AIP P199-CRSAD-Activity 2: BeeProbio: Sustainable Improvement of Honey Bee Health and Productivity with Probiotics

Final Report from AAFC Beaverlodge 2015-16 – S. Pernal

Summary:

Bactocell® is a probiotic marketed by Lallemand, Inc. that is currently used as a feed additive in animal systems for the prevention of disease. The lactic acid bacteria in this product have been shown to prevent vertebral compression syndrome in fish, and have been shown to improve resistance to *Vibrio spp.* infections and increase overall survival in shrimp. Experiments were conducted to determine if Bactocell® had a positive impact on honey bee health, and whether or not it could act as a viable treatment option for the fungal parasite, *Nosema ceranae.* Based on analysis of survival data, caged honey bees fed Bactocell® in sugar syrup exhibited slightly higher mortality than honey bees fed untreated sugar syrup. For bees inoculated with *N. ceranae,* there was no significant difference in survival among untreated bees and bees treated with Bactocell® therapeutically, but slight improvement in survival when a combination of prophylactic and therapeutic applications were used in the same treatment. Impacts on survival from this study must be weighed against the concurrent effectiveness of *N. ceranae* suppression and effects on resident gut microflora. These latter analyses, employing high-throughput next-generation sequencing and qPCR techniques, will be completed by our project collaborators at Laval University.

Introduction:

In North America, control protocols for opportunistic pathogens of honey bee colonies recommend the use of antibiotics (Alippi et al. 2005). Nevertheless, the causative agents of the major opportunistic diseases (*Nosema* disease, American foulbrood, and European foulbrood) are becoming increasingly resistant to the antimicrobial treatments that are currently in use (Evans et al. 2003; Tian et al. 2012; Huang et al. 2013). In addition, some of those treatments promote mortality of brood and young bees (Thompson et al. 2005). Consequently, there is an urgent need to develop effective, sustainable, and specific alternative strategies for controlling the diseases responsible for major bee colony losses. There is no question that the probiotic approach satisfies those requirements fully. According to the World Health Organization (WHO) and the Food and Agriculture Organization of the United Nations (FAO), probiotics are "live micro-organisms which, when administered in adequate amounts, confer a health benefit on the host, beyond the common nutritional effects."

In honey bees, it is suspected that gut bacteria have an effect on both nutrition and defence against pathogens (Engel et al. 2012). Recent studies have highlighted the presence of two bacterial genera, *Lactobacillus* and *Bifidobacterium* (Martinson et al. 2012; Moran et al. 2012; Vasquez et al. 2012; Mattila et al. 2012), which are heavily used in veterinary medicine for their probiotic activity in other animal systems (Fang et al. 1996; La Ragione et al. 2004; O'Mahony et al. 2005; Dowd et al. 2008; Gatesoupe 2008). The same phenomenon has been found in *Bombus* species, in which the gut flora transmitted by faeces protects against the trypanosome *Crithidia bombi* (Koch et al. 2011). Many pathogens take advantage of a physiological disorder in the host or a change in the gut microbiome, known as dysbiosis, to become virulent. The

concept of dysbiosis is also recognized in many cases of pathogenesis in bees (Hamdi et al. 2011; Cornman et al. 2012) and the use of probiotic bacteria is a preferred method for controlling pathogens in such cases (Sokol et al. 2008).

Probiotics are increasingly recommended because of their many advantages over conventional treatments (Courvalin 2006). In bees, the use of a mixture of different commercial strains of *Lactobacillus* and *Bifidobacterium* bacteria has already been shown to provide beneficial effects in terms of activation of the immune response against the pathogen *Paenibacillus larvae* (Evans et al. 2004). In addition, the use of intestinal lactic acid bacteria from bees was shown *in vitro* to have antagonistic effects against European foulbrood (Vasquez et al. 2012). Lastly, a study in *Apis cerana* found that a high prevalence of the microsporidian *Nosema ceranae* was correlated with dysbiosis of the host's gut microflora, characterized by a significant decrease in lactic acid bacteria of the genus *Bifidobacterium* (Li et al. 2012). That result suggests that supplementing with probiotic *Bifidobacterium* strains should help prevent *Nosema* infections by maintaining or re-establishing the homeostasis of the gut microflora in bees.

Bee-specific candidate probiotics are safe in terms of human consumption as well as the environment, and have the potential to form a cornerstone of a new Integrated pest management strategy for beekeeping in Canada. In this study, the utility of using a commercially-produced probiotic against the microsporidian parasite *N. ceranae,* a factor associated with colony losses in Canada and in other parts of the world, will be examined.

Objective:

The objective of this study is to compare the *in vivo* effectiveness and target animal safety of a probiotic bacterial strain marketed by Lallemand Inc., namely Bactocell®, against the microsporidian parasite, *Nosema ceranae*.

Methods:

A. Collection of bees.

Frames of sealed brood with newly-eclosing bees (*Apis mellifera*) were obtained from eight healthy colonies at Agriculture and Agri-Food Canada's Research Farm, in Beaverlodge, Alberta, (55° 18' N; 119° 17' W). Colonies were repeatedly tested to be free from both *N. apis* and *N. ceranae*, using light microscopic and molecular methods, described in Section C. Frames were kept in an incubator (Percival Model 136NLC9, Percival Scientific Inc., Perry, IA, USA) and maintained at hive temperature (33±0.5°C) and relative humidity (70±5%). After 24 h, newly-emerged adult workers were shaken into a wooden nucleus box that was previously disinfected with NaClO (10% household bleach) with the inner sides of the box lined with aluminum foil. The nucleus box containing the newly-emerged bees was maintained in an incubator for 48 h and was provisioned with a frame containing freshly-capped honey.

B. Caging of bees.

When bees were 3 days old, the nucleus box was removed from the incubator and the bees shaken into a sterilized plastic bin. One hundred and twenty-five bees were then counted into individual wooden screened cages (8.0 x 9.5 x 12.0 cm I.D.). Cages were assigned randomly into each treatment group and were fed treatments according to the protocol (Table 1). Each of the 10 treatments was replicated 6 times with the duration of the experiment being 29 d.

C. Preparation of Inoculum.

After collecting workers from *N.* ceranae-infected colonies, abdomens were separated from the remainder of the bodies and placed in a plastic stomacher bag with 1ml distilled water added per bee. Abdomens were then macerated for 1 min at medium speed using a stomacher (Seward Stomacher® 80 Biomaster, Seward Laboratory Systems Inc., Davie, FL, USA) with the resulting crude suspension filtered through honey straining cloth into a 15 ml disposable centrifuge tube.

For determination of spore concentration, 6 µL of spore macerate was loaded onto a Helber Z30000 counting chamber (Hawksley, Lancing, UK), and counted according to the generalized methods of Cantwell (1970), under phase contrast microscopy at 400× magnification.

For confirmation of *Nosema* species, DNA extraction was performed using the DNeasy® Blood & Tissue Kit (Qiagen®, Valencia, CA, USA). The concentration of the extracted DNA was determined spectrophotometrically (NanoDrop 2000C, Thermo Scientific, West Palm Beach, FL, USA), after which 50-100 ng was amplified using polymerase chain reactions (PCR).

A multiplex system that co-amplified the 16S rRNA gene of *N. apis* and *N. ceranae* (Martín-Hernández et al. 2007) as well as the honey bee ribosomal protein RpS5 gene (Thompson et al. 2007) was used within the same reaction. All PCR reactions were performed using a Mastercycler® proS thermocycler (Eppendorf, Mississauga, Canada) and utilizing the Illustra™ PuReTaq Ready-To-Go™ PCR beads (GE Healthcare Life Sciences, Baie d'Urfe, Quebec, Canada). PCR beads were reconstituted to a 25 µL final volume by adding sterile H_2O, 0.5 µL of 20 mM forward and reverse primers (a final concentration of 0.4 mM) and the DNA. All PCR products were visualized on a 2% agarose gel and stained with SYBR® Safe DNA gel stain (Life Technologies, Carlsbad, CA, USA).

D. Mass inoculation:

Cages containing bees were randomly assigned to each treatment group and, where required, mass inoculated with freshly prepared *N. ceranae* macerate. Cages were stocked with 125 worker bees per cage and inoculated with 6,250,000 spores (50,000 per bee). Suspensions were prepared in 5 mL (1:1 w:w) sucrose syrup, administered via gravity feeders fashioned from disposable 15 mL centrifuge tubes (Cat. #93000-020, VWR International, Radnor, PA, USA). Timing of spore inoculations followed Table 1.

E. Bactocell® Preparation:

To achieve a concentration of 10^9 cell/ml, we dissolved 0.1 g Bactocell ($1*10^{10}$ CFU/g) per mL of 1:1 (w:w) sugar syrup. The treated or untreated sugar solutions were prepared weekly, and applied as required. At each replenishment, 10 mL of the Bactocell preparation or unformulated sugar syrup was administered to the caged bees using gravity feeders.

F. Variables Monitored:

Bee mortality was recorded every other day starting at day 0 (the day bees were caged), until the experiment was terminated on day 28. Dead bees were stored at -80°C.

To follow the progress of the *Nosema* infection in bees throughout the experiment, 20 live bees were collected from each cage into 15 ml disposable centrifuge tubes on days 7, 14, 21, and 28 and were stored at – 80°C.

The effectiveness of the treatments will be confirmed by examining the prevalence and abundance of bacterial microflora and *Nosema ceranae* parasites collected from the guts of bees, using high-throughput next-generation sequencing and qPCR techniques. This work will be completed by collaborator Dr. Nicolas Derome of Laval University.

Results:

Raw data for the cumulative mortality of bees in all treatments can be found in the attached file, "Beaverlodge 2015 Probio Cumulative Mortality.xlsx". Data from three cages, across different treatments, have been censored from the dataset because of uncontrolled losses of bees from cages or feeder leakages leading to bee mortality.

Cumulative mortality across the ten treatments is displayed in Fig. 1. Using survival analysis, significant differences in worker bee survival was detected among all treatments [Log-rank (Mantel-Cox): $\chi^2=594.5$; df=9; $P=0.0001$].

Other trends observed in the data were that the neutral control, containing no Bactocell, had greater survivorship than the either the continuously administered Bactocell control treatment ($\chi2=35.67$; df=1; $P<0.0001$) or the prophylactic Bactocell control treatment ($\chi2= 238.9$; df=1; $P<0.0001$) (Fig. 2). Hence, Bactocell consumption appears to have a slight negative effect on worker bee survivorship.

In examining the effect of *Nosema* inoculation on survivorship (Fig. 3), bees in control treatments fed only sugar syrup with no *Nosema* spores had greater survival compared with that of bees infected with *N. ceranae* on day 0-1 ($\chi2=208.9$; df=1; $P<0.0001$) or infected on day 7-8 ($\chi2= 117.1$; df=1; $P<0.0001$). This resulted was anticipated, as *N. ceranae* infection is known to reduce the lifespan of workers. Similarly, the control treatment containing continuous Bactocell administration also had greater survivorship than the two nosema-inoculated control treatments.

Survival of untreated worker bees infected with *N. ceranae* spores on days 0-1 was found to be similar to that of workers infected with *N. ceranae* on days 0-1 and treated therapeutically with Bactocell ($\chi 2=3.575$; df=1; $P<0.0587$) (Fig. 4). This suggests little to no improvement in survival of infected bees after therapeutic applications of the product.

Finally, worker bees infected with *N. ceranae* spores on days 7-8 of the experiment that received a combination prophylactic and therapeutic treatment of Bactocell had improved survival over bees similarly infected, but receiving no treatment ($\chi 2=19.02.1$; df=1; $P<0.0001$) (Fig. 5).

Our results suggest that prophylactic treatments using Bactocell have a slight negative impact on worker bee survival for bees not infected by *N. ceranae,* but that for infected bees, under certain applications (i.e a combination of pre- and post-infection treatment), the product can contribute to improved survivorship

Based on our previous work with *N. ceranae* infections, we observed considerably less mortality by the end of this experiment than when we have used an infective dose of 100,000 spores per bee. Future studies might consider this higher spore dose as it may prove to be more discriminatory.

These data will be combined with those from qPCR, when available, to determine effects on *Nosema* reproduction in treatments.

Personnel:

During this project, resources were used to hire a coop summer student, Mr. Justin Mufford from the Thompson Rivers University who assisted with all aspects of the experiment as well as general colony maintenance. Conduct of the experiments was supervised by AAFC technician Dr. Abdullah Ibrahim, from the apiculture program at Beaverlodge Research Farm.

Budget:

A budget report for the use of industry funds is attached. See file "AIP-P199 Expenditure Report 2015-16".

The industry portion of this project for which CRSAD is responsible totals $20,375, which has fully been expended. Industry funds have been spent according to the following non-pay operating budget sub-categories:

	Expended	Budget Allocation
Overhead:	$ 2,657	$ 2,531
Variable Costs:	$ 844	$ 970
Salary (for Student):	$12,038	$10,352
Travel:	$ 4,354	$ 6,522
Supplies:	$ 482	$ 0
TOTAL:	$20,375	$20,375

Justification of Expenditures:

Overhead / Variable Costs: Institutional

Salary: Cost of employing one co-operative education summer student from May-August 2015.

Travel: Cost for travel to two scientific meetings:

1) Joint meeting of Canadian Association of Professional Apiculturists and Saskatchewan Beekeepers Association, Saskatoon 2-5 December 2015, Saskatoon, SK. Presentation of project results in CAPA Research Symposium by collaborator Giovenazzo and project-related meetings.
2) American Bee Federation Conference and Tradeshow and American Bee Research Conference, 5-9 Jan 2016. Meetings with USDA colleagues working with probiotics for the control of *Nosema* spp. to review project results and compare findings.

Supplies: Miscellaneous molecular biology consumables used for identification of *N. ceranae.*

Variances: No change in overall budgeted NPO expenditures occurred. Small deviations from nominal budget allocation sub-categories within the overall NPO envelope occurred, in that more funds were spent on student support ($+1,686), less than projected on travel ($-2,168) and a minor amount was incurred for lab consumables ($+482).

Outputs:

Giovenazzo P, Derome N, Pernal SF (2015) BeeProbio: Improving Honey Bee Health with Probiotics. *Canadian Association of Professional Apiculturists Research Symposium and SBA Annual Convention*, 2-5 December 2015, Saskatoon, SK.

Table 1. Experimental Treatments.

Group	Description	N (cages)
1	**Control Neutral :** day 0 to 28 = sucrose syrup 1:1	6
2	**Control Bactocell:** days 0 to 28 = sucrose syrup 1:1 + Bactocell® 10^9 cell/mL,	6
2A	**Control Bactocell Prophylactic:** days 0-6= sucrose syrup 1:1 + Bactocell® 10^9 cell/mL; days 7-28 = sugar syrup 1:1	6
2B	**Control Bactocell Therapeutic:** days 0-13 = sucrose syrup 1:1 ; days 14-28 sucrose syrup 1:1 + Bactocell® 10^9 cell/mL	6
3	**Control Nosema 1 :** days 0-1= inoculation Nosema 50,000X125=6,250,000 spores / cage of 125 bees; days 2 to 28 = sucrose syrup 1:1;	6
4	**Control Nosema 2** : days 0 to 6 = sucrose syrup 1:1; days 7-8 = inoculation Nosema 6,250,000 spores / cage of 125 bees; days 9-28 sucrose syrup 1:1	6
5	**Therapeutic Bactocell 1 :** days 0-1= inoculation Nosema 6,250,000 spores / cage of 125 bees; days 2-13 sucrose syrup 1:1 ; days 14-28 sucrose syrup 1:1 + Bactocell® 10^9 cell/mL	6
6	**Therapeutic Bactocell 2 :** days 0 to 6 = sucrose syrup 1:1; days 7-8 = inoculation Nosema 6,250,000 spores / cage of 125 bees; days 9-13 sucrose syrup 1:1 ; days 14-28 sucrose syrup 1:1 + Bactocell® 10^9 cell/mL	6
7	**Prophylactic Bactocell 1 :** days 0-6 = sucrose syrup 1:1 + Bactocell® 10^9 cell/mL; days 7-8 sucrose syrup 1:1; days 7-8 = inoculation Nosema 6,250,000 spores / cage of 125 bees; day 9-28 = sucrose syrup 1:1	6
8	**Prophylactic Bactocell 2 :** days 0 to 6 = sucrose syrup 1:1 + Bactocell® 10^9 cell/mL; days 7-8 = inoculation Nosema 6,250,000 spores / cage of 125 bees; days 9-28 sucrose syrup 1:1 + Bactocell® 10^9 cell/mL	6

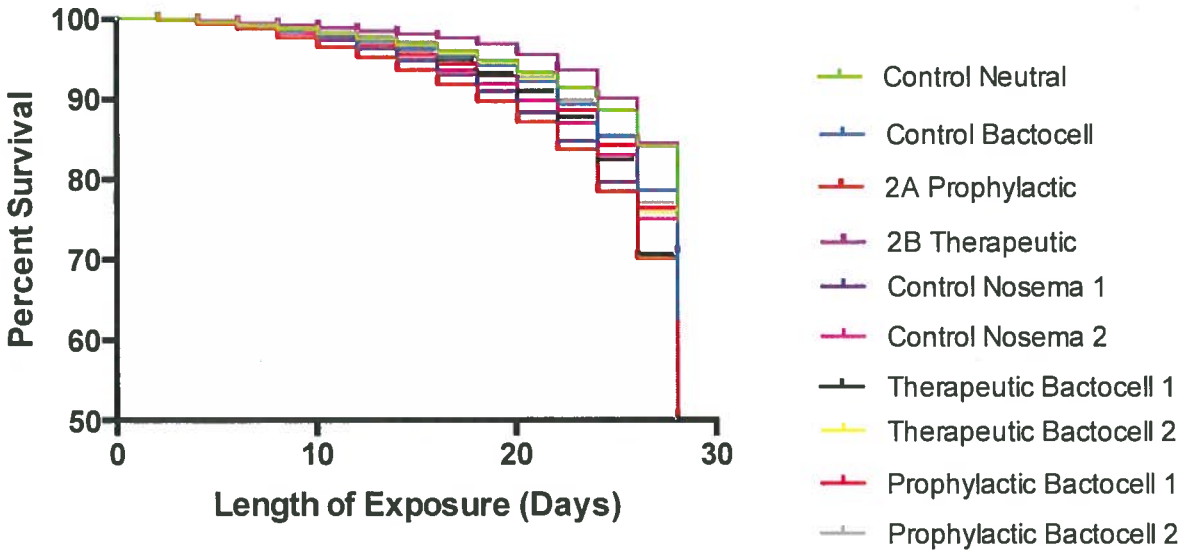

Figure 1. Survival of worker bees subjected to experimental treatments. (For explanation of treatments, refer to Table 1). Treatments had a significant effect on survival across all treatments [Log-rank (Mantel-Cox): $\chi 2=594.5$; df=9; $P=0.0001$].

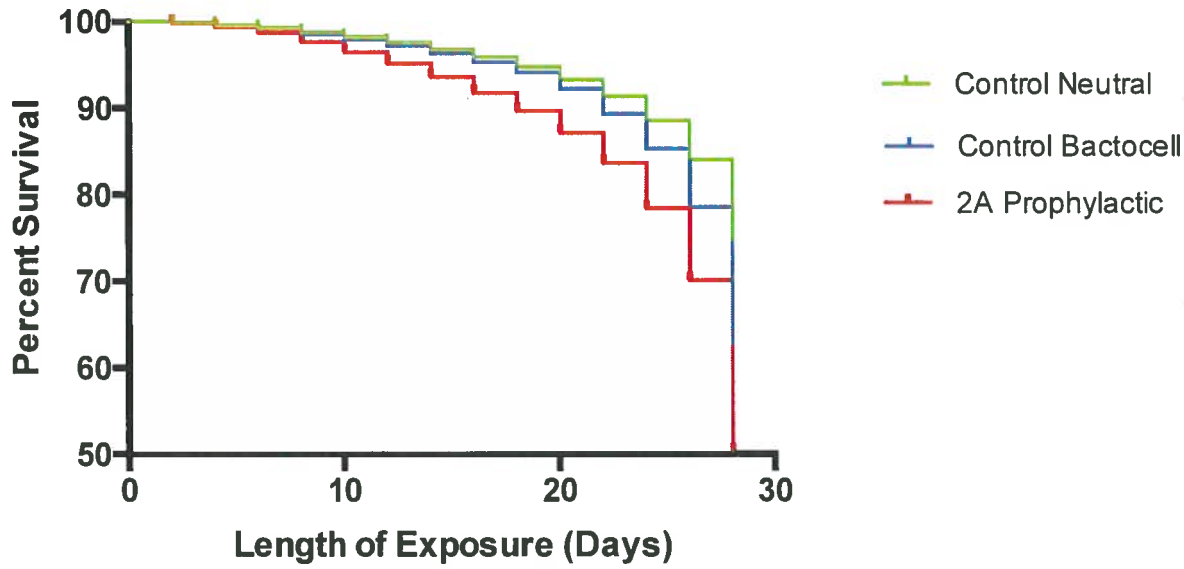

Figure 2. Survival of worker bees subjected to a control treatment of only sugar syrup (Control Neutral) compared with continuous Bactocell treatment (Control Bactocell) or prophylactic applications of Bactocell (2A Prophylactic). Survival in the neutral control treatment was superior to that of continuous Bactocell treatment ($\chi 2=35.67$; df=1; $P<0.0001$] or the prophylactic Bactocell treatment ($\chi 2= 238.9$; df=1; $P<0.0001$).

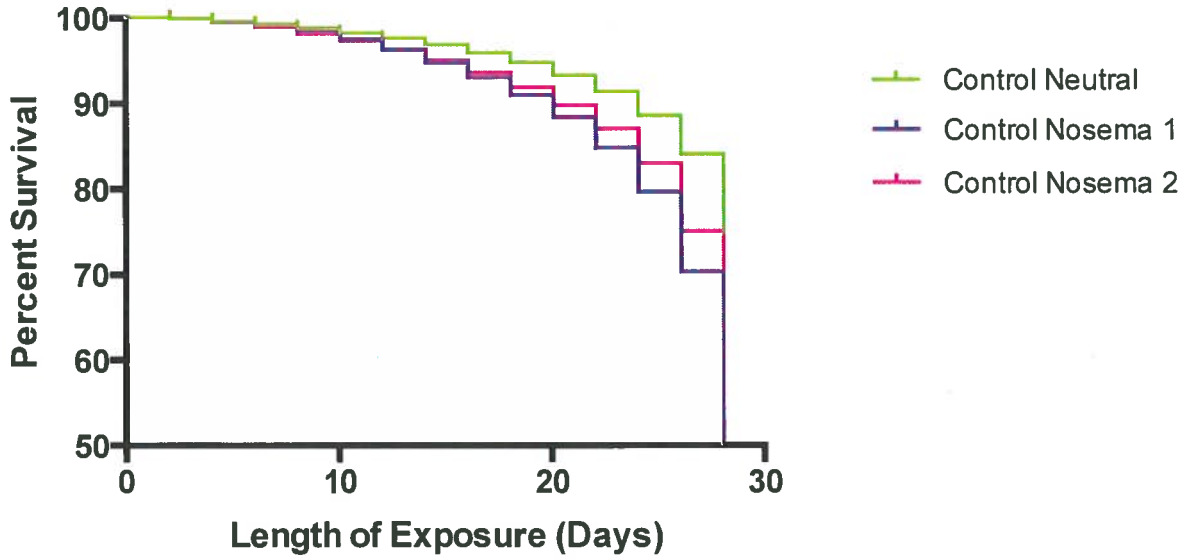

Figure 3. Survival of worker bees subjected to a control treatment of only sugar syrup (Control Neutral) compared with untreated workers infected with *N. ceranae* spores on days 0-1 (Control Nosema 1) or untreated workers infected on days 7-8 (Control Nosema 2). Survival in control treatments was superior to that of bees infected with *N. ceranae* on day 0-1 ($\chi 2=208.9$; df=1; $P<0.0001$) or those infected on day 7-8 ($\chi 2= 117.1$; df=1; $P<0.0001$).

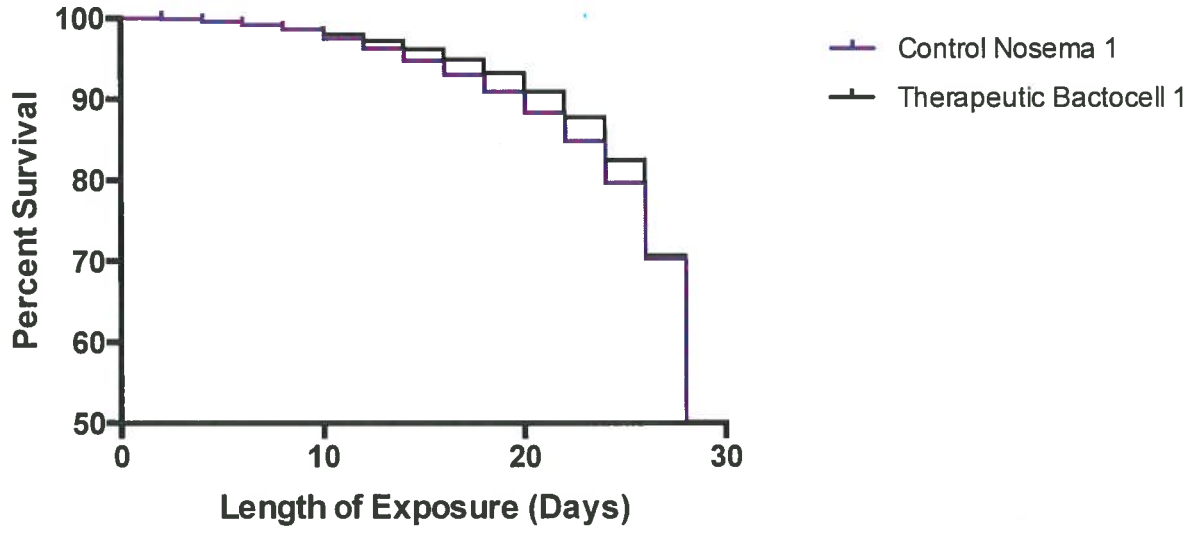

Figure 4. Survival of untreated worker bees infected with *N. ceranae* spores on days 0-1 (Control Nosema 1) compared with workers infected with *N. ceranae* spores on days 0-1 and treated with Bactocell therapeutically (Therapeutic Bactocell 1). Survival in the infected control treatment was similar to that of infected bees treated with Bactocell ($\chi 2=3.575$; df=1; $P<0.0587$). .

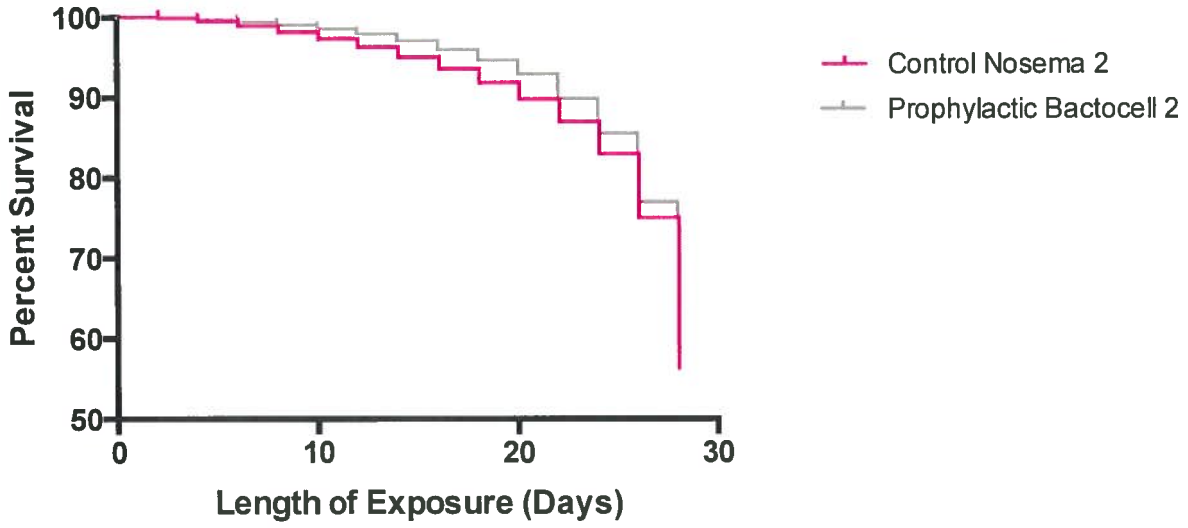

Figure 5. Survival of worker bees infected with *N. ceranae* spores on days 7-8 (Control Nosema 2) compared with bees similarly infected, but receiving a combination prophylactic and therapeutic treatment of Bactocell (Prophylactic Bactocell 2). Survival in of infected bees treated with Bactocell was greater than that of non-treated bees ($\chi 2=19.02.1$; df=1; $P<0.0001$).

References:

- Alippi MA, Albo NG, Ryenaldi JF, De Giusti RM (2005). *In vitro* and *in vivo* susceptibility of the honeybee bacterial pathogen *Paenibacillus larvae* subsp. *larvae* to the antibiotic tylosin. Vet. Microbiol. 109:47-55.
- Cantwell, GE (1970). Standard methods for counting nosema spores. Am. Bee J. 110, 222-223
- Cornman RS, Tarpy DR, Chen Y, Jeffreys L, Lopez D, Pettis JS, vanEngelsdorp D, Evans JD. (2012). Pathogen webs in collapsing honey bee colonies. PLoS One. 2012;7(8):e43562.
- Courvalin P (2006). Antibiotic resistance: The pros and cons of probiotics. Digestive and Liver Disease 38 Suppl 2: S261-265.
- Engel P, Martinson VG and Moran NA (2012). Functional diversity within the simple gut microbiota of the honey bee. Proceedings of the National Academy of Sciences 109: 11002-11007.
- Evans JD (2003). Diverse origins of tetracycline resistance in the honey bee bacterial pathogen *Paenibacillus larvae*. Journal of Invertebrate Pathology 83: 46-50.
- Evans JD and Lopez DL (2004). Bacterial probiotics induce an immune response in the honey bee (Hymenoptera: Apidae). Journal of Economic Entomology 97: 752-756.
- Fang W, Shi M, Huang L, Chen J and Wang Y (1996). Antagonism of lactic acid bacteria towards *Staphylococcus aureus* and *Escherichia coli* on agar plates and in milk. Veterinary Research 27(1): 3-12.
- Gatesoupe FJ (2008). Updating the importance of lactic acid bacteria in fish farming: Natural occurrence and probiotic treatments. Journal of Molecular Microbiology and Biotechnology 14(1-3): 107-114.
- Hamdi C, Balloi A, Essanaa J, Crotti E, Gonella E, Raddadi N, Ricci I, Boudabous A, Borin S, Manino A, Bandi C, Alma A, Daffonchio D and Cherif A (2011). Gut microbiome dysbiosis and honeybee health. Journal of Applied Entomology 135(7): 524-533.
- Huang WF, Solter LF, Yau PM, Imai BS. (2013). *Nosema ceranae* escapes fumagillin control in honey bees. PLoS Pathog. 9:e1003185.
- Koch H and Schmid-Hempel P (2011). Socially transmitted gut microbiota protect bumble bees against an intestinal parasite. Proceedings of the National Academy of Sciences 108(48): 19288-19292.
- La Ragione RM, Narbad A, Gasson MJ and Woodward MJ (2004). In vivo characterization of *lactobacillus johnsonii* fi9785 for use as a defined competitive exclusion agent against bacterial pathogens in poultry. Letters in Applied Microbiology 38(3): 197-205.
- Li J, Quin H, Wu J, Sadd BM, Evans JD, Peng W, Chen Y. (2012). The prevalence of parasites and pathogens in Asian honeybees *Apis cerana* in China. PLoS one 7:e47955.
- Mattila HR, Rios D, Walker-Sperling VE, Roeselers G, Newton IL. (2012). Characterization of the active microbiotas associated with honey bees reveals healthier and broader communities when colonies are genetically diverse. PLoS One. 2012;7(3):e32962.
- Martín-Hernández, R., Meana, A., Prieto, L., Salvador, A.M., Garrido-Bailón, E., Higes, M. (2007) Outcome of colonization of *Apis mellifera* by *Nosema ceranae*. Appl. Environ. Microbiol. 73, 6331-6338.
- Martinson VG, Moy J and Moran NA (2012). Establishment of characteristic gut bacteria during development of the honeybee worker. Applied and Environmental Microbiology 78(8): 2830-2840.

- Moran NA, Hansen AK, Powell JE and Sabree ZL (2012). Distinctive gut microbiota of honey bees assessed using deep sampling from individual worker bees. PLoS ONE 7(4): e36393.
- Morrow LE, Gogineni V, Malesker MA. (2012). Probiotic, prebiotic, and synbiotic use in critically ill patients. Curr Opin Crit Care. Epub ahead of print.
- Naug D. (2009). Nutritional due to habitat loss may explain recent honeybee colony collapses. Biol Conserv. 142: 2369-2372.
- O'Mahony L, McCarthy J, Kelly P, Hurley G, Luo F, Chen K, O'Sullivan GC, Kiely B, Collins JK, Shanahan F and Quigley EMM (2005). *Lactobacillus* and *Bifidobacterium* in irritable bowel syndrome: Symptom responses and relationship to cytokine profiles. Gastroenterology 128(3): 541-551.
- Sokol H, Pigneur B, Watterlot L, Lakhdari O, Bermúdez-Humarán LG, Gratadoux JJ, Blugeon S, Bridonneau C, Furet JP, Corthier G, Grangette C, Vasquez N, Pochart P, Trugnan G, Thomas G, Blottière HM, Doré J, Marteau P, Seksik P, Langella P. (2008). Faecalibacterium prausnitzii is an anti-inflammatory commensal bacterium identified by gut microbiota analysis of Crohn disease patients. Proc Natl Acad Sci U S A. 105:16731-16736.
- Thompson, G.J., Yockey, H., Lim, J., Oldroyd, B.P. (2007) Experimental manipulation of ovary activation and gene expression in honey bee (*Apis mellifera*) queens and workers: testing hypotheses of reproductive regulation. J. Exp. Zool. 307A, 600-610.
- Thompson HM, Waite RJ, Wilkins S, Brown MA, Bigwood T, Shaw M, Ridgway C, Sharman M. (2005). Effects of European foulbrood treatment regime on oxytetracycline levels in honey extracted from treated honeybee (*Apis mellifera*) colonies and toxicity to brood. Food Addit Contam. 22:573-578.
- Tian B, Fadhil NH, Powell JE, Kwong WK, and Moran NA (2012). Long-term exposure to antibiotics has caused accumulation of resistance determinants in the gut microbiota of honeybees. mBio 3(6):e00377-12. doi:10.1128/mBio.00377-12.
- Trapecar M, Leouffre T, Faure M, Jensen HE, Granum PE, Cencic A, Hardy SP (2011). The use of a porcine intestinal cell model system for evaluating the food safety risk of *Bacillus cereus* probiotics and the implications for assessing enterotoxigenicity. APMIS. 119:877-84.
- Vásquez A, Forsgren E, Fries I, Paxton RJ, Flaberg E, Szekely L, Olofsson TC. (2012). Symbionts as major modulators of insect health: lactic acid bacteria and honeybees. PLoS One. 7:e33188.