Use of plant extracts as an alternative, novel, and natural agent for the treatment of Canadian bee colonies affected by foulbrood diseases

Author: J. Ross and G. Bedie
November 30, 2022
Doc# 21063-008-IR1

CHINOOK CONTRACT RESEARCH

NRCS

Project Background:

The honeybee industry faces a major threat with the prevalence of the Gram-Positive bacteria *Paenibacillus larvae* and *Melissococcus plutonious*—respectively, the causative agents of American and European Foulbrood (AFB and EFB). Economically, these pathogens represent the most important diseases in honeybees, causing huge damage worldwide. Overall economic value dependent on insect-mediated pollination service in the United States totaled 34.0 billion USD in 2012 and, in 2019, colony losses cost the US beekeeping industry an estimated $250M (REF#2). In Alberta, the cost of foulbrood outbreaks were estimated at $10.13 million for AFB and $22.23 million for EFB (REF#3). As in the food industry—where chemical sanitizers or disinfectants have long been used to fight against microbial contamination of food and to provide longer shelf life for the derived manufactured food products—antibiotics (such as tetracyclines, tylosin, and lincomycin) have been used to treat diseased bee colonies in Canada and the US. The effectiveness of these chemicals have proven to be limited, with the consequences of their use being the development of resistance by the bee pathogens to these antibiotics and the contamination of honey and bee products with antibiotic residues. This, in turn, represents a risk for consumers, as human pathogens may also develop antibiotic resistance when people consume these products. In addition, the microflora that compete with the pathogens in the gut of adult bee and larvae may be wiped out by the antibiotics, leaving the hosts vulnerable to any other microbial attack. Another factor that reduces the efficacy of chemical or antibiotic treatment may come from the development of a protective biofilm by the foulbrood agents on equipment. In the face of these increasing concerns, the **Nature Recombined Sciences (NRCS)** solution focuses on the use of practical, novel, and natural food-safe plant extracts as an alternative to the chemical and antibiotic treatment of foulbrood diseases. Not only are these extracts effective, but they are selective against Gram-positive endospore and vegetative bacteria. They are non-antibiotics and can be organically sourced from crops grown locally in Alberta, supporting the up-shifting of value in raw food ingredients.

This presentation summarizes the methods and data of early independent testing of several of NRCS's lead compounds.

Technology Background

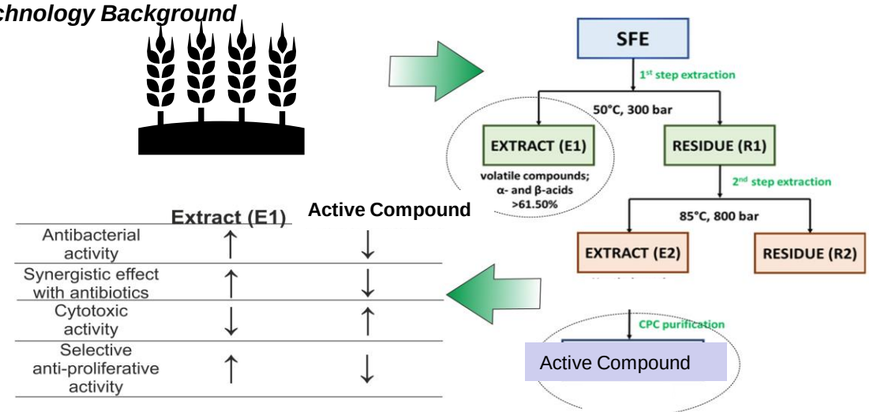
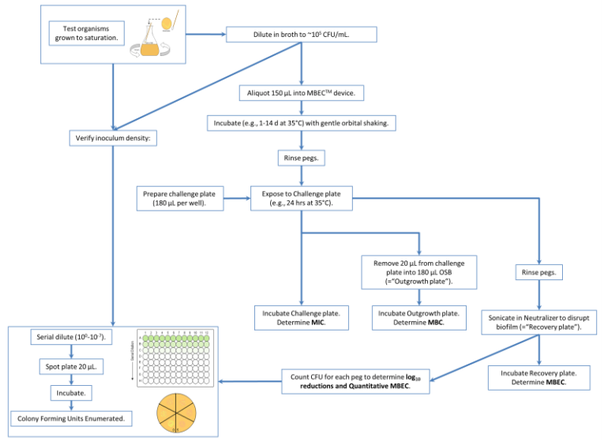


Figure 1 (adapted from Ref #4)

Nature ReCombined Science's natural antimicrobial plant extract solution is currently at Technology Readiness Level (TRL) 4. The antimicrobial and anti-biofilm efficacy of the extracts have been established and repeatedly validated against microbial pathogens including multi-drug resistant *Staphylococcus aureus* and *Listeria monocytogenes* in 3rd party labs in the past two years.

Figure 2. Minimum Biofilm Eradication Concentration (MBEC) Test Method Overview



The MBEC™ method uses a device consisting of a plastic lid with 96 pegs and a corresponding receiver plate with 96 wells that are filled with 150 µL of inoculum. After incubation, biofilm forms on the pegs, which are placed in a new receiver plate containing test antimicrobials for a challenge period. The biofilm is then dislodged by sonication in a recovery medium and incubated for turbidity ("visual") reading to assess the MBEC. For quantification, some of the recovery medium is plated on organism specific agar and the colony forming units (CFUs) counted. The lowest concentration of test antimicrobial yielding a >99.9% reduction is used as the MBEC value.

Conclusions & Discussion

- All four tested NRCS plant extracts have potent efficacy against "planktonic" (free-floating) *P. larvae* cells and against *P. larvae* biofilms.
- Preliminary data also indicate potency against a spore-enriched *P. larvae* culture.
- Each tested NRCS extract was bactericidal against "planktonic" (free-floating) *M. plutonius* at a concentration as low as 10%.
- Preliminary data indicated up to a 96% reduction of *M. plutonius* biofilms, although this data was limited by the poor experimentally simulated biofilm formation by the type strain utilized in these preliminary experiments.
- **Future work will explore the following:**
  - Optimization of extract formulation
  - Efficacy assessment against isolated *P. larvae* spores
  - Repeated biofilm experiments with different strains of *M. plutonius*.
  - Repeated biofilm experiments with different surfaces.

Preliminary P. larvae data

Table 1. Antibacterial and anti-biofilm activity of NRCS extracts against *P. larvae*

Organism	Test Article	Turbidity ("Visual") Assays			Quantitative (Plate Count) Assay	
		MIC (Inhibits Growth)	MBC (Kills Bacteria)	MBEC (Eliminates Biofilms)	% Reduction	P-value
P. larvae (ATCC 9545)	NRCS-011	<MTC	<MTC	<MTC	99.99	0.12
	NRCS-019	<MTC	<MTC	<MTC	99.99	0.12
	NRCS-014	<MTC	<MTC	<MTC	100.00	0.00
	NRCS-009	<MTC	<MTC	<MTC	100.00	0.00

Note: <MTC = "Below Minimum Tested Concentration", which means the extract yielded no visible growth at its lowest tested concentration (1.56% v/v).

This work comprised an assessment of the Minimum Inhibitory Concentration ("MIC"), Minimum Bactericidal Concentration ("MBC"), and Minimum Biofilm Eradication Concentration ("MBEC") of various NRCS extracts against vegetative *P. larvae* cells. The MIC, MBC, and MBEC measure "bacteriostatic" (growth-inhibiting), "bactericidal" (bacterial killing), and antibiofilm activities, respectively; they are "semi-quantitative" measurements of the lowest tested concentration yielding no visible growth. The antibiofilm activity was also measured "quantitatively" (i.e., $\log_{10}$ reduction assay) by plating on agar to enumerate viable colony forming units (CFU) and calculating the percent reduction relative to an untreated growth control.

Table 2. Antimicrobial efficacy of NRCS extracts against a spore-enriched culture of *P. larvae*

Organism	Test Article	Tested Concentration	% Reduction	P-value
P. larvae (ATCC 9545)	NRCS-011	10%	99.96	0.0000
	NRCS-019	1%	99.97	0.0000
	NRCS-014	1%	99.98	0.0000
	NRCS-009	1%	99.91	0.0060

This work comprised a quantitative assessment (i.e., $\log_{10}$ reduction assay) of the antimicrobial efficacy of NRCS extracts against a spore-enriched preparation of *P. larvae*. In this Quantitative Carrier Test (QCT), the test organism was suspended in a tripartite "soil load" and dried onto the surface of a plastic carrier before challenging for 24 hours at 37° C with a broad range of extract concentrations. A neutralizing broth was then added, and the mixture was serially diluted and plated to enumerate viable colony forming units (CFU).

Preliminary M. plutonious data

Table 3. Anti-bacterial and anti-biofilm activity of NRCS extracts against *M. plutonius*

Organism	Test Article	Turbidity ("Visual") Assays			Quantitative (Plate Count) Assay	
		MIC (Inhibits Growth)	MBC (Kills Bacteria)	MBEC (Eliminates Biofilms)	% Reduction	P-value
M. plutonius (ATCC 35311)	NRCS-019	10%	10%	>100%	60.19	0.70
	NRCS-014	10%	10%	100%	95.93	0.17

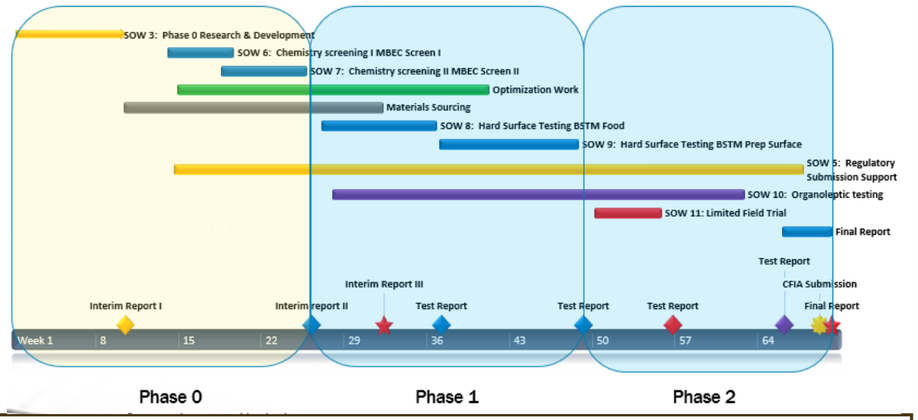
Notes: MIC, MBC, and MBEC are the lowest tested concentrations of each extract (in % v/v) that yielded no visible growth.
% Reduction is the percentage of biofilm cells eliminated during the MBEC assay, which was actually underestimated here due to poor biofilm recovery from the untreated growth control.

This work comprised an assessment of the Minimum Inhibitory Concentration ("MIC"), Minimum Bactericidal Concentration ("MBC"), and Minimum Biofilm Eradication Concentration ("MBEC") of various NRCS extracts against vegetative *M. plutonius* cells. The MIC, MBC, and MBEC measure "bacteriostatic" (growth-inhibiting), "bactericidal" (bacterial killing), and antibiofilm activities, respectively; they are "semi-quantitative" measurements of the lowest tested concentration yielding no visible growth. The antibiofilm activity was also measured "quantitatively" (i.e., $\log_{10}$ reduction assay) by plating on agar to enumerate viable colony forming units (CFU) and calculating the percent reduction relative to an untreated growth control.

Use of plant extracts as an alternative, novel, and natural agent for the treatment of Canadian bee colonies affected by foulbrood diseases

Author: J. Ross and G. Bedie
November 30, 2022
Doc# 21063-008-IR1

High Level Program Plan



References

1. *Jordan, A., H. M. Patch, C. M. Grozinger, and V. Khanna. 2021. Economic Dependence and Vulnerability of United States Agricultural Sector on Insect-Mediated Pollination Service. Environ. Sci. Technol. 55: 2243–2253.*
2. Tauber, P. J., Collins, W. R., Schwarz, R. S., Chen, Y., Grubbs, K., Huang, Q., Lopez, D., Peterson, R., and J. D. 2019. Natural Product Medicines for Honey Bees: Perspective and Protocols. *Insects* 10 (10), 356-381.
3. Laate, E. A., J. P. Emunu, A. Duering, and O. Lynae. 2020. Potential Economic Impact of European and American Foulbrood on Alberta's Beekeeping Industry. URL: https://open.alberta.ca/dataset/029a345b-8621-4986-ad78-7fc6ddcd8b17/resource/25f7b78d-a359-428c-9648-9175c3634720/download/af-potential-economic-impact-european-american-foulbroodon-albertas-beekeeping-industry.pdf.
4. Klimek, K.; Tyśkiewicz, K.; Miazga-Karska, M.; Dębczak, A.; Rój, E.; Ginalska, G. Bioactive Compounds Obtained from Polish "Marynka" plant Variety Using Efficient Two-Step Supercritical Fluid Extraction and Comparison of Their Antibacterial, Cytotoxic, and Anti-Proliferative Activities In Vitro. *Molecules* **2021**, *26*, 2366. https://doi.org/10.3390/molecules26082366

How to contact us

www.ccr01.com

Dr. Joe Ross
joe.ross@cc01.com
Phone: 403-948-9911

Dr. Gerard Bedie
gerard@ccr01.com

www.naturecombined.com

Mingyang Sun
ming@naturerecombined.com
Phone: 604-356-6676

Guan Lim
guan@naturerecombined.com

Len Sarna
len@naturerecombined.com

Next Stages of Development

The Nature Recombined Sciences solution focuses on the use of practical, novel, and natural food-safe plant extracts as an alternative to the chemical and antibiotic treatment of foulbrood diseases. This program particularly aligns well with Results Driven Agriculture Research (RDAR) as it provisions for translational up-shifting value in raw food ingredients (namely the raw plant extract), provides an opportunity for niche food commodity claims (Antibiotic Free Treatment), and enables several opportunities for improved food safety in the farm-to-fork value chain.

We are seeking a small matching investment of $375,000 from various funding sources to support the Phase 0 development of our novel family of plant-based extracts program to develop the first-in-class Natural Plant Based Hard Surface Disinfectant with an Anti-Biofilm claim with a No Rinse Limit.

This project will focus on the following five objectives:

1. Investigating biofilm formation with *M. plutonious* on modified surfaces such as Microplate pegs coated with beeswax and paraffin wax.
2. Conducting mock field studies with the novel treatment to understand the product use (human factor and User Experience activities).
3. Network with the National Bee Diagnostic Centre (NBDC) to do the following:
   a. Conduct surveys of producers asking how they would like to use the product.
   b. Perform in-lab tests of the new product on hard surfaces such as a hive frames and tools used in beekeeping.
4. Complete compound formulation optimization and design for manufacture (DFM) activities
5. Complete efficacy and safety studies in support of a regulatory application to CFIA and Health Canada

Product Valuation:

The global food disinfection market size is estimated to be valued USD 12.2 billion in 2020 and is expected to reach a value of USD 14.9 billion by 2025, growing at a Compound Annual Growth Rate (CAGR) of 4.1% during the forecast period.
Plant extracts are natural, and their antimicrobial effect on foulbrood disease agents represents a major alternative to conventional treatments of bee colonies with antibiotics, thus eliminating the risk of antibiotic resistance development by the bee pathogens and the contamination of the bee products, such as honey, with the chemical. The natural state of the extracts as preservatives may represent a potent labelling advantage for Canadian producers seeking niche or Antibiotic-Free Treatment label claims. The high activity in low concentrations with the absence of toxicity, added to the low processing cost of the extracts, exhibits a major economic and food safety advantage compared to antibiotic treatments.
A first-in-class Natural Plant Based Hard Surface Disinfectant with an anti-biofilm claim with a no Rinse Limit represents a disruptive technology in the traditional disinfectant market segment. For reference, the Global Surface Disinfectant Market size was over USD 5.3 billion in 2019 and is estimated to grow over 7.54% CAGR from 2020 to 2026.

About CCR

Chinook Contract Research Inc. (CCR) is a small, privately held, Canadian Contract Research Organization based in Alberta, specializing in antimicrobial and pharmaceutical research. CCR provides antimicrobial claims support to medical device developers and provides large animal field trial services for pharmaceutical manufacturers in the food animal production chain and in the food industry in general. Recently, much of this work has been focused on the evaluation of the antimicrobial activity of various plant extracts against a broad spectrum of pathogenic organisms, including Gram-negative bacteria and Gram-positive bacteria, in determining their minimum inhibitory concentration, minimum bactericidal concentration, and minimum biofilm eradication concentration with its $\log_{10}$ reduction.

About NRCS

Nature Recombined Sciences Inc. (NRCS) is a privately held small Canadian biotechnology company based in British Columbia. NRCS focuses on harnessing natural, clean power from plants to address emerging global concerns surrounding pandemics, antibiotic abuse, use of synthetic disinfectants, and personal health. NRCS has developed a protocol to prepare aqueous extracts from certain plant species and has generated preliminary data indicating antimicrobial activity against certain significant pathogens of interest. To determine whether their extracts possess antimicrobial activities against biofilms, NRCS has previously contracted Chinook Contract Research Inc. (CCR) to conduct a preliminary screen of several NRCS extracts against a panel of microbial biofilms; indeed, the extracts showed promise against biofilms—particularly those formed by Gram-positive pathogens, such as *Staphylococcus aureus* and *Listeria monocytogenes*.

778-941-2268
info@naturerecombined.com
3160 853 SeaBorne Avenue
Port Coquitlam, British
Columbia V3B0N9 CANADA