The Role of *Lactobacillus micheneri* in Alfalfa Leafcutting Bee Nutrition

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**Introduction:**
In previous studies, we identified several bacteria that commonly associate with the alfalfa leafcutting bee (McFrederick et al. 2014), but how these bacteria affect bee health remains a mystery. During the summer of 2014, we conducted research showing that the bacterium *Lactobacillus micheneri* (which we previously referred to as *L. kunkeei* but have recently proposed as a new species), either by itself or in combination with other microbes, does not lower the abundance of *Acospheara aggregata* cells in infected larvae when compared to controls that lacked bacteria. *Lactobacillus micheneri*, however, did reach high abundance in the *M. rotundata* larval gut (Figure 1.)

Although *L. micheneri* does not appear to prevent chalkbrood, our research suggested a promising, new research avenue. All bees that we inoculated with only *L. micheneri* survived to the pre-pupal stage (N=18) while 8/10 bees reared on sterile provisions survived to the pre-pupal stage. This suggests that *L. micheneri* may benefit *M. rotundata* in some way other than protection from *A. aggregata*. An additional line of evidence was the incredible abundance of this bacterium in the larval gut; *L. micheneri* abundance averaged 3.9 x 10^7 across all larvae, with a high of 3.87 x 10^8 cells found in one larva’s gut (Fig 1).

We have sequenced the genome of a strain of *L. micheneri* that we originally isolated from the sweat bee *Halictus ligatus*, but that we have also detected in association with *M. rotundata*. Amongst other interesting genes we found two genes that encode proteins that can degrade pectin, a component of pollen walls that makes pollen difficult to digest (Roulston and Cane 2000). How most bees digest pollen is poorly understood (Roulston and Cane 2000), but megachilid bees in the genus *Osmia* larvae do not digest pollen with thick inner walls (intine) as readily as pollen with thin intines (Suarez-Cervera et al. 1994). The intine contains mostly pectin and cellulose, so the presence of pectin degrading genes suggests that *L. micheneri* may benefit nutrition of *M. rotundata* by aiding pollen digestion.

**Objective:** Test for bacterial-mediated effects on *Megachile rotundata* nutrition. We tested whether *L. micheneri* aids in pollen wall breakdown, and if this in turn benefits *M. rotundata* nutrition. Prediction: *Lactobacillus micheneri* increases pollen digestibility by...
facilitating nutrient release.

**Hypothesis:** Larvae are able to more completely digest pollen when lactobacilli are present than in the absence of lactobacilli.

**Methods:**
To test this hypothesis, we set up an experiment with four different treatments:

1) Sterile pollen
2) Sterile pollen and *L. micheneri*
3) Sterile pollen, suspended bacteria from fresh pollen provision, and *L. micheneri*
4) Unsterilized provisions

Treatments 1-3: each received 20 uL of liquid containing 5 uL MRS+F (a lactobacillus growth medium) with overnight growth of *L. micheneri* for treatments 2 & 3 and sterile for treatment 1, 5 uL Physiologic Saline (a buffer to suspend microbes in without risk of osmotic shock) with wild microbes for treatment 3 and sterile for 1&2, and 10 uL sterile H2O for all three treatments. Treatment 4 was unmanipulated. We weighed every brood cell at the beginning and end of the experiment, in order to roughly quantify the amount of pollen the larvae consumed.

For sterile pollen, we collected newly constructed *M. rotundata* nests from the McFrederick lab’s alfalfa plot on the campus of the University of California, Riverside. We removed eggs or first instar larvae, both of which had not fed on the provision. We then exposed the eggless provisions to ~28 Kgy in a γ-irradiator. To sterilize *M. rotundata* eggs we collected eggs from *M. rotundata* nests that were overnighted to us from the ARS Logan Bee Lab in Logan Utah. We sterilized eggs for treatments 1, 2, and 3, but left eggs intact on unsterilized pollen for treatment 4.

For treatments 2 & 4, we applied 5 uL of overnight liquid culture of *L. micheneri* to the pollen provisions, making sure to evenly spread the liquid broth across the surface of the pollen provision, and agitating the broth between treatments to keep the bacteria evenly suspended. Our plating counts indicated that we applied 3.6 x 10³ *L. micheneri* cells per pollen provision. For treatment 1, we applied 5 uL of sterile MRS+F (the medium which we grow *L. micheneri* in), so that each brood cell had the same amount of MRS+F added. We also preserved some of the broth at -80 °C for later genetic analysis and plated 20 uL of 1, 10, 100, and 1000 X dilutions in order to determine the number of *L. micheneri* cells we applied to each brood cell.

While transferring sterilized eggs to new, treated brood cells we kept the treatment plate (i.e. the plate we transferred eggs into with sterilized pollen and treatments 1-3) in a plastic tub on its side, sterilized with 1% bleach followed by ~95% EtOH, with a flame bulb burning in front of it to keep out airborne microbes. Each treatment was kept in its own plate, and we rotated the plates during the experiment to control for possible microclimate effects. Over the duration of the experiment, we kept the plates in sterilized plastic tubs, with paper towels (that we had exposed to UV radiation to sterilize) lightly moistened with sterile water. We kept the plastic tub at 27 °C with 50% relative humidity.

We then monitored larvae daily, taking notes on mortality. Once larvae had finished defecating and spinning their cocoons, we collected them, weighed each larvae individually, weighed the empty brood cell, collected frass from each brood cell, and stored the larvae and frass at -80 °C until we were ready to conduct the protein analyses. For each treatment, we collected a minimum of 30 replicate larvae.
Protein analysis:
To extract proteins from pre-pupae, we placed each larva in 300ul of sterile water, with 2 steel 3mm beads and then homogenized the pre-pupae in a Qiagen Tissue Lyser II for 6 minutes at 30Hz.

We used the Pierce BCA Protein Assay kit from Thermo Fisher for protein quantification. Optimization of this protocol concluded that 50x dilution of our samples produced consistent results within the standard curve. We therefore diluted all samples by 50x in the same sterile water they were initially homogenized in.

We followed the Pierce BCA Protein Assay kit “Microplate Procedure” which requires 1:8 mix of sample/standard to working reagent. One initial set of standards was mixed according to the Pierce BCA protocol and was used for each plate of samples. Samples and standards were assayed in triplicate.

We then mixed the samples with working reagent in tissue culture U-bottom plates, incubated the plates at 37 °C for 30 minutes, cooled them to room temperature, then took absorbance measurements at 562nm with the Thermo Scientific Varioskan Lux using the SkanIt software.

Statistical analysis: To determine if larval weight, frass protein content, and larval protein content differed by treatment, we conducted one-way ANOVA analyses in the program R. We checked the residuals for heteroscedasticity, and used log transformations as appropriate. To test for differences between treatments, we used Tukey’s honest significant differences test.

Results
Larval weight differed significantly by treatment ($F_{3,106} = 9.499, P < 0.0001$) with the unmanipulated group weighing significantly more that the other treatments, which did not differ in weight (Fig. 2). The protein content in the frass did not significantly differ by treatment (Fig. 3, $F_{3,106} = 0.526, P = 0.666$), with each treatment group containing appreciable amounts of pollen. Larval protein concentration, however, did differ significantly by treatment (Fig.4, $F_{3,116} = 5.534, P < 0.002$).
**Discussion**

*Aerococcus micheneri* does not function as a nutritional mutualist, nor does it appear to compete for protein or carbohydrates with its larval host. Given the impressively high numbers of *L. micheneri* cells that we measured in a previous study using similar protocols, this is a surprising result. The function of this bacterium remains elusive, although when a bacterium is present in such high numbers it is hard to imagine that there is no effect on host fitness.

While *L. micheneri* presence did not result in greater protein assimilation by the larvae, the presence of a natural microbial community (= microbiome) did result in less protein being assimilated by the larvae. Before analyzing the prepupae, we carefully checked for signs of chalkbrood and did not find any symptomatic larvae. The lower protein content in larvae that grew in the presence of a natural microbiome is therefore unlikely to be caused by *Ascospaera aggregata*, and may be the result of some unknown member or members of the microbiome that compete with the larvae for protein, or somehow otherwise inhibit protein assimilation by the larvae.

The amount of protein that a larval bee consumes can have a large effect on its body size (Roulston and Cane 2002), which has knock-on effects on foraging range (Greenleaf et al. 2007). Protein assimilation can therefore affect bee health. Future work identifying and targeting the microbe or microbes underlying this effect may lead to production of larger, better pollinating bees.

None of the manipulated treatments resulted in larvae that significantly differed in weight. The unmanipulated brood cells, however, resulted in larvae that weighed significantly more than the other treatments. As we did not measure dry weight, this may represent differences in water content. The sterilization process resulted in pollen provisions that appeared much drier than fresh provisions. We rehydrated the sterilized provisions, but likely not to the same extent as the fresh provisions in the unmanipulated treatment group.
That the frass did not significantly differ in protein content suggests that an equal amount of protein was extracted from the pollen in each treatment group. The bees assimilated less of that protein when the natural microbiome was present, however. Even in the unmanipulated treatment, which resulted in prepupae that weighed more than prepupae in the other treatments, protein assimilation was less than sterile or sterile & *L. micheneri* inoculated provisions.

Although we found that *L. micheneri* is not a nutritional symbiont, we were able to detect a possible means by which we may be able to improve the nutrition of *M. rotundata*. By identifying microbes that compete with the larvae for protein, we may be able to help improve the nutrition of the alfalfa leafcutting bee.


