Project: Preservation and distribution of probiotic *Parasaccharibacter apium* (Acetobacteraceae Alpha 2.2) for use by beekeepers.

Dates covered by award: FY2016-2017

PIs: Vanessa Corby-Harris and Kirk Anderson

Aim: *Parasaccharibacter apium* (Acetobacteraceae Alpha 2.2) is a bacteria found primarily in the hive environment and in larvae. *P. apium* strain C6 confers a survival benefit to honey bee larvae (Corby-Harris et al., 2014). With funds granted by the NHB in 2014 (Corby-Harris, 2015; Corby-Harris et al., 2016), we also found that this strain of *P. apium* survives in pollen patty, travels from pollen patty throughout the hive, increases hive strength in the spring, and increases forager immunocompetence and *Nosema* resistance (Corby-Harris, 2015; Corby-Harris et al., 2016). The aim of the current project was to further develop *P. apium* strain C6 for commercial use. Our four main objectives were to determine whether 1) *P. apium* can be freeze dried and subsequently revived, 2) freeze dried and then revived *P. apium* survives in pollen patty, 3) *P. apium* is beneficial to beekeeper hives in multiple locations, and 4) foragers from *P. apium*-treated hives contain fewer *Nosema* in their guts than foragers from untreated hives.

Main results: 1) *P. apium* can be freeze dried and subsequently revived, 2) freeze dried and then revived *P. apium* survives in pollen patty, 3) freeze dried *P. apium* does not provide a benefit in beekeeper hives, and 4) forager-aged bees from *P. apium*-treated hives contain fewer *Nosema* in their guts than foragers from untreated hives.

Aim #1: Can *P. apium* be freeze dried and subsequently revived? For *P. apium* to be distributed to and used by beekeepers, it needs to be in a safe and stable state so that it can be mailed and then easily added to supplemental diet. Our first objective was to test whether *P. apium* can be freeze dried (lyophilized) and subsequently revived. This was done with the assistance of an outside company with experience lyophilizing bacteria. The company, OPS diagnostics, developed a protocol for freeze drying *P. apium* that we subsequently used in our lab.

Methods: 50 ml of a stationary phase culture of *P. apium* was pelleted and the growth medium was poured off. Fresh freeze drying medium was added to the pellet and the culture was re-suspended. Three aliquots of this re-suspended culture were diluted (10^{-5}). 100 µl of each aliquot was plated twice on Sabouraud Dextose (SD) media. The average growth for the two plates after 48 hours was calculated for each of the three aliquots. This value was used to calculate the initial pre-freeze drying number of colony forming units (CFUs) of *P. apium*. 500 µl of each pre-freeze drying culture was then freeze dried following the recommendations of OPS Diagnostics (Lebanon, NJ). Briefly, a liquid culture of *P. apium* was pelleted by centrifugation and the liquid media was replaced with freeze drying media. These cultures were gently mixed and placed at -80°C for 3 days. The vials were then placed into a freeze drying apparatus (Labconco Freezone 1). The freeze dried cultures were then reconstituted: 500 µl of distilled water was added to the vial, the vial was gently mixed by flicking, and let to sit at room temperature for 30 minutes. Two 10^{-5} aliquots in SD liquid media were prepared from each vial; from these vials two plates were prepared by plating 100 µl of this aliquot onto two SD plates as above. The average growth for the two plates after 48 hours was calculated for each of the aliquots. The average value of the two aliquots per vial was used to calculate the average number of CFUs recovered per vial. This value was used to calculate the post-freeze drying number of colony forming units (CFUs) of *P. apium*.

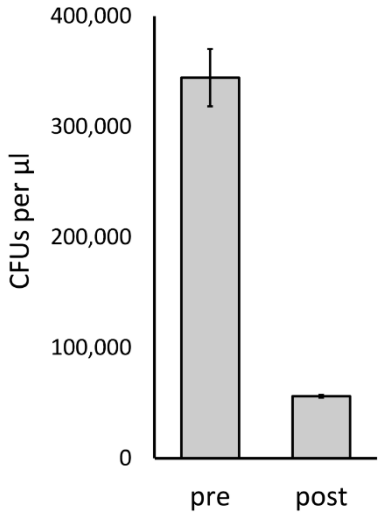

Figure 1. *P. apium* colony forming units (CFUs) per µl of culture before (pre) and after (post) freeze drying. Average counts for three vials are presented ± S.E.

Results: Freeze drying *P. apium* resulted in approximately 83.5% mortality (Figure 1). The average number of CFUs per µl of culture pre-freeze drying was 344,333 ± 25,900 S.E. and the average number post-freeze drying was 56,167 ± 1,176 S.E.

Aim #2: Does freeze dried and then revived *P. apium* survive in pollen patty? In our initial experiments, fresh *P. apium* added to natural pollen patty and then fed to the colony was consumed by the workers (Corby-Harris, 2015; Corby-Harris et al., 2016). We wanted to know whether a similar delivery method could be used with freeze dried *P. apium*. We tested whether *P. apium* that had been freeze dried and then reconstituted with distilled water survived in natural pollen patty.

Methods: A large batch of *P. apium* was grown and freeze dried using the methods described above. In three of the freeze dried vials, 500 µl of distilled water was added to reconstitute the culture as described above. For each vial, three aliquots (10^{-5}) were prepared from each vial in SD liquid media. From each vial, two plates were prepared by plating 100 µl of this aliquot on two SD plates as above. The plates were incubated at 27°C for 48h in order to obtain a starting number of CFUs per µl of revived culture. This number was multiplied by 500 to obtain a total number of CFUs per freeze dried vial. Next, 10 pollen patties were prepared (equal parts sucrose, Drivert® sugar, and natural pollen combined and mixed with water to a cookie batter consistency) and weighed (~15g each). 10 vials of freeze dried *P. apium* were prepared as described above and each one was added to a different patty. One core was obtained from each patty using a straw according to the methods described in (Corby-Harris, 2015; Corby-Harris et al., 2016). The core was vortexed into sterile SD broth and the sample was gently centrifuged at a low speed to pellet the pollen patty. A sample of the supernatant was diluted to 10^{-5} and 100 µl of this was plated twice to obtain an average number of CFUs per sample. The patties were re-weighed to obtain the weight of the core via subtraction. The number of CFUs per patty obtained above was divided by the weight of the core to obtain an estimate of the number of CFUs per gram of patty. The patty was then incubated at 27°C for 24h. The coring was repeated, the core weight was calculated, and the number of CFUs in the core was again obtained. The value for the number of CFUs per gram of patty after 24h was calculated.

Results: The average number of CFUs per µl of revived culture was 16,056 ± 4,227 S.E. CFUs, meaning the number of CFUs revived from each vial was approximately 8 million. The average weight of the 10 patties was 15.42 g, meaning that approximately 520,473 CFUs were added per gram of patty (Figure 2a). This was approximately twice (2.15 times) the amount of live bacteria added to patties in our previous experiments with fresh bacteria (242,000 CFUs/g patty in 2014 study; (Corby-Harris, 2015; Corby-Harris et al., 2016); Figure 2a). Almost immediately after being mixed into the patty, the number of CFUs that could be recovered per gram of patty was approximately 554 ± 59 S.E. CFUs, meaning that only about 0.1% of the freeze dried bacteria survived the initial stress of being put into the pollen patty (Figure 2b). After 24h in the patty, approximately 316 ± 33 S.E. CFUs survived per gram of patty (Figure 2b). This was comparable to the number of bacteria recovered from patty after 24h for the fresh bacteria study in 2014, where 154 ± 27 S.E. CFUs were recovered per gram of

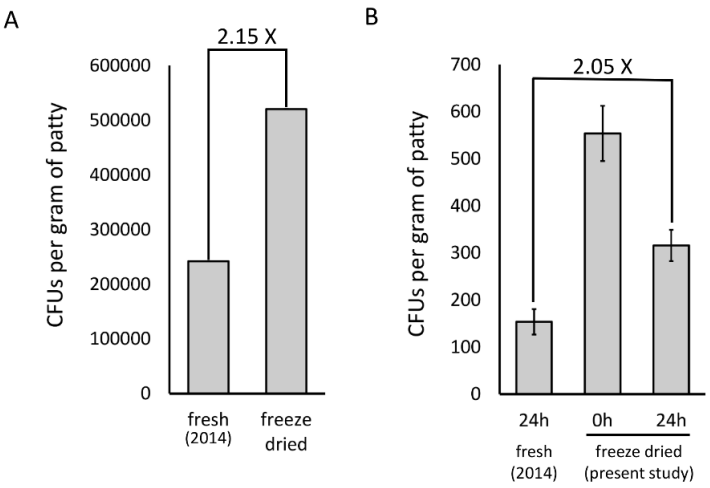

Figure 2. Amount of inoculum placed into pollen patty (A) and amount of bacteria recovered (B) in the 2014 versus the present. The 2014 study used fresh bacteria, whereas in the present study patties were inoculated with bacteria that were freeze dried and then revived. Panel A shows the number of bacteria initially added to the patty, calculated from the initial dose and weight of the patty. Panel B shows that number of bacteria recovered from patties 0h or 24h after incubation. Note the differences in the scale of the Y-axes.

patty (Figure 2b). In other words, we recovered about twice the number of bacteria after 24h in the present study using freeze dried bacteria, but in the fresh bacteria study only half of the starting inoculum was put into the patty. So, the freeze dried bacteria fared as well as fresh bacteria in pollen patty after 24h.

Aim #3: Is *P. apium* beneficial to beekeeper hives in multiple locations? *P. apium* is abundant in fall pollens gathered from the environment and stored in the hive (Anderson et al., 2014). In our 2014 study with fresh *P. apium* added to pollen patty, we did not observe any difference in hive strength due to supplementation. However, we did see that bees from supplemented hives were more resistant to *Nosema*. We wondered if hives from other parts of the country where *Nosema* is more of an issue (i.e., cooler temperate climates) could benefit from *P. apium* supplementation. We therefore distributed freeze dried *P. apium* to beekeepers in these climates and asked whether beekeepers saw increased survival of their hives through winter if *P. apium* was provided to their hives in the fall, when it would normally be found in the pollination environment and collected by bees.

Methods: Information on this project that we named the "*P. apium* Project" was distributed to beekeepers through talks at the American Beekeeping Foundation and American Honey Producer's Annual Convention in 2016 and various listserv groups, including "Catch the Buzz" and "The Bee Informed Partnership (BIP)". In these initial solicitations, beekeepers were directed to a project website. From these announcements, we obtained interest from 193 beekeepers who were interested in participating. Interested participants were then asked to fill out an online spreadsheet (Appendix A). This led to a smaller list of 39 beekeepers. From this list, we recruited 12 beekeepers from 10 states (ID, WI, MN, KS, PA, NY, NJ, GA, NC, and TX), who were responsive to our emails and stated that they could test the treatments on a minimum of 8 hives each, yielding a total of 152 hives for the study. In our initial online surveys, we could not obtain good information about *Nosema* incidence from beekeepers. This could be because *Nosema*, especially the more prevalent *N. ceranae*, is difficult to diagnose. Therefore, we could not target our study towards beekeepers with high levels of *Nosema* in their hives as we had originally hoped. Instead, we decided to select more beekeepers from areas where *Nosema* is an issue (cooler, temperate areas) in order to target hives that could benefit more from the treatment. Beekeeper hives were divided equally into treatment (*P. apium*) and control groups. Each beekeeper was given 4 vials per hive of freeze dried *P. apium* (the treatment) and 4 vials per hive of freeze dried inoculum that contained only freeze drying medium and no *P. apium* (negative control). Both of the vials looked identical, except for being labeled green (negative control) or pink (*P. apium*). The participating beekeepers were also provided with a "kit" containing all of the materials needed for reconstituting the vials along with detailed written instructions for reviving the cultures and adding it to patty. Written and video instructions were also provided on the *P. apium* Project website. Detailed instructions that were sent to beekeepers are provided in Appendix B. Beekeepers provided their own patties to inoculate with the treatment or control. All participants stated that the directions were easy to follow. Participants were asked to provide the treatments in patties to each hive for 4 weeks in the fall when they would normally feed in preparation for winter. Participants were also asked to enter data about the status of their hives pre- and post- treatment on a private online spreadsheet provided on the *P. apium* Project website. Pre-treatment data related to hives in the late summer and fall, while post-treatment data related to hives in the spring. The questions we asked for each hive were:

1) Pre-treatment questions: brood levels (above average, average, below average), adult population (frames of bees), any known disease (yes/no, if yes what type), Varroa (yes/no), using natural pollen patty or pollen substitute.
2) Dates the treatments were added in the fall (should be 4 times)
3) Were the directions clear? Were you able to follow the directions given the components of the kit?
4) Post-treatment questions:
   a. Overall, did you think that one treatment group did better than the other? If so, which one (pink or green)?

b. Post-treatment time point #1 (ideally in the spring): date of assessment, brood levels (above average, average, below average), adult population (frames of bees), any known disease (yes/no, if yes what type), Varroa (yes/no)
c. Post-treatment time point #2 (ideally in the spring): date of assessment, brood levels (above average, average, below average), adult population (frames of bees), any known disease (yes/no, if yes what type), Varroa (yes/no)

Results: Twelve beekeepers were sent *P. apium* treatment (pink) and control (green) vials. One beekeeper with 8 colonies returned a complete questionnaire, however this beekeeper experienced a 100% loss that winter and so could not say whether one treatment was more beneficial than the other. Five additional beekeepers with 80 hives reported qualitative information about their success rates (whether the *P. apium* did better than the control) but no detailed information on hive strength. Six beekeepers (64 hives) did not respond with data. Of the five beekeepers that responded, 2 reported no difference (with 52 hives), 2 (with 20 hives) reported that the hives fed the control performed better than hives fed *P. apium*, and 1 beekeeper (with 8 hives) reported higher survival for colonies fed *P. apium*. None of the beekeepers that were sent vials indicated that the directions were unclear; several responded that the directions were clear and easy to follow. Overall, this experiment provided no indication about whether hives fed freeze dried *P. apium* fared differently than control-fed hives.

Aim #4: When *P. apium* is freeze dried and mixed into natural pollen patty, do foragers from *P. apium*-treated hives contain fewer *Nosema* in their guts than foragers from untreated hives? One of the main results from the 2014 study was that bees collected from hives supplemented with *P. apium* had lower (~42%) levels of *Nosema* when challenged with a controlled dose compared to bees from un-supplemented hives (Corby-Harris, 2015; Corby-Harris et al., 2016). We repeated these experiments with freeze dried *P. apium* to determine whether they were also beneficial to the bee with respect to *Nosema* resistance.

Methods: The freeze dried *P. apium* (or the negative control containing no *P. apium*) were added to natural pollen patty as described above. 10 hives were supplemented with *P. apium* (5 hives) or the negative control (5 hives) for 6 weeks in June and July of 2017. During this period, one supplemented hive lost their queen and was removed from the study. From each of the remaining 9 hives, frames of emerging brood were cleared of adults and placed into a temperature and humidity controlled room. Bees were allowed to emerge overnight, for approximately 15-17 hours. These bees were collected and kept on the bench top in a covered bin at room temperature for an additional ~4 hours. (Prior experience suggests that this increases the bees' appetite for the inoculum.) The bees were then inoculated with 2 µl of a 50% sucrose that contained 5,000 spores/µl of *Nosema*, for a total of 10,000 spores fed per bee. Approximately 200 bees were inoculated with the treatment and 200 with the control. The inoculated bees were distributed in groups of 50 bees into plexiglass cages and were fed water, 30% sucrose w/v in water, and natural pollen patty *ad libitum* (Corby-Harris et al., 2016). In total, there were 4 cages of each treatment. Nine days later, at 10d of age, the bees were sacrificed and their guts were dissected. *Nosema* levels were measured for each individual as described in (Cantwell, 1970). The data were analyzed with a GLM using the Poisson distribution and the log link function, accounting for overdispersion. Treatment and cage

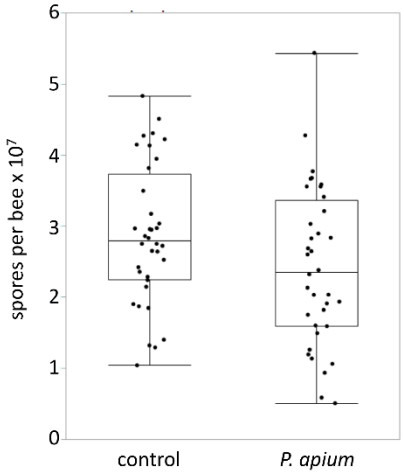

Figure 3. *Nosema* spores per bee for individuals from hives supplemented with freeze dried and then revived *P. apium* or a negative control consisting of medium alone. Bees were fed 10,000 spores at 1d of age and sacrificed for spore counts 9 days later. There was a significant effect of treatment on spore count ($p = 0.0125$).

nested within treatment were treated as fixed effects.

Results: *Nosema* levels differed significantly among cages nested within treatment ($X^2_6 = 41.86$, $p < 0.0001$) and also due to treatment ($X^2_1 = 6.24$, $p = 0.0125$). The average number of *Nosema* spores decreased 15.7% in bees from hives supplemented with *P. apium* compared to the negative control (Figure 3; average number of spores in control bees: 28,761,806 ± 1,632,488 S.E., average number of spores in *P. apium*-fed bees: 24,253,472 ± 1,860,608 S.E.). This was less than the ~42% decline in *Nosema* titer observed when bees were from hives supplemented with fresh *P. apium* as part of our 2014-2015 study (Corby-Harris, 2015; Corby-Harris et al., 2016).

Conclusions

1) *P. apium* can be freeze dried and subsequently revived. Freeze drying is a stressful process for bacteria and can affect different species in various ways. Indeed, we observed that freeze drying itself incurred an appreciable stress on *P. apium*. Mortality was approximately 83.5%. We suspect that this mortality could be lowered with future adjustments to the freeze drying protocol.
2) Freeze dried and then revived *P. apium* survives in pollen patty. Freeze dried *P. apium*, for the most part, did not survive the initial stress of being added to the pollen patty very well. However, the we recovered about the same number of freeze dried *P. apium* as fresh *P. apium* after a 24h incubation in the patty. This suggests that those freeze dried bacteria that do survive the initial stress of being added to the patty survive as well as their fresh counterparts after 24h.
3) *P. apium* did not provide a benefit to beekeeper hives. Many beekeepers expressed a willingness to help us with our study. After factoring in the limitations of the study, we distributed equal amounts of freeze dried *P. apium* and a control inoculum to 12 beekeepers around the U.S. in 10 states. At the conclusion of the study, we had a response rate of ~50%, or ~58% of the intended hives in the study. The beekeeper responses did not indicate any difference between the treatments that would suggest that *P. apium* provided a benefit in hives. Many factors could explain this, most notably that the *Nosema* levels in the hives that were selected for the study may not have been that high. More work must be done to determine whether *P. apium* is beneficial in beekeeper tended hives.
4) Freeze dried and then revived *P. apium* helps adult workers resist *Nosema*. When challenged with a fixed dose of *Nosema*, bees from supplemented hives were more able to resist *Nosema* than bees from un-supplemented hives. We note, however, that the benefit of freeze dried *P. apium* was much lower than that observed when fresh *P. apium* was added to hives in earlier studies.

Future directions: At this point, we cannot say whether freeze dried *P. apium* could benefit the beekeeping community. Freeze drying was obviously harmful to the bacteria, resulting in significant mortality. Survival of freeze dried *P. apium* could likely be improved with further adjustment of the freeze drying protocol. This is something to look into in future efforts. *Nosema* prevalence in supplemented hives was significantly less than unsupplemented hives, but these supplemented bees did not perform as well as earlier studies using fresh *P. apium* in the patty. This could be due to the freeze drying, which may have attenuated the actions of the bacteria at some level. We did not observe any additional mortality in pollen patty due to the freeze drying protocol. This suggests that the differences in *Nosema* resistance between fresh and freeze dried supplemented hives was probably not due to differences in inoculation. And finally, our successes working with beekeepers was mixed. We appreciated the highly positive initial response to our project, but our ultimate response rate was lower than we expected and possibly too low to conclude whether *P. apium* was beneficial. We understand the numerous pressures that beekeepers are under and that their circumstances change often. We hope that the beekeeping community will be willing to work with us in the future, where we will take these lessons into account. Overall, we hope that this work will continue. These initial steps provided a great deal of information that we hope can be used to further refine this method so that *P. apium* can be used by beekeepers in the future.

References

Anderson, K.E., Carroll, M.J., Sheehan, T., Mott, B.M., Maes, P., Corby-Harris, V., 2014. Hive-stored pollen of honey bees: many lines of evidence are consistent with pollen preservation, not nutrient conversion. Molecular Ecology 23, 5904-5917.

Cantwell, G.E., 1970. Standard methods for counting Nosema spores. American Bee Journal 110, 222-223.

Corby-Harris, V., 2015. Report to the National Honey Board on FY 2014 project entitled "Probiotic use of Acetobacteraceae Alpha 2.2 for improving honey bee colony health".

Corby-Harris, V., Snyder, L., Meador, C.A., Naldo, R., Mott, B., Anderson, K.E., 2016. *Parasaccharibacter apium*, gen. nov., sp. nov., improves honey bee (Hymenoptera: Apidae) resistance to *Nosema*. Journal of Economic Entomology 109, 537-543.

Corby-Harris, V., Snyder, L.A., Schwan, M.R., Maes, P., McFrederick, Q.S., Anderson, K.E., 2014. Origin and effect of Alpha 2.2 Acetobacteraceae in honey bee larvae and description of *Parasaccharibacter apium* gen. nov., sp. nov. Applied and Environmental Microbiology 80, 7460-7472.