National Honey Board
Final report

The probiotic potential of *Lactobacillus kunkeei* for honey production

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Objectives:

- Test the ability of *Lactobacillus kunkeei,* administered as a probiotic in supplemental feed, to increase colony productivity
- Test the ability of *Lactobacillus kunkeei,* administered as a probiotic in supplemental feed, to increase individual bee survival.

Problem and its significance

The bacterium *Lactobacillus kunkeei* is commonly found in the honey bee crop, honey, corbicular pollen, and on the inner surfaces of a honey bee colony (Corby-Harris et al. 2014). The effects of *L. kunkeei* on colony health and individual bee survival, however, are unknown. *Lactobacillus kunkeei* and some of its relatives are known to inhibit the growth of the honey bee pathogens *Melissococcus plutonius* and *Paenibacillus larvae*, the causative agents of European and American foulbrood (Forsgren et al. 2010, Vásquez et al. 2012), and lactobacilli in general are used as food preservatives (Stiles 1996). Several *Lactobacillus* species are commonly associated with fermented foods that form a major part of human diets across the world (Walter 2008). We therefore hypothesized that supplementation with *L. kunkeei* will increase colony

productivity and individual bee survival.

Materials and Methods

We planned to feed probiotic *L. kunkeei* to honey bee colonies throughout the winter of 2014-2015. Due to overwintering colony losses at UC Riverside and delays in transfer of funds from University of California, Riverside (UCR) to the Carl Hayden Bee Research Center (CHBRC), however, we conducted a whole-colony experiment at UCR and replicated cage experiments at both UCR and CHBRC.

Whole colony experiment

For the whole colony experiment, we obtained 15 packages with Italian queens from C.F. Koehnen and Sons in April 2015. Before installing these packages, we assigned half to receive supplemental *L. kunkeei* and half to receive no supplemental microbes. To apply supplemental microbes we mixed 25 mL of liquid *L. kunkeei* culture per 1 L of sugar syrup added 1L of this mix to in-hive feeders as well as sprayed hive surfaces with this mix. For control colonies, we added 50 mL of sterile culture per 1 L sugar syrup and followed the same protocol. To continue the treatments, we fed sterile of *L. kunkeei* inoculated sugar syrup in April, May, and June. We added the same amount of sugar syrup to each colony, and also weighed all components of the hove bodies so that we could accurately measure colony growth.

To measure colony productivity, we weighed each colony at 6 time points from mid-April 2015- late-July 2015. To measure the number of adult bees in each colony, we took digital photos of each frame, and used imageJ to calculate the number of cm^2 of bees per frame (Delaplane et al. 2013). We used repeated measures ANOVA to test for differences between non-supplemented and *L. kunkeei* supplemented colonies.

Cage experiments

We conducted replicated cage experiments at UCR and CHBRC. We used the same methods for both experiments, but different strains of *L. kunkeei.* To characterize the *L. kunkeei* strains, we are currently sequencing their genomes. We have assembled and annotated one genome, while the second genome is currently in line to be sequenced. Once the genome sequences are in hand, we will determine how different the strains are, and identify genes that encode proteins that may be either beneficial or harmful to the host.

We randomly assigned each cage to one of four treatments: No bacteria, 10^2 colony forming units of *L. kunkeei* (CFUs) per gram of supplemental protein patty, 10^3 CFUs/g and 10^4 CFUs/g. To apply these treatments, we fed bees protein supplement (BeePro) laced with bacteria. To prepare these protein patties, we used slightly different methods. At CHRBC, we stored a master stock of a *L. kunkeei* in ~300 separate freezer stocks (1ml MRS media and 20% glycerol). Each freezer stock contained the same amount of bacterial CFUs/ml (10^7). Prior to incorporation into the protein supplement the freezer stocks were thawed, spun down to pellet the bacterial cells, and the nutrient rich media was decanted. The bacterial pellet was resuspended in 1ml of a sterile 50% sugar solution (25% sucrose and 25% fructose) and added into the sugar solution that was used to form the protein patty. At UCR, we used similar methods, except that we used fresh cultures.

To obtain newly emerged bees we took capped brood frames were taken from 10 healthy colonies at Carl Hayden Honey Bee Research Facility in Tucson AZ and 8 healthy colonies at UCR. We housed the developing pupae (capped brood frames) in a room (CHRBC) or incubator (UCR) controlled for humidity (~50%) and temperature (~35˚ C) and allowed the pupae to emerge naturally overnight. We randomized individuals sourced from different colonies, homogenizing newly emerged individuals in a single container following emergence. We then

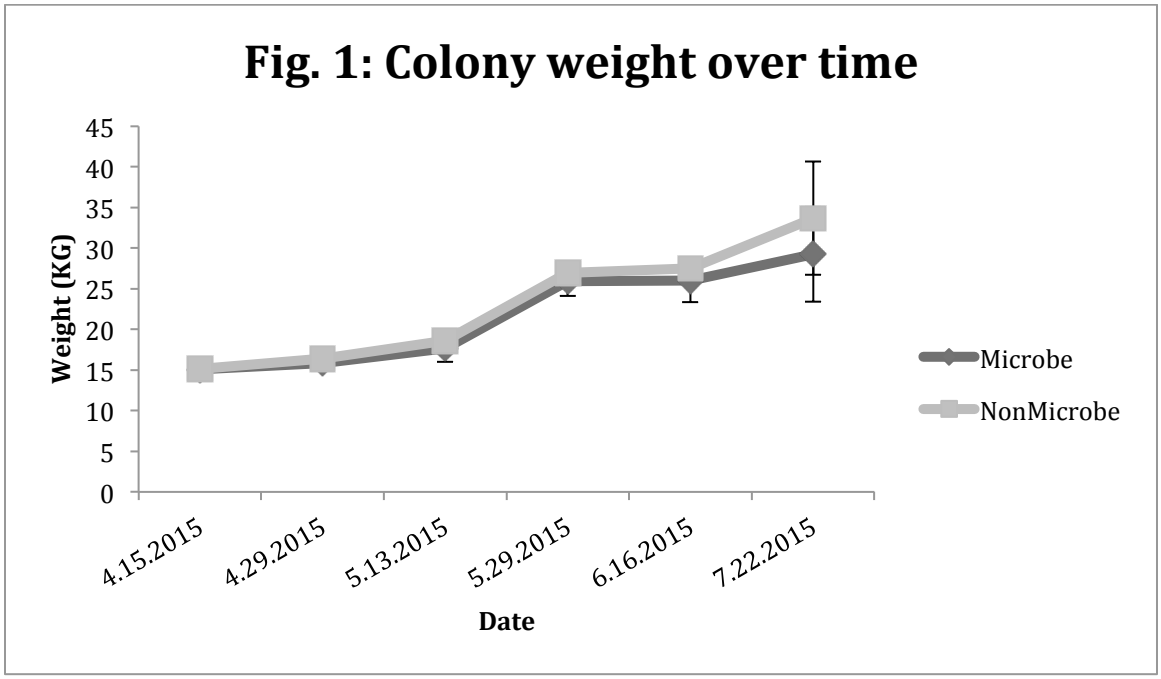
randomly selected 100 newly emerged bees from the larger group and placed them into cages. Each treatment consisted of 10 replicates for a total of 40 cages and 4000 bees at CHRBC and 12 replicates per treatment for a total of 48 cages and 4800 bees at UCR.

We supplied each cage with fresh, treated protein supplement every seven days until the end of the experiment. We checked mortality on a daily basis (at approximately the same time each day) until all of the caged bees had perished. We analyzed the resulting data using Kaplan Meier Survival Analysis.

Results

Whole colony experiment.

Lactobacillus kunkeei supplementation had no significant effect on colony-level fitness. Colony weight did not significantly differ between treatments, although at the end of the experiment the average weight of colonies that did not receive supplemental *L. kunkeei* was slightly higher than those that did (Fig. 1, $F_{1,68} = 3.559$, $P = 0.062$).



Cage experiment.

In the replicate of the cage experiment conducted at CHBRC, *L. kunkeei* supplementation significantly decreased survivorship (Fig. 2, Log-rank- $X^2_{3,1445} = 20.65$, $p < 0.0001$, Wilcoxon- $X^2_{3,\ 1445} = 12.48$, $p < 0.0059$. Caged bees avoided eating protein patties supplemented with *L. kunkeei* ($F_{3,36} = 0.78$, $p < 0.0001$). In the replicate experiment conducted at UCR, *L. kunkeei* supplementation had no significant effect on survivorship (Figure 3, $X^2_3 = 5.2$, $P = 0.16$).

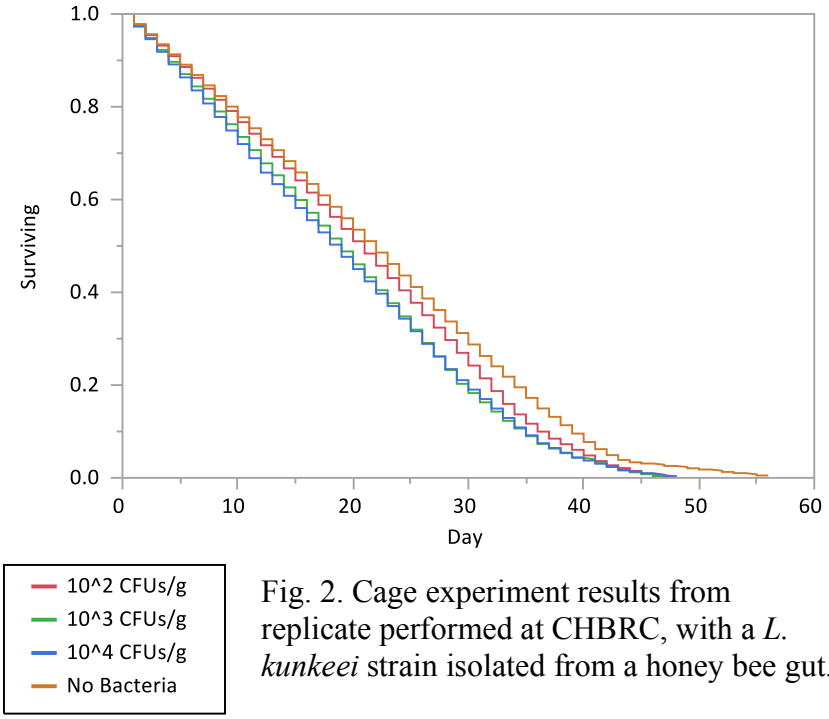
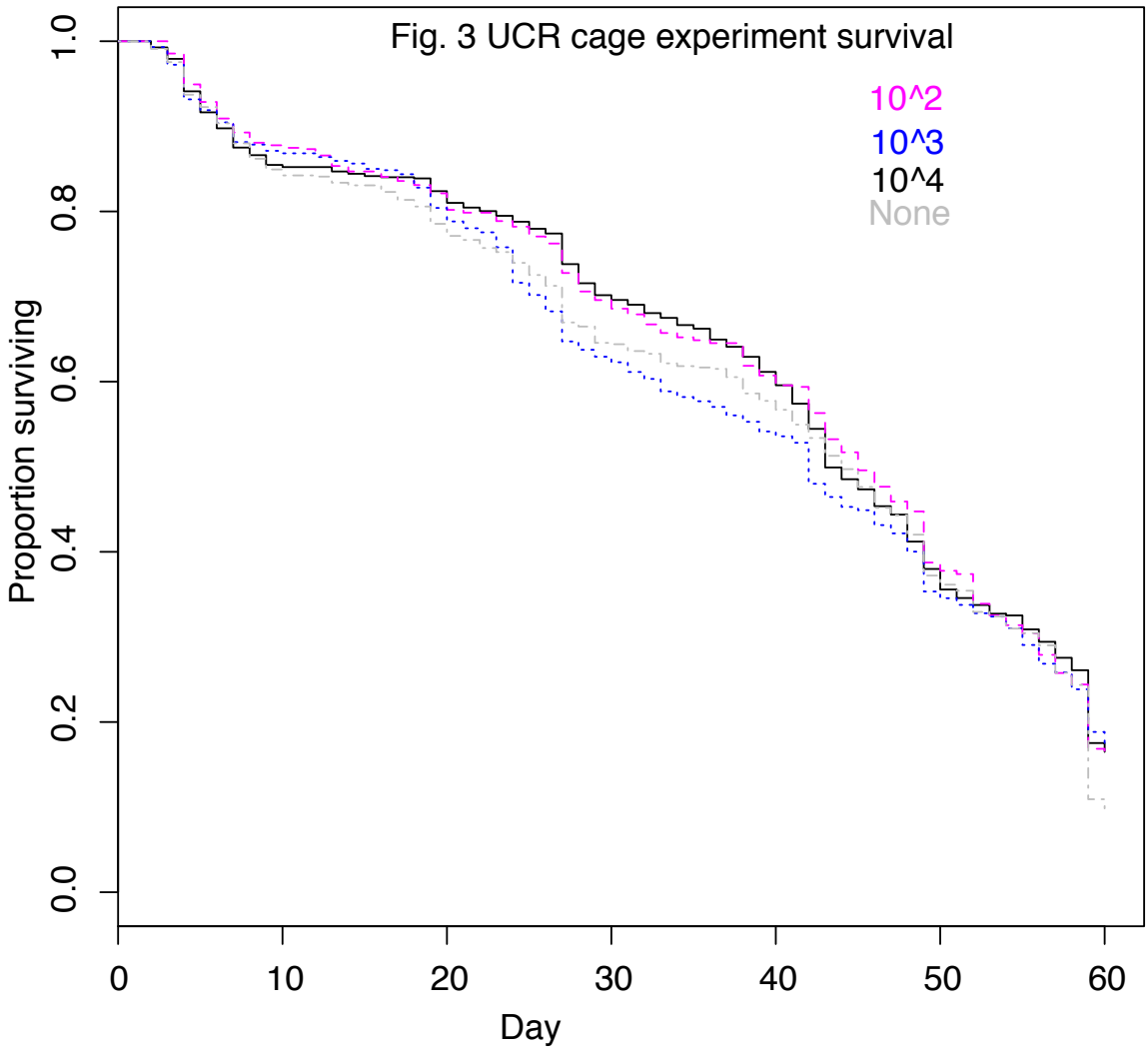


Fig. 2. Cage experiment results from replicate performed at CHBRC, with a *L. kunkeei* strain isolated from a honey bee gut.

Discussion

Contrary to our expectations, *L. kunkeei* supplementation either had no effect on honey bee colony growth and survival or had a negative effect on honey bee survival. We currently do not have an explanation regarding why one strain negatively affected honey bee survival while a different strain had no effect. We are eagerly awaiting our genome sequencing results, in the hopes that these data will provide insight into whether certain strains of *L. kunkeei* contain genomic features of pathogenic bacteria while others do not.

Our results do not suggest that we should completely abandon *L. kunkeei* as a possibly beneficial bacterium for honey bees. Our data do, however, show that the *L. kunkeei* strain matters. One of our strains appears to be possibly pathogenic, while the other has no effect on



bee health. While it is entirely possible that beneficial *L. kunkeei* strains exist in nature, our data caution against placing untested strains in colonies as supplements. Careful experiments are necessary before any bacterial treatment can be recommended as a honey bee probiotic.

Literature Cited:

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