Nova Scotian Bees as sources of antimicrobial compounds against American Foulbrood Disease

By

Prashansa Kooshna

A Thesis Submitted to

Saint Mary's University, Halifax, Nova Scotia

in Partial Fulfillment of the Requirements for

the Degree of Bachelor of Science with Honours in Chemistry.

April, 2020, Halifax, Nova Scotia

Approved: Dr Clarissa Sit

Assistant Professor,

Supervisor.

Approved: Jason Masuda

Department Chair

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Abstract

Honey bees, *Apis mellifera*, face many parasites and pathogens such as *Paenibacillus larvae*, the causal agent of American Foulbrood disease (AFB), a highly contagious disease. Honey bees rely on a diverse set of individual and group-level defenses to prevent disease. One route by which honey bees might combat disease is through the protective effects of their microbial symbionts. This study focuses on microbial interactions in bees that help in fighting AFB through the inhibition of *P. larvae*.

Honey bees and wild bees in Nova Scotia were sampled for microbial isolation and screened against *P. larvae* using pairwise antimicrobial assay. Isolates showing good inhibition were chosen for extraction of metabolites with ethyl acetate and 1:1 chloromethane-methanol to obtain antimicrobial compounds that inhibit *P. larvae*. The extracts were analysed using LC/DAD and UHPLC/MS.

Novel microbial species such as *Pseudomonas chloroaphis, Debaromyces prosopidis* and *Paenibacillus lactis* along with previously reported *B. cereus* and *B. subitilis*, were isolated from beehive swabs and showed strong inhibition against *P. larvae*. Chloroform: Methanol extracts of E8 that is a mixture of at least 6 microbial strains and of A12 showed strong inhibition while ethyl acetate extracts showed moderate inhibition against *P. larvae.* Our findings show great potential for discovery of novel antagonistic compounds against AFB.

24 April 2020

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A12	Isolate from beehive swabs of honeybee
ACN	Acetonitrile
AFB	American foulbrood disease
ATCC	American type culture collection
BHIT	Brain heart infusion with thiamine HCl
BLAST	Basic local alignment search tool
BLIS	Bacteriocin-like inhibitory substances
CM	1:1 Chloroform: methanol
ddNTPs	Dideoxyribonucleotide
DNA	Deoxyribonucleic acid
E8	Isolate from beehive swabs of honeybee
EA	Ethyl Acetate
ERIC	Enterobacterial repetitive intergenic consensus
ESI	Electrospray ionization
HILIC	Hydrophilic interaction liquid chromatography
HPLC	High performance liquid chromatography
IR	Infrared spectroscopy
ITS	Internal transcribed spacer
LC/DAD	Liquid chromatography with photodiode array detection
LC/MS	Liquid chromatography with mass spectrometry
LPMOs	Lytic polysaccharide monooxygenases
mRNA	Messenger ribonucleic acid
MS	Mass spectrometry
NA	Nutrient agar
NAA	Nutrient agar with ampicillin
NAC	Nutrient agar with cycloheximide
NARP	Non-aqueous reversed phase liquid chromatography
NB	Nutrient broth
NMR	Nuclear magnetic resonance
PCR	Polymerase chain reaction

PM	Peritrophic matrix
PYEG	Peptone yeast extract glucose media
qTOF-MS	Quadrupole time of flight mass spectrometry
RP	Reverse phase
rRNA	Ribosomal ribonucleic acid
RT	Room temperature
UHPLC	Ultra- high performance liquid chromatography

1. Introduction

1.0. Importance of honey bees

Honey bees (*Apis mellifera*) contribute to the ecosystem and economy through their pollination services, benefiting humans both directly and indirectly. It was reported by Mendleson that honey bee pollination is worth about $1.7 billion in Canada.[1] Around 380 000 beehives in Canada, which is half the stock of Canadian beehives, are responsible for the pollination of canola seeds only. Moreover, fifty-two of the 115 leading global commodities depend on honey bee pollination. For instance, 80% of the world's supply of almonds are pollinated by millions of beehives. Some honey bee-dependent commodities can face a decline of more than 90% in their yield without honey bees.[1]

1.0.1 The status quo honey bee population

While global honey bee populations have been increasing, the rate of increase is not keeping pace with demand. The proportion of land dedicated to the production of pollinator-independent crops has shrunk when compared to land used for cultivation of pollinator-dependent crops. Furthermore, in spite of the global increase in honey bee populations, some parts of Europe and North America have been facing declines in honey bee populations.[2,3]

Managed honey bee populations are influenced by many factors including diseases, parasites, pesticides, the environment, and socio-economic factors. American

foulbrood disease (AFB) is the most serious bacterial disease of the honey bee.[4] Of all diseases affecting honey bees, AFB has had the greatest impact on the industry. In 2000, annual economic loss attributed to AFB infection in US was around USD $5 million.[5]

1.1 American Foulbrood disease

American Foulbrood disease is a worldwide problem; it has been reported that *Paenibacillus larvae* spores, the causal agent of the disease, were found in honey samples in regions of US, sub Saharan Africa and South America.[5,6,7] Prevention and control of the disease are challenging because *P. larvae* form spores that can survive environmental adversities for long periods of time.[8] AFB affects honey bees at a larval stage and can wipe out a whole generation, through larval death within only 6 to 12 days.[8] Antibiotics tylosin and oxytetracycline were approved by the FDA to be used against *P. larvae*.[9,10,11] However, the pathogen developed drug resistance against both antibiotics. Moreover, higher doses of those antibiotics are harmful for human consumption of the honey. Thus, beekeepers resort to burning infected hives to contain the disease.

1.1.1 Pathogen, Paenibacillus larvae

Paenibacillus larvae, which is part of the genus *Paenibacillus*, is a rod-shaped, facultative anaerobic, spore forming and gram-positive bacteria.[8,12] *P. larvae* has been classified into four different genotypes (Enterobacterial Repetitive Intergenic Consensus ERIC I–IV).[13] These genotypes differ phenotypically with respect to spore and colony morphology, metabolism, and most importantly virulence.[14,15,16] The genotypes ERIC I and ERIC II are regularly isolated from infected colonies worldwide, whereas ERIC III and ERIC IV are only represented by few historical isolates in type culture collections.[17]

Based on genomic data, it has been proposed that *P. larvae* ERIC I and II evolved different strategies to achieve invasion of the hemocoel. The hemocoel is the body cavity in bees, comprising a series of interconnected spaces between tissues and organs, through which a mixture of blood and lymphatic fluid (hemolymph) flows freely.[18]

It has been demonstrated that the genotype ERIC II is more virulent on the larval level than ERIC I. It commonly kills bee larvae within 6–7 days, while ERIC I strains need up to 12 days to kill all infected larvae.[19] However, these differences on the individual larval level have different implications for virulence on the colony level, because the earlier larvae die, the more efficiently they can be removed by nurse bees engaged in brood hygiene. This is a part of the social immune response of honey bees.[20] The social immune response is better adapted to contain ERIC II infections rather than ERIC I infections. This leads to the paradoxical situation that *P. larvae* ERIC II is less virulent on the colony level than ERIC I.

Several species from *Paenibacillus* genus are known for their useful *Paenibacillus*-derived antimicrobial compounds such as polymyxins and fusaricidins. Moreover, many *Paenibacillus* genus species yield a range of enzymes that have several applications in medicine, food, textiles and biofuel.[12] On the other hand, *Paenibacillus larvae* which is a pathogen releases a chitinase, that is responsible for the pathogen's invasion of the midgut of honey bee larvae.[4] Understanding the mechanism of the infection can provide better guides to solutions against AFB.

1.2 Mechanism of pathogenicity

1.2.1 Pathobiology of *P. larvae:* chitin-degrading enzymes

In most invertebrates like bees, the role of mucus is incurred by peritrophic matrix (PM), the lining of the gut epithelium and provides a protective barrier against pathogens.[22,23] The PM consist of a network of chitin containing microfibrils in a matrix of proteins, glycoproteins, and proteoglycans. The major constituents are the fibrils made of chitin, an insoluble linear beta (1,4)-linked N-acetylglucosamine.[24]

Invasive pathogens like *P. larvae*, need to breach the midgut epithelium before they can interact with the epithelial cells. Hence, degradation of the PM in the larval midgut is a key step in the pathogenesis of *P. larvae*.[25] The mechanism of infection by *P. larvae* is shown in figure 1. It was found that the chitin-degrading enzyme *PlCBP49* responsible for the degradation was not a classical chitinase as the genomic sequences of *P. larvae* did not reveal functional genes for classical chitinases. Henceforth, *PlCBP49* was classified as a novel member of auxiliary activity 10 (AA10) family of lytic polysaccharide monooxygenases (LPMOs).[26,27,28] Members of the AA10 family are capable of degrading recalcitrant polysaccharides like crystalline chitin via a novel, copper-dependent, oxidative enzymatic mechanism.[14,29,30]

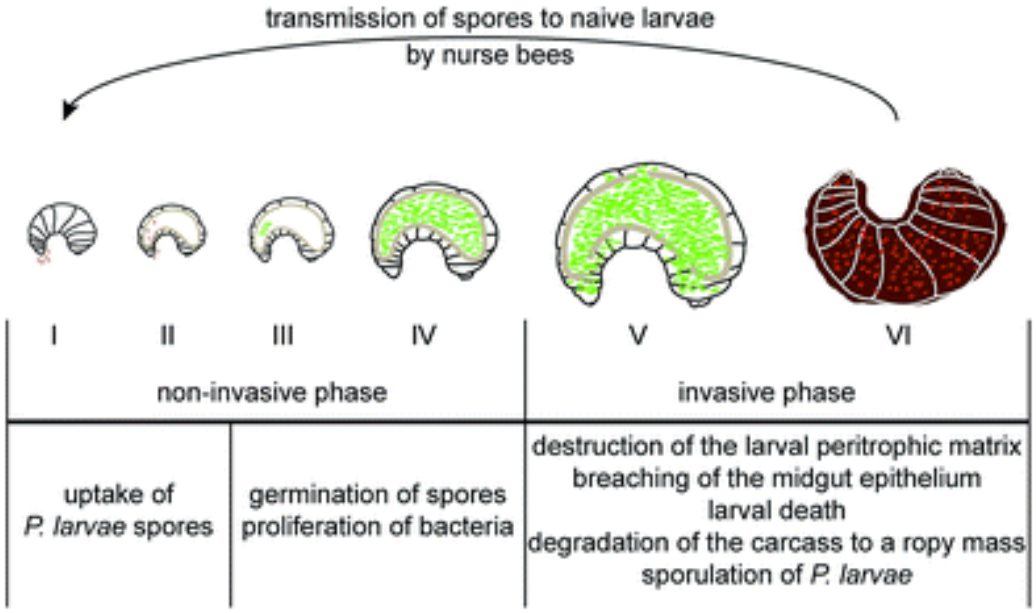


Figure 1. Pathogenesis of *P. larvae* infections in honey bee larvae. From stage I to II, honey bee larvae ingest the *P. larvae* spores in the contaminated food. Exposure to excess of nutrients in the midgut promotes spore germination (III) and allows the vegetative bacteria to proliferate until they occupy nearly the entire midgut lumen (IV).[4] The invasive phase of infection is initiated by the total destruction of the midgut PM enabling the bacteria to cross the epithelial barrier and invade the hemocoel (V). At stage V, the infected larva is dead and *P. larvae* totally degrades the cadaver to a ropy mass and eventually starts forming spores again. Nurse bees trying to clean the brood cell become contaminated and transmit the spores to other uninfected larvae when feeding them. Along with the chitin degrading enzyme, certain toxins/secondary metabolites released by *P. larvae* also participate in invading of the hemocoel (Muller et al.).[8] Permission for use of image was granted.

1.2.2 Pathobiology of *P. larvae*: toxins/secondary metabolites

P. larvae secondary metabolites exhibiting antimicrobial activity may play a role during *P. larvae* proliferation in the midgut lumen. Only ERIC I will be discussed here as it is more virulent at colony level and is the one studied in this honours project.[31] ERIC I-genome harbors several functional toxin genes. Two of the ERIC I-specific toxins Plx1, Plx2 are novel AB-toxins. AB-toxins consist of two subunits, an enzymatically active A subunit and a B subunit that assist in the translocation of subunit A into the host cell. The A subunit inhibits normal cellular functions by modifying its target. [32]

These AB-toxins, Plx1 and Plx2 most likely act on the epithelial cells, once the protective PM has been degraded. Fünfhaus et al. (2013) shed light on some possibilities, but the actual mechanism of toxins is yet to be confirmed. They found that the Plx1 B subunit contained four ricin-B-like domains with characteristic motifs that have been shown to be involved in carbohydrate binding.[32] Thus, Plx1 might be able to bind to glycoproteins or glycolipids present on honey bee larval midgut epithelial cells, facilitating the entry of the toxin into host cells. [33] As for Plx2, from the observed sequence and structural features of Plx2 A subunit, they hypothesized that activity of Plx2 in the host cell results in loss of the actin cytoskeleton that may lead to the observed rounding up of host cells in the midgut epithelium of AFB-infected larvae.[33,34]

Also, *P. larvae* proliferating in the larval midgut will encounter microbial competitors as soon as the larval food is supplemented by honey and pollen containing bacterial spores, bacteria, and fungi. Other secondary metabolites like paenilamicin or the paenilarvins will enable *P. larvae* to defend its niche and outcompete saprophytes during

degradation of the larval cadaver, thus ensuring that a pure culture of *P. larvae* prevails in the end. [35,36]

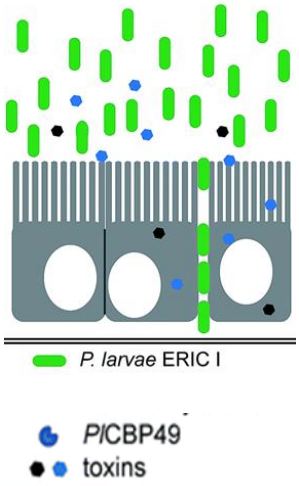


Figure 2. Role of toxins Plx1 and Plx2 in virulence of *P. larvae*. These toxins most likely act on epithelial cells once the protective PM has been degraded. *P. larvae* then breaches the epithelial layer and invades the hemocoel (Muller et al. 2014).[8] Permission for use of image was granted.

1.3 Solutions

1.3.1 Progress on fighting against AFB so far

There is an ongoing search for antimicrobial compounds against the pathogen, *P. larvae*. Several antibiotics have been tested in vitro and in vivo against AFB such as oxytetracycline and tylosin. It was found that AFB was controlled by oxytetracycline hydrochloride, tylosin and terramycin but in all cases, colonies exhibited disease recurrence from five to 10 months after treatment.[36] Many other potential antimicrobial compounds against the honey bee pathogen have recently been discovered from various sources such as tea tree oil, poplar resins, tilmicosin and fermented materials.[38-41] For instance, 4 active dihydroflavonols from poplar resins (pinobanksin-3-butyrate, pinobanksin-3-isopentanoate, pinobanksin-3-hexanoate, pinobanksin-3-octanoate) were

found to inhibit *P. larvae*. (IC_{50}: 17-68 µM) where an increasing antimicrobial activity against *P. larvae* was observed, with longer acyl groups.[39]

Studies have also demonstrated the efficiency of probiotics in strengthening honey bee's resistance against AFB. Lactic acid bacteria in genera Lactobacillus and Bifidobacterium, originating from honey stomach exhibited resistance both in vitro and in vivo against *P. larvae* and was proposed as candidates for use as probiotics against AFB.[42] *Lactobacillus plantarum* (ATCC 14917), *Lactobacillus rhamnosus* (ATCC 55826), and *Lactobacillus kunkeei* (previously isolated from a healthy honey bee hive) were tested as probiotics to provide resistance against AFB among honey bees and found to reduce pathogen load, upregulate expression of key immune genes, and improve survival during *P. larvae* infection.[43] These findings show that the use of probiotics supplement can be a practical and affordable solution for beekeepers. The use of probiotics can be a cheaper alternative compared to antibiotics as the cost of synthesis of antibiotics is likely to be higher.

1.3.2 Microbiome of honey bee as source of active compounds

A study found that seven strains showed strong inhibitory activity against *P. larvae* out of 35 isolates from the digestive tract of the Japanese honey bee, *Apis cerana japonica*. Most of the antagonistic bacteria belonged to Bacillus species and the strong inhibitory strains were closely related to the *subtilis* and *cereus* subspecies.[44] Another study by Alippi and Reynaldi[45] tested 242 isolates from apiarian sources in Argentina from which 49% produced no inhibition, 28% produced medium inhibition, 12% produced good inhibition, and 11% produced very good inhibition. Within those 11%

(26) strains that showed very good inhibition, 10 strains were selected and identified as: *Bacillus subtilis, Bacillus pumilus, Bacillus licheniformis, Bacillus cereus, Bacillus megaterium, Brevibacillus laterosporus, Bacillus laterosporus.*

Furthermore, a bacterial strain showing a high level of antimicrobial activity against *P. larvae* ATCC 9545 was isolated from honey samples and identified as *Paenibacillus polymyxa*. [46]

1.3.3 Microbiome of wild bees as source of active compounds

It was found by Keller and co-workers that *Paenibacillus* strains with genes encoding the virulence factors or chitinases of American Foulbrood disease were not found among 13 species of wild bees.[47] Hence, it is possible that wild bees have co-evolved with some immune responses against AFB. For instance, Olofsson and Vasquez[42] (2008) reported a novel bacterial flora composed of lactic acid bacteria of the genera *Lactobacillus* and *Bifidobacterium*, in *Apis mellifera* honey stomach. However, Evans and Armstrong[48] (2006) failed to find *Lactobacillus* species in *A. mellifera*, suggesting that the gut microbial population is not constant even within the same species. Therefore, it can be hypothesized that wild bees have different gut microbiomes too. Their gut microbiomes can also be potent sources of antimicrobial compounds if wild bees have evolved with a microbiome against diseases such as AFB.

1.4 Theory behind methods

1.4.1 Finding potential inhibitors

There are countless microorganisms thriving in the bee microbiome that may be producing potent antimicrobial compounds. The isolation of microbes depends on several factors such as nutrient availability, nature of substrate, oxygen levels and temperature. Also, the presence and nature of competitors (other surrounding microbes) can affect the chemical composition of the media as they produce secondary metabolites that can inhibit or promote growth of other strains. Many of those factors, such as presence of competitors, cannot be controlled at the beginning stage. Therefore, the choice of media and physical conditions are crucial in maximizing the number of isolates.

General media allow the growth of non-fastidious microorganisms while selective media will support fastidious organisms, which need a specific set of requirements met, to grow. Nutrient agar (NA) was used as general media, while nutrient agar with antibiotic ampicillin (NAA) was used to favor fungal growth and nutrient agar with fungicide cycloheximide (NAC) was used for selective growth of bacteria.

Streaking colonies aseptically leads to the isolation of individual colonies, which are a group of microbial cells that came from one single progenitor microbe.[54] An antimicrobial assay that can screen several strains at once, is needed to find potential producers of antimicrobial compounds. Twelve well plates pairwise assay allow us to conduct several pairwise tests at once.

1.4.2 Identification of inhibitors

DNA extraction and primers used

For DNA (deoxyribonucleic acid)

extraction, cells and their nuclei need to be broken open. This can be accomplished by mechanical methods, such as grinding, or by chemical methods that break apart cell walls and cell membranes. The use of ethanol helps in precipitating DNA and removing salts by solvating them better than DNA.

For bacterial identification, 16S ribosomal RNA (16S rRNA) is targeted and sequenced as it is a highly conserved region of the bacterial genome. 16S rRNA is the component of the 30S small subunit of a prokaryotic ribosome that binds to the Shine-Dalgarno sequence. The Shine-Dalgarno sequence is a ribosomal binding site in bacterial and archaeal messenger RNA, (mRNA) that helps to recruit mRNA for protein synthesis.[49,50,51]

Fungi also have an internal transcribed spacer (ITS), which is a highly conserved cluster present in the rRNA. This cluster encodes three subunits of ribosomal RNA; 18S (small subunit), 5.8S and 28S (large subunit) and ITS region. An important attribute of the ITS sequences for molecular phylogenetic research is that they show significant variations between closely related fungi, and sometimes between populations within a single species. These variations are caused by insertions, deletions, and point mutations, which are conserved in the ITS region.[52,53]

Sanger Sequencing

This sequencing procedure which is dideoxy sequencing was invented by Frederic Sanger and his colleagues in 1977. With a few modifications and automation, this method is still used today in genomics, allowing large sequencing centers to read over 1,000 bases of DNA sequence per second.[55]

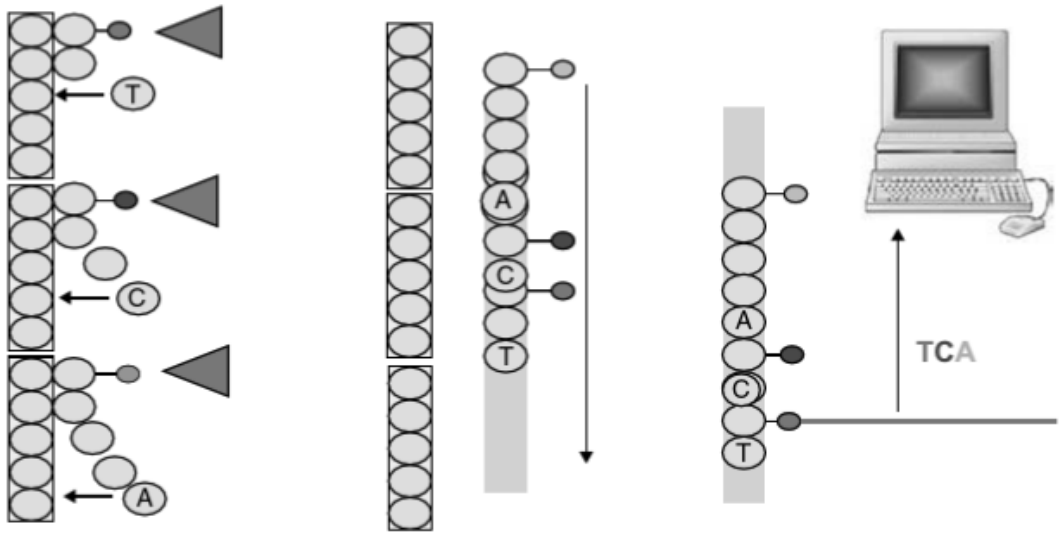
Sanger sequencing test samples consist of the extracted DNA of test organism, primers that are oligonucleotides of around 20 base pairs long and complementary to the target DNA The sample mixture is added to four tubes containing one of the four dNTPs (deoxyribonucleotides), their corresponding ddNTPs (dideoxyribonucleotide) and DNA polymerase.

Each tube first undergoes heating to separate the double stranded DNA by disrupting the hydrogen bonds and van der Waals forces of attraction. The DNA primer is then annealed at one end of the sequence of interest on the DNA strands, which act as template strands for DNA polymerase.

DNA polymerase extends the oligonucleotide, using the template strand to guide incorporation of dNTPs. Randomly, a ddNTP will be incorporated into the growing DNA strand. Because it is missing the 3’ hydroxyl group, the ddNTP will prevent the DNA chain from being extended further.

In addition, each ddNTP has a different color label. Consequently, each terminated DNA chain is colored according to the nucleotide at its end. When the DNA

strands are separated by length by capillary electrophoresis, individual chains of increasing length can be identified by their color.[55]



Various samples of DNA are subjected to the dideoxy reaction, but a different colour dye (shown by arrows) is attached to each primer	**The resulting fragments are separated in a single lane of gel, where they move down quicker or slower according to size**	**A laser source at the bottom of the lane detects the colours and sends the corresponding information to a computer, which deduces the sequence.**

Figure 3. Scheme showing how the ddNTPs causes termination in Sanger sequencing by stopping DNA polymerase from elongating DNA strand and can be used to sequence DNA.[54] Permission for use of image was granted.

Analysis of sequence

A sequence similarity search allows scientists to deduce the function of a sequence from similar sequences. The sequence received from Sanger sequencing can be analysed with various bioinformatic tools. BLAST (Basic Local Alignment Search Tool) is a well-known program that finds regions of local similarity between sequences. The program compares nucleotide or protein sequences to sequence databases and calculates the statistical significance of matches. This can be used to help identify members of gene families or infer functional and evolutionary relationships between sequences.
The BLASTN nucleotide-nucleotide search looks for more distant sequences while the megaBLAST nucleotide-nucleotide search is optimized for very similar sequences in the same or in closely related species. MegaBLAST first looks for an exact match of 28 bases, and then attempts to extend that initial match into a full alignment.[55]

1.4.3 Target secondary metabolites and extraction

Antimicrobial activity is caused by the production of an array of secondary metabolites of bacteria and fungi such as peptides, lipopedtides, β-lactams, glycopeptides, lantibiotics, surfactins and many more.[56,57,58] The method of extraction varies according to the nature of target compounds. A common point observed from previous studies is the use of methanol or precipitation by ammonium sulphate or concentrated acid to extract antimicrobial compounds. Acetone and chloroform were also used to extract antimicrobial compounds from microbes.[59,60,61]

Bacillus subtilis, isolated from honey and bee gut samples showed very good inhibition in Alippi and Reynaldi's study[45] (2006), and is known for producing surfactin.

Natural surfactin produced by *B. subtilis* is a mixture of isoforms with slightly different properties as a result of substitutions in amino acids and the aliphatic chain. Cell free supernatants of the *B. subtilis* cultures were precipitated with concentrated HCl and was then extracted with methanol. Vegetative cells of *P. larvae* were affected as soon as they came in contact with the surfactin sample in their antimicrobial assay.[45]

Bacillus cereus, another strain that has been showing very good inhibition in previous studies, has been fairly well investigated for its useful or toxic secondary metabolites. Bizanni and Brandelli (2002) identified a bacteriocin produced by the bacterium *Bacillus cereus 8 A* that could be used in food safety. Conversely, it was also found that *Bacillus cereus* produces food poisoning toxins such as an emetic that causes vomiting. The emetic toxin has been named cereulide and consists of a ring structure of three repeats of four amino and/or oxy acids: [D-*O*-Leu-D-Ala-L-*O*-Val-L-Val]$_3$. This dodecadepsipeptide has a molecular mass of 1.2 kDa and is chemically closely related to the potassium ionophore valinomycin, which is a potent antibiotic that translocates K^+ ions across cell membranes.[62,63]

Another study isolated bacteriocin-like inhibitory substances (BLIS) that are antagonistic to *P. larvae* from *B. cereus* isolated from apiarian sources. Both BLIS have a narrow activity range and highly inhibit the growth of *P. larvae*. An electrophoretic analysis of the proteins in a BLIS molecule, showed three bands having apparent molecular weights of about 6.2, 14.4 and 17.1 kDa respectively.[61]

Paenibacillus polymyxa isolated from honey samples, produced an antimicrobial compound of molecular mass 1168.78 Da, matching that of polymyxin E1.The

antimicrobial compound was purified by 80% saturated ammonium sulfate precipitation followed by carboxymethyl-sepharose chromatography and characterised by reverse-phase HPLC and electrospray ionization -quadrupole time of flight mass spectrometry (ESI-qTOF MS).[46]

1.4.4 Purification & analysis of active compounds

High Performance Liquid Chromatography Mass Spectrometry (HPLC/MS)

The target molecules of this project are small antimicrobial compounds with molecular weights below 2 kDa. Reversed phase HPLC (RP-HPLC) successfully separates both polar and nonpolar neutral molecules with molecular weights below 2000 Daltons.

RP- HPLC is characterized by a situation in which the mobile phase used is more polar than the stationary phase. For neutral analytes, the mobile phase consists of water (the more polar component) and an organic modifier, which is commonly known as the organic phase. The organic modifier lowers the polarity of the mobile phase leading to a variation in the retention of analytes. In reverse phase column chromatography, stationary phase is usually comprised of C-18 column. Water is usually used as the aqueous mobile phase and methanol or acetonitrile as organic modifiers. When ionic analytes are present, other additives such as buffers or ion pairing reagents can be added to the mobile phase to control retention and reproducibility. Formic acid is commonly used as an additive.

Chromatographically, in RP-HPLC water is the ‘weakest’ solvent as, being the most polar, it repels the hydrophobic analytes into the stationary phase more than any other solvent, hence lengthening retention times.

When the organic modifier is added, the hydrophobic part of the analyte is no longer as strongly repelled into the stationary phase, spends less time in the stationary phase, and therefore elutes earlier.

Alternative methods if RP-HPLC cannot separate compound depends on the nature of the compound. normal phase or hydrophilic interaction liquid chromatography (HILIC) can be used for very hydrophilic compounds which may have too short retentions in RP.

On the other hand, non-aqueous reversed phase chromatography (NARP) can be used if analyte is very hydrophobic which is indicated by strong retention under reversed phase conditions and may require the use of non-aqueous conditions.

LC/DAD

UV-Vis chromatography uses light over the ultraviolet range (185 - 400 nm) and visible range (400 - 700 nm) of electromagnetic radiation spectrum. From literature, it was found that isolated antimicrobial compounds tend to be in the ultraviolet range which is why samples are analysed at wavelengths of 212, 225, 254, 275 and 350 nm to detect peptide compounds in the range of 200 to 225 nm and aromatic compounds higher than 225 nm.[64]

1.4.5 Testing extracts

Several well- known bioassays such as disk-diffusion, well diffusion and broth or agar dilution are commonly used, but others such as flow cytofluorometric and bioluminescent methods are not widely used as they require specialised equipment. This makes the latter methods less appealing for preliminary tests of activity from unknown compounds even if they can provide rapid results of the antimicrobial agent's effects.

The agar well diffusion method is commonly used to evaluate the antimicrobial activity of microbial extracts. The agar plate surface is inoculated by spreading a volume of the microbial inoculum to be tested against over the entire agar surface. Then, a hole is punched aseptically with a 1000 µl pipette tip, and the antimicrobial agent or extract solution at desired concentration is introduced into the well. Agar plates are subsequently incubated under suitable conditions depending upon the test microorganism. The antimicrobial agent diffuses in the agar medium and inhibits the growth of the microbial strain tested. The radius of inhibition can then be used to quantitatively compare the activity of the extracts. [65]

1.5 Objectives

The objective of this study is to find and characterise antimicrobial compounds against AFB that are harmless to both humans and bees using the microbiome of bees. Since testing the found antimicrobial compounds on human and animals is out of this study’s scope, the antimicrobial compounds showing high inhibition at low

concentrations will be ideal, in the hopes that they will also be less toxic. We are also open to finding isolates that has the potential to be used as probiotics against AFB.

Moreover, we are interested in investigating if wild bees have some strong inhibitory microbes against AFB, as it was reported in a study (Keller et al.)[47] that the pathogen *P. larvae* was not found among any of the wild bees sampled.

For honey bees, the exteriors, beehive swabs and guts will be isolated, while for the wild bees, the guts and exteriors only will be isolated. Bombus species (bumble bees) and Andrena species (mining bees) will be used as a model for wild bees due to the ease of their collection. The strains isolated from the samples will be tested against *P. larvae* in a pairwise assay in 12 well plates. Strains showing inhibition will then be inoculated in larger scale to extract secondary metabolites using ethyl acetate (EA), methanol and chloroform as solvents. The extracts will be fractionated and tested against *P. larvae*. The active fractions will then be characterized using LC-MS, IR and NMR spectroscopies.

The activity of the extracts will be tested against *P. larvae* using well diffusion assay. An average of the radius of inhibition will be recorded and compared with a negative control such as sterile water and extracts of nutrient agar.

2. Experimental

2.1 Sample Collection and processing of bee samples

Along with colleagues, Morgan Crosby and Julie Anne Dayrit, honey bees and beehive swabs were collected from 3 different apiaries across Nova Scotia and stored in sterile centrifuge tubes during summer. The locations covered were Middle

Musquodoboit, Middle Stewiacke and Truro. The samples were processed with the help of Morgan Crosby. The honey bees were anesthetized by leaving the tubes in the freezer for half an hour to an hour before processing. The samples were processed by first vortexing in nutrient broth to culture the microbes on the exterior of the bees. The exterior ones were left to incubate for 2 weeks before re-streaking on agar plates. The beehive swabs were cultured in nutrient broth and left to incubate for 7 days before streaking on solid media. Nutrient broth (NB) was made of 5g Tryptone, 5g NaCl, 3g yeast extract and 1L of deionized water. Nutrient agar had the contents of nutrient broth along with 15 g of agar. The media were sterilized in a Getinge Vacuum Steam Sterilizer (Model 533Ls) at 121°C for 15 minutes.

Bombus species (bumble bees) and Andrena species (mining bees) were collected as a model for wild bees in South End Halifax, with the help of Abdurrahman Elajmi. Those bees were chosen for collection as they could be easily found in the locality. The wild bees would be out in hot summer days, especially around noon and early afternoon.

The wild bees were collected using butterfly nets and stored in sterile centrifuge tubes until processing. Processing and dissection of the wild bees were performed by Abdurrahman Elajmi and myself. Pictures of the wild bees were taken for identification and records. The exteriors of the bees were cultured using the same method as the sample processing of honey bee exteriors.

2.1.1 Dissection and processing of honey bee and bumblebee guts

The Adrena bees weren't dissected due to their narrow body size. Dissection tools were sterilized by first washing with 10 % bleach solution, sterile water and then 70

% ethanol. The ethanol was allowed to evaporate before dissecting the bees. Firstly, the thorax was cut with a sterile scalpel to access the gut. The whole gut was pulled along with the honey stomach with sterile tweezers, as it was hard to isolate the guts without bursting the honey stomach. The gut samples were cultured in Peptone Yeast Extract Glucose (PYEG) broth (5 µg/ml Hemin, 0.5 µg/ml Vitamin K_1 , 0.5 µg/ml Vitamin K_2, 20 g tryptone, 10g yeast extract, 10 g D-Glucose, 0.5g L-cysteine, 0.4g $NaHCO_3$, 0.08g NaCl, 0.04g KH_2PO_4, 0.04g K_2HPO_4) for one week at 37 ˚C until growth was seen. The microbial cultures were then streaked on PYEG agar and incubated at 37 ˚C in 2.5 L anaerobic jars (Mitsubishi AnaeroPack Rectangular Jar by Thermo Scientific, R685025). An anaerobic indicator, Oxoid Resazurin Anaerobic indicator from Thermo Scientific (BR0055B) was used to monitor the anaerobic conditions of the jar. Pink colour indicates the presence of oxygen while white shows anaerobic conditions.

2.2 Strain Isolation

Colonies were isolated according to visible morphological characteristics such as shape (figure 4), margin or edge of colony (figure 5), colour, texture (powdery, smooth, fluffy) and opacity (translucent, clear, opaque).

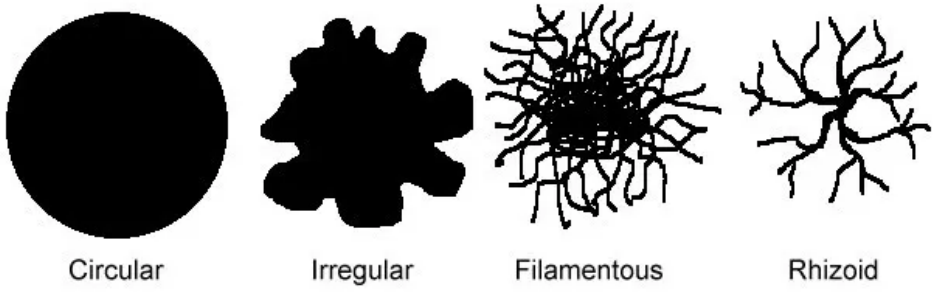


Figure 4. Types of shapes of bacterial colonies

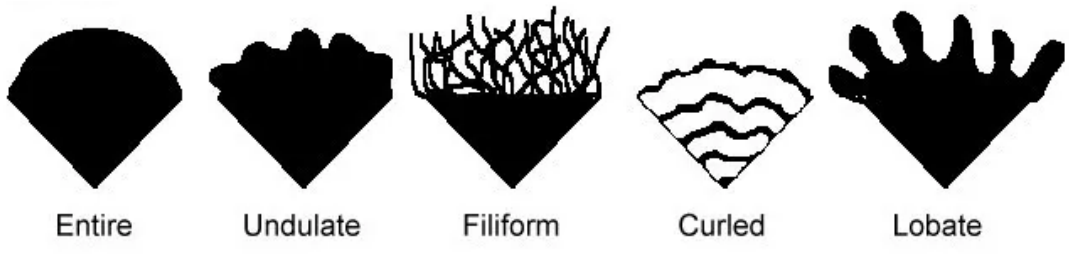


Figure 5. Types of edges of colonies

2.2.1 Honey bees

This part of the project was done with the collaboration of Morgan Crosby and Abdurrahman Elajmi. At the end of the incubation periods of the first cultures, the samples were re-streaked in 3 different media to maximize the number of strains isolated. Plain nutrient agar (NA), nutrient agar with 1 mg/ml cycloheximide (NAC), nutrient agar with 1 mg/ml ampicillin, (NAA). Ampicillin was added when the agar cools down until it is comfortable to touch to prevent degradation of ampicillin.

2.2.2 Wild bees

Twenty µl of the first cultures of the guts of the bees were re-streaked on NA incubated under aerobic conditions and on PYEG agar incubated in anaerobic jars at 37 ˚C. The first cultures of the exterior of the wild bees were re-streaked on NA. Only general media, NA, was used to reduce volume of work due to time constraints.

2.3 Antimicrobial screening

A general antimicrobial screening was performed using 12 well plates to find potential inhibitors. The metabolites of candidates showing inhibition would then be extracted to further test inhibitory activity.

2.3.1 Revival of Paenibacillus larvae

Paenibacillus larvae (ATCC 9545) was obtained from ATCC, American Type culture Collection. The entire pellet was rehydrated with 500 µL of Brain Heart Infusion with thiamine HCl (BHIT) broth. (37 g Brain Heart Infusion extract, 1L deionized water, 0.1 mg/L Thiamine HCl). The entire content was transferred to a 3 ml tube of BHIT broth (primary culture). Additional tubes of 5 ml BHIT broth were inoculated with 500 µL of the primary culture and left to incubate at 37°C for 48 to 72 hours under aerobic conditions. The primary culture was also streaked on BHIT agar and left to incubate under the same conditions. The *P. larvae* grew in the broth rather than agar after a few days. *P. larvae* liquid cultures were re-streaked on BHIT agar and on NA later, for use in antimicrobial assay. The appearance of the growth of the strain did not differ when media was switched from BHIT agar to NA. However, the *P. larvae* got contaminated and a new batch was ordered.

Upon revival of the new batch using the aforementioned method, the *P. larvae* grew very slowly, slower than the first batch. It was also growing differently from the first batch.

2.3.2 General antimicrobial screening

This part was also done in collaboration with Abdurrahman Elajmi. Antimicrobial screening of honey bee and wild bee gut isolates was carried out against *P. larvae* (ATCC 9545) using a pairwise assay on 12 well plates as shown in figure 6. The temperature conditions were used according to the incubation conditions of the test isolates during isolation ie beehive swabs strains isolated at room temperature and 30 ˚C, exterior of bees at RT, gut strains which were incubated at both aerobic and anaerobic conditions at 37 ˚C. For anaerobic isolates, 12 well plates were placed in the 2.5 L AnaeroPack jar (Thermo Scientific) with Resazurin anaerobic indicator (Thermo Scientific).

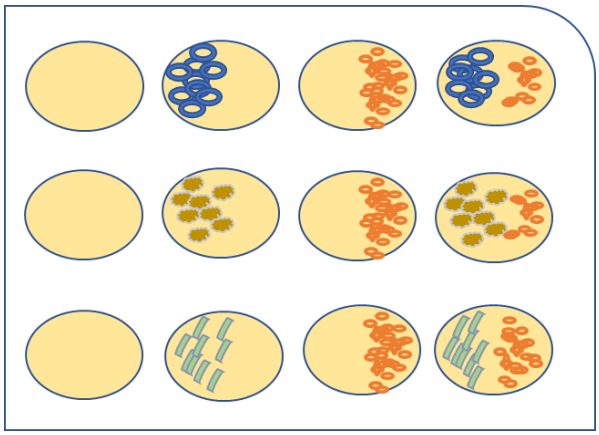


Figure 6. Pairwise antimicrobial assay using 12 well plates to test inhibition among bee isolates (test isolates) against *P. larvae*. The first column is the control which is plain NA, second and third column is streaked with test strain and *P. larvae* respectively and the last column is the pairwise test.

2.3.3 Modified pairwise assay

For pairwise assays where inhibition wasn't clear, the test bee isolates were inoculated first and left to incubate for one to two weeks depending on the speed of growth of the test isolate. This is done to allow the test strain to produce secondary metabolites that may inhibit *P. larvae*. The *P. larvae* was then streaked in its control column and opposite to the test strain in the pairwise test column. Comparison of the growth of *P. larvae* in the pairwise test column and control was taken after 3 days and one week.

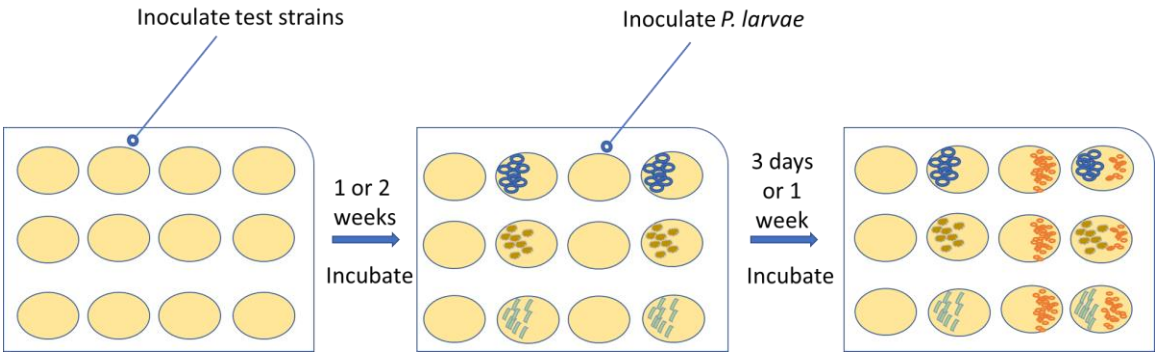


Figure 7. Modified 12 well plates pairwise assay to test isolates from bee samples against *P. larvae*. The first column is the negative control NA, second and last column is streaked with test isolates first and left to incubate for one to two weeks to allow test isolates to produce secondary metabolites. Lastly, *P. larvae* is streaked in the third which acts as a positive control for *P. larvae* and last column which is the pairwise test. Growth of *P. larvae* after 3 days or 1 week is compared with the control of *P. larvae* (third column).

2.3.4 Preparing cultures to further test inhibition

Two inhibitory strains, A12 (a honey bee swab isolate from RT) and E8 (a honey bee swab isolate from 30˚C), were first selected to further test inhibition as they showed really good inhibition in the pairwise assays. They were cultured in 10 sterile 15 ml centrifuge tubes of 5 ml of NB and incubated at 30 ˚C. A12 was inoculated on NA in four 12 well plates and incubated at RT for 4 weeks.

After finding out E8 is a mixture of strains, some strains isolated from E8 namely E8.4 and E8.3A were inoculated each on NA in four 12 well plates at RT for 4w.

2.3.4 Extraction of Metabolites

The liquid cultures were centrifuged to pellet cells and extracted with EA followed by methanol. The solid cultures were extracted with EA and 1:1 methanol: chloroform. The agar was transferred to Erlenmeyer flasks and left to sit in ca. 100 ml EA for 24 h. The agar in the EA mixture was then filtered by gravity. The filtrate which is the EA extract was evaporated using vacuum while the solid cultures were re-extracted with 1:1 chloroform: methanol (CM) solution and left to sit for 24 h before gravity filtration and vacuum rotary evaporation (figure 8).

Extracts were evaporated and stored in the refrigerator at 4˚C, away from light, until further use to prevent contamination and degradation of extracts. Around 15 to 30 mg of extracts were then dissolved in HPLC grade methanol for LC/DAD and LC/MS analysis.

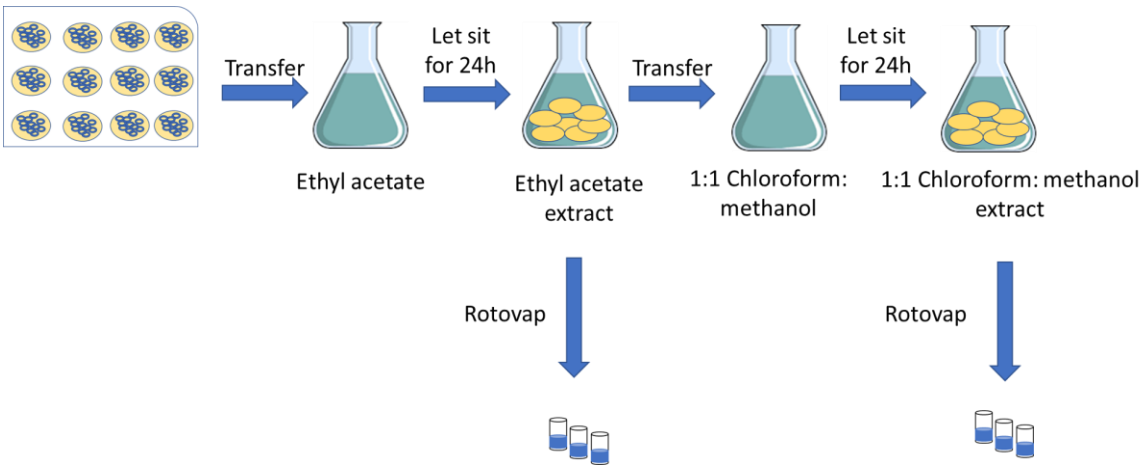


Figure 8. Scheme of method of extraction of solid cultures.

2.3.5 Preliminary tests for activity (Well Diffusion Assay)

Twenty µl of *P. larvae* in sterile deionized water at 0.5 OD was spread evenly over the surface of NA using a sterile cotton swab. Wells were made using sterile 1000 µl pipette tips and 20 µl of the crude extracts with approximate concentrations of 15- 30 mg/ml were added to the wells to get preliminary activity results of the extracts. Distilled water and plain NA extracts were used as negative controls. The extracts were tested in duplicates or triplicates depending on the yield of the extracts. The diameters of the inhibition were recorded after 24h and used to compare activity.

Figure 9. Graphical representation of well diffusion assay extracts against *P. larvae* (dark blue). Average radius of inhibition can be calculated from average diameter of area of inhibition (pale blue). Average radius = Average diameter/2

2.3.6 Analysis of Extracts

The methods were developed for general separation of secondary metabolites with the help of Patricia Granados at the Saint Mary's University Centre for Environmental Analysis and Remediation (CEAR) Lab, Halifax, NS.

Crude extracts were analyzed using an Agilent 1100 series LC-MS equipped with an iontrap mass spectrometer (Agilent 110 Series LC/MSD Trap) and a diode array detector. The 25 mg/ml samples of the extracts were filtered using a 0.22 µm syringe and run through a reverse phase chromatography with C-18 column (Zorbax Eclipse XDB C-18, 4.6 x 75 mm, 3.5 microns), acetonitrile (ACN) with 0.1% formic acid as organic phase and milliQ water with 0.1% formic acid as aqueous phase. Two gradient methods were used during method development. The gradient method A involves elution of sample at a flow of 1ml/min through a gradient of 20% ACN for 25 mins, followed by 80% ACN for 3 mins and 100% ACN for another 5 mins. The gradient method B involved an isocratic flow of 20% ACN: milliQ water for 15 mins. The column was kept at a temperature of 30ºC and maximum pressure of 100 bar. UV chromatography was carried out at 212, 225, 254, 275 and 350 nm for each sample. Since the mass spectrometer was malfunctioning, only the LC-DAD was used to detect the presence of compounds in the samples that were then analysed with another mass spectrometer.

Method development of UHPLC/ESI-qTOF MS for active extracts analysis

An Agilent Technologies 1290 Infinity II series mass spectrometer equipped with a quadrupole time of flight (qTOF) and coupled with an UHPLC was used to further analyse some extracts.

Samples were run through a reverse phase C-18 column (Poroshell 120 EC-C18, 3.0 x 150 mm, 2.5 micron) with acetonitrile with 0.1% formic acid as organic mobile phase and milliQ water with 0.1% formic acid as aqueous mobile phase at a flow of 0.6 ml/min. The samples were run through the column using both gradient method A and B. The column was maintained at a temperature of 30ºC and maximum pressure of 600 bar. Samples were analyzed from 100 m/z to 2200 m/z using + Electrospray Ionization quadrupole time-of-flight mass spectrometry with a drying gas flow of 11 L/min at 350 ºC.

2.4 Fractionation of extracts

The crude extract of each of the pure isolate strains (1 ml of 25 mg/ml in methanol) was fractionated using a gravity microcolumn with C18 silica (6.5 cm height, approximately 1 g) and an acetonitrile/water gradient (10 ml each of 0%, 10%, 20%, 60%, 80%, and 100% acetonitrile in water). Each fraction was collected in scintillation vials and stored in the refrigerator at 4°C away from light for subsequent bioactivity testing and analysis. The fractions were tested using the well diffusion assay and analyzed as explained in section 2.3.3 and 2.3.4 respectively.

2.5 Identification of inhibitors

Fifteen randomly selected inhibitory strains were revived and incubated for at least 24 h for PCR preparation. DNA was extracted by mixing 750 µl of 70% ethanol, glass beads and an inoculation loopful of the sample. The mixture was then vortexed 6 times at high speed for 10 seconds each time.

For sample preparation, 2 µl of 10 µM 16S rRNA FOR primer (AGA GTT TGA TCC TGG CTC AG) and 10 µM 16S rRNA REV primer (ACG GCT ACC TTG TTA CGA CTT) for bacterial samples, 2µl of 10 µM ITS 1F and 2 µl of 10 µM ITS 4 primers for fungal samples, 19 µl nuclease free water, 25 µl master mix (GoTaq® DNA Polymerase which is supplied in 2X Green GoTaq® Reaction Buffer (pH 8.5), 400 µM dATP, 400 µM dGTP, 400 µM dCTP, 400 µM dTTP and 3mM $MgCl_2$) were used along with 2 µl of extracted DNA.[34] PCR products were examined by using agarose gel electrophoresis and visualized using ethidium bromide and UV light. The DNA samples were sent to McGill University and Génome Québec Innovation Centre for Sanger Sequencing.

Results

3.1 Strain isolation

From the honey bee samples, about 359 isolates were obtained using NA, NAA and NAC. 235 isolates comprising 99 from the exterior, 69 from beehive swabs incubated at 30 ˚C and 69 from beehive swabs incubated at RT were found using NA. 86 isolates comprising of 69 from the exterior and 17 from beehive swabs at 30 ˚C were

isolated using NAC and 38 isolates comprising of 25 from the exterior and 13 from beehive swab at 30 ˚C were obtained using NAA.

Table 1. Summary of number of isolates found using different media and conditions. Isolation of beehive swabs at RT was not completed with use of NAA and NAC.

Media	Honeybee exterior	Beehive Swabs, 30 ˚C	Beehive swabs, RT
NA	99	69	69
NAA	25	13	-
NAC	69	17	-

From wild bees, about 140 isolates have been found so far using NA. (The selective media such as NAA and NAC were not used due to time and workload limitations) 24 strains were isolated from guts of bumble bees under anaerobic conditions.

3.2 Potential inhibitors

3.2.1 General antimicrobial screening

About 102 out of 359 isolates from honey bee samples showed inhibition against *P. larvae*. The pairwise assay using 12 well plates gave only qualitative insights on the inhibitory activity of the isolates. Moreover, these observations cannot show us if inhibition is caused by production of antagonistic compounds that harm *P. larvae* or competition for nutrients. Therefore, some isolates (A12 and E8) showing good inhibition were chosen to further test inhibition and find antimicrobial compounds.

From the 24 strains isolated from bumble bee guts, 3 strains showed strong inhibition. Those strains did not grow on NA under aerobic conditions, indicating that the strains may be obligate anaerobe. Further tests needed to be done to confirm this observation. Frozen stocks of the isolates were revived for further testing. Upon revival the second time, less colony growth was seen and the appearance of the colonies also changed, which indicated that the viability of the cells decreased. Also, due to the slow growth of both test strains and *P. larvae* on NA under anaerobic conditions, it was difficult to observe cases of moderate inhibition. The modified pairwise assay may be more suitable to test inhibition of the test strains.

From the modified pairwise assay performed on wild bee exterior isolates, inhibition could not be tested due to a reduction in the cell viability of *P. larvae.* The loss in cell viability was observed from unusual patterns and slowness of growth in *P. larvae* upon re-streaks or revival from frozen stocks.

3.2.2 Identification of selected inhibitors

Of the previously selected candidates, A12 was identified as *Bacillus Cereus* (percent identity ≥ 99%) and E8 could not be identified due to high noise level in the sequence reported by Sanger Sequencing. The high noise level was indicative of the presence of more than one strain in E8. E8 was re-streaked to isolate the different strains. Subsequently E8 was found to contain at least 6 strains; 1 from *Bacillus subtilis* group (E8.1A), 2 belonging to *Pseudomonas chloroaphis* group (E8.1B, E8.3A), 1 as *Debaromyces prosopidis* (E8.2A) and a mixture of strains (E8.4). E8.4 contains at least 2 unknown strains as the sample showed bands for both 16S and ITS primers in gel electrophoresis. The high noise level observed during its sequencing confirms the presence of more than one strain in E8.4.

Out of the 15 selected inhibitors sent for Sanger sequencing, 9 were successfully identified (percent identity ≥99%) while the other 6 had a high noise to signal ratio and could not be sequenced. The 9 strains comprised of 1 related to *Raoultella terrigena*, 4 in *Pseudomonas chloroaphis* group, 2 to *Debaromyces prosopodis*, 1 *Paenibacillus* as *Paenibacillus lactis* and 1 belonging to the *Bacillus subtilis* group.

3.3 Assessment of bioactivity and analysis of extracts

3.3.1 Bioactivity of first batch of extracts

From the first trial of well diffusion assay of crude extracts of A12 and E8 against *P. larvae*, the CM extract of 4w old A12 at RT, methanol extracts of 4w old liquid cultures of A12 and E8 showed really good inhibition with the former showing the highest activity (table 1). However, on the second trial of testing the extracts, the control NAP

showed inhibition, which indicated that the extracts were contaminated. Since the extracts got contaminated, they were no more viable for further analysis.

Table 2. Average radius of inhibition of agar well diffusion assay of extracts of solid and liquid cultures of A12 and E8 at different temperatures for 4 weeks. a- solid culture at RT. b- liquid culture at 30 ˚C.

Extract	Average radius (mm)
Control NAP, EA[a]	0
Control NAP, CM[a]	0
A12, CM[a]	3.9
A12, EA[a]	1.5
A12, methanol[b]	3.0
A12, EA[b]	0
E8 liq. Methanol[b]	4.1
E8 liq. – EA[b]	1.9

3.2 Activity of A12 (identified as *B. cereus*)

The CM extract of the solid cultures of A12 showed strong inhibition against *P. larvae* (figure 10). Therefore, 4w old A12 cultured on NA (solid culture) were extracted

again for further analysis. LC/DAD with an isocratic gradient was run on the CM crude extract. The UV chromatogram was compared to that of the CM crude extract of NA (figure 11). Several peaks from A12 was detected indicating that fractionating the extract will be needed to find the anti- *P. larvae* antagonistic compounds. The crude extract was fractionated and analysed using UHPLC/qTOF-MS. Preliminary results are included in Appendix C

Unfortunately, the activity of the extracts and fractions from the second extraction could not be tested against *P. larvae* due to loss of cell viability.

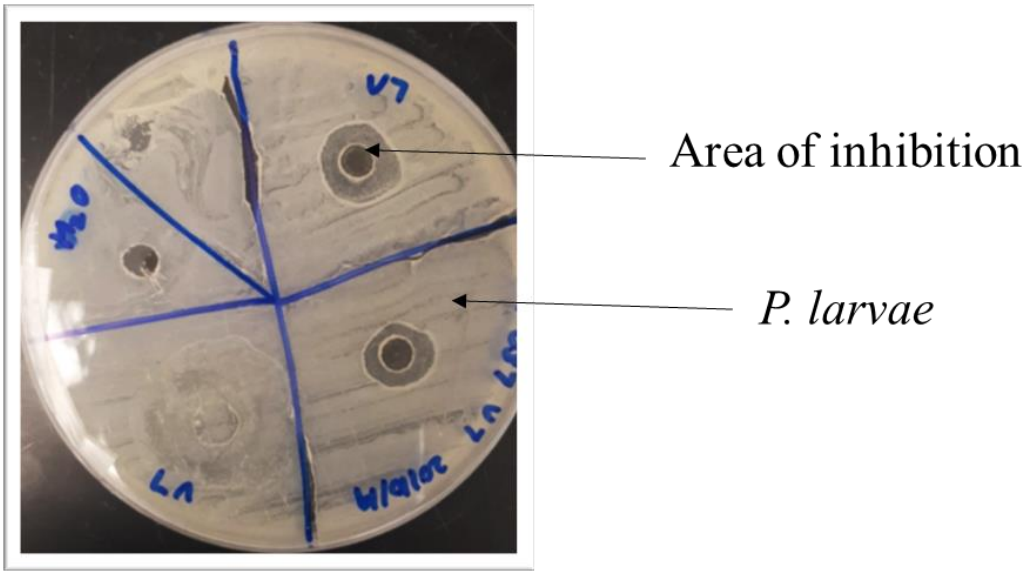


Figure 10. Well diffusion assay of first batch of extracts; CM extract of 4w old A12. The assay is divided into 2 parts. One part is the well diffusion assay which is divided into 3 fractions comprising of water as a control and 2 replicates of the test extracts. The other part is another test method, that involve dropping the test extract directly over the plate

streaked with *P. larvae*. It is comprised of a water control and one replicate of test extract. Inhibition is more visible through the well diffusion assay.

◆ : Present in control

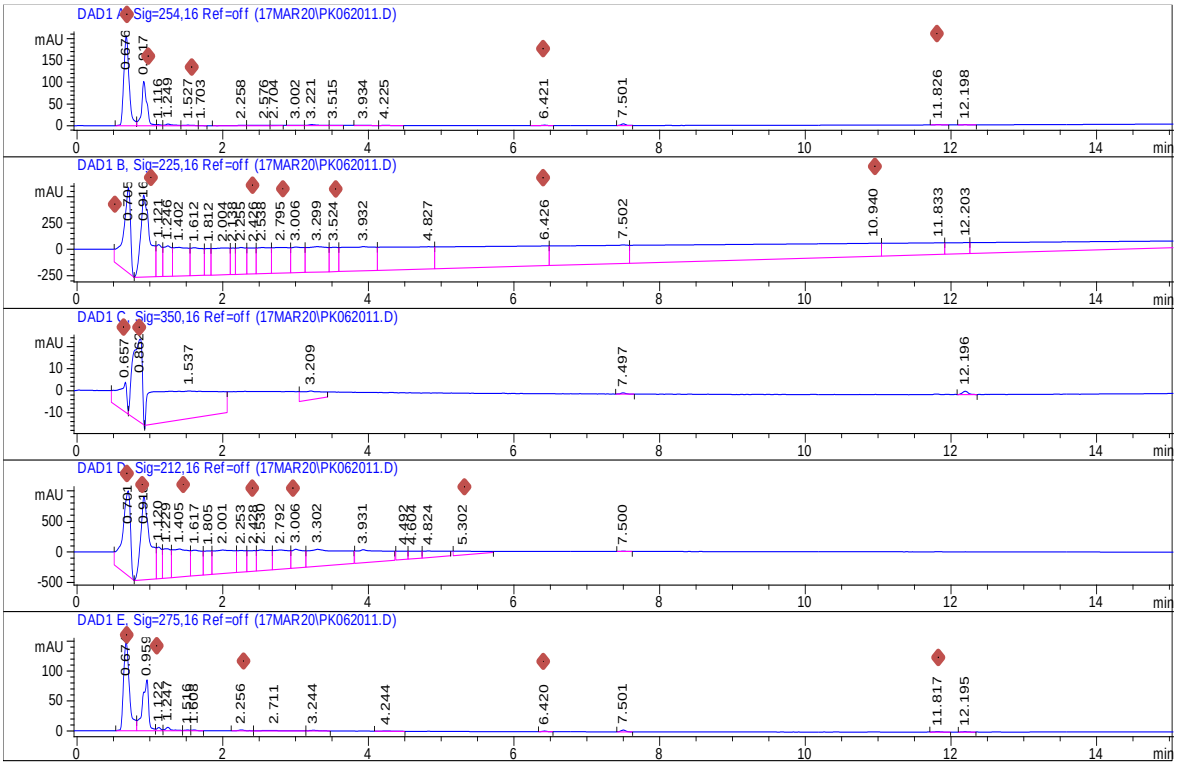


Figure 11. UV chromatograms of CM extract of A12 4w old ran under isocratic gradient of 20% acetonitrile: water for 15 mins at λ= 254, 225, 350, 212, 275 nm.

3.3.3 Activity of E8 (mixture of strains)

The extracts of E8 showed strong to moderate inhibition against *P. larvae* (figure 12). However, it was discovered that E8 is a mixture of strains.

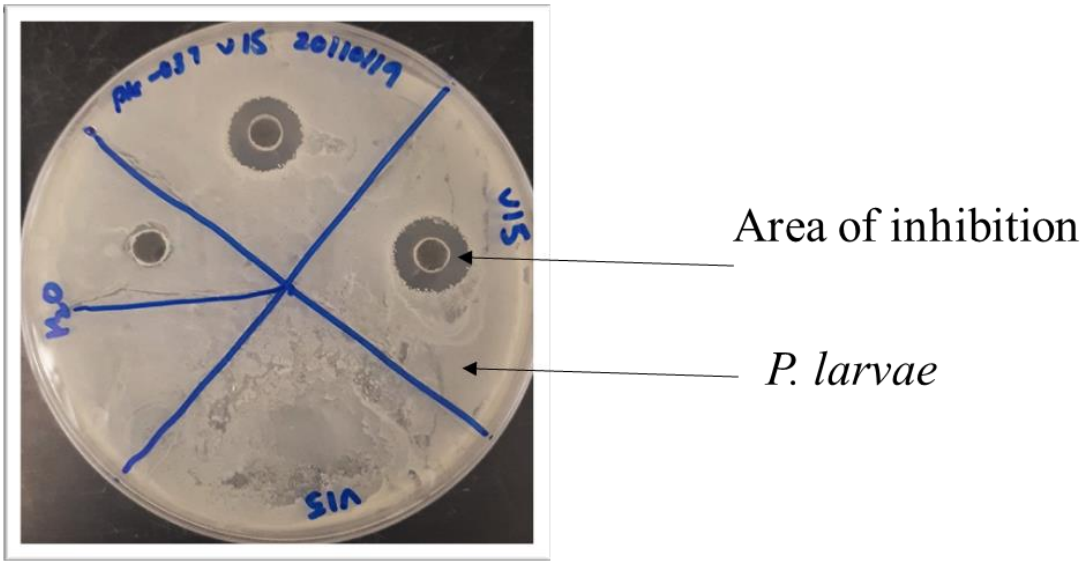


Figure 12. Well diffusion assay of first batch of extracts; EA extract of 4w old E8 against *P. larvae*. The assay is divided into 2 parts. One part is the well diffusion assay which is divided into 3 fractions comprising of water as a control and 2 replicates of the test extracts. The other part is another test method, that involve dropping the test extract directly over the plate streaked with *P. larvae*. It is comprised of a water control and one replicate of test extract. Inhibition is more visible through the well diffusion assay.

Since, E8 showed promising activity, isolates from E8 (E8.4 and E8.3A) were selected for further analysis. 4w old E8.4 and E8.3A solid cultures were extracted for further analysis. LC/DAD with an isocratic gradient was run on the crude extracts. The

labelled peaks in the chromatograms below show peaks that were either not found in the or were present at lower absorbance units at their respective retention times in the controls.

Therefore, the labelled peaks may be indicative of the metabolites produced by the microbes. From the UV chromatograms, the retention time of the CM extracts of both E8.3A and E8.4 are very short (less than 4 mins) and the peaks are not well separated. However, the isocratic gradient seems to be working better with the EA extracts of E8.4 and E8.3A. Activity of the extracts could not be tested against *P. larvae* due to loss of its viability.

LC/DAD of E8.3A (identified as *Pseudomonas chloroaphis*)

◆ : Absent in control

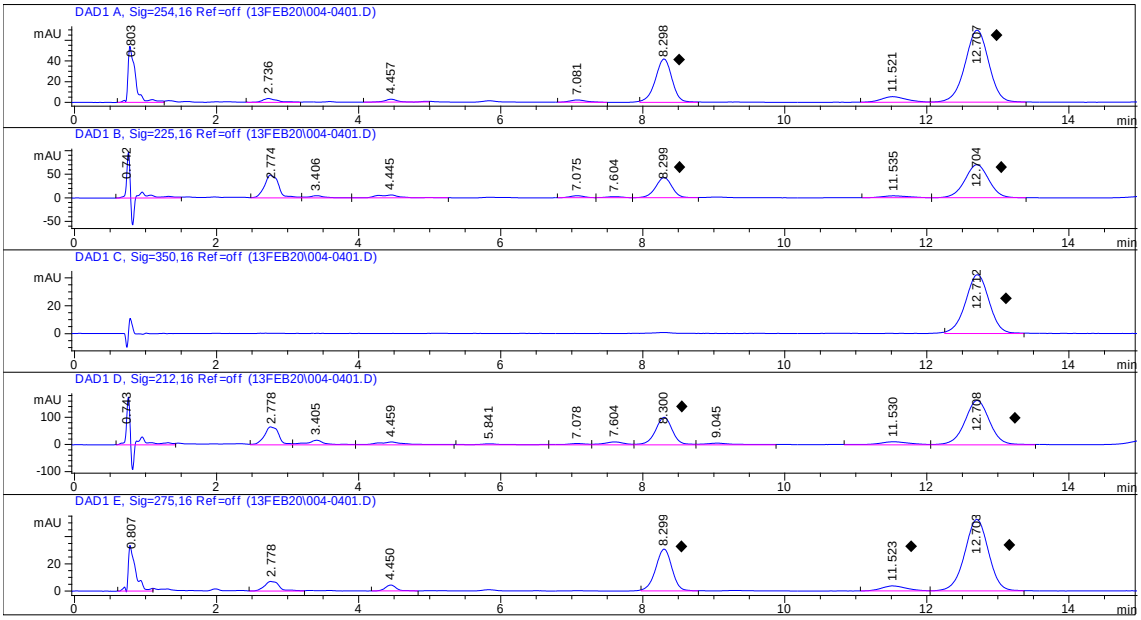


Figure 13. UV chromatograms of EA extract of E8.3A ran under isocratic gradient of 20% acetonitrile: water for 15 mins at ʎ= 254, 225, 350, 212, 275 nm.

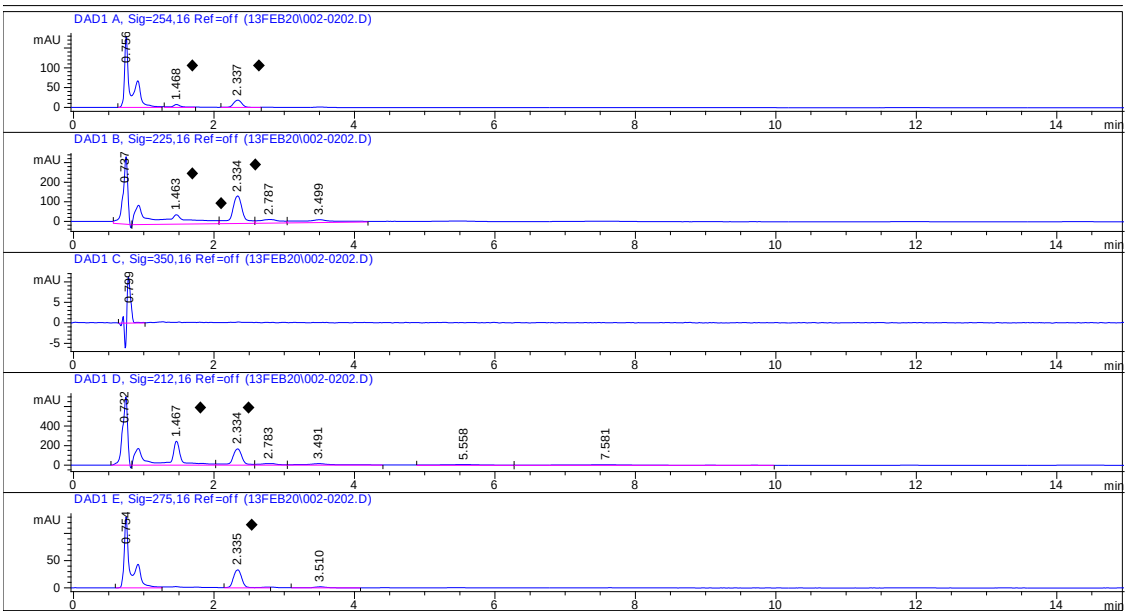


Figure 14. UV chromatograms of CM extract of E8.3A ran under isocratic gradient of 20% acetonitrile: water for 15 mins at λ= 254, 225, 350, 212, 275 nm.

LC/DAD of E8.4 (mixture of unknown strains)

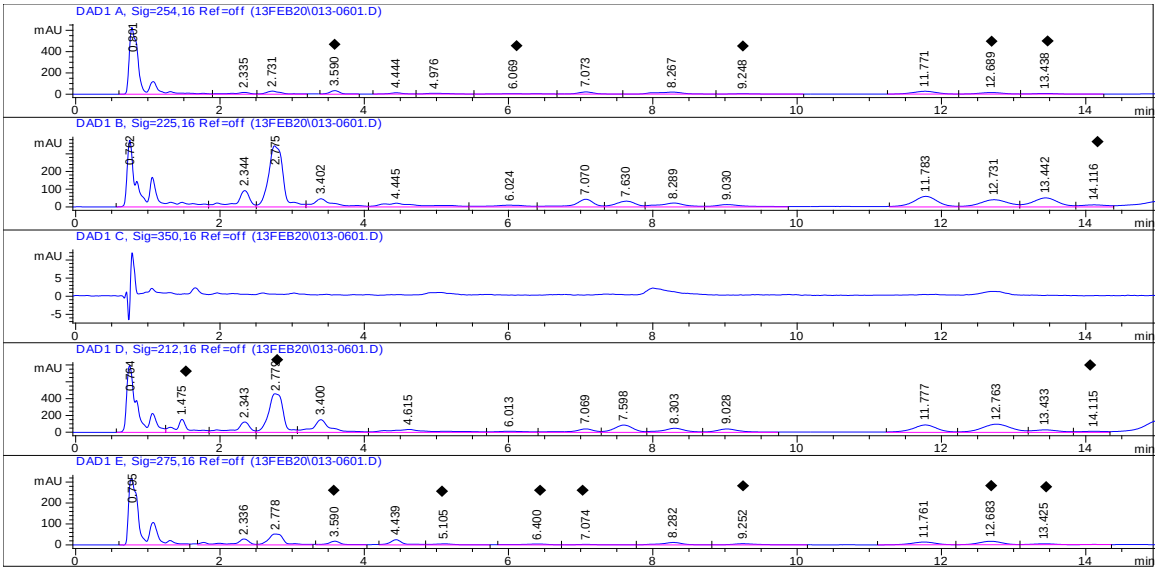


Figure 15. UV chromatograms of EA extract of E8.4 ran under isocratic gradient of 20% acetonitrile: water for 15 mins at λ= 254, 225, 350, 212, 275 nm.

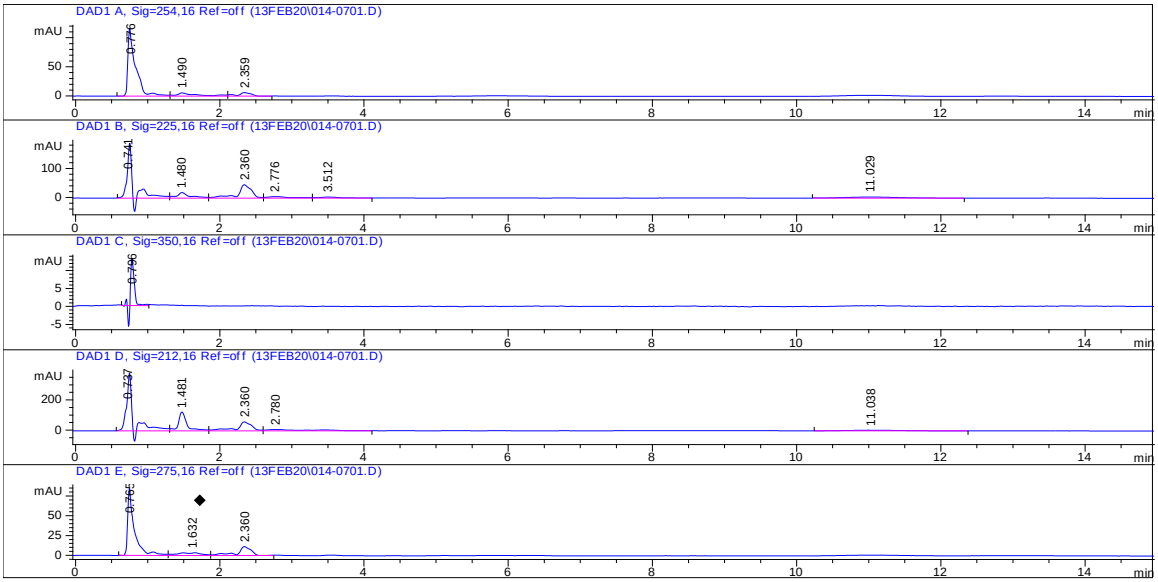


Figure 16. UV chromatograms of CM extract of E8.4 ran under isocratic gradient of 20% acetonitrile: water for 15 mins at λ= 254, 225, 350, 212, 275 nm.

3.4 Well diffusion assay of the second batch of extracts of A12, E8.3A & E8.4

No growth of P. larvae was observed on the plate after 3 days and very little growth was seen after 1 week. *P. larvae* did not grow homogeneously and grew unusually slow in a different pattern.

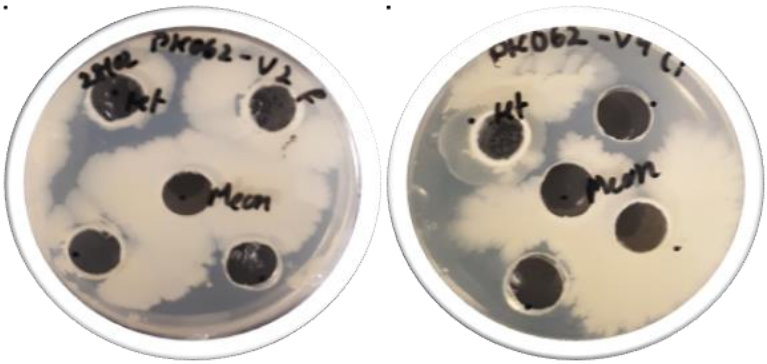


Figure 17. Post 2 weeks observations of well diffusion assay of second batch extracts against newly ordered *P. larvae*. Methanol and water were used as negative controls. This trial of well diffusion assay was unsuccessful as *P. larvae* did not grow homogeneously to allow for assessment of the extracts.

4. Discussion

4.1 Strain isolation

Interestingly, isolates were obtained mostly from the exterior of bees relative to swabs of the beehives. The exterior isolates were slow growers, specially at the beginning. They were left to incubate for longer than the swabs which may have allowed for more microorganisms to grow.

4.2 Finding inhibitors

Twelve well plates pairwise assay was suitable for screening a large amount of strains in a time efficient manner, as the activity of 3 test strains can be assessed per plate. However, it was challenging to determine the activity of test strains in some cases as the growth of test strains and *P. larvae* in the pairwise test would not differ much from their respective controls.

Another general antimicrobial screening assay, perpendicular streak method was used in previous studies whereby a 20 mm streak of the test strain is made with a sterile cotton swab across the plate and incubated for a few days to allow the production of antagonistic substances. Then 3 to 5 streaks of *P. larvae* are made perpendicular to the test strain's streak and left to incubate for a few days to assess inhibition.[45] This method would give more insights on whether the metabolites of the test strains are causing inhibition as it allows test strains to produce potential antagonistic substances before inoculating *P. larvae*. If the metabolites of the test strains cause inhibition, restricted growth of *P. larvae* will be observed in the pairwise test. The modified 12 well plates pairwise assay provides the same advantage as the perpendicular streak method and on top of that, it allows assess activity of more than one test strain at the same time.

Inhibitors from wild bees could not be found yet because inhibition could not be assessed using the modified 12 well plate pairwise assay as the *P. larvae* was growing in neither the control nor the pairwise test. We confirmed that it is not due to contact independent inhibition i.e. inhibition caused by gaseous antagonistic compounds, because the *P. larvae* was not even growing well on a separate control. Comparison of the growth

pattern of our *P. larvae* (figure 19) with literature (figure 18), confirms that our *P. larvae* is behaving strangely which may due to loss in cell viability.

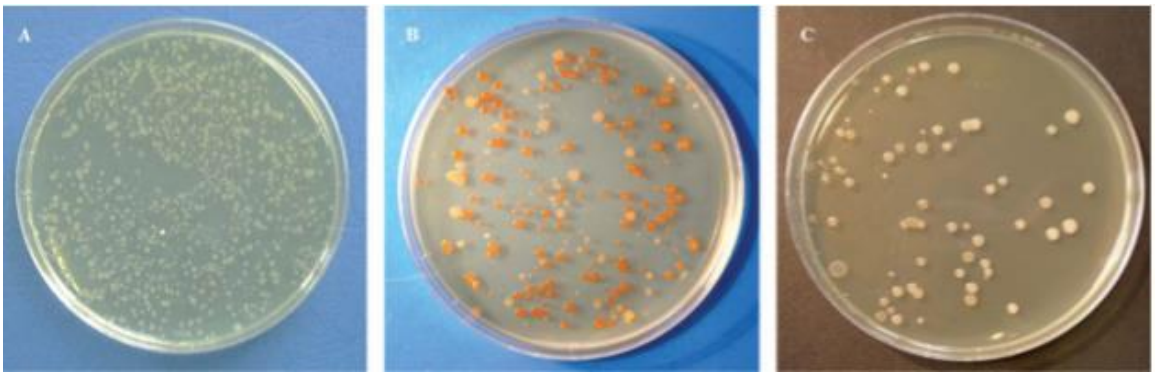


Figure 18. Physical appearance of colonies of *P. larvae* genotype ERIC I (a) and ERIC II (b) and (c). The *P. larvae* was isolated from AFB outbreaks in Italy [66] (Permission for use of image is granted)

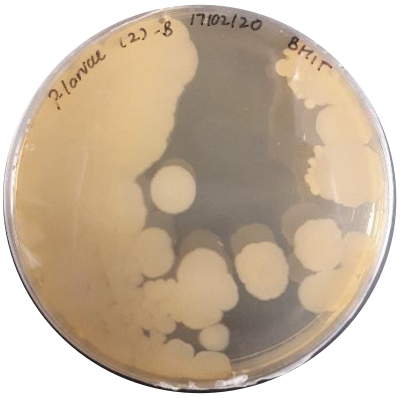


Figure 19. *P. larvae* re-streak from newly revived ATCC 9545 *P. larvae* strain (ERIC I) with biofilm production. No biofilm is visible in any of the reported ERIC I and ERIC II genotypes of *P. larvae* isolated from AFB outbreaks in Italy (fig.18)

The reason for loss of viability of *P. larvae* and the unusual growth pattern such as formation of biofilms is unknown. Normally, cells change their genetic expression, resulting in changes in appearance or formation biofilms when they are highly stressed.

A bacterial community can induce death in a part of the population in response to various stress conditions to favour the survival of the colony, including: oxidative stress, radiation exposure, nutrient deprivation, phage infections, and many others.

Biofilms protect bacteria from stressing conditions as well as from other microorganisms that live in the same environment. Spore-forming bacteria produce both biofilm and endospores being able to respond more swiftly to environmental stresses. Moreover, biofilm is an optimal environment for sporulation.[67] Since *P. larvae* is spore forming bacteria, the formation of biofilm of the bacteria can indicate that the bacteria were transformed due to exposure to stress conditions.

4.2.1 Identification of inhibitors

Some samples showed a high level of noise, which is indicative of the presence of nucleotides from different sources in the sample. It is very likely to occur due to the presence of more than one microbial strain in the isolates sequenced. Moreover, during Sanger Sequencing of a sample batch, an unexplained error of the presence of Indel homopolymer microsatellite contaminated short fragment, was reported in all the samples which affected the quality of the sequences. Since it was found in all the samples, it is highly possible that the Go Taq green master mix has degraded or was contaminated. Nonetheless, the presence of the Indel homopolymer microsatellite contaminated short fragment still enabled acquisition of good quality short sequences for BLAST analysis.

For the strains with approved quality of sequences, the ones with the highest number of hits and highest percent identity were chosen. All 9 identified strains can be found in the environment, such as in soil. These findings match with the isolates' samples of origin which are beehive swabs of honey bees.

In most cases, all the top hits were found to belong to the same group of species. A group of bacterial species comprises of genetically closely related individuals. In some cases, such as identification of A12, it was difficult to distinguish between species due to some scores being the same, showing that the species were closely related to each other.

For further identification, species specific primers can be used. In some cases, biochemical tests also can be used to distinguish among candidate species. For instance, E8.2A was identified by BLAST as *Debaromyces prosopidis* and *Debaryomyces subglobosus* with *Debaromyces hasenii* as the second closest hit. Debaryomyces prosopidis can be differentiated phenotypically from both varieties of *Debaromyces hasenii, D. hansenii var. hansenii* and *D. hansenii var. fabryi* by lack of growth on cellobiose after 2 weeks incubation and from the variety *hansenii* by a higher maximum temperature for growth.[68]

The top hit for A12 was *Bacillus Cereus* and the other close hits belonged to the Bacillus Cereus group. The *Bacillus cereus* group contains closely related gram-positive bacteria such as *B. anthracis* but exhibit highly divergent properties. They are genetically very similar, but scientists have not classified them as one species as their metabolomics and behavior are significantly different. There is a wide difference in behaviours *of B. Cereus* strains themselves. Some strains of *B. cereus* can cause food-borne disease, while

some are also plant growth promoter and animal probiotics. [69] *B. cereus* strains can produce beta-lactamases and bacteriocins such as emetic toxins.[70] On the other hand, *B. cereus* isolated from apiarian sources have been found in many studies to inhibit *P. larvae* by producing bacteriocin-like inhibitory substances that has the potential to be combined with an Integrated Pest Management approach.[62]

The fact that closely related *B. cereus* species can have different metabolomics suggests that it is highly possible that A12 is producing antimicrobial compounds that are yet to be discovered.

16S rRNA is limited in its ability to differentiate the *B. cereus* group bacteria. The nucleotide sequences of the 16S rRNAs of the *B. cereus* group exhibited very high levels of sequence similarity (>99%) Likewise, Ash and Collins reported that even the 23S rRNA gene sequences of *B. anthracis* and an emesis-causing *B. cereus* strain were almost identical. However, a study was able to examine the phylogenetic relationships of *B. cereus* group strains using nucleotide sequences of *groEL* and *sodA* genes.
The *groEL* genes, encode highly conserved housekeeping proteins that assist in proper protein folding (chaperons).[71]

E8.4 contains at least one bacterial and fungal strain as genetic material was isolated when 16S and ITS primers were used respectively. E8.3A belongs to the *Pseudomonas chloroaphis* group and, more specifically, as *Pseudomonas chloroaphis subsp aurantica. Pseudomonas chloroaphis* has been found to be a biocontrol agent against plant pathogens caused by *Fusarium graminearum. Pseudomonas chloroaphis* produces phenanzines and oxysporumphenazine-1-carboxamide, an antifungal metabolite

that is required for biocontrol of plant diseases such as tomato foot and root rot.[72] It will be interesting to find anti-*P. larvae* compounds as well from this strain.

4.3 Separation and MS analysis of extracts

The isocratic gradient provided better separation with both CM and EA extracts of A12. However, the E8.3A and E8.4 CM extracts were not well separated and had short retention times. This shows different gradient methods may be needed for extracts of different isolates. If retention times stay short after adjusting gradient, normal phase chromatography may be used for further analysis.

The CM extract of A12 was fractionated, run through the isocratic gradient (Gradient method B) in UHPLC and analysed with qTOF-MS. Although the isocratic method was efficient using HPLC, the gradient does not seem efficient in separating the compounds using UHPLC, given short retention times (less than 4 mins) were observed. Gradient method A showed better separation in the crude extracts of A12 compared to the isocratic gradient. Therefore, gradient method A can be used as an attempt to better separate the fractions of A12. Preliminary results of mass spectra of the crude extracts and fractions of the CM extract of A12 are attached in Appendix C.

Conclusion

Novel microbial species such as *Pseudomonas chloroaphis, Debaromyces prosopidis* and *Paenibacillus lactis* isolated from beehive swabs have been found to inhibit *P. larvae.* Along with the mentioned novel species, *B. cereus* and *B. subtilis* that were heavily reported to show good inhibition against *P. larvae* were also isolated as A12

and E8.1A from our samples. CM extracts of A12 and E8 showed strong inhibition while EA extracts of E8 showed moderate inhibition against *P. larvae.* Since E8 showed stronger inhibition than A12, it will be interesting to see whether the inhibitory activity is due to a synergistic activity of the different strains together against *P. larvae* or the strains independently inhibit *P. larvae.* Reproducibility of the activity of extracts can be influenced by factors such as presence of contaminants, concentration of extracts and stability of compounds in the extracts.

Future work

Future work involves ensuring *P. larvae* recovery and quality from the newly ordered batch to allow further testing of extracts. The minimum inhibitory concentrations of the active fractions need to be determined to lower risks of toxicity. The CM fractions of A12 can be analysed to detect novel antimicrobial compounds produced by *B. cereus* as high inhibition was already observed from the first batch of extracts. Gradient method A will be performed for UHPLC/qTOF-MS on the fractions of A12 to elucidate compounds that inhibit *P. larvae*.

It will be interesting to compare activity of E8 isolates with E8 to determine if inhibition is gone or reduced when the strains in E8 are separated. If A12 and E8.1 A produce novel inhibitory compounds that have not been reported in literature yet, more specific primers will be needed to characterize the *Bacillus cereus* and *Bacillus subtilis* species respectively.

Moreover, there should be a focus on finding new compounds from the novel bacteria that were found to inhibit *P. larvae.* This can be helpful to avoid isolating already discovered antimicrobial compounds. Biochemical tests to distinguish between *Debaryomyces prosopidis* and *Debaromyces hasenii* can be pursued given their simplicity.

The bumblebee microbial re-streaks for isolation need to be completed. The isolates should be tested against *P. larvae* using the modified 12 well plates pairwise assay to find inhibitors for discovery of anti-*P. larvae* compounds. Also, we could see if the inhibitors found can be used as probiotics for honey bees against *P. larvae* given the promise of probiotics in strengthening honey bee's immunity.

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Appendix A

Table 3. Identification of *P. larvae* inhibitor samples from honey bee isolates

Sample ID, SS*	ID of inhibitors			
	Group	Species	Query cover, # Hits	Percent ID (%)
A12, 61.36	*Bacillus Cereus*	*Bacillus Paramcycoides*	100, 1	100
		Bacillus cereus	100, 6	100

		Bacillus albus	100, 1	100
		Bacillus luti	100, 1	100
		Bacillus nitratireducens	100, 1	100
		Bacillus tropicus	100, 1	100
		Bacillus anthracis	100, 1	100
w5, 60.19	*Raoultella*	*Raoultella terrigena*	100,3	99.55 (2), 99.41
		Klebsiella oxytoca	100,5	99.26,99.55 (2), 99.41
E8.1B, 60.58	*Pseudomonas chloroaphis*	*Pseudomonas subsp aurantica*	100,2	99.87
		Pseudomonas chloroaphis	100,4	99.87
		Pseudomonas sesami	100,1	99.87
		Pseudomonas baetica	100,1	99.87
		Pseudomonas jessenji	100,1	99.87

		Pseudomonas umsongensis	100,1	99.87
E8.3A,61.09	*Pseudomonas chloroaphis*	***Pseudomonas subsp aurantica***	100,2	99.87
		Pseudomonas sesami	100,1	99.87
		Pseudomonas baetica	100,1	99.87
		Pseudomonas jessenji	100,1	99.87
E8.2A,63.8	*Debaromyces*	***Debaromyces prosopidis***	**99,1**	**100**
		Debaryomyces subglobosus	**99,1**	**100**
		Debaromyces hasenii	99,1	99.87
E2A, 61.97	*Pseudomonas chloroaphis*	*Pseudomonas subsp aurantica*	100,2	99.87
		Pseudomonas chloroaphis	100,4	99.87
		Pseudomonas sesami	100,1	99.87

		Pseudomonas baetica	100,1	99.87
		Pseudomonas jessenji	100,1	99.87
		Pseudomonas umsongensis	100,1	99.87
E8.1A	Bacillus subtilis	*Bacillus velezensis*	100,2	99.58
		Bacillus subtilis	100,3	99.58
		Bacillus mojavensis	100,4	99.58
		Bacillus vallismortis	100,2	99.58
		Bacillus tequilensis	100,1	99.58
		Bacillus halotolerans	100,4	99.58
w21, 63.70	*Debaromyces*	***Debaromyces prosopidis***	**100,1**	**100**
		Debaromyces hasenii	100,1	99.83
w20, 51.36	*Paenibacillus*	*Paenibacillus lactis*	100,1	99.39
		Paenibacillus lautus	100,3	93.42(2), 98.17

Sequences of identified inhibitors along with their chromatograms

A12 from 40 to 782

Agcttgctcttatgaagttagcggcggacgggtgagtaacacgtgggtaacctgcccataagactgggataactccgggaaac

cggggctaataccggataacattttgaaccgcatggttcgaaattgaaaggcggcttcggctgtcacttatggatggacccgcgt

cgcattagctagttggtgaggtaacggctcaccaaggcaacgatgcgtagccgacctgagagggtgatcggccacactggga

ctgagacacggcccagactcctacgggaggcagcagtagggaatcttccgcaatggacgaaagtctgacggagcaacgccg

cgtgagtgatgaaggctttcgggtcgtaaaactctgttgttagggaagaacaagtgctagttgaataagctggcaccttgacggta

cctaaccagaaagccacggctaactacgtgccagcagccgcggtaatacgtaggtggcaagcgttatccggaattattgggcgt

aaagcgcgcgcaggtggtttcttaagtctgatgtgaaagcccacggctcaaccgtggagggtcattggaaactgggagacttga

gtgcagaagaggaaagtggaattccatgtgtagcggtgaaatgcgtagagatatggaggaacaccagtggcgaaggcgacttt

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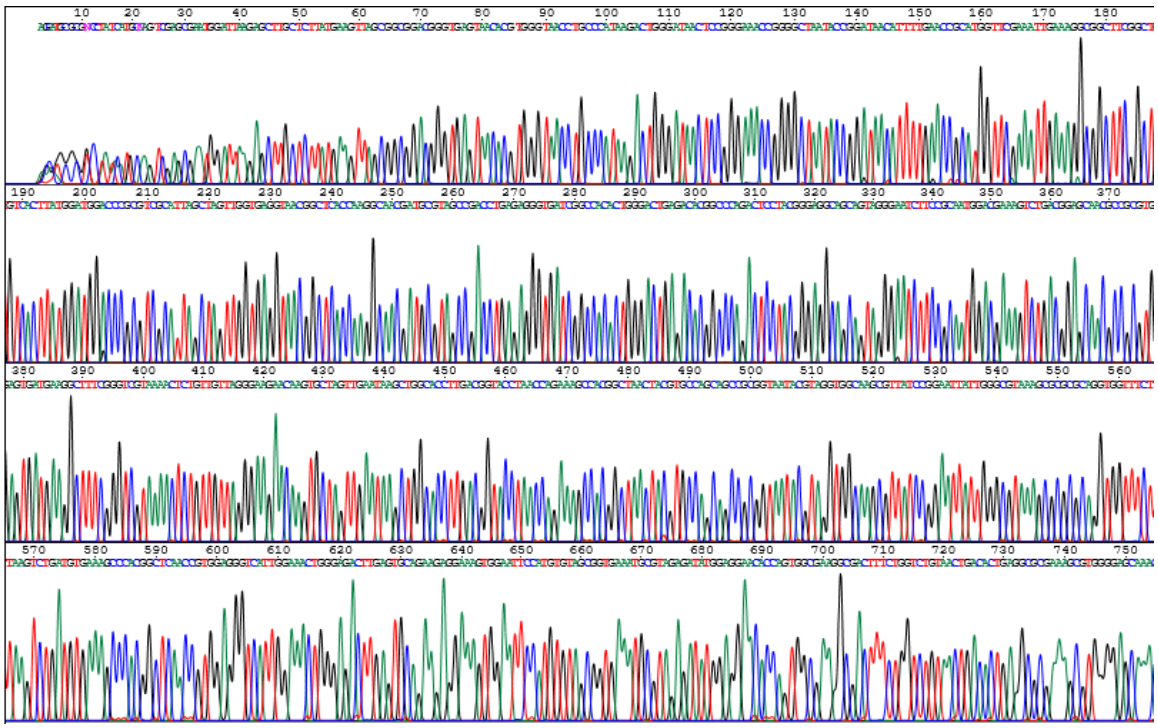

Figure 20. Chromatogram of Sanger sequencing of A12.

E8.1B from 45 to 801

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gattactagcgattccgacttcacgcagtcgagttgcagactgcgatccggactacgatcggttttatgggattagctccacctcg

cggcttggcaacccctttgtaccgaccattgtagcacgtgtgtagcccaggccgtaagggccatgatgacttgacgtcatccccac

cttcctccggtttgtcaccggcagtctccttagagtgcccaccattacgtgctggtaactaaggacaagggttgcgctcgttacgg

gacttaacccaacatctcacgacacgagctgacgacagccatgcagcacctgtctcaatgttcccgaaggcaccaatccatctct

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cgtcaattcatttgagttttaaccttgcggccgtactccccaggcggtcaacttaatgcgttagctgcgccactaagagctcaaggc

tcccaacggctagttgacatcgtttacggcgtggactaccagggtatctaatcctgtttgctccccacgctttcgcacctcagtgtca

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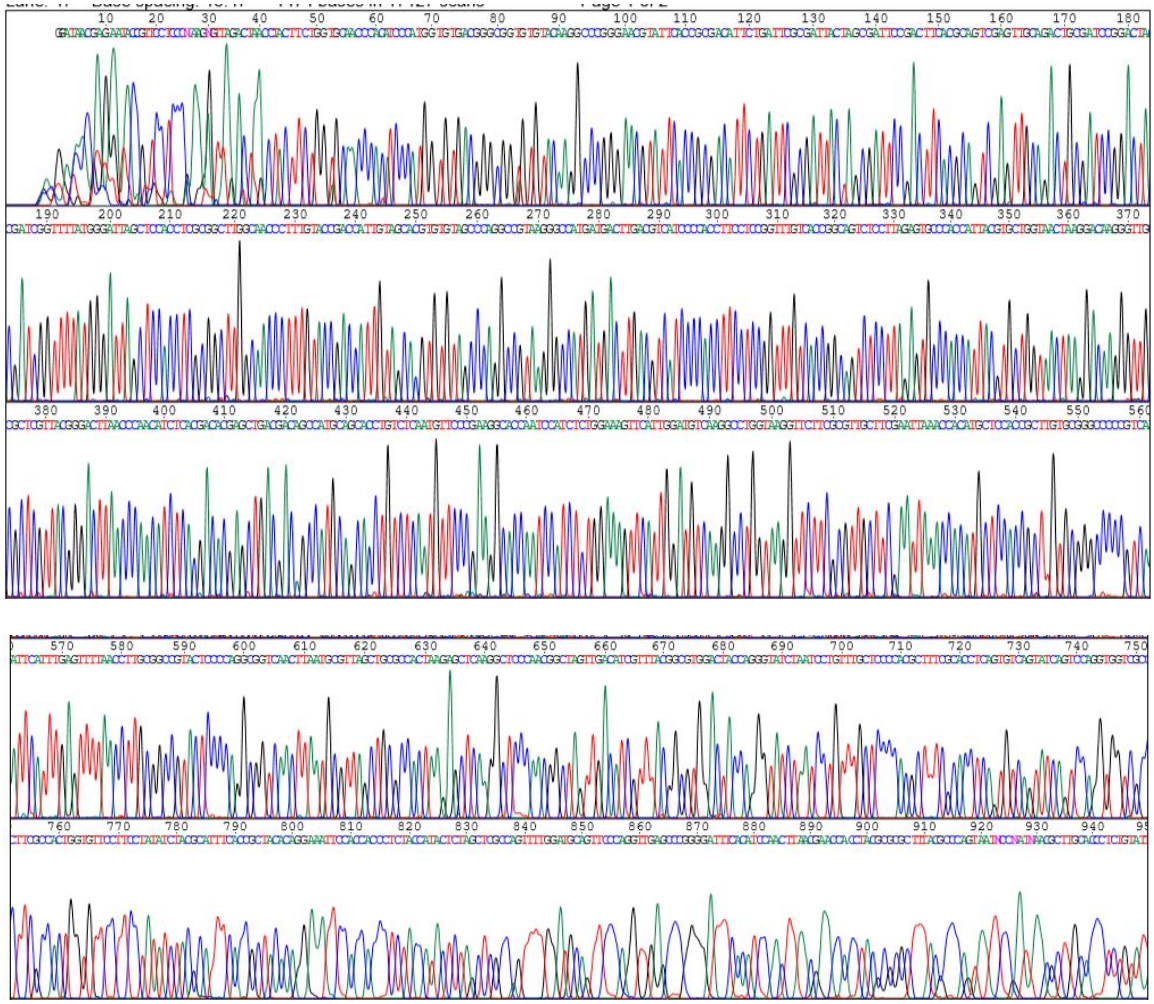

Figure 21. Chromatogram of Sanger sequencing of E8.1B.

E8.1A from 34 to 508

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atccgcgattactagcgattccagcttcacgcagtcgagttgcagactgcgatccgaactgagaacagatttgtgggattggctta

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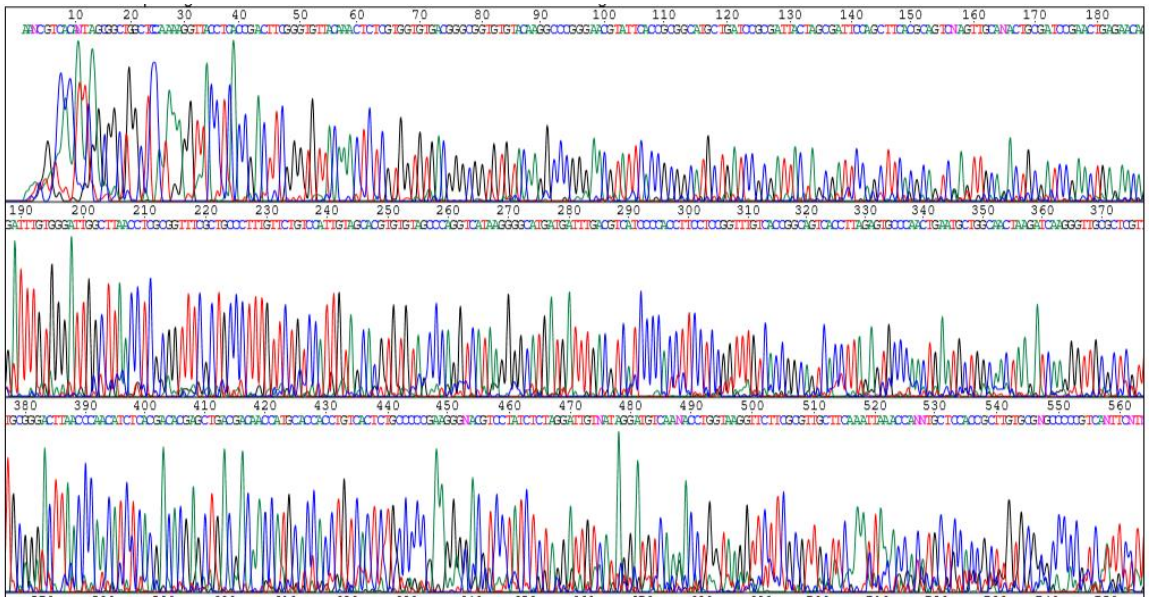

Figure 22. Chromatogram of Sanger sequencing of E8.1A.

E8.2A 26 to 633

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aagtcatatagcactatccagtaccactcatgccaatacatttcaagcaaacgcctagttcgactaagagtatcactcaataccaaa

cccgaaggtttgagagagaaatgacgctcaaacaggcatgccctctggaataccagagggcgcaatgtgcgttcaaagattcg

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agcgctggcaaaaagaatactgtaatgatccttccgcaggttcacctacggaaaccttgttacgacttttacttcctcta

Figure 23. Chromatogram of Sanger sequencing of E8.2A.

E8.3A from 66 to 808

cccactcccatggtgtgacgggcggtgtgtacaaggcccgggaacgtattcaccgcgacattctgattcgcgattactagcgatt
ccgacttcacgcagtcgagttgcagactgcgatccggactacgatcggttttatgggattagctccacctcgcggcttggcaacc
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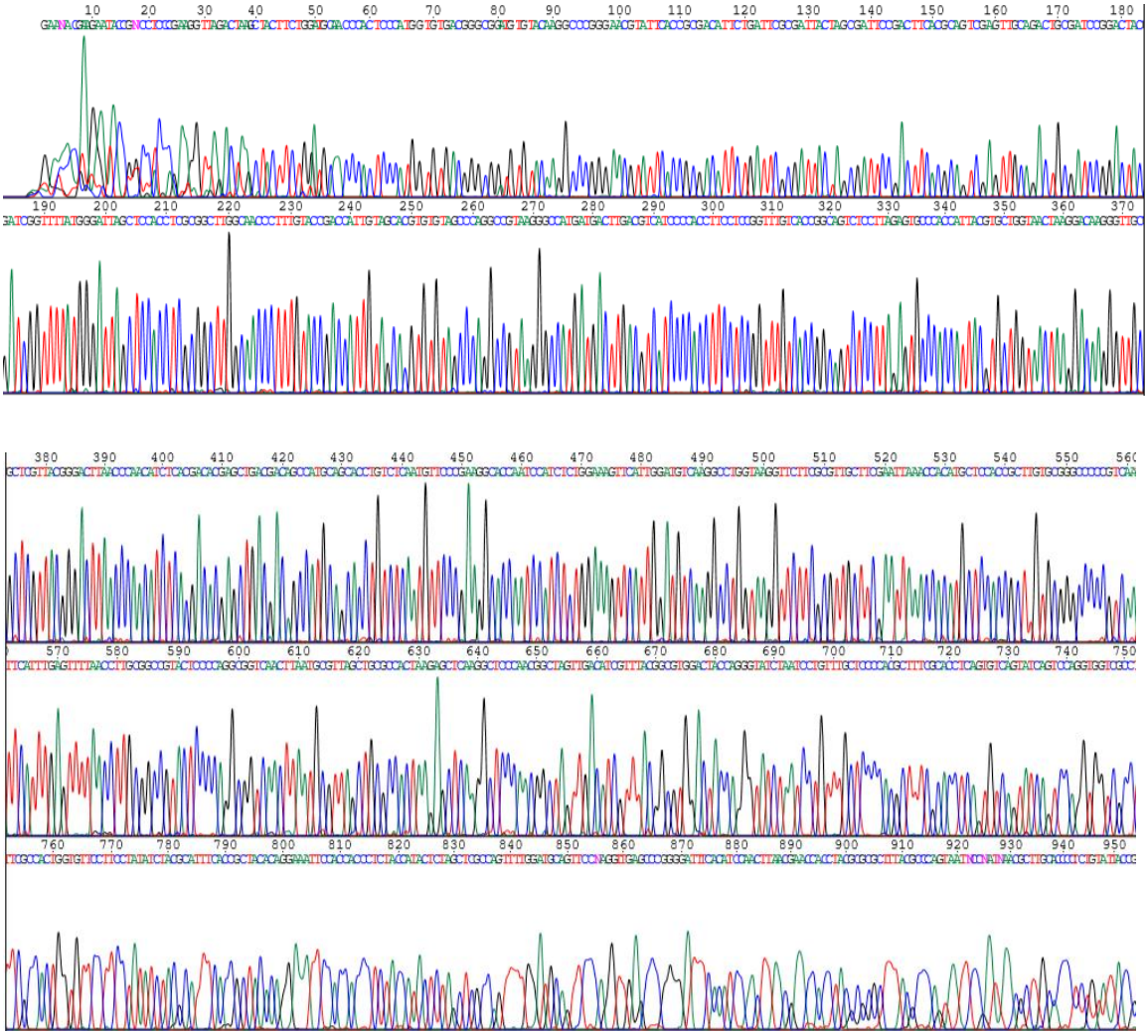

Figure 24. Chromatogram of Sanger sequencing of E8.3A.

w20 from 35 to 853

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tgatccgcgattactagcaattccgacttcatgcaggcgagttgcagcctgcaatccgaactgagactggctttttaggattggctc

cacctcgcggcttcgcttcccgttgtaccagccattgtagtacgtgtgtagcccaagtcataaggggcatgatgatttgacgtcatc

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ctcctcttctgcactcaagttccccagtttccagtgcgtcccg

Figure 25. Chromatogram of Sanger sequencing of w20.

w21 from 50 to 639

tgtaaggccgagcctagaataccgagaaatataccattaaactattcaacgagttggataaacctaatacattgaaagtcatatagc

actatccagtaccactcatgccaatacatttcaagcaaacgcctagttcgactaagagtatcactcaataccaaacccgaaggtttg

agagagaaatgacgctcaaacaggcatgccctctggaataccagagggcgcaatgtgcgttcaaagattcgatgattcacgaa

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tctagtccagaccaaagcaaaagttcttgtaataacaaaaaacactgtgtgtaaggtttttttcgccgcgcaattaagcgctggcaaa

aagaatactgtaatgatccttccgcaggttcacctacggaaaccttgttacgacttttacttcc

Figure 26. Chromatogram of Sanger sequencing of w21.

w5 from 49 to 835

ctacctacttcttttgcacccactcccatggtgtgacgggcggtgtgtacaaggcccgggaacgtattcaccgtggcattctgatc

cacgattactagcgattccgacttcacggagtcgagttgcagactccgatccggactacgacatactttatgaggtccgcttgctct

cgcgaggtcgcttctctttgtatatgccattgtagcacgtgtgtagccctactcgtaagggccatgatgacttgacgtcatccccac

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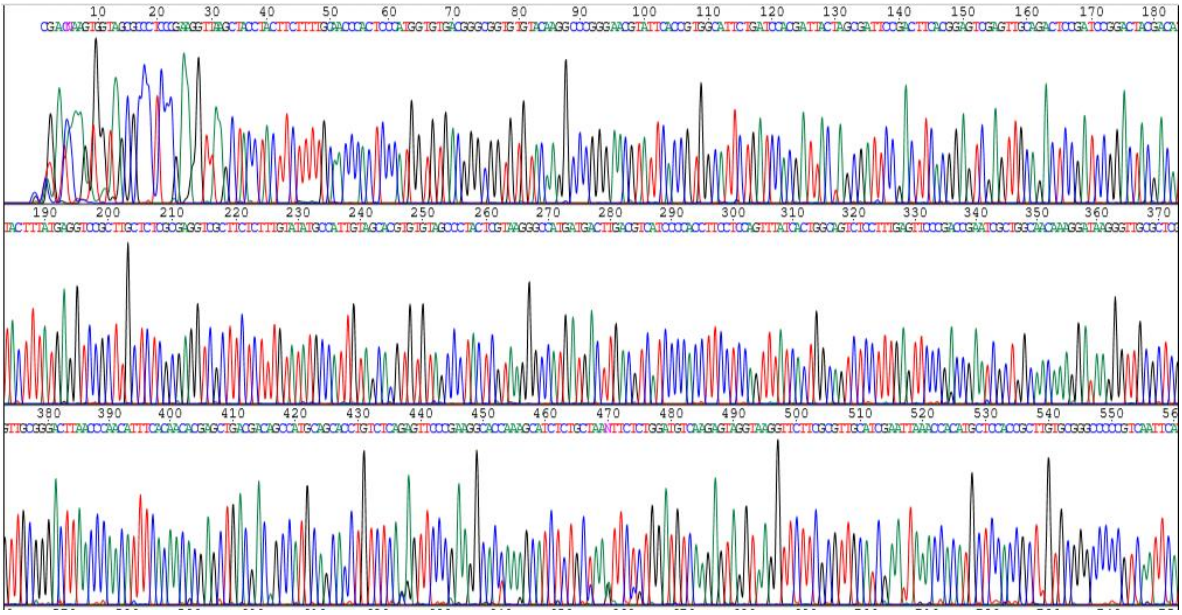

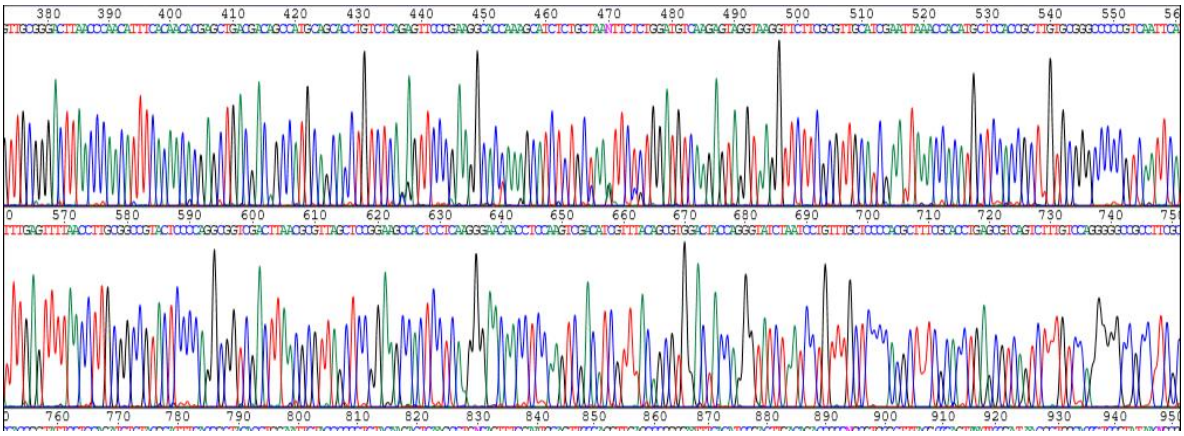

Figure 27. Chromatogram of Sanger sequencing of w5.

E2A 67 to 808

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cgacttcacgcagtcgagttgcagactgcgatccggactacgatcggttttatgggattagctccacctcgcggcttggcaaccct

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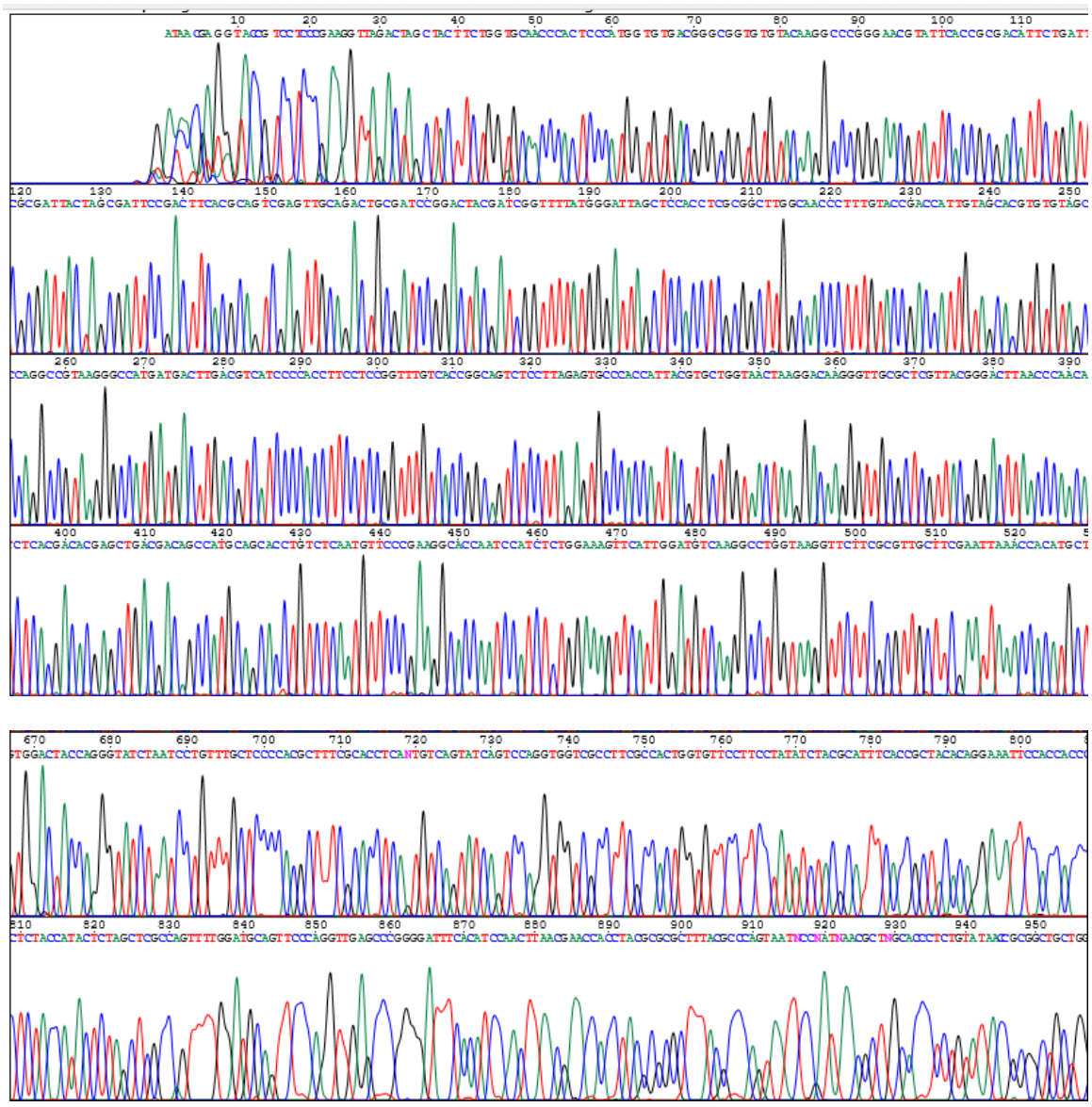

Figure 28. Chromatogram of Sanger sequencing of E2A.

Appendix B

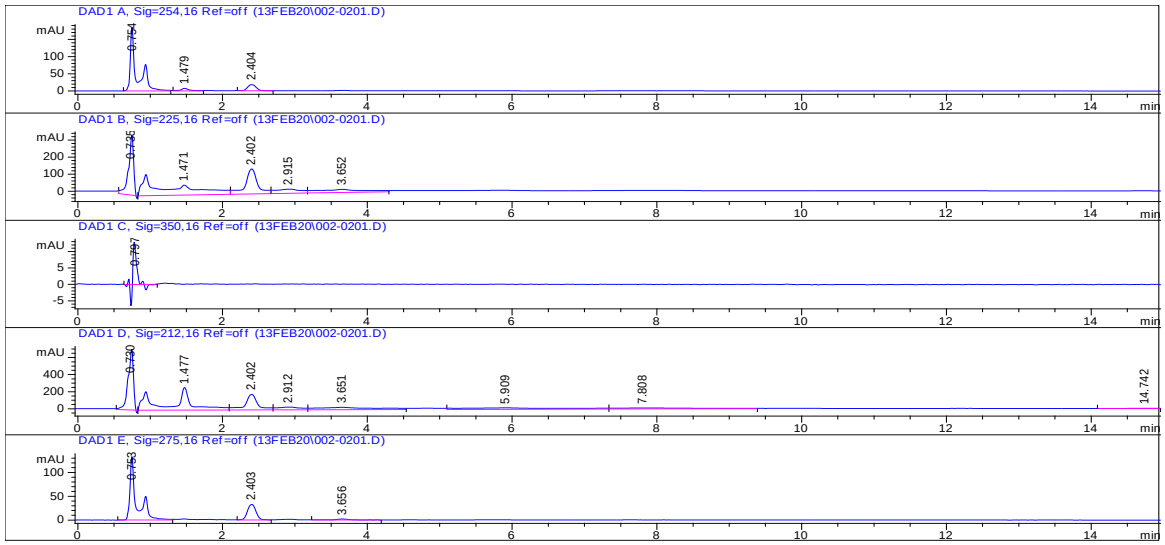

Figure 29. UV chromatograms of CM extract of control NA ran under isocratic gradient of 20% acetonitrile: water for 15 mins at ʎ= 254, 225, 350, 212, 275 nm.

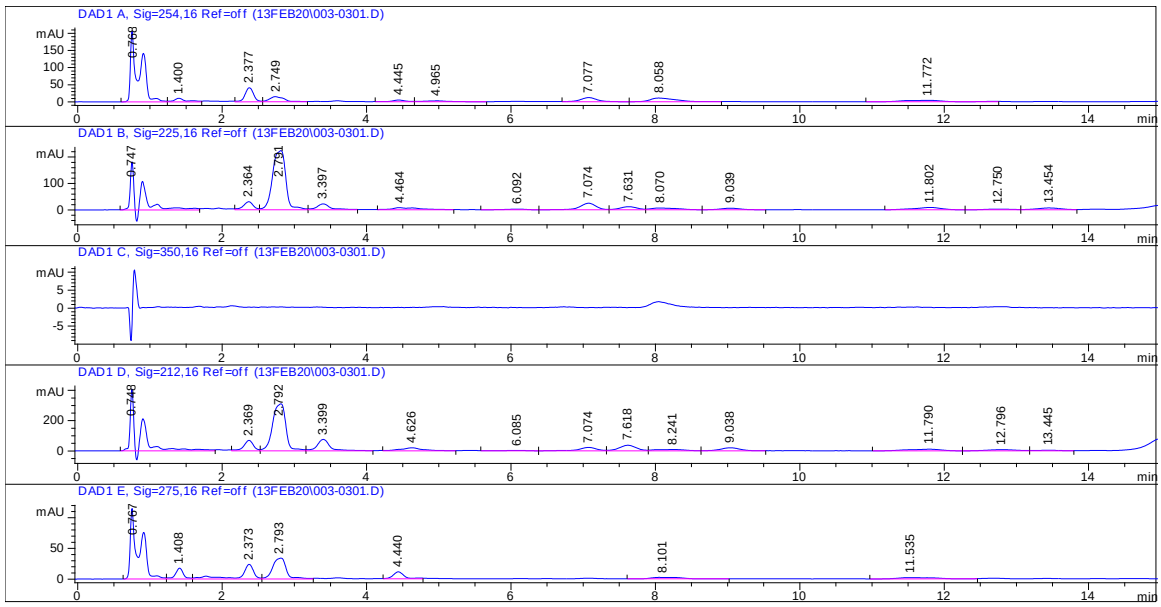

Figure 30. UV chromatograms of EA extract of control NA ran under isocratic gradient of 20% acetonitrile: water for 15 mins at ʎ= 254, 225, 350, 212, 275 nm.

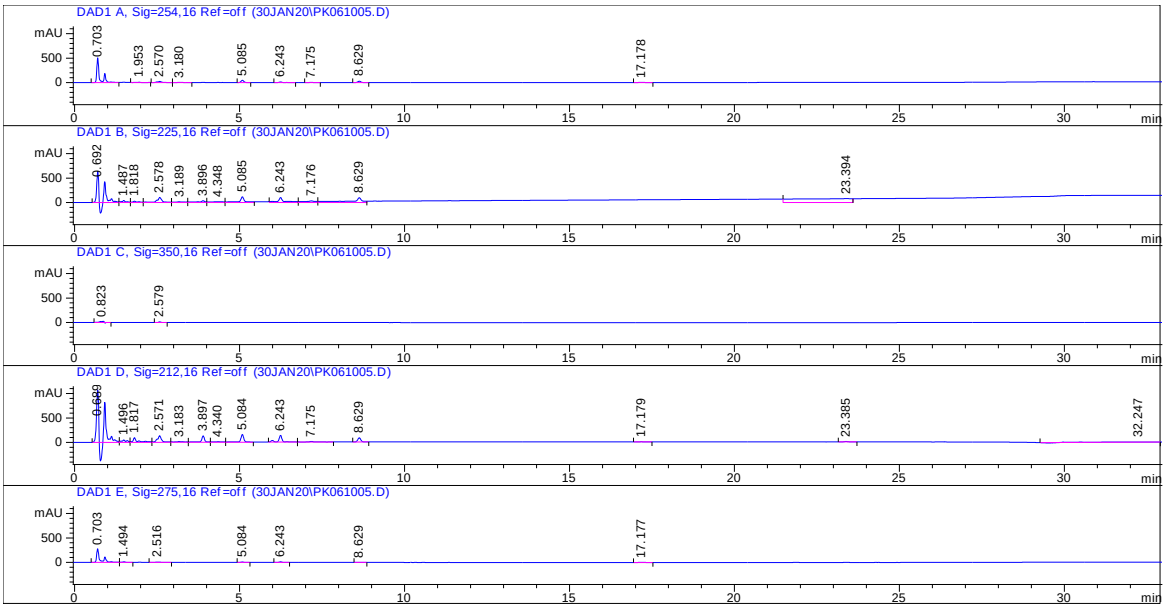

Figure 31. UV chromatograms of CM extract of A12 of 4w old ran under gradient method A for a total of 33 mins at ʎ= 254, 225, 350, 212, 275 nm.

Appendix C

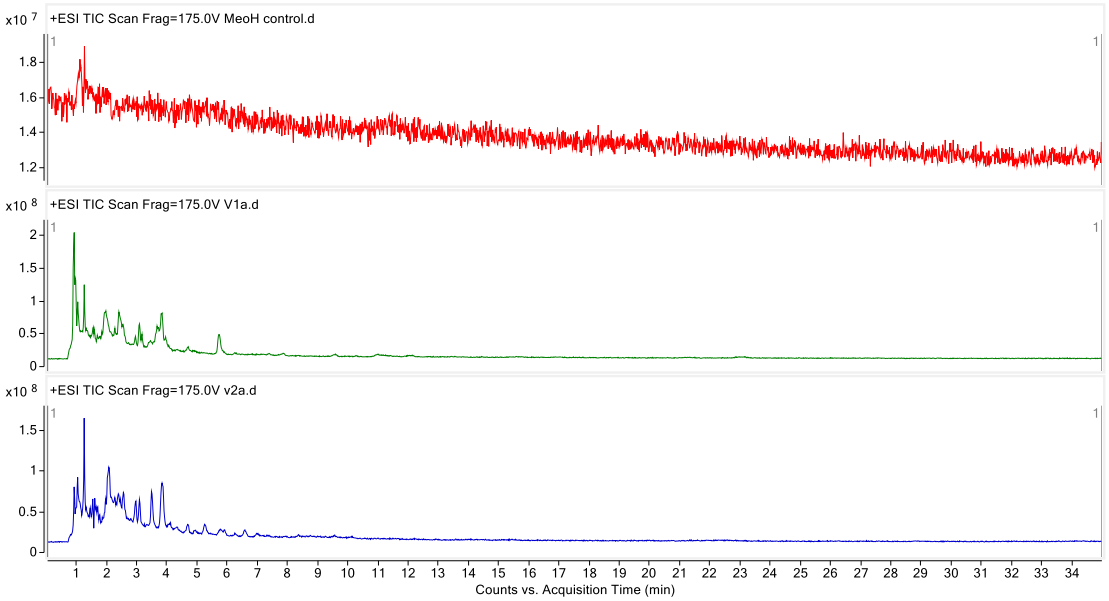

Figure 32. Total Ion Chromatograms of A12 crude extracts using gradient method B- 20% acetonitrile isocratic gradient for 15 mins.

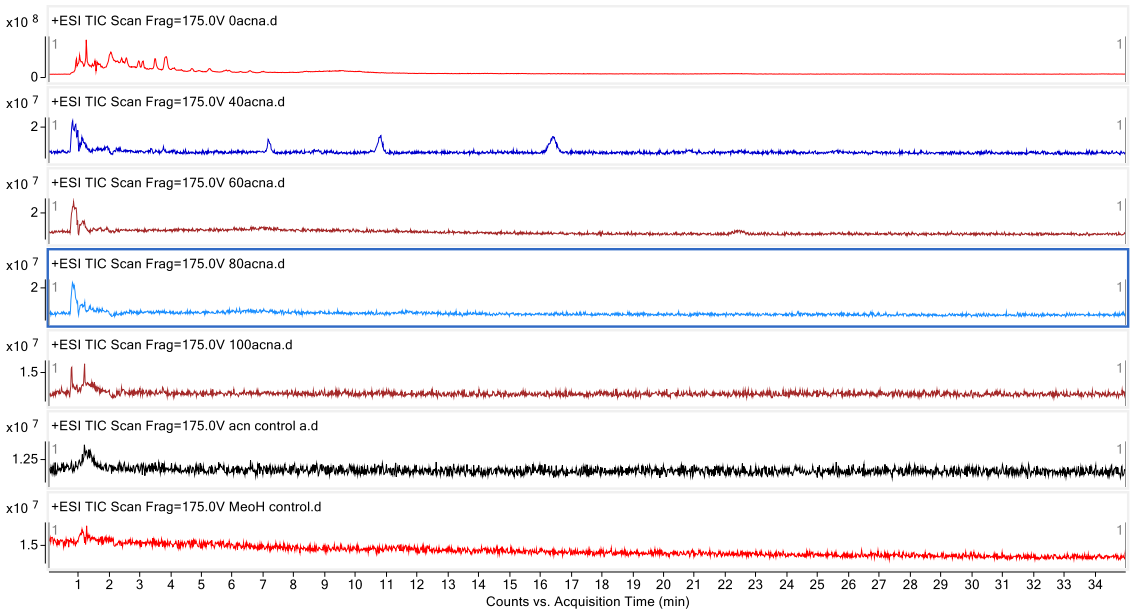

Figure 33. Total Ion Chromatograms of fractions of A12 using gradient method B- 20% acetonitrile isocratic gradient for 15 mins.

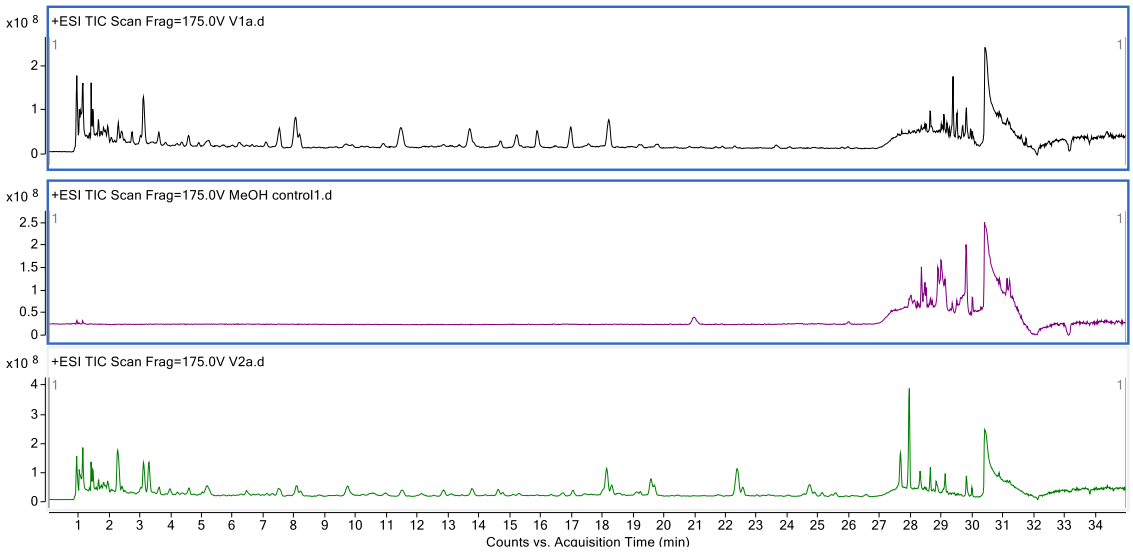

Figure 34. Total Ion Chromatogram of A12 crude extracts with method gradient A. v1 is the EA crude extract, v2 is the CM crude extract and methanol which is the solvent of the extracts.