provide insight into how the bacterial community interplays with the rest of the leaf-cutter ant system.

Introduction: Leaf-cutter ants of the tribe Attini represent a highly successful group of herbivores in tropical forest ecosystems, capable of utilizing up to 17% of all plant biomass in the *cerrado* biome of Brazil (Costa *et al.* 2008). This considerable amount of plant biomass is not directly consumed by the ants themselves, but is instead given to a symbiotic fungus (*Leucoagaricus gongylophorus*) that supplies the ants and their brood with needed sustenance through specialized fungal projections known as gongylidia (Chapela *et al.* 1994). The fungal cultivar exudes a variety of biomass degrading enzymes to process this plant material (Silva *et al.* 2006a, Silva *et al.* 2006b, Schiøtt *et al.* 2008).

Although *L. gongylophorus* can produce its own digestive enzymes, within ant colonies it also coexists with a variety of bacteria (Aylward *et al.* 2012). The community composition of these bacteria is conserved between ant colonies and within vertical strata of the fungus, with a large proportion of the community being Gammaproteobacteria (Scott *et al.* 2010, Aylward *et al.* 2012). The function of most of the bacteria in this arrangement remains unclear, but several strains have been implicated in nitrogen fixation (Pinto-Tomas *et al.* 2009). Some earlier studies

have suggested that the bacterial community in these fungus gardens is responsible for plant biomass deconstruction (Suen *et al.* 2010, Bacci *et al.* 1995). However, more recent studies indicate that the fungus is primarily responsible for biomass breakdown (Aylward *et al.* 2013, Khadempour *et al.* 2016), and this, combined with the low abundance of bacteria in the gardens, indicates that the bacteria are likely playing another role in the system.

The addition by bacteria of nitrogen and other nutrients such as vitamins into the leaf-cutter ant system may potentially increase the overall health of the community through affecting growth of the fungal cultivar. In order to investigate the possible roles that bacteria may be playing within the fungal cultivar of the ants, several different experiments were conducted. We hypothesized that bacteria within this system were benefitting the fungal cultivar either directly or indirectly. To test this hypothesis, we evaluated the ability of bacterial co-cultures to facilitate fungal growth and we measured the response of the overall community to the introduction of antibiotics to the system. If bacteria within the fungus garden are beneficial to the leaf-cutter ant system, then we would expect some bacterial strains to be able to increase fungal growth in co-culture assays and that lowering of bacterial abundance using antibiotics will reduce overall community health.

Methods:

Infusion of leaf-discs with antibiotics

Leaf-discs (11m diameter) were cut from black oak, to exclude large leaf veins. Leaf discs were then suspended in a vacuum flask in a solution containing 0.1% Tween 20 and 500 µg/mL of either chloramphenicol, gentamicin, tetracycline, kanamycin, streptomycin, or a control solution containing only 0.1% Tween 20. Infusion of leaf-discs occurred for two hours until all discs had

fallen to the bottom of the vacuum flask, indicating that liquid penetration of the discs had occurred.

Setup of leaf-cutter ant sub-colonies

All *Atta* cephalotes colonies used were originally collected from Costa Rica, with colonies collected from Alajuela and Heredia provinces in Costa Rica. Colonies were maintained by provision of oak and maple leaves three times a week in a temperature and humidity controlled building within the Microbial Sciences Building at the University of Wisconsin-Madison. Eighteen total sub-colonies were created from 8 *Atta cephalotes* colonies, with 2 sub-colonies created from each parent colony except for 3 colonies from which 3 sub-colonies were created . Each *Atta cephalotes* ant sub-colony consisted of one cylindrical chamber of fungus garden and the ants contained within that chamber upon its extraction from laboratory colonies. Each colony additionally had a single weigh boat in which leaf-discs were provided to the ants.

Feeding experiment

To stabilize the sub-colonies prior to treatment, they were provided control leaf-discs for one week prior to the provision of experimentally treated leaves. After this initial week, each sub-colony was randomly assigned to either the control group or one of the five antibiotic treatments. Due to a complication in which the sub-colonies from one of the original ant colonies had to be returned to the original colony prior to the start of the experiment, all treatment groups had 3 associated sub-colonies except for those treated with gentamicin, streptomycin, and kanamycin which only had 2 sub-colonies each. Ants had *ad libitum* access to leaf-discs, and food consumption was monitored daily based on number of leaf-discs incorporated into the fungus garden.

Evaluation of fungal cultivar growth rate

Prior to the beginning of growth trials, three samples of fungal cultivar isolated from leaf-cutter ant colonies were plated onto potato dextrose agar (PDA) to ensure that fungal isolates are capable of growth on this type of media. Plates were grown in darkness at 25°C. Plugs of fungus were extracted from these three plates and re-plated onto fresh plates of PDA. Three replicates were created for each condition, consisting of controls grown in isolation, as well as similar plates with the addition of a co-culture of either *Klebsiella*, *Pantoea*, unclassified Enterobacteriaceae, *Serratia*, or *Pseudomonas* after one month of fungal growth. Initial surface area of the sample was noted based on the approximation of the sample as a circle with formula $SA = 4\pi r^2$, with r representing the radius of the circle. Radius of the circle was determined as an average of four linear measurements along the axes of each plate using ImageJ software (Figure 1). Surface area measurements were made twice a week for two months following the addition of the bacterial co-cultures, with growth rate noted as change in surface area.

Figure 1: Representation of analysis of the dimensions of fungal growth. All plates were analyzed using ImageJ software, and the surface area of fungal growth was determined based on an average radius as measured along the four drawn axes. The segmented line shown represents a measurement along one of the axes, including faint outer ring growth.

Design of primers specific to bacterial genera

Representative sequences of genes of interest were compared using the program CLC Sequence Viewer 7. Regions showing promise for use as a specific primer were then analyzed for primer properties using the program Primer 3. As these primers are intended for use in quantitative PCR, the overall targeted amplicon length was around 200 bp and targeted annealing temperature of about 58°C.

Isolation of bacterial DNA

Ten representative strains of each of the genera *Enterobacter*, *Pantoea*, *Klebsiella*, *Serratia*, and *Pseudomonas* were cultured on yeast malt extract agar(YMEA) media. Bacterial DNA was isolated using a Macherey-Nagel NucleoSpin Plant II kit using the standard instructions.

Testing of designed primers

All primers were synthesized at the UW-Madison Biotechnology Center. Primer sequences then had their annealing temperature verified using thermal gradient PCR. This was followed by analysis of the ability of designed primers to amplify a variety of strains within a genus of interest. Finally, cross-reactivity of the primer with off-target genera was evaluated.

Results:

Antibiotic infused leaf-disc feeding experiment

After two weeks of treatment with antibiotics, the overall appearance of fungal gardens within sub-colonies did not grossly change (Figure 2). However, the number of leaf-discs that ants in antibiotic treated colonies incorporated into the fungus garden decreased over time (Figure 3).

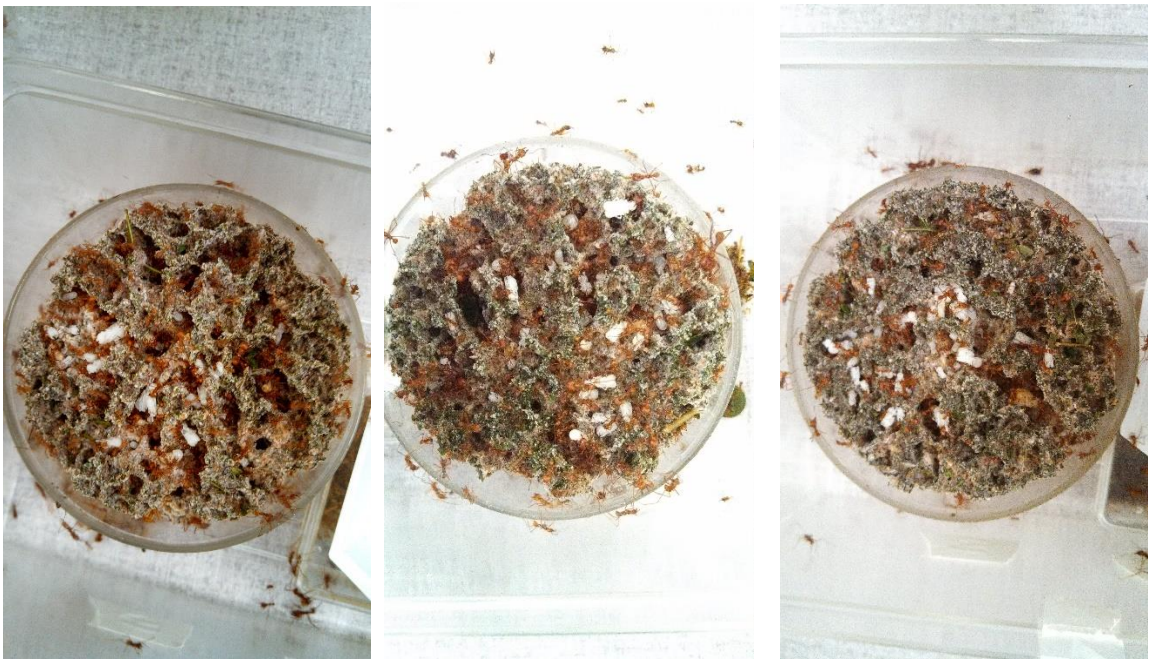

Figure 2: Images of a leaf-cutter ant fungus garden treated with tetracycline over the course of two weeks. The image on the far left is before antibiotics were added to the system, the center image one week after start of antibiotic addition, and the image on the right is two weeks after the start of antibiotic addition.

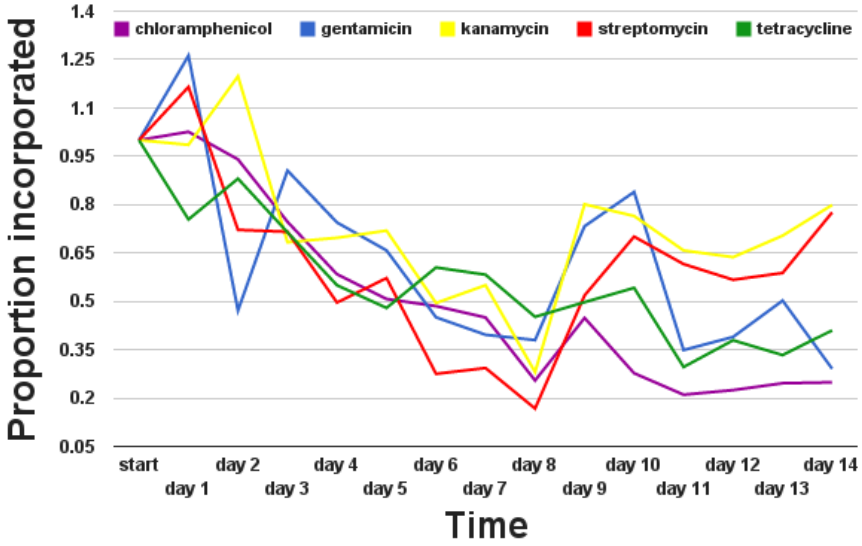

Figure 3: Feeding behavior of leaf-cutter ants in sub-colonies treated with different antibiotics. All colonies were given leaves infused with one of five antibiotics over the two-week

experimental period. Feeding behavior was measured based on leaves integrated into the fungus garden normalized to the number of leaves utilized by control populations.

Bacterial effect on fungal growth rate

The addition of all tested bacteria to fungus samples grown on PDA led to declines in fungal growth overall (Figure 4). However, following addition of bacterial isolates to fungal plates fungal growth rate increased for plates on which *Pantoea* was applied (Figure 5).

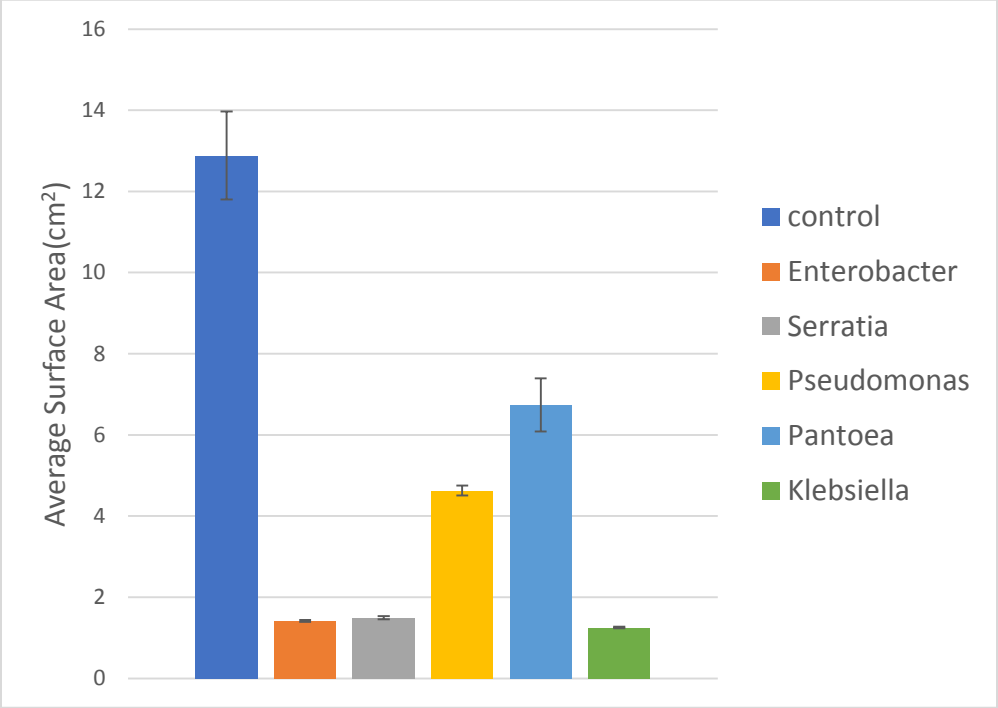

Figure 4: Growth of fungal cultivar on PDA from leaf-cutter ant fungus gardens in the presence or absence of bacterial isolates. Surface area was determined using an average radius determined along four axes of growth and assuming that growth is circular. Error bars are ± 1 SE.

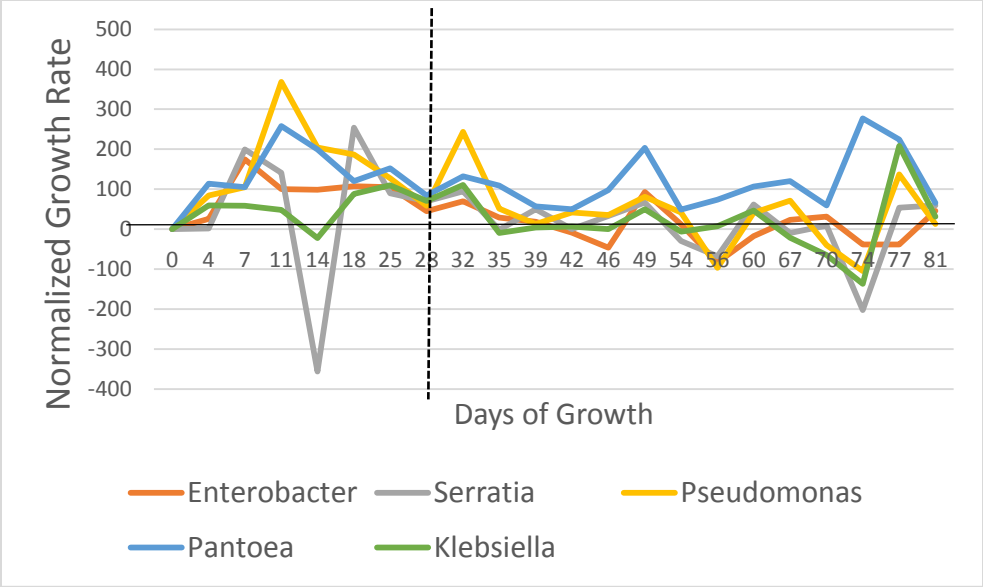

Figure 5: Growth rates of fungal cultivar grown in the presence of pure bacterial isolates. The dashed vertical line indicates the date of bacterial addition to cultures. All growth rates are normalized to the average growth rate for control plates that had no bacteria.

Primer design

All three primers tested for specificity showed the specificity to their targeted sequences (Figures 6,7,8). Pseu1/Pseu2 targets *Pseudomonas* strains, Flux/Rlux targets *Serratia* strains, and EnPaKl1/EnPaKl2 targets strains in the genera *Enterobacter, Pantoea*, and *Klebsiella*.

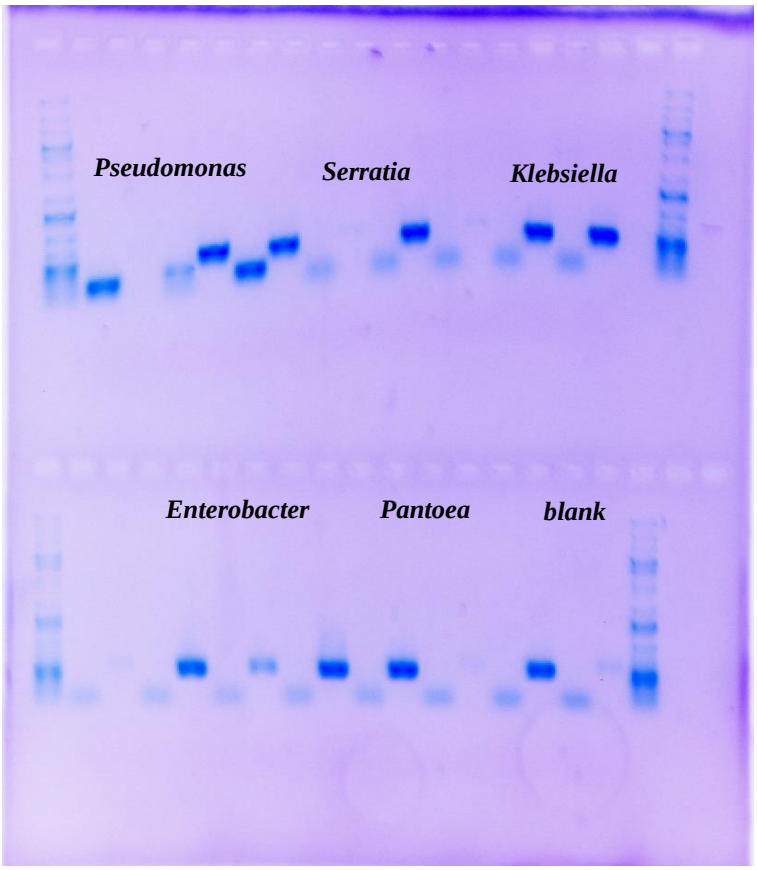

Figure 6: Testing of the specificity of Pseu1/Pseu2 primers. Each strain was tested as an adjacent pair of bands, bands on the left are amplified using Pseu1/Pseu2 and bands on the right are amplified using primers that target the 16S gene. Three strains of each genus of interest were tested adjacent to each other, starting with *Pseudomonas* and *Serratia* on the top row followed by two strains of *Klebsiella*. The third *Klebsiella* strain is the first pair of bands in the bottom row, and this is followed by three strains each of *Enterobacter* and *Pantoea*. Blank lanes were used as negative controls.

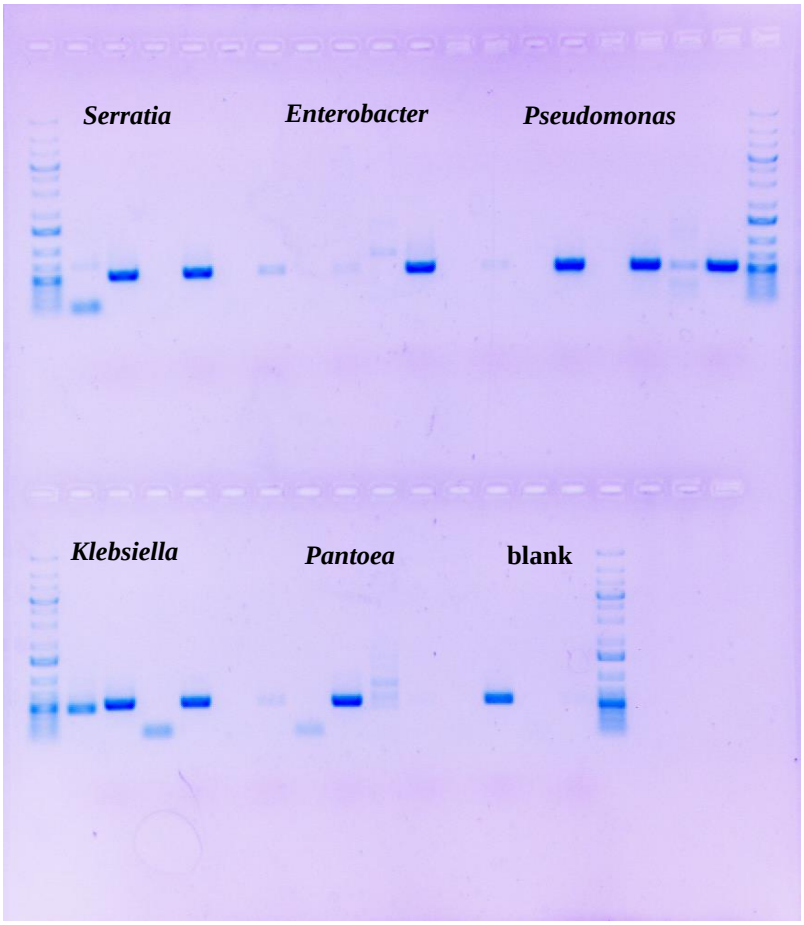

Figure 7: Testing of the specificity of Flux/Rlux primers. All strains were tested in pairs of adjacent bands. Bands on the left of a pair were amplified using Flux/Rlux, bands on the right were amplified using primers that target the 16S gene. Three strains of each genus of interest were tested adjacent to each other, starting with *Serratia, Enterobacter,* and *Pseudomonas* on the top row. The bottom row contains three strains each of *Klebsiella* and Pantoea. Blank lanes were used as negative controls.

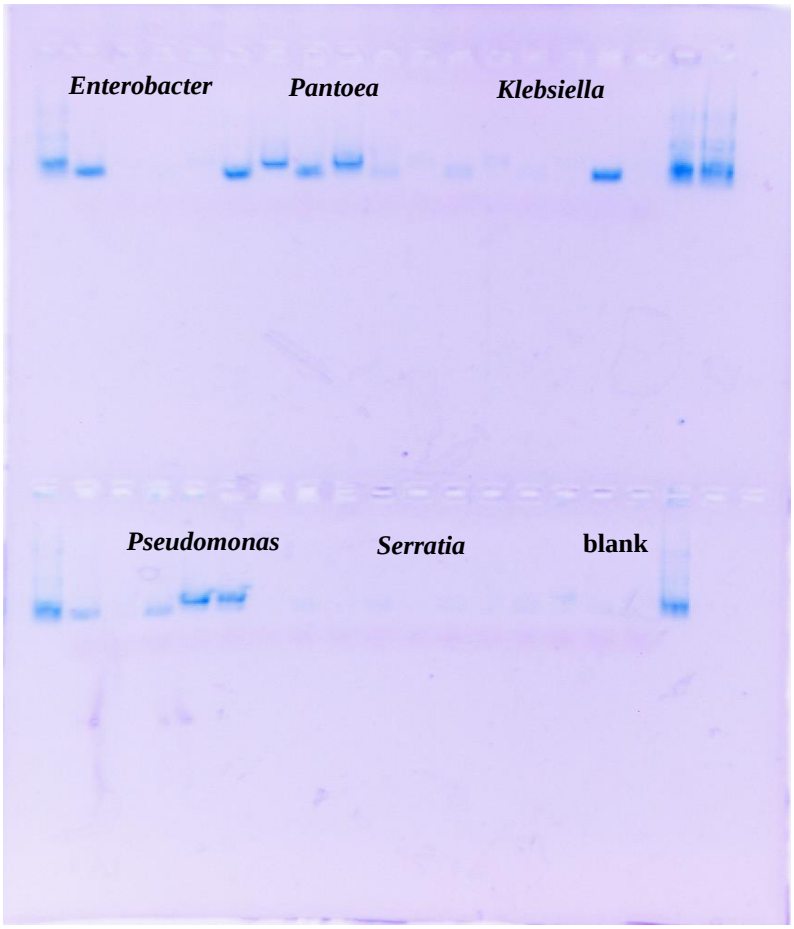

Figure 8: Testing of the specificity of the EnPaKl1/EnPaKl2 primers. All strains are tested in pairs of adjacent bands. Bands on the left are amplified using EnPaKl1/EnPaKl2 primers, while bands on the right are amplified using primers that target the 16S gene. Three strains of each genus of interest were tested adjacent to each other, starting with *Enterobacter* and *Pantoea* on the top row followed by two strains of *Klebsiella*. The third *Klebsiella* strain is the first pair of bands in the bottom row, and this is followed by three strains each of *Pseudomonas* and *Serratia*. Blank lanes were used as negative controls.

Discussion:

Based on the results of these experiments thus far, we cannot definitively conclude whether the bacteria in leaf-cutter ant fungus gardens are essential to the system. Some of the evidence suggests that they are important for fungal health while others contradict it. For this reason, we have begun the work to use QPCR in the fungus gardens to further our insights.

Lack of visual change in the fungus garden during two weeks in the antibiotic feeding experiment suggest that bacteria are not important within the overall leaf-cutter ant system. This observation is further supported by the result that co-cultures of bacteria decreased the overall growth of the fungal cultivar. These two observations suggest that bacteria within the leaf-cutter ant system are mainly present to take advantage of excess nutrients associated with plant matter breakdown by the fungal cultivar (Aylward *et al.* 2013).

However, other results within these experiments suggest evidence of some mutualisms between the bacteria and the fungal cultivar. Antibiotic treatment of leaf-cutter ant fungus gardens led to a reduction in the rate at which ants brought leaves into the fungus garden, suggesting a role for bacteria in the overall health of the colony. Additionally, the addition of *Pantoea* to plates of fungal cultivar increased their overall growth rate. *Pantoea* strains have already previously been noted to provide nitrogen to the fungal cultivar system, but these results open the possibility that the bacteria are providing other nutrients that are in low concentration in or not found in PDA (Pinto-Tomas *et al.* 2009)

Multiple attempts were made to design primers specific to the separate genera *Enterobacter*,*Pantoea*, and *Klebsiella*. However, the close phylogenetic relationships between these three genera complicated the design of genus specific primers (Paradis *et al.* 2005). Instead, a more generalized primer set targeting *Enterobacter*, *Pantoea* and *Klebsiella* simultaneously was created. The combination of this primer set along with primers targeting

Psuedomonas and *Serratia* specifically will be able to provide insight in future analysis as to the extent that ant behavior seen in the feeding experiment reflects changes in bacterial abundance. This analysis will provide insight into which bacterial genera when limited within leaf-cutter ant fungus gardens cause the most negative impacts. These primers can additionally be used in the future to evaluate the effects of a variety of different disturbances in the leaf-cutter ant system on the overall bacterial community within the fungus garden, such as changes in food substrates provided to the fungus or the addition of antifungal compounds.

The distinction between different modes of symbiosis can often be poorly delineated (Leung and Poulin 2008). The overall inhibition of growth of the fungal cultivar by all tested bacterial strains suggest that the bacteria within the system may be to some extent parasitic and limiting fungal growth. However, the conservation of the bacterial community within the fungal garden between different species of leaf-cutter ants and the feeding behavior of ants when given antibiotic infused leaf-discs suggests that some selective pressure is promoting the persistence of these strains within the community (Aylward *et al.* 2012). One possible role of the bacteria in the system may be in resisting colonization by other bacteria that may harm the system. Colonization resistance by gut microbiota is an important health function within the human microbiome, and physiology of the host plays an important role in determining which bacteria are found within the gut (Eren *et al.* 2015, Forsythe and Bienenstock 2010). Future experiments testing the susceptibility of the fungus garden community to invasion by novel bacteria following knockdown of the resident bacterial community offers the opportunity to provide insight into this potential role for the bacteria within the fungus garden.

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