# ANTIMICROBIAL RESISTANCE AND GENOMIC CHARACTERIZATION OF NORTH AMERICAN *MELISSOCOCCUS PLUTONIUS* ISOLATES

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**ABSTRACT**

European foulbrood (EFB) is a disease of honey bee larvae caused by *Melissococcus plutonius*. Western Canada has seen a recent emergence of EFB disease despite oxytetracycline (OTC) use, resulting in economic losses for beekeepers. In North America, antimicrobials are widely used for treatment and control of bacterial brood diseases, including both American foulbrood (AFB) and European foulbrood (EFB) disease. In contrast, prophylactic antimicrobial-use is prohibited in beekeeping in the European Union. Thus, antimicrobial resistance (AMR) poses an imminent threat to North American apiaries, especially for cases of EFB disease since only a single antibiotic, oxytetracycline (OTC), is approved for use. In contrast, tylosin (TYL) and lincomycin (LMC) are also registered for use against AFB. The most recently published antibiotic sensitivity testing for *M. plutonius* was performed approximately 20 years ago on *M. plutonius* isolates from outside North America. Moreover, little is known about the resistance profiles of *M. plutonius* and how these are related to the efficacy and ability of drugs to clear infection in honey bee larvae.

To address these issues, we sought to investigate OTC, TYL, and LMC as potential treatment options for EFB disease using laboratory-reared larvae infected with *M. plutonius*. Moreover, our study aims to determine if antibiotic resistance to OTC TYL, or LMC exists in North American *M. plutonius* isolates. Genomic characterization of Canadian isolates is lacking as well, and thus we also aimed to characterize a subset of isolates we did antimicrobial susceptibility testing for.

Firstly, the utility of OTC, TYL, and LMC were compared through an experimental design that either mimicked Metaphylaxis or antimicrobial intervention, using concentrations that corresponded to previous studies. We found that all concentrations prevented clinical signs of EFB disease following infection with *M. plutonius* 2019BC1 *in vitro*. Interestingly, *M. plutonius* 2019BC1, following antimicrobial susceptibility testing was characterized as resistant to OTC, with a minimum inhibitory concentration (MIC) of 32 µg/mL.

Accordingly, we then compared the utility of the three drugs using four *M. plutonius* isolates that demonstrated a range of resistance phenotypes. Additionally, treatment doses were determined through corresponding MICs. We demonstrate that infections and the efficacy of antimicrobial treatments in honey bee larva are concordant with in vitro MIC measurements and

that the survival outcomes of larva are not dependent on *M. plutonius* clearance but a strain-dependent reduction of their numbers within larva.

To assess the range of antimicrobial resistance present in North America, a total of 67 *M. plutonius* isolates. We found that a majority of isolates had a minimum inhibitory concentration that indicated resistance to OTC (> 16 μg/mL), while all isolates had MICs that corresponded to sensitivity against tylosin and lincomycin (< 4 μg/mL). Interestingly, resistant isolates showed sensitivity to OTC when different media was used, indicating that resistance is media-dependent.

53 of these isolates were used for whole genome sequencing and genomic comparison with 17 publicly available *M. plutonius* genomes. Through genomic characterization, we did not identify a resistance gene to explain the resistance seen for OTC. Moreover, phylogenetic analysis demonstrated that OTC resistance was not clustered together. However, similar clonal complexes cluster together.

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**LIST OF ABBREVIATIONS**

AB Alberta

AFB American foulbrood

AMR Antimicrobial Resistance

ARG Antimicrobial Resistance Gene

AST Antimicrobial sensitivity testing

BC British Columbia

CAPA Canadian Association of Professional Apiculturists

CARD Comprehensive Antibiotic Resistance Database

CC Clonal Complex

CFU Colony forming unit

CLSI Cinical and Laboratory Standards Institute

EFB European foulbrood

ETX Epsilon Toxin

GC Grafting Control

HGT Horizontal Gene Transfer

IC Infection Control

IPM Integrated Pest Management

LMC Lincomycin

MBC Minimum Bactericidal Concentration

MFS Major Facilitator Superfamily

MI Michigan

MIC Minimum Inhibitory Concentration

$MLS_B$ Macrolide, Lincosamides, and Type B Streptogramins

MLST Multilocus Sequence Typing

MtxA Melissotoxin A

NCBI National Center for Biotechnology Information

OR Oregon

OTC Oxytetracycline

PBS Phosphate Buffered Saline

QU Quebec

ST Sequence Type

SK Saskatchewan

TYL Tylosin

TX Texas

UT Utah

# CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

## 1.1 General overview of honey bees and their importance

European honey bees (*Apis mellifera)* are the most common managed bees in the world and are responsible for one third of global food production and the pollination of major crops worldwide, such as blueberries, almonds, apples, and alfalfa.[1–3]. 90% of commercial agricultural pollination is performed by managed honey bee colonies, including the use of 1 million honey bee colonies for the pollination of almond trees in California each year [4]. In addition to providing pollination services, honey bees also contribute economic value through the production of honey and other hive products such as royal jelly, propolis, and beeswax.[5] Approximately 74.4 million pounds of honey was harvested in 2022 in Canada, valued at 253.5 million dollars[6]. Thus, honey bees play a significant role in both the economy and food production.

Despite their importance to our food and agricultural systems, the health of honey bee populations has been demonstrated to be declining in recent years. The Canadian Association of Professional Apiculturists (CAPA) reported a 45.5% overwinter honey bee colony loss in Canada in 2022, which is double the average annual colony loss between 2007 and 2021 (25.8%)[7]. Factors contributing to these unsustainable overwintering losses include infectious diseases, such as Nosema, Varroa mites, American and European foulbrood.[8] Other factors include poor queen quality and environmental stressors including pesticide exposure and lack of nutritional/forage diversity.[8]

## 1.2 Honey bee disease

### 1.2.1 Bacterial diseases of honey bees

Currently, only two bacterial pathogens are described in honey bees, *Melissococcus plutonius* and *Paenibacillus larvae*, which cause European foulbrood (EFB) and American foulbrood (AFB) disease, respectively.

### 1.2.2 American foulbrood disease overview

AFB is a disease of honey bee larvae and pupae (brood) and is considered to be one of the most destructive honey bee diseases, which inevitably leads to death of the entire colony[9,10]. Clinical disease occurs when as few as 10 infectious endospores of *P. larvae* are ingested by susceptible larvae and germinate within the larval midgut to produce over a billion vegetative bacteria which ultimately breach the midgut epithelium leading to bacteremia and death in the

prepupal to pupal stage.[11,12] After pupal death, the vegetative bacteria sporulate into resilient endospores which form a characteristic scale on the wall of the brood cells [13]. These endospores are able to remain viable in the hive for up to 40 years where they are easily transferred within the hive and between colonies through adult bees which act as asymptomatic carriers. Other routes of inter-colony transmission include robbing behavior, where foraging bees from one colony steal endospore-contaminated honey from a weak or dead colony affected with AFB, or through management practices of beekeepers, such as the sale or exchange of infected colonies and equipment. In North America, antibiotic metaphylaxis with one of the three approved antimicrobials (oxytetracyline, tylosin, and lincomycin) has been widely used for prevention of clinical AFB disease; however, antibiotic therapy is only effective against the vegetative form of *P. larvae* and does not eliminate infectious endospores from the colony. Accordingly, antibiotic treatment is not recommended for colonies with clinical signs of AFB. Instead, clinically affected colonies should be burned or irradiated to destroy *P. larvae* endospores.

### 1.2.3 European foulbrood (EFB) disease overview

Despite its name, EFB is not restricted to Europe, and is known to affect honey bees worldwide[14]. The causative bacterium, *Melissococcus plutonius*, is known to infect European honey bees (*Apis mellifera)*, as well as Asian honey bees (*Apis cerana*) and Himalayan giant honey bees (*Apis laboriosa*).

EFB, like AFB, is a bacterial disease of honey bee larvae*;* however, unlike in AFB, EFB is considered a stress-associated disease with colonies capable of spontaneous recovery when colony stressors are mitigated. While *M. plutonius* is not a spore-forming bacteria, *M. plutonius* can persist within the hive[15], leading to emergence of clinical disease when colonies experience nutritional or environmental stress. Often, EFB arises in early spring when colonies typically have a low population of nursing bees and a high population of honey bee larvae, as well as limited pollen and nectar foraging resources in the surrounding environment[16,17]. Suboptimal brood care and low access to food increase a colonies susceptibility to EFB disease[15] Other factors, such as genetic, weather, and geography, may also be significant in predisposing a colony to disease. Clinical EFB results in larval death leading to reduced populations of adult bees, decreased honey production, and in some cases, colony death[15].

Transmission of *M. plutonius* typically occurs when newly-hatched, susceptible larvae are fed contaminated brood food by nurse bees[14,15]. *M. plutonius* multiplies within the midgut of

infected larvae and are thought to compete with the larvae for nutrients resulting in larval starvation and sometimes death. Larval death is dependent on the amount of ingested pathogen, as well as the quality of brood, nursing care, and nutrition[15,18]. In the presence of adequate brood care and larval nutrition, larvae are able to survive the disease and emerge as asymptomatic adult honey bees.

Larvae succumbing to infection with *M. plutonius* are often co-infected with a number of secondary bacterial invaders including *Paenibacillus alvei, Lactobacillus kunkeei, Achromobacter Euridice,* and *Enterococcus faecalis*[14,15]. Interestingly, *P. alvei* is an aerobic bacterium that is typically unable to survive in the gut of a living larva, suggesting that *P. alvei* colonization occurs after larval death from EFB. Secondary bacterial invaders contribute to the clinical symptoms of EFB, including the presence of a sour smell, as well as, in some cases, 'pseudoropiness' which is the ability to macerate an infected larva and draw it out of the cell using a stick. However, with EFB, larval remains cannot be drawn out more than 2 cm like with AFB[19].

#### 1.2.3.1 *Clinical diagnosis and differentiation of foulbrood diseases*

Both EFB and AFB can be diagnosed in the field based on a visual inspection of a colony, however, these two diseases share many clinical symptoms. One of these clinical symptoms is an uneven and spotty brood pattern, which is referred to as a shotgun brood pattern. This abnormal pattern can be seen in a variety of diseases such as EFB, AFB, as well as certain issues such as a failing queen[20]. In foulbrood diseases, this pattern is caused by the death of infected larvae, which are removed from the hive. However, other larvae, that are either uninfected or infected but still alive, go on to be capped. Overall, while this symptom is indicative that something is wrong in the colony, it is not a definitive diagnosis of a foulbrood disease. Typically, EFB disease will cause larvae to die before capping, while AFB will cause larvae to die after capping. Thus, when a hive has EFB, diseased larvae that are discolored yellow or even brown are noticeable. Additionally, these larvae are quite deflated when compared to other white, plump, healthy larvae. With AFB, rather than seeing uncapped dead larvae, changes to the cappings may indicate disease[21]. Typically, if a diseased larva has been capped, the capping will appear discoloured, sunken, and will most likely be punctured. Honey bees display hygienic behaviour and can identify and remove diseased larvae from the hive[22].

The presence of an irregular hole in a colony with AFB indicates that the diseased larva has been identified by the honey bee and will be removed from the hive.

Additional foulbrood disease symptoms sometimes include a bad smell. Foulbrood diseases get their name from the foul odour that is typically present during the progression of the disease[20,21]. With EFB disease, if a smell is present, it is said to be slightly sour and resembling sauerkraut. The smell accompanying AFB disease is described as putrid and resembles rotten fish.

A test that can be carried out in the field to diagnose AFB from EFB is referred to as the "ropiness" test[23,24]. Essentially, a probe is used to macerate the larval remains and draw them out of the cell. With AFB, the remains should draw out at least 2 cm, while with EFB, they will not. Co-infection of larvae does occur in EFB, and this may allow the larval remains to draw out further than normal, which is referred to as "pseudo-ropiness"[24]. However, these remains will still not draw out as far as AFB infected larval remains will. Thus, the ropiness test is required to differentiate AFB symptoms from EFB symptoms in the field.

Additional symptoms that are present in colonies that have EFB disease is the presence of larval scale[21]. Larvae with EFB disease will typically form a rubbery scale that does not adhere tightly to the cell[20]. Additionally, these larvae may be twisted within the cell. In contrast, larvae with AFB will remain flat and adhere tightly to the lower wall of the cell[21]. The larval scale produced by AFB is also typically quite brittle.

Other protocols to distinguish between the two foulbrood diseases include rapid field tests as well as laboratory diagnosis through bacterial culture or polymerase chain reaction (PCR). Rapid field tests, such as the Vita AFB Diagnostic Test Kit (Vita Bee Health) allows the diagnosis of AFB in the field, and has been demonstrated to reliably diagnose this disease in the field[25]. This diagnostic kit consists of a lateral flow immunoassay test allowing for AFB disease to be confirmed rapidly. However, other antibody-based methods have been offered in the past including the use of an enzyme-linked immunosorbent assay (ELISA)[26]. Vita Bee Health also offers a lateral flow device that can be used for the detection of EFB disease in a hive. Similar to the AFB test, the EFB test is capable of rapidly identifying the presence of the causative agent, *M. plutonius*[27].

Bacterial culture is also commonly used to cultivate both *M. plutonius* and *P. larvae*, further confirming symptoms seen in the field. Due to the fact that *P. larvae* is a spore-forming

bacterium, its cultivation in the lab often begins with a heating step to eliminate the chance of other bacteria outcompeting *P. larvae* in media[24]. Commonly, MYPGP agar (media containing Mueller-Hinton broth, yeast extract, glucose, K2HPO4, sodium pyruvate, and agar[28]) is used to cultivate *P. larvae* in a laboratory[29]. Incubation of the bacteria in this medium does not require $CO_2$ to be present, however, 5% $CO_2$ significantly increases the growth of the bacterium. Nalidixic acid is added to inhibit the growth of common contaminants such as bacteria belonging to the following genera: *Bacillus, Brevibacillus,* and *Paenibacillus*. *M. plutonius* is a fastidious organism and requires microaerophilic to anaerobic conditions to grow[30]. Commonly, this organism is isolated in basal media (which is adapted from EFB media) or KSBHI[18,30,31]. Due to the different types of *M. plutonius* (see 1.2.3.3), different growth conditions are sometimes required. For example, typical isolates require anaerobic conditions as well as a Na:K ratio below 1. Atypical isolates, however, are capable of growing in less stringent conditions. Regardless, colony morphology of typical and atypical isolates remains similar, with atypical isolates growing to be slightly larger than typical isolates.

Finally, PCR is widely used to confirm the presence of *P. larvae* and *M. plutonius* in suspected diseased colonies. 16S PCR is commonly used in the detection of both organsims[30,32]. With the determination of *P. larvae,* PCR protocols exist to determine the specific genotype of the isolate[29].

**Table 1.1** – Differences between American and European foulbrood symptoms

| **European foulbrood** | **American foulbrood** |
|---|---|
| Colony is capable of spontaneous recovery | Fatal disease |
| Death occurs before capping of infected larvae | Death occurs after infected larvae have been capped – cell cappings appear sunken and may have the presence of irregular holes. |
| Ropiness test – with the presence of secondary invaders may exhibit "pseudoropiness" however larval remains will draw out < 2 cm. | Ropiness test – larval remains can be drawn out > 2 cm after maceration |
| Twisted, rubbery scale found on the bottom of the cell | Flat, brittle scale found on the side of the cell |
| Sour odour – resembles saurkraut | Rotten odour – resembles rotting fish |

### 1.2.4 *Melissococcus plutonius:* the etiological agent of EFB

*Melissococcus plutonius,* the causative agent of EFB disease in honey bee larvae, is a Gram-positive lanceolate coccus[33,34]. This bacterium is typically 0.5 x 1.0 µm and can be found individually, in pairs, or in chains of varying lengths[15,35].

This organism was first named *Bacillus Y* in 1908 after AFB was determined to be caused by *Bacillus larvae* (now known as *Paenibacillus larvae)*[18]. The EFB causing organism was then renamed *Bacillus pluton* in 1912. In 1956, the isolate was once again renamed to *Streptococcus pluton* after identifying that EFB was induced in bees with coccus-shaped isolates. Finally in 1981, the organism was renamed *Melissococcus pluton* after determining that this isolate had a distinct GC content profile when compared to isolates belonging to *Streptococcus*[36]. Moreover, *M. plutonius* has since been revealed to be a closer relative to the genus *Enterococcus* than to *Streptococcus*[15].

*M. plutonius*, as it is now known, is a fastidious organism that requires microaerophilic to anaerobic conditions for growth[31]. Additionally, carbon dioxide is required as well as a Na:K ratio less than 1. *M. plutonius* isolates are thought to be highly similar and have been demonstrated to be similar in their whole cell proteins, immunoreactive antigens, and DNA

restriction endonuclease profiles, despite being isolated from geographically distant regions, including Australia and the UK[37].

#### 1.2.4.1 *M. plutonius pathogenesis*

EFB disease is the result of an *M. plutonius* infection in honey bee larvae[15]. This infection occurs through the consumption of contaminated food provided to larvae by adult honey bees. Digestion of *M. plutonius* first results in the asymptomatic colonization of the larval gut. As little as fifty colony forming units of *M. plutonius* have been successful in initiating infection in honey bee larvae using *in vitro* models[38,39]. The bacterium will then multiply in the midgut where it is thought to compete with the larva for nutrients leading to larval starvation and sometimes larval death[35].

There is limited empirical evidence to support distinct and well-defined determinants of *M. plutonius* infection and virulence, however, a few processes have been linked to *M. plutonius* pathogenesis. For example, the production of tyramine is toxic to larvae and contributes to the melanisation that infected larvae undergo[40]. Additionally, genomic analyses have revealed the presence of a plasmid named pMP19 that encodes for an extracellular matrix-binding protein and melissotoxin A (MtxA)[41–43]. MtxA is similar to the epsilon toxin (ETX) found in the *Clostridium* genus and a mosquitocidal toxin (Mtx2) that is found in the *Bacillus* genus. Epsilon toxins act by changing the cells permeability to ions through the formation of membrane pores[44]. A recent study also found that a protein belonging to the ETX/Mtx2 family may play an important role in virulence in ERIC III and IV type *P. larvae* isolates[45]. Thus, MtxA may play a role in increasing the virulence of *M. plutonius* through pore formation in host cells resulting in cell, and ultimately, larval death. Indeed, infection of larvae with *M. plutonius* belonging to clonal complex 3 (CC3) that were previously cured of pMP19 significantly decreased larval mortality, indicating that this plasmid plays a role in pathogenesis[42]. However, it is important to note that *M. plutonius* isolates belonging to CC12 remained pathogenic despite being cured of plasmid pMP19. Moreover, isolates belonging to CC13 remained avirulent even when plasmid pMP19 was present.

#### 1.2.4.2 *M. plutonius transmission and association with adult bees*

Transmission of *M. plutonius* occurs within a colony (intra-colony) and between different colonies (inter-colony). Persistence of *M. plutonius* within the brood comb occurs when infected individuals are able to survive the infection and emerge as asymptomatic adult bees[15]. These

asymptomatic individuals will deposit the bacteria into the cell when they pupate, where *M. plutonius* will now remain viable for a long period of time. Adult worker bees isolates from brood nests were demonstrated to have a higher bacterial load than worker bees sampled from flight entrances[34]. Additionally, worker bees sampled from the brood nest of healthy colonies (colonies that displayed no clinical symptoms of EFB disease) also had a relatively high *M. plutonius* bacterial load. These honey bees are able to act as carriers of EFB disease within a colony through the role of the nursing bee who are responsible for feeding honey bee larvae.

Asymptomatic honey bees are also capable of transmitting this *M. plutonius* to other colonies and apiaries as well. The spatial distribution of colonies may be important to the spread of EFB[46]. As the distance to diseased colonies (display clinical symptoms) increased, the proportion of honey bee samples testing positive for *M. plutonius* decreased. However, while this trend was noted, the distance was not significant. Additionally, this study demonstrated that healthy colonies located in "EFB" free areas tested negative for *M. plutonius,* while the healthy colonies located in area with EFB tested positive for *M. plutonius* in all but one sample. Thus, demonstrating the ability of adult honey bees to become carriers for this bacterium as well as the ability to spread the bacteria between different colonies.

Finally, honey found within colonies has been demonstrated to be contaminated with *M. plutonius*[47]. Thus, robbing, the act of honey bees stealing honey from other colonies, may further contribute to the spread of *M. plutonius* from one colony to another[15].

#### 1.2.4.3 *Multilocus sequence typing and clonal complexes*

Multilocus sequence typing (MLST) is a nucleotide sequence-based approach, first proposed in 1998, used to characterize strains of the same bacteria using a number of key housekeeping genes[48]. A typing scheme was first introduced for *M. plutonius* by Haynes et al. (2013) allowing differentiation of various *M. plutonius* sequence types (STs)[49]. Traditionally, MLST schemes contain 6-10 housekeeping genes. Each gene represents a different locus and any sequence variation within the gene represents a different allele. The use of housekeeping genes is important because they are highly conserved with the genome and will be present in all of the strains[48]. While these genes are conserved, strain differences may still be present allowing for the characterization of different strains. A number is given for each gene depending on the allele that is present, and these different numbers allow strains to be grouped by ST. The housekeeping genes used in the *M. plutonius* are *argE, galK, gbpB,* and *purR*[49].

The first two genes, *argE* and *gbpB*, correspond to coding regions of the genes, acetylornithine deacetylase and putative secreted antigen, respectively. Acetylornithine deacetylase may be involved in arginine metabolism in *M. plutonius.* While the product of *gbpB* is related to other proteins which have been demonstrated to be virulence factors in cocci. For example, GbpB, which is an essential protein for *Streptococcus mutans* may be involved in mediating cell-surface interactions with glucan[50]. Moreover, immunization with this protein gives protection against dental caries in animal models which demonstrates that GbpB is associated with virulence in *S. mutans*[51].

The last two loci, *galK* and *purR,* include intergenic regions, which is atypical of MLST schemes[49]. The first loci, *galK,* includes a region between two galactokinase fragments which are separated by stop codons. The final loci, *purR,* begins upstream of the 5' end of the *purR* coding region which encodes for the purine operon repressor.

Previous studies have demonstrated a spread of STs globally, however, the sample sizes are limited[33]. For example, one study found that ST3 and ST12 were common in *M. plutonius* isolates found in many countries including Japan and Europe[52]. To date, there are currently 47 known STs for *M. plutonius* isolated from 17 different countries across North and South America, Europe, Asia, Australia, and Africa[53].

#### 1.2.4.4 *Typical vs atypical strains*

Depending on the ST of an *M. plutonius* isolate, they are grouped into three different clonal complexes (CC): CC3, 12, or 13[33]. Isolates belonging to CC3 and CC13 are considered to be "typical", while those belonging to CC12 have been characterized as "atypical". While typical and atypical isolates have been demonstrated to have various phenotypic differences, morphologically they are similar. One of the major differences between typical and atypical isolates are growth requirements[31]. Typical isolates require a Na:K ratio to be $\leq 1$, however, atypical isolates are able to grow despite differences in this ratio. In fact, atypical isolates have been demonstrated to grow in media that was both supplemented and not supplemented with potassium salt, unlike typical isolates[31].

Additionally, biochemical characteristics were also found to be different in typical and atypical isolates. Typical isolates were shown to use glucose, fructose, and D-mannose only to produce acid. Contrastingly, atypical isolates were able to use the three aforementioned sugars as well as L-arabinose, D-cellobiose, and salicin. Few atypical isolates were also able to utilize

lactose. Furthermore, atypical isolates displayed evidence for ß-glucosidase activity while typical isolates did not. Finally, atypical isolates displayed strengthened phosphatase and ß-galactosidase activity compared to typical isolates.

Experimental reproduction of EFB disease using an *in vitro* larval model also differed between typical and atypical isolates[31]. When infecting larvae with a typical isolate, DAT606, EFB disease was not successfully established in larvae. While some larvae died, the mortality was similar to the uninfected control group. In contrast, when larvae were infected with an atypical isolate, DAT561, EFB disease was successfully established. Growth in these larvae stopped 2 to 3 days after being fed the infectious diet. By 5 days post infection, 94.3% of the larvae had died and the mortality of larvae infected with a typical isolate was significantly higher than control and typical *M. plutonius* infected larvae. The deceased larvae displayed symptoms typical to EFB disease including a change in body elasticity and colour.

#### 1.2.4.5 *Attempts to connect geno- and phenotypes*

Previously, *M. plutonius* was thought to be a highly similar bacterial species, however, it is now known that two distinct types of *M. plutonius* exist: typical and atypical[31]. Moreover, the different STs of *M. plutonius* can be grouped into three CCs: CC3, 12, and 13, with CC3 and CC13 isolates belonging to the typical group and CC12 isolates belonging to the atypical group[33]. Phylogenetic analysis of these three CCs resulted in each complex clustering separately, demonstrating that the different complexes are indeed genetically distinct enough to warrant grouping them differently[33,43].

Typical and atypical isolates were differentiated *in vitro* due to typical isolates failing to produce infection in larvae[31]. However, when comparing the three CCs and field pathology, CC3 was found to have highly infected colonies when compared with CC12 and CC13[33]. Interestingly, this study took place in England and Wales only. Additionally, it is not known whether the bacterial load was comparable across hives, as disease intensity was assessed visually.

A genetic analysis of various *M. plutonius* revealed 132 proteins which are present in the atypical strain but not all of the typical strains[43]. Many of these proteins may be involved in virulence, such as cell surface and adhesion-associated proteins and capsule/cell envelope forming proteins. Additionally, many isolates belonging to CC13 lacked tyrosine decarboxylase, which is required for tyramine production. Tyramine has been demonstrated to produce a classic

symptom of EFB where larvae turn brown/yellow after infection[40]. This could indicate that this CC is less virulent, which has previously been demonstrated[33,42]. Interestingly, genes encoding for bacteriocin biosynthesis was missing in the atypical isolate, however, only one atypical genome was analyzed[43]. Moreover, bacteriocins may not play a role in pathogenesis or virulence if a CC12 isolate is still able to produce disease in larvae without the genes necessary for bacteriocins.

Interestingly, typical and atypical isolates were also shown to differ in energy and sugar metabolism[43]. The atypical isolate was able to use a variety of different sugar substrates for energy through glycolysis, the pentose phosphate pathway, and the Entner-Duodoroff pathway. These sugars are all present within a honey bee colony (honey, royal jelly, and pollen) and indicates that atypical isolates may be better suited for survival within the larval gut compared to typical isolates. This analysis indicates that the faster growth of atypical isolates may be due to its potential to be better adapted to sugars found in a colony, which may in turn be the reason it is considered to be more virulent than typical isolates.

Two plasmids, pMP1 and pMP19, are known to exist in *M. plutonius* isolates[42,43]. Plasmid pMP1 exists in every single isolate and is comprised of ~200 kbp, while plasmid pMP19 exists only in a few isolates and is comprised of ~20 kbp[43,54]. Plasmid pMP19 is thought to be highly virulent due to the fact that it encodes for melissotoxin A (MtxA), which may be important in *M. plutonius* pathogenicity. Previous studies have demonstrated that curing CC3 isolates of this plasmid renders them avirulent, however, CC12 isolates have been shown to remain virulent with and without this plasmid[42]. This may be due to the lack of proteins (that may be important in virulence) are missing from typical strains but are present in atypical strains[43]. Interestingly, CC13 isolates have been shown to remain avirulent in the presence and absence of this plasmid[42]. Overall, this plasmid is thought to play an important role in *M. plutonius* pathogenesis.

### 1.2.5 Treatments for foulbrood disease

Integrated pest management (IPM) techniques are key in preventing foulbrood diseases and ensuring honey bee colonies remain healthy[14]. Firstly, colonies should be inspected for any honey bee diseases at least twice a year. The first inspection would occur right after winter before the first nectar flow and the second inspection should occur after the last honey pull before colonies are prepared for winter. However, it is important to carry out frequent

inspections for early detection of EFB and AFB. Additionally, the movement of beekeeping equipment, such as frames and supers, should be minimized between colonies and apiaries to prevent the movement of any pathogens within the beekeeping equipment. EFB disease is typically induced most commonly due to nutritional stress[15]. Thus, it is important to provide colonies with food during a nectar dearth to prevent stress on the hive[14].

In North America, antimicrobials are widely used in the treatment of both AFB and EFB disease. However, only one antimicrobial is currently approved for treatment of EFB, oxytetracycline hydrochloride. Along with oxytetracycline (OTC), tylosin tartrate, and lincomycin hydrochloride are approved for the treatment of AFB disease[55]. Contrastingly, antimicrobials are not approved for the treatment of either EFB or AFB in the European Union due to concerns about antibiotic residues in the honey as well as the rise antibiotic resistant strains of both *P. larvae* and *M. plutonius*[13].

Metaphylactic treatment for AFB occurs twice a year in North America where 200 mg of OTC is applied to a single colonies for a total of three treatments that occur 4 to 5 days apart[55]. Alternatively, lincomycin (LMC) or tylosin (TYL) may be used for AFB treatment and these are applied at either 100 or 200 mg/colony, respectively, every 7 days for 3 weeks.

Treatment for EFB is typically only administered when there is evidence of disease, unlike AFB where treatment is administered to prevent disease. Like AFB, EFB is treated by administering 200 mg OTC to the colony 3 times every 4 to 5 days.

#### 1.2.5.1 *Antibiotics approved for use in management of EFB and AFB*

##### 1.2.5.1.1 *Oxytetracycline: mechanisms of action and resistance*

Oxytetracycline, initially known as terramycin, is a bacterial natural product that was among the first discovered tetracyclines, a group of antibiotics first discovered in the 1940s[56]. OTC was discovered as a product of *Streptomyces rimosus*. Tetracycline antimicrobials are broad spectrum antibiotics that have activity against both Gram-positive and Gram-negative bacteria. OTC, as well as other tetracyclines, belong to the polyketide family of natural products and are considered a type II polyketide[57,58]. Type II polyketides are also known as bacterial aromatic polyketides. The compounds are naturally produced in bacteria using a poly-ß-ketone as an intermediate which is involved in the formation of a polycyclic product that has at least one aromatic ring.

This family of antimicrobials exhibit their activity through the inhibition of protein synthesis. Synthesis is inhibited by preventing aminoacyl-tRNA from attaching to the ribosomal acceptor (A) site[56]. Therefore, to successfully inhibit bacterial growth, tetracyclines must cross one or more membranes in order to interact with its target. In Gram-negative bacteria, tetracyclines cross the outer membrane through the OmpF and OmpC porin channels. Whereas in Gram-positive bacteria, the crossing of tetracyclines across the cytoplasmic membrane is dependent on the ΔpH component of the proton motive force.

Tetracyclines have been widely used in both human and veterinary medicine, and resistance is widespread[56]. After their discovery in the 1940s, the first resistant isolate, *Shigella dysenteriae,* was isolated not long after in 1953. Resistance to tetracyclines occur through the acquisition of *tet* or *otr* genes. Resistance genes may encode for a series of protective functions such as efflux pumps, ribosomal protection proteins, and antibiotic destruction.

Efflux proteins, which are the best studied Tet proteins, belong to the major facilitator superfamily (MFS)[56]. Efflux pumps encoded by *tet* genes are all membrane-associated proteins found in both Gram-positive and Gram-negative bacteria. Efflux pumps reduce the concentration of tetracycline within the cell by exporting the tetracycline out through the pump. This lowered concentrations effectively protects the ribosomes due to the reduced number of tetracyclines available to bind to it. Efflux genes have been divided into six groups based on the sequence similarity. Group 1 includes TetA-E, G-J, Z, and 30. Within this group, only TetZ is found in Gram-positive bacteria. Group 2 includes TetK and TetL, which are found primarily on plasmids in Gram-positive bacteria, such as *P. larvae*[59]. Group 3 includes OtrB and Tcr3, while group 4 includes TetA (P)[56]. Group 5 includes TetV while group 6 includes unnamed determinants of resistance.

Ribosomal protection proteins are cytoplasmic proteins that confer a wide spectrum of resistance to tetracyclines[56]. TetM and TetO are most studied proteins within this group of resistance proteins. These proteins have ribosome dependent GTPase activity. When GTP is present with TetM or TetO, tetracycline binding to the ribosome is significantly reduced. Currently, it is thought that the binding of a ribosomal protection protein to the ribosome results in a change in the ribosomal conformation which blocks tetracycline binding and prevents protein inhibition. GTP hydrolysis may be significant in providing the energy required for the conformational change.

Finally, enzymatic inactivation of tetracyclines may also confer resistance to an organism[56,60]. However, this class of tetracycline resistance is rarer than resistance through ribosomal protection and efflux pumps. While TetX, a well characterized antibiotic degradation protein, has been reported in many human pathogens, it has not been documented to cause tetracycline resistance in a clinical case yet[60]. TetX is a member of class A flavin monooxygenases (FMOs) which are flavoprotein hydroxylases that use FAD cofactors along with NAD(P)H electron donors for oxidation of substrates. In the context of tetracycline resistance, TetX modifies tetracyclines in the presence of oxygen and NADPH which prevent the antibiotics from exhibiting their activity against the bacteria[56].

**1.2.5.1.2** ***Tylosin: mechanisms of action and resistance***

Tylosin (TYL) is a member of a group of antibiotics known as macrolides, which are considered to have narrow spectrum activity against mainly Gram-positive cocci[61,62]. However, they can also act against Gram-positive bacilli and Gram-negative cocci[63]. Macrolide antibiotics are polyketides that typically contain a 14-16-membered macrocyclic lactone ring which have other sugars and side chains linked[63,64]. TYL specifically is a 16-membered macrolide with three sugar substituents including mycaminose, mycinose, and mycarose[65].

Macrolides have been used to treat both humans and animals since the late 1950s, however, TYL is almost exclusively used in veterinary medicine[65]. This group of antibiotics work by inhibiting protein synthesis. This inhibition occurs by binding to the 23S rRNA in the 50S (large) subunit of the 70S ribosome[64]. Specifically, they target the nascent peptide exit tunnel which is the tunnel used by the synthesized protein to leave the ribosome[66]. In the past, it was though that macrolides inhibit all protein synthesis by blocking this passage and thereby blocking all newly synthesized proteins from leaving, however, it is now believed that macrolides can allow some proteins to leave. Thus, they inhibit synthesis of only a subset of proteins.

Due to the usage of macrolides, resistance to these antibiotics have developed. The major mechanisms of resistance are modification of the antibiotic target, efflux pumps, and inactivation of the antiobiotic[63]. Moreover, macrolides belong to a major antibiotic group known as macrolides, lincosamides, and type B streptogramins ($MLS_B$) antibiotics due to their similar mechanism of action[67]. Thus, cross resistance between these three types of antibiotics can occur.

Firstly, $MLS_B$ resistance may occur due to modification or mutations in 23S rRNA[64]. The most common mutations observed in 23S rRNA that confer resistance are typically at A2058 and A2059 in *rrl*, the gene that encodes for the 23S rRNA. Moreover, *rrl* alleles containing a mutation at A2059 have been demonstrated to increase the minimum inhibitory concentration of erythromycin[68]. Mutations occurring in genes encoding ribosomal proteins L4 and L22 have also been demonstrated. A major mechanism of macrolide resistance is through the acquisition of *erm* genes which encode for rRNA methyltransferases[64]. These enzymes add one or two methyl groups to A2058. These enzymes confer macrolide resistance, however, resistance to lincosamides and streptogramin B is also obtained.

Macrolide resistance can also be acquired through efflux pumps. Mef family pumps belong to the major facilitator superfamily[64]. These pumps can bind macrolides and undergo a conformational change where the macrolide is removed from the cell in exchange for a proton. Primarily, *mef* genes are found in Gram-positive bacteria. Additional efflux pumps exist in the Msr family, encoded for by *msr* genes. MsrA is described as a class 2 ABC-transporter, however, the mechanism is not completely known.

Finally, macrolide resistance may be conferred through macrolide inactivation. This is achieved through macrolide esterases which specifically inactivate 14- and 15-membered macrolides through hydrolytic inactivation[64]. The genes for these esterases are typically plasmid-encoded through *ere* genes. Additionally, macrolide phosphotransferases also act to inactivate macrolides. These enzymes are able to inactive the 14-, 15-, and 16-membered macrolides by transferring the gamma phosphate of nucleotide triphosphate to the 2'-OH group of the macrolide. This transferral prevents the macrolides interaction with the 23S rRNA. These enzymes are encoded for by the *mph* genes which are typically found on mobile genetic elements containing other antibiotic resistance genes.

#### 1.2.5.1.3 *Lincomycin: mechanisms of action and resistance*

Lincomycin (LMC) is a naturally occurring lincosamide antibiotic that shares its mechanism of action with that of TYL and other macrolides[69]. Like macrolides, lincomycins work primarily against Gram-positive cocci, while Gram-negative bacteria are typically intrinsically resistant to lincosamides[70]. Additionally, both Gram-positive and negative anaerobic bacteria are typically sensitive to lincosamides. While the mechanism of action between macrolides and lincosamides are similar, there are a few differences. Firstly, lincosamides

interact with the A- and P-site on the 50S ribosomal subunit which inhibits formation of a peptide bond.

Due to the fact that lincosamides share their mechanism of action with macrolides and streptogramin B, cross resistance can develop between the three groups of antibiotics[70]. Moreover, $MLS_B$ resistance is the major way bacteria can develop resistance against lincosamides. $MLS_B$ resistance is encoded for by genes, such as *erm* genes, that encode for methyltransferases where the common target site of these three antibiotic groups, the 23S rRNA, is modified. However, lincosamide resistance can also be conferred through the enzymatic inactivation of lincosamides. These enzymes are encoded for by *lin* genes and can be found on plasmids[71]. The enzyme inactivates lincomycin through nucleotidyation. Finally, lincomycin resistance can also arise through genes encoding for efflux pumps, such as the *msr* genes, similar to TYL and other macrolides[70].

#### 1.2.5.2 *Antibiotic residues in honey*

Antimicrobials are widely used across North America to manage honey bee diseases[55]. Following label treatments, honey cannot be harvested until 54 to 57 days after OTC treatments due to residues in the honey[55]. This 57-day period includes the 12-15 day treatment period, as well as a 42 day post treatment withholding period where honey supers are not added to the colony. Additionally, many countries have a maximum residue limit in place for food. For example, the European Union has a list of limits, however, due to the ban on antibiotic use in honey bees, there is no limit in place for honey[72]. Moreover, the US also does not have a maximum residue limit in place for either OTC, TYL, or LMC in honey, nor does it have recommended concentrations. Contrastingly, Canada has a limit of 300 µg/kg for OTC and 200 µg/kg for tylosin. However, there is no limit currently set for LMC. Despite a waiting period after treatment, OTC and other tetracycline residues have been reported in honey. For example, approximately 29% of Greek honey samples were determined to have drug residues, while 20% were determined to contain more than one tetracycline derivative[73]. Additionally, tetracycline residues in honey have been reported in China, India, and France[74–76]. While studies are limited, TYL residues have also been reported in Spain[77]. Overall, reports of various antibiotics, including OTC and TYL, have been documented.

When treating hives with OTC, beekeepers must wait up to 6 weeks after treatment before they can harvest honey from a treated colony[55]. However, studies have found that OTC

residues last in honey up to 8 weeks after treatment, at concentrations of 3700 µg/kg, which is far in excess of the Canadian maximum residue limit of 300 µg/kg[72,78]. However, this residue analysis was determined through a liquid treatment. When the treatment was administered in a powder form, residue levels were 450 µg/kg, which is only slightly above the maximum residue limit in Canada. Another study demonstrated that the half-life OTC in four types of honey was 15-19 days, while in the fifth type of honey, OTC degradation was not seen even after 60 days. In fact, the degradation was expected to occur after 116 days[79]. Studies have been performed to determine the degradation of TYL in honey as well. TYL was demonstrated to convert to demycosin with a half-life of 4 months at 34°C[80]. Due to the long half-life of TYL, spring treatment of colonies with this antimicrobial is not recommended.[81] Moreover, this study showed that desmycosin was very stable in honey, and that the concentration of both TYL and desmycosin only decreased slightly over 9 months. Finally, LMC was found to persist in hives at a concentration of 380 µg/kg after 129 days after treatment and continued to be present in samples even 290 days after treating the hives[82]. While there is no maximum residue limit in place for LMC, this study had a higher residue level than the maximum limit for both OTC and TYL in honey in Canada[72].

Antibiotic residues in honey may be cause for concern. The major concern involves the development of resistance to these antibiotics. According to the World Health Organization, antibiotic resistance is one of the biggest threats to global health and agriculture[83]. Additionally, resistance to OTC, TYL, and LMC can be spread through exchange of plasmids.[84,85] The development of antimicrobial resistant bacteria can be exposed to human bacterial flora through the consumption of honey containing antibiotic residues. This can encourage the flow of antimicrobial resistance genes and can be spread to human pathogens as well.

#### 1.2.5.3 *Antibiotic resistance in the context of honey bees*

Historically, *M. plutonius* isolates have been considered to be sensitive to OTC. The first studies carried out to determine if any potential OTC resistance existed in *M. plutonius* isolates yielded all sensitive isolates. Minimum inhibitory concentration testing yielded United Kingdom and Australian isolates sensitive to OTC at ~4 µg/mL and 1-2 µg/mL, respectively[86,87]. Interestingly, these studies were carried out over 20 years ago. Since these studies, no further antimicrobial susceptibility testing of *M. plutonius* isolates have been carried out. Additionally, North American isolates have never been used in the few studies that do exist.

Contrastingly, antimicrobial susceptibility testing of *P.* larvae isolates is abundant, and OTC-resistant isolates of *P. larvae*, the causative agent of AFB, have been identified in Canada, USA, and Argentina.[88,89] OTC-resistance in P. larvae is mediated via plasmid-encoded *tetL and tetK genes*, which encode tetracycline efflux pumps[85].

Like OTC, no TYL or LMC-resistant *M. plutonius* isolates have been reported to date. However, a recent study found that *M. plutonius* became resistant to the macrolide antibiotic, mirosamicin through a frameshift mutation in the rRNA large subunit methyltransferase gene *rlmA* II[90]. Concerningly, $MLS_B$ resistance can occur through the acquisition of *erm* genes which encode for rRNA methyltransferases[64,69]. Thus, TYL resistance arising in *M. plutonius* isolates may be possible. Moreover, TYL and LMC-resistant *P. larvae* isolates have been identified in North America[91]. Additionally, it has been demonstrated *in vitro* that TYL and LMC resistance can be conferred to *P. larvae* through the acquisition of *ermC* on a mobilizable plasmid[85]. Remarkably, this plasmid was maintained by *P. larvae* for 10 passages under non-selective culture conditions in the laboratory.

Concerningly, antimicrobial resistance genes (ARGs) have also been identified in numerous non-pathogenic bacteria found in honey.[85,92]. One study analyzed 77 *Bacillus cereus* isolates from honey samples and found that 77% had at least one tetracycline resistance determinant, including *tetK, tetL, tetM,* and *otrA.* Another study investigating *Bacillus* and *Paenibacillus* isolates from Japanese honey identified resistance plasmids encoding the ARGs *ermC* (which encodes for macrolide resistance) and *tetL*[85] These results indicate that in-hive bacterial communities may serve as a reservoir for ARGs with potential for transfer to *M. plutonius* or *P. larvae*.

#### *1.2.5.4 Setting Clinical Breakpoints*

Clinical breakpoints are important to have in order to indicate which populations of bacteria are considered resistant or sensitive to an antibiotic[93]. Several methods are used to define these breakpoints which are dependent on both antimicrobial activity of the drug as well as the pharmacology of the drug. Both the Clinical and Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) provide similar definitions for three categories of breakpoints: sensitive, intermediate, and resistant. Microorganisms defined as sensitive are associated with a high chance of successful treatment with the corresponding antibiotics. Intermediate microorganisms are associated with uncertain

therapeutic effect. Treatment of infection with an intermediate isolate may only be possible in areas where the drug is concentrated to higher levels. Finally, microorganisms categorized as resistant when there is a higher chance of failure when treating with that antibiotic.

The British Society for Antimicrobial Chemotherapy (BSAC) Working Party considers three features when determining breakpoints: (i) the distribution of MIC measurements (ii) pharmacological properties of the antimicrobial (iii) clinical outcome data[94]. The distribution of MIC measurements for most bacterial populations are typically either unimodal, where the bacteria have innate susceptibility or resistance, or bimodal, where there are two populations of bacteria: those that are susceptible and those that possess a resistance mechanism. These distributions are taken into account when deriving clinical breakpoints for antibiotics for bacterial populations. CLSI believes that using MIC distributions to create susceptibility breakpoints should not be dependent on the bacterial population but should be more dependent on the correlation between clinical outcomes and MICs[93,95], Thus, their approach follows an examination of the MIC distribution in a clinical database.

The process of actually setting a breakpoint for BSAC involves obtaining antibiotic pharmacokinetic information, followed by MIC measurements of at least 500 strains to determine the MIC distribution[94]. The MICs are determined using a disk diffusion assay, the results of which are used to inform the breakpoint selection. CLSI, along with disc diffusion assays, additionally use dilution tests (such as broth micro-dilution assays) to determine these breakpoints[95].

### 1.2.6 Review of experimental models of EFB disease

#### 1.2.6.1 *In vitro vs colony models*

The use of *in vitro* larval models to produce and study EFB disease is well-established. In fact, many studies have utilized the *in vitro* larval model to study this disease[38,39,96,97]. Honey bee larvae are generated using field colonies by trapping the queen with an empty brood frame. This allows the generation of age synchronized honey bee larvae. Immediately after hatching, these larvae are transferred (‘grafted’) to 48-well plates to commence larval rearing in the laboratory at 35°C and a relative humidity of ~94%. Grafting controls are used to confirm that healthy larvae are able to survive in a lab setting with a minimum of 75% survival.

*M. plutonius* infection in lab reared larvae can be reliably induced with as little as 50 CFU *M. plutonius* given in the larval diet[38,39] although, many studies infect with up to $10^9$ CFU

*M. plutonius*/mL of diet[96]. Despite differences in infectious dose given to lab-reared larvae, inducing clinical signs of EFB *in vitro* is highly reproducible. In contrast reproducing clinical EFB disease in field colonies is more difficult and without established protocols. Consequently, most studies involving EFB in field colonies involve natural disease outbreaks [27,98,99].

#### 1.2.6.2 *Advantages and disadvantages of the in vitro and colony models*

A major advantage of using an *in vitro* larval model for studying EFB disease is that highly controlled and replicated experiments can be conducted during a short (6-day) time period, with opportunity to include multiple treatment groups, using low-cost and simple methodology. In contrast, in colony-level experiments are expensive, time-consuming, and difficult to replicate. The major disadvantage of the *in vitro* model of EFB is the absence of colony-level social behaviors which may influence disease outcomes in field colonies. For example, social immunity is a defense against pathogens and it influences disease progression through the production of antimicrobial peptides and the display of hygienic behavior, wherein worker honey bees can identify and remove diseased brood from the hive.[100] While colony-level models of EFB benefit from realistic colony conditions, ironically the presence of social immunity within the hive makes clinical disease challenging to reproduce experimentally.

### 1.3 Unconventional EFB disease occurrence in western Canada

While EFB is reported worldwide, there has been a recent re-emergence of this disease in western Canada[101]. Typically, because EFB disease is often stress-induced, colonies are able to spontaneously recover, especially when colony stressors have been alleviated[15]. However, recent EFB emergence has included unconventional cases and poor recovery of colonies without intervention, leading to increased disease transmission[101]. These unconventional cases have been described as more virulent and difficult to treat. Moreover, many colonies have not been able to spontaneously recover even after hive stress has been reduced.

The re-emergence of this disease is costly to the western Canadian beekeeping industry and is estimated to cost beekeepers up to 600 CAD per hive depending on the timing of the outbreak (with fall outbreaks being less costly than a spring outbreak)[101]. However, using an industry-wide perspective, EFB outbreaks are estimated to cost up to 35,000 CAD depending on the timing of the outbreak (fall versus spring).

A Canadian national honey bee health survey reported the prevalence of EFB disease in four Canadian provinces (British Columbia (BC), Alberta, Manitoba, and Ontario) in 2015[102].

The lowest prevalence of this disease was 0% in Manitoba and highest was reported to be 1.7% in BC. Alberta and Ontario were determined to have a disease prevalence of 0.6% and 0.7%, respectively. The same survey was carried out in 2017 and reported a national average of 0.6%, compared to the average of 0.75% in 2015. However, it is important to note that the 2015 survey only included 4 provinces, while the 2017 survey included 8 provinces. Thus, the national averages from 2015 and 2017 may not be comparable. Individual comparisons of the provinces may be a better comparison instead. The 2017 survey reported the EFB incidence in BC rose to 3.0%, while AB prevalence decreased to 0.2%. Both Manitoba and Ontario had a reported 0% EFB incidence. More recently, in 2020, Alberta reported an increased prevalence of virulent EFB cases[101].

One hypothesis for the rise of these unconventional cases includes the presence of hypervirulent strains of *M. plutonius* circulating in western Canada. A recent investigation of three EFB disease outbreaks in western Canada revealed an *M. plutonius* isolate belonging to a novel multi-locus ST (36)[17]. Additionally, the three *M. plutonius* strains isolated from the outbreaks all belonged to CC12, which is believed to be the most virulent CC clinically.

Another hypothesis for the presence of unconventional cases of EFB theorizes that antimicrobial resistance to conventional OTC treatment for EFB may be the reason for the re-emergence of EFB disease. This is especially concerning as OTC is the only approved treatment for EFB disease in North America[55].

### 1.4 Rationale of project and objectives

Currently there is only one approved treatment for EFB disease in North America: OTC. However, no studies have been carried out on North American *M. plutonius* isolates to determine if antimicrobial resistance to OTC exists. Additionally, the last antimicrobial susceptibility testing done using *M. plutonius* isolates was carried out over 20 years ago on Australian and European isolates only[86,87]. The lack of knowledge of antimicrobial resistance in North America is concerning due to the re-emergence of EFB disease in western Canada[101]. Currently, other honey bee pathogens, such as *P. larvae* have been demonstrated to carry tetracycline resistance on a plasmid, thus, *M. plutonius* is also at risk of acquiring tetracycline resistance through horizontal gene transfer[59].

The first aim of my MSc. research project is to investigate the use of TYL and LMC as treatments for EFB. These two antimicrobials are approved in the treatment of AFB disease in

honey bees and may serve as potential alternatives to OTC for treatment of EFB [55]. To evaluate and compare the ability of OTC, TYL, and LMC to clear *M. plutonius* infections, I will use *in vitro* larval rearing trials, by slight modification (addition of antibiotics) to an otherwise well-established protocol[38,97]. I will first evaluate whether these antimicrobials are adequate in saving larvae from *M. plutonius* infections using concentrations of antibiotics that have been used in previous studies for EFB and AFB[96,103]. I will then evaluate the use of these antimicrobials at concentrations that reflect the minimum inhibitory concentrations (MIC) of the strain to OTC, TYL, and LMC. Additionally, I will test concentrations corresponding to four times below the MIC, as well as four times above the MIC.

The second aim of my MSc project will involve the isolation of ~60 *M. plutonius* isolates from samples collected from suspected EFB outbreaks from North American apiaries. There is a lack of current antimicrobial susceptibility testing for *M. plutonius* isolates in North America. Accordingly, the clinical isolates acquired in this project will be used to determine the MIC and minimum bactericidal concentration (MBC) for each strain and each of the three antibiotics.

The third aim of my MSc research is to correlate *in vitro* antimicrobial resistance (AMR) phenotypes to *M. plutonius* genotypes. Whole genome sequencing of the aforementioned isolates will be performed in order to identify AMR genes. I anticipate that the widespread spring and fall use of OTC for metaphylaxis of AFB in Canada has inadvertently imposed selective pressure for resistant isolates to emerge in *M. plutonius*. Tetracycline resistance mechanisms have been well studied in other bacteria, and dissemination through mobile genetic elements is common[56,59]. OTC resistance in *Paenibacillus larvae*, the causative pathogen of AFB, has developed, in part, due to plasmids which carry these tetracycline resistance genes. *M. plutonius* are known to carry plasmids, though genome sequencing efforts to date have largely focused on strains from regions that forbid antimicrobial use at apiaries (*e.g.* Europe)[43]. Additionally, it has been demonstrated that non-pathogenic strains found in honey carry tetracycline resistance genes, and that honey may act as a reservoir for ARGs[85]. In contrast to this work, the resistome (collection of ARGs) of *M. plutonius* has not been explicitly investigated. Thus, my project represents the first effort to systematically pair AMR phenotypes to *M. plutonius* genotypes.

The final aim of my MSc research project is to compare the ~50 whole genome sequences of the clinical M. plutonius isolates acquired during this project, as well as publicly available *M. plutonius* genomes, using a pangenome pipeline to determine the core and

pangenome of *M. plutonius*. Additionally, a phylogenetic analysis will be carried out to determine how North American and non-North American isolates are related to each other.

Taken together, my *in vitro* and genomic characterization of the antimicrobial susceptibility of *M. plutonius* will inform evidence based EFB management strategies, which will in turn, enhance the health and productivity of the Canadian beekeeping industry.

# CHAPTER 2. EVALUATING APPROVED AND ALTERNATIVE TREATMENTS AGAINST AN OXYTETRACYCLINE-RESISTANT BACTERIUM RESPONSIBLE FOR EUROPEAN FOULBROOD DISEASE IN HONEY BEES

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## Author Contributions

**Conception and design of the study**: FM, ES, AR, SW; **Methodology:** FM, JT, ES, AR, SW; **Field work:** FM, JT, AC, IK, MZ, SB, ES, SW; **Acquisition of data:** FM, JT, AC, JL; **Formal Analysis:** FM, JT, AC, IK, MZ, SB, ES, AR, SW; **Funding Acquisition:** ES, AR, SW; **Resources:** FM, JT, AC, MMG; **Writing – original draft:** FM, AR, SW; **Writing – review and editing:** FM, JT, AC, IK, MZ, SB, JL, MMG, ES, AR, SW.

## 2.1 Abstract

European foulbrood (EFB) is a disease of honey bee larvae caused by *Melissococcus plutonius*. In North America, oxytetracycline (OTC) is approved to combat EFB disease though tylosin (TYL) and lincomycin (LMC) are also registered for use against American foulbrood disease. Herein, we report and characterize an OTC-resistant *M. plutonius* isolate from British Columbia, Canada, providing an antimicrobial sensitivity to the three approved antibiotics and studying their abilities to alter larval survival in an in vitro infection model. Specifically, we investigated OTC, TYL, and LMC as potential treatment options for EFB disease using laboratory-reared larvae infected with *M. plutonius*. The utility of the three antibiotics were compared through an experimental design that either mimicked metaphylaxis or antimicrobial intervention. At varying concentrations, all three antibiotics prevented clinical signs of EFB disease following infection with *M. plutonius* 2019BC1 in vitro. This included treatment with 100 μg/mL of OTC, a concentration that was ~ 3× the minimum inhibitory concentration measured to inhibit the strain in nutrient broth. Additionally, we noted high larval mortality in groups treated with doses of OTC corresponding to ~ 30× the dose required to eliminate bacterial growth in vitro. In contrast, TYL and LMC were not toxic to larvae at concentrations that exceed field use. As we continue to investigate antimicrobial resistance (AMR) profiles of *M. plutonius* from known EFB outbreaks, we expect a range of AMR phenotypes, reiterating the importance of expanding current therapeutic options along with alternative management practices to suppress this disease.

## 2.1 Introduction

Honey bees (*Apis mellifera)* are the most economically significant pollinators of major crops worldwide[3]. It is estimated that roughly one third of food that is consumed is either directly or indirectly dependent on honey bee pollination[4]. Safeguarding honey bee health through the management of infectious diseases of honey bees is therefore critical to the success of global agriculture. In North America, antimicrobials are widely used for treatment and control of bacterial brood diseases, including both American foulbrood (AFB) and European foulbrood (EFB) disease. In contrast, prophylactic antimicrobial-use is prohibited in beekeeping in the European Union[104]. Thus, antimicrobial resistance (AMR) poses an imminent threat to North American apiaries, especially for cases of EFB disease since only a single antibiotic,

oxytetracycline (OTC), is approved for use[55]. EFB is the result of an infection of honey bee larvae by the Gram-positive bacterium *Melissococcus plutonius*, occurring through the consumption of contaminated food provided by adult bees[15]. Adult bees are asymptomatic carriers that can transmit the pathogen within and between colonies. The high density of bee populations within colonies and apiaries, as well as the density of apiaries within a region, facilitates the transmission of *M. plutonius* to larvae, which can result in immense brood loss leading to weakened colonies and even colony collapse[15,105]. Upon ingestion, *M. plutonius* multiplies in the developing midgut and competition for nutrients between host and bacterium has been hypothesized to result in clinical symptoms of EFB disease that ultimately results in death through starvation[15,106].

EFB disease has been reported worldwide and has recently seen a re-emergence in western Canada[101]. In this region, honey bee colonies have historically been characterized by a capacity to spontaneously recover from EFB disease, however, recent disease emergence has included unconventional cases and poor recovery without intervention[107]. The increase in honey bee colonies unable to spontaneously recover from EFB has translated not only to a disease state that is increasingly difficult to eradicate but also increased transmission[101]. These distinctly new cases of EFB disease are hypothesized to be caused by hypervirulent strains of *M. plutonius* circulating in western Canada. An alternative hypothesis posits that resistance to conventional antimicrobial therapies explains the recalcitrance of recent EFB disease outbreaks in western Canada. While some geno- and phenotypic characterization of *M. plutonius* has been reported from Europe and Asia, the bacteria responsible for recent outbreaks in Canada remain to be characterized at the same level[43,87].

Currently, oxytetracycline (OTC) is the only approved antimicrobial used for treatment of EFB disease in Canada and it is applied to individual hives at a concentration of 200 mg per 20 g of icing sugar, for three consecutive doses, 4-5 days apart[55]. OTC is a bacterial natural product that was among the first discovered tetracyclines, a drug class that has been widely used in both human and veterinary medicine since the late 1940s[56]. The tetracyclines are broad-spectrum, bacteriostatic antibiotics that inhibit protein synthesis by preventing aminoacyl-tRNA attachment to the A site of the ribosome. Accordingly, resistance to OTC and other tetracyclines are now common in animal pathogens with a widespread distribution of AMR genes transferred through bacterial mechanisms of genetic exchange. These genes, which have a colloquial naming system

based on discovery, can encode for a series of protective functions such as efflux, ribosomal modification proteins, and antibiotic destruction. In the context of honey bee health, OTC-resistance has been observed in *Paenibacillus larvae*, the causative agent of AFB disease, resulting from plasmid-encoded tetracycline resistance through the *tetL* gene[85]. Additionally, a series of *Bacillus cereus* strains isolated from honey were found to carry tetracycline resistance genes, including *tetL* and *tetK*[92]. These results indicate the presence of AMR genes in pathogens and non-pathogenic organisms within colonies that may act as reservoirs of tetracycline resistance genes.

The risk of *M. plutonius* developing resistance to OTC is heightened by its use to treat AFB disease[104]. Alternative treatments to suppress AFB disease are currently approved in Canada, including tylosin (TYL) and lincomycin (LMC). Notably, TYL is a macrolide antibiotic that is widely used in western Canada to treat livestock animals that are commonly raised near apiaries in the region[108]. LMC is a lincosamide antibiotic that, like the macrolides, inhibit protein synthesis by binding to the 23S subunit of the ribosome[63,70,108]. Due to similarities in their mechanism, including overlapping drug binding sites, bacteria can develop cross-resistance to both TYL and LMC via macrolides, lincosamides, and streptogramin B ($MLS_B$) resistance[64,109]. $MLS_B$ resistance is typically associated with genes that encode for methyltransferases that modify a common target site of the antibiotic. For example, *erm* genes, a major source of $MLS_B$ resistance, encode for rRNA methyltransferases that add one or two methyl groups onto the 23S rRNA, preventing the antibiotics from binding their target[64]. While no TYL or LMC-resistant *P. larvae* or *M. plutonius* have been identified, bacteria isolated from honey encode for $MLS_B$ resistance genes, including plasmid-borne 23S rRNA methyltransferases such as *ermC*[85]. Additionally, a recent study found *M. plutonius* isolates resistant to the macrolide antibiotic, mirosamicin[90]. Thus, there exists a possibility of *M. plutonius* developing $MLS_B$ resistance. Antimicrobial use in honey bees has been shown to negatively impact the honey bee microbiome, leading to dysbiosis[110–112]. Microbial imbalance can lead to changes in gene expression, metabolism, and allow for the overgrowth of opportunistic pathogens. Nevertheless, antibiotic use can be beneficial in the case of treating and preventing foulbrood diseases[112].

In this study, we evaluated the potential utility of both EFB and AFB disease-approved treatments in protection against infection by *M. plutonius* using an *in vitro* model. Prophylactic treatment of EFB in honey bees in north America is prohibited[55]. Thus, our experimental design

considered both metaphylaxis (concurrent infection and treatment) and therapeutic (treatment after infection) antimicrobial interventions of larvae infected with a previously described isolate from British Columbia named *M. plutonius* 2019BC1[38]. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the three antibiotics against *M. plutonius* 2019BC1 were determined and antibiotic concentrations within and above the range of environmental exposures were evaluated for their ability to protect against EFB and harm honey bee larvae *in vitro*. Our results are discussed within the context of EFB disease and the North American beekeeping industry.

## 2.3 Materials and methods

### 2.3.1 Determination of Minimum Inhibitory Concentrations

The minimum inhibitory concentrations (MICs) of OTC (TCI, 00475), TYL (Thermo Scientific, AC463070050), and LMC (Thermo Scientific, AAJ6125106) were determined using both a broth microdilution method in a 96-well format and growth on an agar surface supplemented with antibiotics. KSBHI (brain heart infusion supplemented with 0.15 M $KH_2PO_4$ and 1% soluble starch) or KSBHI with 1.5% agar were used to support bacterial growth[31]. For the 96-well formatted broth assay, antibiotics were prepared as 2-fold serial dilutions in 200 μL of KSBHI. Dilution series includes 128 μg/mL to 2 μg/mL OTC and 16 μg/mL to 0.25 μg/mL TYL or LMC. To measure the MICs, a culture of *M. plutonius* 2019BC1 was inoculated into KSBHI with and without antibiotic at an optical density of $5 \times 10^{-4}$, measured at a wavelength of 600 nm. Growth was evaluated after a 72-h incubation in microaerophilic conditions (AnaeroPack System, Mitsubishi Gas Chemical Company, Inc.) at 37°C, shaking at 200 rpm. The MIC was defined as the lowest concentration of antibiotic in which no *M. plutonius* growth was visible. For each MIC determination, three technical replicates were performed on three biological replicates of *M. plutonius* 2019BC1. For the agar plate method, antibiotics were also prepared as 2-fold serial dilutions in KSBHI agar as above, though 1 mL volumes were used to generate agar surfaces in a 48-well plate. A culture of *M. plutonius* 2019BC1 was diluted to an optical density of $5 \times 10^{-4}$ before inoculating agar surfaces with 2 μL. Growth was evaluated after a 96-h incubation in microaerophilic conditions at 37°C. For each MIC determination, three technical replicates were performed on three biological replicates of *M. plutonius* 2019BC1.

### 2.3.2 Determination of minimum bactericidal concentrations.

The minimum bactericidal concentrations (MBCs) of OTC, TYL, and LMC were determined by inoculating 2 mL of fresh, antibiotic-free KSBHI media with samples from 96-well MIC plates that had no visible *M. plutonius* growth after 72 h. The growth of surviving bacteria was then evaluated after a 72-h incubation in microaerophilic conditions at 37°C, shaking at 200 rpm. The MBC was defined as the lowest concentration of antibiotic in which no *M. plutonius* growth was observed after 72 h in the fresh outgrowth media.

### 2.3.3 Source of honey bee larvae

Six well-established, healthy, experimental field colonies in Saskatoon, SK, Canada, were used as the source of honey bee larvae. Freshly hatched larvae used for *in vitro* larval rearing experiments were generated by placing empty brood frames in a cage with a laying queen. Twenty-four hours later, frames were removed from the queen cages and placed in adjacent uncaged space in the brood chamber. After three days, the frames, which contained first-instar larvae, were transported to the laboratory in a portable incubator kept at 35°C. Genetically unrelated queens were used as host genetics contribute to the outcome of the disease progression[113].

### 2.3.4 Larval rearing, infection and antimicrobial treatments *in vitro*

The *in vitro* larval rearing protocol was adapted from Schmehl et al (2016)[97]. Field-collected larvae were transferred (grafted) from brood frames into 48-well sterile tissue culture plates (considered day 0, d0, of the experiment). Briefly, each well contained a 1 cm diameter cups and 10 μL of pre-warmed diet A (44.25% royal jelly, 5.3% each of glucose and fructose, 0.9% yeast extract, and 44.25% water), and were maintained at 35°C throughout the grafting process. After grafting, an additional 9.5 μL of diet A mixed with either 0.5 μL PBS (used for the grafting control) or 0.5 μL PBS containing 50 CFU of *M. plutonius* 2019BC1 (sequence type 19, clonal complex 12), isolated from a honey bee colony with clinical signs of EFB in B.C, Canada, was added[38]. Plates were incubated at 35°C in an environment humidified by 0.9 M $K_2SO_4$. Larvae were fed increasing amounts of diet throughout the experiment with the exception of d1 when no additional food was provided. Plates were monitored daily using a stereomicroscope, and dead larvae were removed immediately. Larval death was determined based on melanisation, the lack of mobility and spiracle movement[38]. Rearing experiments were terminated 6 days after the grafting date (d6).

Treatment and control groups were defined by dividing 48-well plates into four groups of 12 larvae. Each plate included a negative control group (grafting control; GC, n = 12 animals) consisting of uninfected larvae fed control diet. A minimum of two technical replicates (n = 24) and two to five biological replicates (experiments performed using different queens corresponding to distinct genetic lineages) were performed for each treatment group. Treatment groups included 1, 10, 100, and 1000 μg/mL of OTC, and 33, 330, and 3300 μg/mL of both TYL and LMC. To ensure that effects were not caused by variations in grafting (a common technical challenge) or potential poor larval health, only experimental plates with >75% survival in the grafting controls were used in the study. Likewise, experimental infection was determined by using plates only when <50% survival of the I0 control was observed.

Antibiotics were included in diets daily for either 4 or 5 days depending on the experimental design, Thus, total 4 or 5-day doses were of 0.14 to 140 μg or 0.16 to 160 μg OTC and 4.62 to 462 μg or 5.28 to 528 μg of TYL and LMC not accounting for the half-lives of OTC, TYL, and LMC, which in water are 34 h, 200 d, and 30 h, respectively[114–116].

Statistical analyses were performed using Stata 17 with larval survival at d6 compared among treatment groups using a Pearson's chi-square test.

### 2.3.5 Measure of average healthy larval weight

On d6 of *in vitro* larval rearing trials, surviving larvae were rinsed with water to remove any excess diet before being gently patted dry. Dry larvae were then weighed using an analytical balance.

### 2.3.6 Survival of *M. plutonius* in larval diets

*M. plutonius* 2019BC1 was diluted to 5000 CFU/mL in diet A, with or without antibiotics, to mimic preparations used for larval infection. The diets were incubated for 30 minutes at 35°C and subsequently, 50 μL of each diet was plated, in triplicate, and grown in microaerophilic conditions at 37°C for 72 hours. The number of viable of *M. plutonius* were counted and reported as CFU/mL

### 2.3.7 Screening publicly available *M. plutonius* genomes for *tet* resistance genes

17 publicly available *M. plutonius* genomes[43], were downloaded from NCBI and uploaded to the Comprehensive Antibiotic Resistance Database (CARD) to identify known tetracycline resistance genes[117].

## 2.4 Results and discussion

### 2.4.1 *M. plutonius* 2019BC1 is resistant to oxytetracycline

To define the *M. plutonius* 2019BC1 AMR phenotype *in vitro*, the three antibiotics of interest – OTC, TYL and LMC – were evaluated using two-fold serial dilutions in a microbroth dilution assay followed by antibiotic-free outgrowth to define the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC; Table 2.1). *M. plutonius* 2019BC1 was most sensitive to LMC (MIC 0.5 μg/mL), followed by TYL (2 μg/mL). Remarkably, the 2019BC1 isolate was resistant to OTC with an MIC of 32 μg/mL required to suppress visible growth of the strain in KSBHI broth. While *M. plutonius* is considered sensitive to OTC at a cut-off value of 2.5 μg/mL according to Clinical & Laboratory Standards (CLSI) guidelines, there is no information for what constitutes an OTC-resistant isolate[118]. Unlike *M. plutonius*, OTC-resistance in *Paenibacillus larvae*, which causes AFB disease, has been identified.[119–121] According to CLSI guidelines, *P. larvae* is considered resistant to OTC at ≥16 μg/mL using a broth microdilution method[118]. Thus, applying the threshold used to classify *P. larvae*, another honey bee pathogen, *M. plutonius* 2019BC1 can be classified as resistant to OTC. Alternatively, *M. plutonius* may be tolerant to high concentrations of OTC. Bacterial tolerance is defined as survival without replication in the presence of an antibiotic[122]. However, the MIC of a tolerant strain is typically similar to that of a susceptible strain, while the minimum time of killing would be longer for a tolerant strain than a susceptible strain[123]. Thus, the over 12-fold increase in MIC of *M. plutonius* 2019BC1 relative to the CLSI OTC susceptibility cut-off value of 2.5 μg/mL for *M. plutonius* suggests that *M. plutonius* 2019BC1 is an OTC-resistant strain[118]. To the best of our knowledge, this is the first report of an OTC-resistant strain of *M. plutonius* recovered from an EFB disease outbreak. Previous studies of *M. plutonius* isolates from the United Kingdom, where antimicrobial use in apiculture is prohibited, and Australia were sensitive to OTC at ~4 and 1-2 μg/mL, respectively[86,87]. These studies measured MICs on solid agar, prompting our own evaluation of the antibiotics in KSBHI. *M. plutonius* 2019BC1 was resistant to OTC at 16 μg/mL when grown on a solid KSBHI agar surface. The two-fold difference in MIC may have resulted from a difference in bacterial physiology during vegetative versus growth on a solid surface, though variations within a 2-fold dilution are typical in these types of experiments. In either case, extrapolating the CLSI guidelines for *P. larvae* to *M. plutonius*, the 2019BC1 isolate is resistant to OTC[118].

**Table 2.1.** Susceptibility of *M. plutonius* 2019BC1 to antibiotics (μg/mL).

| Antibiotic | MIC (agar) | MIC (broth) | MBC |
|---|---|---|---|
| OTC | 16 | 32 | 128 |
| TYL | 1 | 2 | 4 |
| LMC | 0.5 | 0.5 | 2 |

Classification of *M. plutonius* 2019BC1 as OTC resistant is of particular interest. The resistant phenotype is an expected result considering the frequent use of OTC as a treatment for EFB and AFB in North America[55]. In fact, the concentration of OTC required to inhibit this strain *in vitro* is higher than the expected accumulation of the antibiotic during a standard treatment. For example, honey bee larvae sampled from one or two chamber field colonies, one day after colony treatment with 300 mg OTC (in excess of current label recommendations of 200 mg), were shown to have OTC concentrations of ~13 and 9 μg/g, respectively[124]. While this appears to be a sub-therapeutic dose for infection with *M. plutonius* 2019BC1, social immunity (see below) may influence on colony-level antibiotic efficacy. Nevertheless, this first observation of OTC-resistant *M. plutonius* calls for additional sampling and characterization of *M. plutonius* isolates from North American apiaries, including intensive AMR phenotyping and whole genome sequencing, to uncover the genetic determinants of antibiotic resistance in this pathogen. A survey of 17 other, previously assembled, publicly available *M. plutonius* genomes did not yield significant hits to any known tetracycline resistance genes, suggesting that further studies will need to be done to uncover the genetic determinants of OTC resistance in *M. plutonius.*

### 2.4.2 Antibiotics prevent and control *M. plutonius* infections

To evaluate the effects of antibiotics on larval survival and bacterial infection, we designed an *in vitro* rearing experiment that included two distinct dosing strategies. The first strategy mimics metaphylactic treatment by administering antibiotics at the time of grafting (A0) or co-administration of antibiotic and *M. plutonius* at the time of grafting (IA0). The second strategy involved antimicrobial intervention two days after grafting (A2) or infection (I0A2). Rearing treatments were performed in parallel to demonstrate effects of grafting (GC) and untreated infection (I0). Larval infections were established using 50 CFU of *M. plutonius* 2019BC1 as previously described[38]. Growth of the larvae was monitored for 1 week: typical healthy and infected phenotypes show significant macroscopic differences during this timeframe

(Figure 2.1A, B). The average weight of healthy larva that survived the rearing experiment was 130 ± 20 mg (n = 517), in line with previous studies[113,125]. Experiments were conducted with a range of distinct genetic lineages with larva sourced from different colonies (Table 2.2). The concentrations of OTC were selected based on a previous work that tested its effects on honey bee larvae infected with *M. plutonius in vitro*[96]. The TYL concentrations were selected based on a previous study of the antibiotic treatment for AFB *in vitro,* which also considered field relevant concentrations[103]. The doses of LMC mirrored those of TYL due to the similar applications of these drugs for the treatment of AFB in the field[55].

**Table 2.2** Summary of larval rearing experiments showing survival[a] 6 days after grafting and treatment**.**

| | | | GC | I0 | A0 | IA0 | A2 | I0A2 |
|---|---|---|---|---|---|---|---|---|
| | **No. of queens** | **No. of larvae** | **Grafting control** | **Infection control** | **Metaphylaxis control** | **Metaphylaxis treatment** | **Therapeutic control** | **Therapeutic treatment** |
| 1 µg/mL OTC | 3 | 527 | 91 ± 5 | 28 ± 11 | 92 ± 10 | 56 ± 21 | 83 ± 13 | 40 ± 12 |
| 10 µg/mL OTC | 2 | 571 | 86 ± 7 | 31 ± 15 | 77 ± 8 | 81 ± 9 | 82 ± 10 | 62 ± 13 |
| 100 µg/mL OTC | 5 | 480 | 87 ± 12 | 35 ± 8 | 82 ± 11 | 86 ± 21 | 93 ± 10 | 88 ± 11 |
| 1000 µg/mL OTC | 5 | 625 | 88 ± 9 | 33 ± 33 | 0 + 0 | 0 ± 0 | 9 ± 12 | 8 ± 9 |
| 33 µg/mL TYL | 5 | 431 | 90 ± 6 | 25 ± 7 | 82 ± 16 | 83 ± 13 | 95 ± 5 | 85 ± 15 |
| 330 µg/mL TYL | 4 | 667 | 84 ± 10 | 23 ± 14 | 82 ± 18 | 82 ± 14 | 81 ± 10 | 73 ± 13 |
| 3300 µg/mL TYL | 3 | 324 | 82 ± 7 | 33 ± 8 | 67 ± 35 | 69 ± 15 | 82 ± 11 | 76 ± 13 |
| 33 µg/mL LMC | 2 | 383 | 92 ± 8 | 44 ± 5 | 97 ± 5 | 92 ± 6 | 100 ± 0 | 85 ± 7 |
| 330 µg/mL LMC | 4 | 481 | 88 ± 9 | 35 ± 11 | 90 ± 4 | 78 ± 7 | 85 ± 11 | 84 ± 5 |
| 3300 µg/mL LMC | 3 | 381 | 91± 7 | 22 ± 5 | 81 ± 11 | 86 ± 10 | 100 ± 0 | 86 ± 21 |

*a* – survival is expressed as mean percent ± standard deviation between experiments

All three antibiotics were able to reduce larval mortality from *M. plutonius* infection at their low, medium, and high concentrations (Figure 2.1C and Table 2.2). Antibiotic treatment significantly increased larval survival from *M. plutonius* infection by 12-53%, 36-60%, and 41-64% relative to the infection control group (I0) for OTC, TYL, and LMC, respectively (Figure 2.1C; * $p < 0.1$, ** $p < 0.001$, and *** $p < 0.0001$). We did not observe lineage-specific responses in our assays. This is the first *in vitro* demonstration of the efficacy of TYL or LMC as

treatments against *M. plutonius* infection in honey bee larvae whereas OTC has been previously shown to be effective in treating *M. plutonius* infection of *in vitro*-reared larvae[96].

In this previous study, OTC concentrations ranging from 1 μg/mL to 10 μg/mL reduced larval mortality from *M. plutonius* infection, and treatment with 20 μg/mL OTC was required to reduce larval mortality to the same level as the negative control (non-infected larvae); however, the AMR phenotype of this strain (e.g. MIC of OTC) used was not evaluated[96]. Here we show that concentrations of 1 μg/mL and 10 μg/mL OTC were able to significantly increase larval survival by 12-50% when compared to an infected control (I0; * $p < 0.1$, ** $p < 0.001$, *** $p < 0.0001$; Figure 2.1C), though survival of these groups were significantly lower by 5-51% relative to the uninfected GC group ($p < 0.0001$; Table 2.2). Larval treatment with 100 μg/mL OTC, corresponding to ~3X the MIC *in vitro*, was necessary to reduce larval mortality to the same level as the uninfected GC group. After six days of *in vitro* rearing, larvae treated with 100 μg/mL OTC were calculated to receive a total dose of 105-120 μg/g OTC per larvae, not accounting for the 34-hour half-life of OTC in water[114]. Accordingly, the dose of OTC per larva *in vitro* is ~10X the quantity of OTC previously reported in honey bee larvae from a field colony treated with 300 mg OTC[124]. In the field, we anticipate that concentrations lower than 100 μg/mL will be sufficient due to the presence of social immunity within a colony as a mechanism for pathogen defense. Examples of honey bee social immunity involves the production of antimicrobial peptides, such as defensin-1, grooming, and hygienic behaviour, which refers to the colony's ability to detect and remove diseased brood[100].

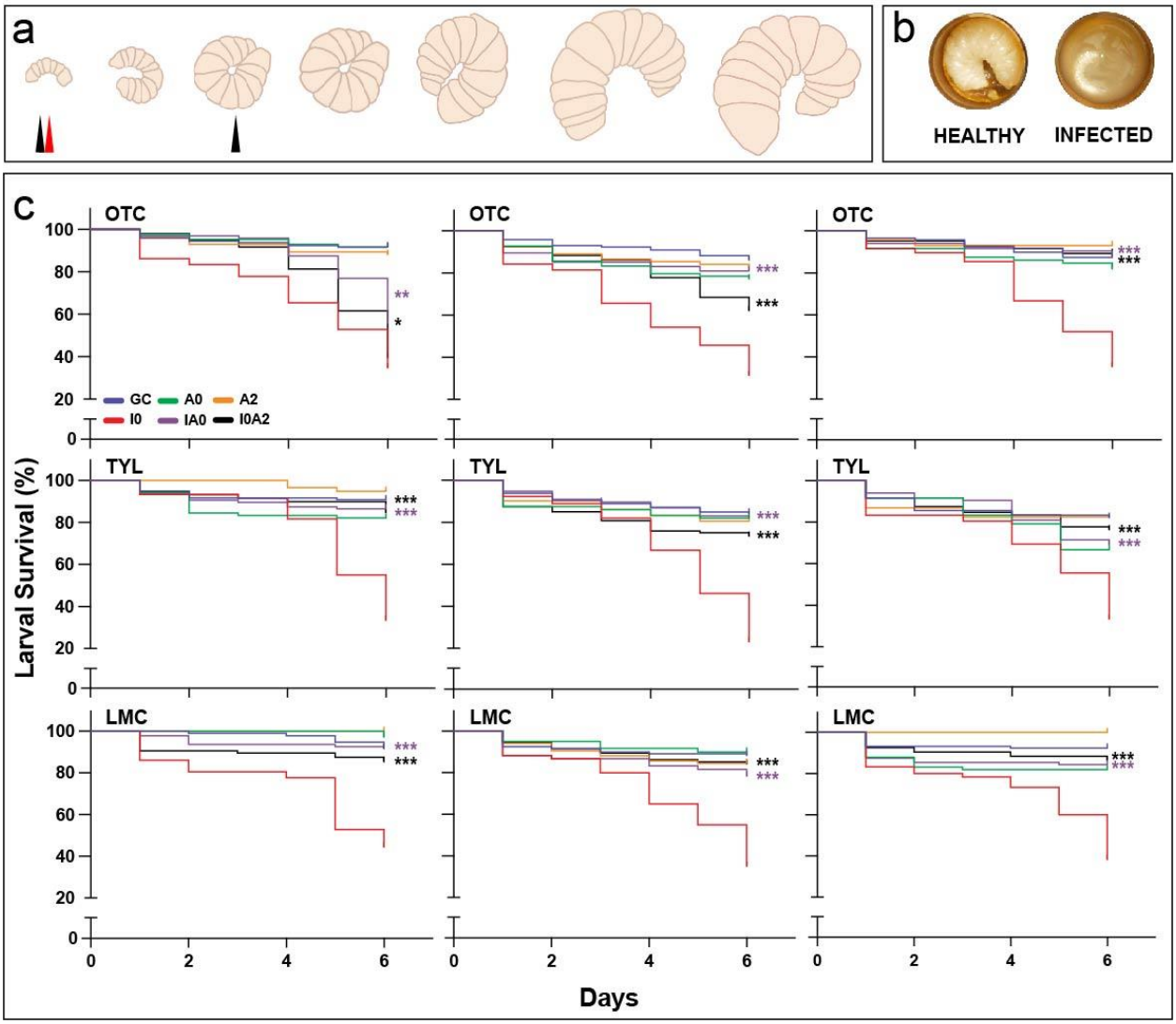


**Figure 2.1**. **Results of *in vitro* larval rearing experiments upon exposure to *M. plutonius* with and without antibiotics. (a)** Cartoon representation of typical growth of healthy larvae during a week-long *in vitro* rearing experiment. The red arrow indicates the day at which larval infection with 50 CFU of *M. plutonius* 2019BC1 occurred (d0), while the black arrows indicate the days on which an antibiotic was first administered (d0 or d2). **(b)** Representative phenotypes of healthy and infected larvae. Healthy d6 larvae are white and plump, while infected d6 larvae are typically smaller in size and typically darker in colour. **(c)** Survival of *in vitro* reared honey bee larvae based on Table 2.2, excluding 1000 μg/mL OTC. The top three panels, from left to right, display survival in experiments conducted with 1, 10, and 100 μg/mL OTC. The middle and bottom panels display survival in experiments with 33, 330, and 3300 μg/mL of TYL and LMC, respectively. Statistical analysis of the survival was performed using a chi-square two-way association test comparing either IA0 or I0A2 with the I0 and significant differences are denoted by asterisks (* $p < 0.1$, ** $p < 0.001$, *** $p < 0.0001$).

In contrast to the protection provided by the 100 μg/mL dose of OTC, we observed significant toxicity to larval brood from the antibiotic at 1 mg/mL. This high dose resulted in nearly complete killing of the larvae with only 0-8% larval survival observed in the antimicrobial

treatment groups. This observation is well-aligned with anecdotal evidence communicated by beekeepers reporting OTC-dependent brood toxicity within colonies. Similarly, previous controlled experiments have demonstrated that OTC can be toxic to honey bee larvae when administered to a colony in a 5 mg/mL sucrose solution or as a powder treatment according to label instructions at 200 mg OTC per 20 g of icing sugar[78,126].

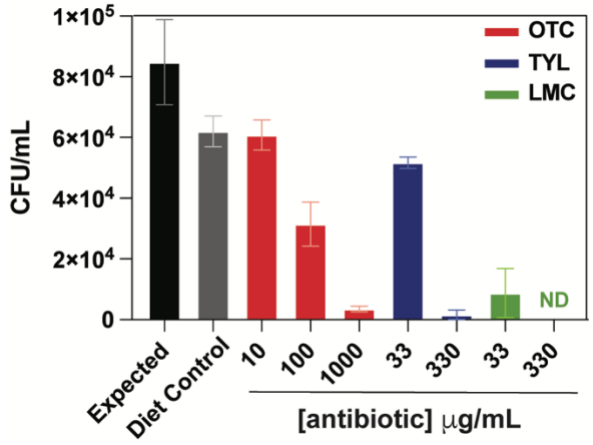


**Figure 2.2 – Survival of *M. plutonius* in larval diet and diets supplemented with antibiotics.**
Recovery of *M. plutonius* 2019BC1 from diets is presented in CFU/mL after a 30 min incubation in control larval diet A (grey) or larval diet A containing various concentrations of OTC (red), TYL (blue), or LMC (green). Expected (black) represents expected recovery of *M. plutonius* in KSBHI with no antibiotic or diet component. The mean and standard deviations are plotted. The detection limit was 20 CFU/mL. ND indicated no detection.

### 2.4.3 Larval diet inhibits *M. plutonius* survival

While the larval rearing experiment provided information related to metaphylactic and curative effects of antibiotics delivered to larvae, we sought to better understand the ability of each drug to affect *M. plutonius* in the laboratory diet. Moreover, our laboratory infection model is atypical in that relatively few CFUs are delivered to larvae in order to establish disease when compared to others[47,96]. Thus, to clearly establish the consequences of concurrent administration of antimicrobials and *M. plutonius* during our *in vitro* rearing experiments, we inoculated antibiotic-containing and antibiotic-free diets with *M. plutonius* for 30 minutes at 35°C before recovering viable bacteria on KSBHI agar (Figure 2.2). In all cases, *M. plutonius* viability decreased, including a 27% loss of *M. plutonius* counts within the antibiotic-free, control diet relative to the expected values of *M. plutonius* in KSBHI. The larval diet itself possesses modest antimicrobial activity and has been previously shown to inhibit *M. plutonius* growth[127,128]. Recently, the antimicrobial activity of the diet has been linked to Royal Jelly, and more

specifically, Major Royal Jelly protein 1 (MRJP1) was shown to be the determinant of *M. plutonius* inhibition[129]. The addition of low doses of TYL and OTC to diets showed similar reductions in *M. plutonius* viability compared to the control diet, while greater 63%-98% reductions in *M. plutonius* were observed in diets containing concentrations of 100 μg/mL OTC and 330 μg/mL TYL. LMC treatment was associated with the most dramatic declines in *M. plutonius* survival in the diet, mirroring the observed MICs *in vitro*. At 330 μg/mL LMC, no viable *M. plutonius* were recovered from the diet after 30 minutes, representing at least a 4-log reduction in the bacterium, considering the detection limit of this assay is 20 CFU/mL (Figure 2.2). Due to the reduction of *M. plutonius* viability in larval diet, co-administration of high doses of antibiotics and bacteria on d0 of *in vitro* larval rearing (Figure 2.1A) likely resulted in a lack of larval infection. Indeed, the effect of the diet, and its preparation with and without antibiotics, may explain the need in other models of EFB to establish larval infection *in vitro* with doses as high as $10^7$ CFU[47,96]. However, CC12 infections require lower CFUs than infections with CC3 or CC13[130]. Nevertheless, the results of our study, which included antibiotic interventions 2 days after grafting – the I0A2 groups – demonstrated the efficacy of OTC, TYL, and LMC in treating infected larva *in vitro*.

**2.5 Conclusion**

In summary, we identified, for the first time, an OTC-resistant isolate responsible for EFB disease in a North American apiary. *M. plutonius* 2019BC1 remains susceptible to TYL and LMC, suggesting that these antimicrobials may be suitable treatments for colonies showing clinical signs of EFB disease due to infection with *M. plutonius* 2019BC1, however, further evaluation is required for approval in Canadian apiculture. In fact, we provide evidence to support further evaluation towards regulatory approval of TYL and LMC, along with OTC, as treatments for OTC-resistant *M plutonius* infection in honey bee larvae. The overlapping molecular target of all three antibiotics, however, remains a significant concern, especially considering the lack of alternative treatment options. Additional research is required to characterize the AMR phenotypes and genotypes of *M. plutonius* strains circulating in North America where antibiotic use is common.

## 2.6 Transition Statement

In the previous chapter, we determined that tylosin and lincomycin could be used as alternatives to oxytetracycline for EFB disease using an *in vitro* larval infection model. However, their utilities *in vitro* were investigated using a single *M. plutonius* isolate (2019BC1) and concentrations of antibiotics used were based on a previous study of American Foulbrood infection (Peng et al., 1996) and not reflect field-relevant doses. Moreover, information related to the AMR profiles of *M. plutonius* clinical isolates is limited and 2019BC1 was the first reported oxytetracycline-resistant strain. Thus, we sought to complete a robust survey of *M. plutonius* AMR phenotypes and genotypes using samples obtained from EFB disease outbreaks across North America. This in turn, guided a revised series of larval infection studies to better evaluate and report on antibiotic protection against *M. plutonius* with varied levels of resistance to oxytetracycline.

# CHAPTER 3. ANTIMICROBIAL RESISTANCE AND GENOMIC CHARACTERIZATION OF NORTH AMERICAN *MELISSOCOCCUS PLUTONIUS* ISOLATES

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**Author Contributions**

**Conception and design of the study:** FM, ES, AR, SW; **Methodology**: FM, JT, TS, MS, MJ, ES, AR, SW; **Field work:** FM, JT, MS, MJ; **Acquisition of data:** FM, JT, TS, MS, MJ; **Formal analysis**: FM, JT, TS, MS, MJ, ES, AR, SW; **Funding acquisition:** ES, AR, SW; **Writing – original draft**: FM, AR, SW; **Writing; review and editing:** FM, ES, AR, SW.

## 3.1 Abstract

*Melissococcus plutonius* is a major bacterial pathogen of honey bee larvae, acting as the causative agent of European foulbrood (EFB) disease. Western Canada has seen a recent emergence of EFB disease despite oxytetracycline (OTC) use, resulting in economic losses for beekeepers. In North America, antimicrobials are used to control disease outbreaks at apiaries. The number of antimicrobials approved for use in honey bees is limited, making antimicrobial resistance a significant concern. Moreover, little is known about the resistance profiles of *M. plutonius* and how these are related to the efficacy and ability of drugs to clear infection in honey bee larvae. Thus, our study aims to determine if antibiotic resistance to OTC exists in North American *Melissococcus plutonius* isolates. Genomic characterization of Canadian isolates is lacking as well, and thus we also aimed to characterize a subset of isolates we did antimicrobial susceptibility testing for. We found that a majority of isolates had a minimum inhibitory concentration that indicated resistance to OTC (> 16 μg/mL), while all isolates had MICs that corresponded to sensitivity against tylosin and lincomycin (< 4 μg/mL). Through genomic characterization, we did not identify a resistance gene to explain the resistance seen for OTC. Additionally, genetic relatedness of isolates did not explain resistance either. Moreover, the utility of OTC, lincomycin (LMC), and tylosin (TYL) were compared through an experimental design that mimicked antimicrobial intervention of honey bee larvae infected with one of four north American *M. plutonius* isolates. We demonstrate that infections and the efficacy of antimicrobial treatments in honey bee larva are concordant with *in vitro* minimum inhibitory concentration measuremed in KSBHI media, and that the survival outcomes of larva are not dependent on *M. plutonius* clearance but a strain-dependent reduction of their numbers within larva.

## 3.2 Introduction

Reduction of antimicrobial-use in agriculture remains a priority to address the global threat of bacterial antimicrobial resistance (AMR)[131]; accordingly, a moratorium exists on antimicrobial application to honey bee colonies in the European Union[104]. Nevertheless, for the past 70 years and continuing today[55], beekeepers in North America rely on indiscriminate colony application of oxytetracycline (OTC) to control the two most common bacterial brood diseases of honey bees: American foulbrood (AFB), caused by larval/pupal sepsis due to *Paenibacillus*

*larvae* infection[10], and European foulbrood (EFB), caused by overgrowth of *Melissococcus plutonius* within the midgut of honey bee larvae[15].

In clinical EFB disease, uncontrolled expansion of an *M. plutonius* population in the larval midgut is thought to deprive the animal from effectively feeding, which ultimately results in death by starvation.[41] Honey bee larvae are exposed to *M. plutonius* through their diet, and microbial community profiling of larvae has revealed variable and significant levels of the pathogen that is related to their food.[132] Moreover, sampling and microbial community profiling of larva collected from apiaries without a history of EFB disease in Arizona demonstrated the presence of this pathogen during the apparently healthy development of animals throughout their first 4-5 days of life.[133]

The existential threat of antimicrobial resistance (AMR) as well as an increasing number of anecdotal reports of recalcitrant cases of EFB disease in western Canada[101] are growing concerns that are potentially linked.

We recently reported that a clinical isolated of *M. plutonius* obtained from an outbreak in British Columbia (BC), Canada, could resist OTC in vitro at 32 µg/mL; however, the publicly available genome sequences of 17 strains were devoid of a known tetracycline resistance gene.[39] Indeed, antimicrobial use has selected for resistant strains of *P. larvae*, including in western Canada, through genetic exchange, mediated largely by plasmid-encoded resistance genes[59,84,85]. Surveys, monitoring and reporting of specific pathogens and AMR is essential to guide antimicrobial use. In the case of EFB disease, there has yet to be a systematic investigation of *M. plutonius* AMR phenotypes. Moreover, there are no standards or established protocols to report potential indicators such as minimum inhibitory concentrations (MICs) across genotypes, which differ in their growth requirements in a laboratory setting.[31] To initiate these studies, we set out to survey the AMR profiles and whole genome sequences of *M. plutonius* that belong to clonal complex 12 (CC12) throughout North America where antimicrobial use is prevalent. The study of bacterial monocultures with known AMR profiles were then compared to antibiotic performance in an animal infection and treatment model. We report that the choice of bacterial cultivation medium can significantly impact the measurement of the MIC of OTC for 67 *M. plutonius* CC12 isolates obtained from 4 Canadian provinces and 4 United States. Nevertheless, the use of laboratory-reared larvae showed a positive correlation between resistance phenotypes measured in brain heart infusion media supplemented with soluble starch and antibiotic doses

resulting in animal survival. The latter, we demonstrate is the result of a reduction in the viable *M. plutonius* population rather than clearance of this enteric pathogen.

### 3.3 Materials and Methods

#### 3.3.1 Isolation of *M. plutonius*.

A total of 66 clinical isolates of *M. plutonius* were obtained from swabs collected by beekeepers with suspected outbreaks of European Foulbrood (EFB) disease in Canada (BC, AB, SK, QU) and the United States (OR, TX, MI, UT). Specifically, beekeepers were instructed to use sterile swabs to macerate larvae displaying clinical symptoms of EFB, which were stored at -20°C until submission to the lab. *M. plutonius* was isolated from individual swabs by adding 2 mL of phosphate buffered saline (PBS), pH 7.4, directly to the swab collection tube, vortexing for 1 min, and then allowing the swab to soak for a 15 min incubation at room temperature before preparing a serial dilution series ($10^{-1}$ to $10^{-4}$) for plating. Next, 100 μL volumes were plated on brain heart infusion agar supplemented with 0.15 M $KH_2PO_4$ and 1% soluble starch (KSBHI) and incubated for 72 h in microaerophilic conditions at 37 °C. Bacterial colonies with common *M. plutonius* morphological traits[31] were selected for isolation. Glycerol stocks (20% v/v) of monoculture were prepared and stored at -80 °C.

#### 3.3.2 Minimum Inhibitory Concentration Measurements.

The minimum inhibitory concentrations (MICs) of OTC (TCI, 00475), TYL (Thermo Scientific, AC463070050), and LMC (Thermo Scientific, AAJ6125106) were determined using a 96-well format broth microdilution method as previously described. Antibiotics were prepared as 2-fold serial dilutions in 200 μL: 128 μg/mL – 2 μg/mL OTC, 16 μg/mL – 0.25 μg/mL TYL and LMC. To measure the MICs, a culture of *M. plutonius* was subcultured at an optical density (OD) of 0.0005 in fresh KSBHI with and without antibiotic. Growth was evaluated after a 72-h incubation in microaerophilic conditions at 37°C, shaking. The MIC was defined as the lowest concentration of antibiotic in which no *M. plutonius* growth was visible. Experiments were performed in biological and technical triplicates. The MIC of *M. plutonius* ATCC 35311 was determined by subculturing it at an OD of 0.0005 in fresh basal media (0.1 M $KH_2PO_4$, 1% yeast extract, 1% glucose, 1% soluble starch, 0.025% L-cysteine, pH 6.7), with and without antibiotics. Growth was evaluated after a 96-h incubation in microaerophilic conditions at 35°C, shaking. Additionally, 4 clinical *M. plutonius* isolates were chosen, 2019BC1, 2021BC16, 2021AB55, and 2021SK40, to determine the MIC in basal media at 37°C for 72 h. Overall, a

total of 66 North American isolates were used to evaluate MICs in KSBHI, while 5 isolates (4 of which overlap with the KSBHI isolates were used to evaluate MICs in basal media. The aforementioned 4 isolates were also used to evaluate MICs in SYPG and EFB media. Moreover, MICs for these 4 isolates were also determined using solid KSBHI, BM, SYPG, or EFB agar. In total, MICs were determined for 67 isolates.

### 3.3.3 Minimum Bactericidal Concentration Measurements.

The minimum bactericidal concentrations (MBCs) of OTC, TYL, and LMC were determined by inoculating fresh KSBHI media containing no antibiotic with wells from 96-well microtiter plates used for MIC determination. Specifically, a sterile loop was used to inoculate 2 mL of KSBHI from wells that had no visible growth of *M. plutonius*. Growth was evaluated after a 72-h incubation in microaerophilic conditions at 37°C. The MBC is defined as the lowest concentration of antibiotic in which no growth was observed in KSBHI, indicating no bacteria survived the treatment.

### 3.3.4 DNA Extraction, Sequencing, and Assembly.

DNA was extracted from 53 *M. plutonius* isolates using the MasterPure Complete DNA and RNA Purification Kit. Draft-level genomes were obtained from Illumina NovaSeq PE 150 or MiSeq PE 250 (Genome Quebec) or Oxford Nanopore (Prairie Diagnostic Services Inc.) sequencing platforms and a closed genome was obtained using PacBio SMRT (University of Maryland Institute for Genome Sciences). The de novo assembly of draft-level genomes from nucleotide sequences obtained using the Illumina platforms was performed using Bowtie, whereas the Oxford Nanopore assemblies were performed using Dragonflye.

### 3.3.5 Bioinformatic screen for Antimicrobial Resistance Genes.

The 53 *M. plutonius* genomes and 17 additional publicly available sequences (PRJDB6526, PRJNA215018, PRJDA61383, PRJNA632212, PRJNA263538, PRJNA263539, PRJNA263544, PRJNA263546, PRJNA263547, PRJNA263541, PRJNA263540, PRJNA263542, PRJNA263545, PRJNA263543, PRJNA263537) were interrogated for the presence of antimicrobial resistance genes (ARGs) using Resistance Gene Identifier (RGI) tool provided by the Comprehensive Antibiotic Resistance Database (CARD)[117].

### 3.3.6 Phylogenetic analysis.

Prior to analysis, genomes were annotated using Prokka[134] and then subjected to the bacterial pangenome analysis pipeline Roary[135]. The annotated protein sequences were clustered

by an all-against-all BLASTP using a sequence identity threshold of ≥ 95%. The resulting newick format phylogenetic tree was visualized using Itol[136].

### 3.3.7 Gene Ontology

Core, soft-core, shell, and cloud genes were analyzed separately using ShinyGO[137] to identify enriched gene ontology (GO) terms and pathways.

### 3.3.8 Larval rearing, infection and antibiotic treatments.

Honey bee larvae were sourced from experimental field colonies in Saskatoon, Saskatchewan, Canada between June and August 2022. *M. plutonius* infections, antibiotic treatments and monitoring of in vitro reared larvae was performed as previously described.[39,97] Treatment groups were defined by dividing a 48-well plate into 4 four groups of 12 larvae each. Each plate included a negative control group (grafting control; GC, n=12 animals) which included larvae that were uninfected and fed a control diet (not containing any antibiotic). Additionally, each plate had a positive control group (infection control; IC, n=12) which included infected larvae that received a control diet. For each treatment, there were two technical replicates (n=24) for the GC and IC, and four technical replicates (n=48) for the treatment group. Antibiotic treatment groups included OTC, TYL and LMC at concentrations that correspond to the MIC of each individual *M. plutonius* isolate, as well as concentrations 4-fold higher and 4-fold lower than the MIC. Larvae were infected with *M. plutonius* on the day of grafting and treated with an antibiotic from days 2 to 6 after grafting. A chi-square test was performed to measure two-way associations between antibiotic-treated groups and untreated, infected controls (IC groups) using Stata 17.

At the end of rearing experiments, three surviving larvae per treatment group were chosen at random to determine viable *M. plutonius* counts. The larvae were subject to surface sterilization using 70% ethanol before maceration and plating a 10 µL aliquot of liquid larval contents in triplicate on KSBHI agar. Counts were obtained after 72 h of growth under anoxic conditions. In parallel, and the remaining set of surviving larvae were all selected for weight measurements, which were obtained using an analytical balance. Prior to weighing, larvae were rinsed with water and patted dry with a Kimwipe. In groups where only few larvae were present on day 6, all larvae were weighed before selecting larvae for CFU counts. An unequal variance t-test with a confidence interval of 95% was used to compare groups to the IC.

### 3.3.9 Checkerboard assay with STIP3-29.

A checkerboard assay was performed using a 96-well format broth microdilution method and varying the concentration of OTC (128 µg/ml – 2 µg/mL) and STIP3-29 (100 µg/mL – 1.56 µg/mL), an antimicrobial peptide STIP3-29 known to permeabilize the membrane of Gram-positive bacteria, in KSBHI.[138] Growth was evaluated after a 72-h incubation in microaerophilic conditions at 37°C, shaking.

## 3.4 Results and Discussion

### 3.4.1 The majority of North American *M. plutonius* isolates are resistant to OTC *in vitro*.

To establish baseline levels of AMR phenotypes of North American (NA) *M. plutonius* isolates to OTC, TYL and LMC, we determined minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) using a two-step microbroth dilution assay in 96-well plates. First, MICs were determined as the lowest concentration of antibiotic that did not reveal macroscopic levels of growth (Figure 3.1A). Next, the contents of wells in which no growth was observed were passaged into antibiotic-free media to determine the MBC (Figure 3.1B). A total of 67 *M. plutonius* isolates were evaluated. All were sensitive to TYL (MICs ≤ 4 µg/mL) and LMC (MICs ≤ 1µg/mL); however, resistance to OTC was prevalent in our collection of North American. An MIC for OTC ≥16 µg/mL was used to define resistance, and while Clinical and Laboratory Standards (CLSI) guidelines for *M. plutonius* suggest a sensitivity breakpoint of ≤2.5 µg/mL for OTC, we used ≤4 µg/mL to define the phenotype sensitivity (Figure 3.1C).[118] Currently, there are no CLSI guidelines to define OTC intermediate or resistant *M. plutonius* strains, thus guidelines from another honey bee pathogen, *P. larvae* has been adapted. The MBCs typically ranged from 0.25 µg/mL to 16 µg/mL for TYL and LMC whereas the for OTC was from 2 µg/mL to 128 µg/mL, reflecting the broad range of MICs measured for the latter. Overall, our collection accounts for a remarkable frequency of OTC resistance (45/67 isolates; Figure 3.1C).

The high prevalence of OTC-resistant ($OTC^R$) *M. plutonius* is a concern. It is also a deviation from previous reports of sensitive isolates from the United Kingdom and Australia, which were performed ~20 years ago.[86,87] These studies measured MICs using different media and on solid agar surfaces, reporting MICs between 1 and 4 µg/mL for OTC across 184 *M. plutonius* isolates. While we previously demonstrated that the MICs of antibiotics for *M. plutonius* on KSBHI agar and in a 96-well KSBHI broth microdilution format were comparable,

varying by just 2-fold[39], the impact of growth media on MICs has not been explicitly measured. Accordingly, four *M. plutonius* isolates were chosen to evaluate the effect of two commonly employed media types for bacteria cultivation (KSBHI and basal media, BM; Table 3.1). While three of the four isolates were OTC-resistant in KSBHI, all four were determined to be sensitive to OTC in BM. Notably, *M. plutonius* ATCC 35311 cannot be propagated in KSBHI, and belongs to a non-pathogenic clonal complex (CC13).[139] The media-dependence of the MIC data provides the most plausible explanation for the deviation between our measurements and those from previous reports. Specifically, the media types employed in previous *M. plutonius* antimicrobial susceptibility were more similar to BM than KSBHI[86,87].

The observed phenotypic variance that results from cultivation media is likely attributed to differences in gene expression and the physiological states of bacteria in distinct environments. The influence of media on the measurements of MICs is a well-established phenomenon; for example, differences of orders of magnitude in the MIC for azithromycin were measured against *Pseudomonas aerugionsa* in various media.[140] Moreover, so-called standard media types often fail to meet diagnostic thresholds as recently exemplified for the poultry pathogen *Avibacterium gallinarum*, which required optimization in order to characterize a collection of clinical isolates[141]. Further studies are clearly required to explain the media-dependence of *M. plutonius* AMR phenotype*s in vitro,* as well as optimize conditions for antimicrobial susceptibility testing across pathogenic and non-pathogenic isolates.

**Table 3.1.** Media-dependence observed for the MICs of OTC

| | MIC (mg/mL) | |
|---|---|---|
| **Isolate** | **KSBHI** | **BM** |
| 2019BC1 | 32 | <2 |
| 2021BC16 | 4 | <2 |
| 2021AB55 | 32 | 4 |
| 2021SK40 | 64 | 4 |

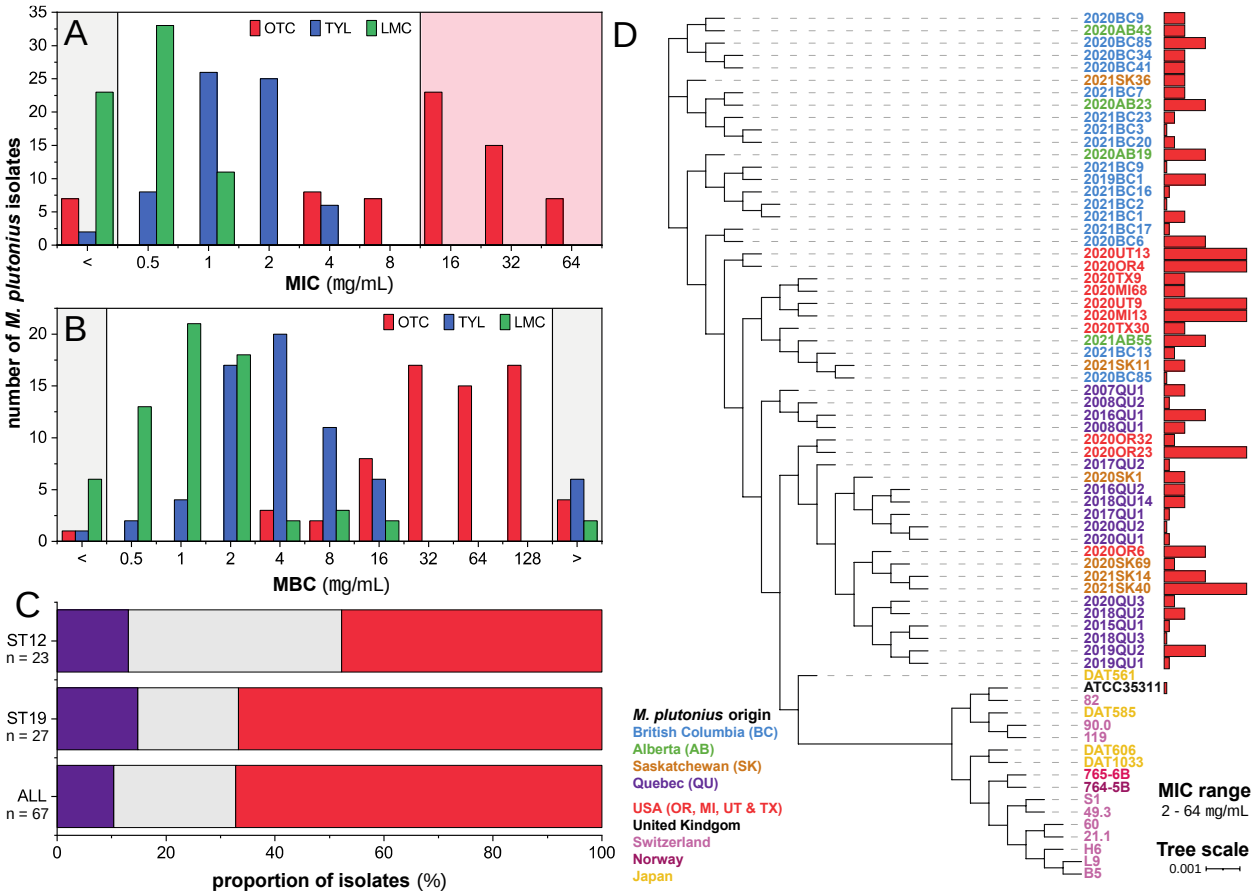


**Figure 3.1 (A)** Histogram showing the distribution of MICs for three antibiotics (OTC, TYL and LMC) evaluated against a panel of 67 *M. plutonius* isolates. The shaded grey area indicates the MIC was below the lowest concentration of antibiotic tested. The red shading highlights OTC-resistant organisms. **(B)** Histogram showing the distribution of MBCs for the same collection of *M. plutonius* with grey shaded areas indicating values that were not measured based on the concentration range of each antibiotic that was assayed. **(C)** Bar graphs showing the proportion of isolates that were deemed to be resistant to OTC based on MIC values determined in KSBHI media. The full collection of 67 isolates is compared to the two most abundance STs that were studied. **(D)** A phylogenetic tree showing the relatedness of 70 *M. plutonius* isolates with the OTC resistance phenotypes of 53 from Canada and the United States shown at the right. Bacteria are color-coded by geographical origin.

### 3.4.2 OTC resistance is not conferred by a single well-established ARG.

In light of the frequency of OTC resistance among *M. plutonius* isolates, we sequenced the genomes of 53 members in our North American collection (Table S1) and searched for ARGs within a collection of 70 organisms that also included 17 previously sequenced strains from outside of North America.[39,43] Importantly, antimicrobial use is permitted in North America and the emergence of OTC-resistance in Saskatchewan has recently been linked to a plasmid-encoded *tet(L)* gene in *P. larvae*[142], which mirror global descriptions of OTC-resistant *P. larvae* and *Bacillus cereus* found in honey.[59,84,85,92] These results indicate a potential reservoir of

tetracycline genes within honey bee colonies, and the continued use of OTC for both EFB and AFB also puts *M. plutonius* at risk for developing OTC resistance.[55] Nevertheless, we could not detect any known tetracycline resistance genes in *M. plutonius* based on sequence similarity to entries in CARD.[117] A query of 3156 genes from 70 genomes did not produce a significant hit to any of the 116 known tetracycline resistance genes (>80% identity; >80% sequence coverage), and no ARGs known to confer resistance to TYL or LMC were detected. In fact, *M. plutonius* genomes were generally devoid of antimicrobial resistance genes (ARGs) with only a single weak hit to an established ARG -*qacG*- based on 45-47% sequence similarity and >97% coverage. QacG is an efflux pump that confers resistance to quaternary ammonium-containing compounds.[143] While the activity of QacG was not assessed, differential expression of this gene in distinct media types as well as across isolates in a single media type is possible; however, our results also suggest that *qacG* expression is not controlled by OTC, which is atypical for a dedicated resistance determinant.

An alternative hypothesis is that distinct *M. plutonius* isolates may be intrinsically resistant to OTC due to membrane permeability. To evaluate this hypothesis, we performed a checkerboard assay using STIP3-29, a membrane-permeabilizing antimicrobial peptide[138], to measure the impact of membrane integrity on OTC activity. Remarkably, while STIP3-29 did not inhibit *M. plutonius* 2019BC1 growth at 100 µg/mL, the presence of the peptide reduced the MIC of OTC 8-fold from 32 µg/mL to 2 µg/mL.

Together with the lack of known OTC resistance genes in phenotypically resistant *M. plutonius* isolates, the potentiating impact of a membrane-permeabilizing agent suggests a multifactorial mechanism of resistance that further studies are required to uncover more precisely how *M. plutonius* isolates resist the action of OTC. This is particularly concerning since OTC is the only approved antibiotic to treat EFB in North America[55], and yet a significant number of the target pathogens, two thirds in our collection, appear to have some level of intrinsic resistance.

### 3.4.3 OTC resistance is not strongly correlated to sequence type.

The lack of a specific ARG for OTC suggests that differences in gene expression or general physiological differences between isolates might better explain the phenotypes. Moreover, the relatively broad distribution of MIC values and moderate resistance phenotypes (no MIC ≥128 mg/mL) and the impact of membrane permeability suggest a multifactorial explanation for OTC resistance. Thus, core- and pan-genome analysis was performed in attempt

to identify a possible relationship between AMR phenotype and the relatedness of *M. plutonius* clinical isolates (Figure 3.1C, D). A total of 70 genomes of varying quality (7 closed, 63 draft; Table S2) and that belong to three *M. plutonius* clonal complexes (CC3, CC12, and CC13) resulted in the identification of 3156 unique genes, including 1384 core genes (universally conserved), 108 genes in the soft core (in 95-99% of genomes), 801 shell genes (in 15-95% of genomes) and 863 cloud genes, which occurred in a single genome (Figure 3.2A, B). The pangenome of the 54 CC12 isolates included 2468 genes, consisting of 1563 core, 41soft core, 389 shell, and 475 cloud genes. Thus, despite representing ~75% of the genomes in the analysis, the CC12 shell and cloud genes accounted for only ~50% of the least conserved genes. This perhaps represents the geographical distribution of the strains: all but one CC12 isolates are from North America (DAT561 is from Japan), whereas the publicly available genomes are CC3 and CC13 and originate from organisms found in Europe and Japan. More specifically, all of the isolates in our North American collection belonged to 5 sequence types (ST) that have been historically used to characterize *M. plutonius*, including ST12, 19, 21, 27, and 36[33]. It should be noted that this analysis, and our sampling strategy in North America is biased for the isolation of CC12, which appears to be endemic. CC12 isolates are a less fastidious group of organisms compared to CC3 and CC13.[31] A direct comparison of *M. plutonius* CC12 isolate relatedness and AMR phenotype were not clearly correlated (Figure 3.1C). This is exemplified by three closely-related isolates from Saskatchewan, 2020SK69, 2021SK11, and 2021SK40, which have MICs of 8, 16, and 64 µg/mL OTC. Similarly, two isolates from Oregon, 2020OR32 and 2020OR23, are most closely related to each other than to any other strains in the collection, yet their MICs of OTC are 8 and 64 µg/mL, respectively. In contrast, there did appear to be a correlation between country of origin and AMR phenotype in that isolates from the United States had higher MICs than those from Canada (Figure S1B). While a wide distribution of MICs was observed in 42 Canadian isolates, all 10 American submissions showed an intermediate or resistant OTC phenotype. Future expanded analysis of isolated from the United States is needed to further evaluate the correlation between OTC AMR and country of *M. plutonius* origin.

In addition to defining the core and accessory genomes of *M. plutonius* GO analyses were performed and, again, the results failed to generate more specific hypotheses on the discordance between geno- and phenotype. There was no particular enrichment of genes involved in cellular membrane composition or function, for example, in the accessory genome. Instead, the GO

analysis served to clearly illustrate the lack of annotation in the shell and cloud genes (Figure 3.2C). Thus, while there may appear to be a lack of evidence for partially conserved accessory genes in resistant versus sensitive *M. plutonius*, this annotation gap precludes any definitive assessments. Accordingly, targeted biochemical and biophysical studies to better define OTC interactions at the *M. plutonius* membrane represent a clearer path to relevant annotation of either core or accessory genes involved in resistance.

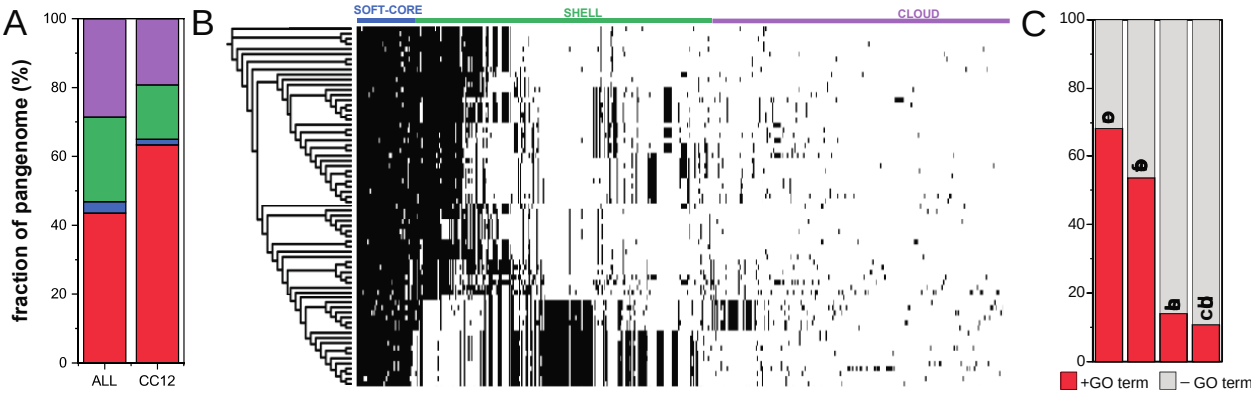


**Figure 3.2 (A)** Bar graphs showing the number of unique genes in the pangenome of 70 *M. plutonius* isolates or the 53 from North America that belong to CC12. **(B)** A plot of gene presence and absence in the 70 isolate *M. plutonius* pangenome. Core genes have been omitted to simplify the visualization. **(C)** Summary of the fractions of the core, soft-core, shell and cloud genes in the 70 isolate *M. plutonius* pangenome that were identified and associated with a specific GO term (red) or that were classified as hypothetical proteins (grey).

Finally, while they were not implicated in AMR in this study, the fact that plasmids are prevalent in *M. plutonius* presents a general concern. Plasmid-mediated antibiotic resistance and, more specifically, the emergence of resistance via plasmids is common throughout the biosphere[144] Moreover, a plasmid was recently described to confer tetracycline resistance to *P. larvae* in Saskatchewan.[142] An ~178 kb plasmid named pMP1 was universally conserved in our collection of *M. plutonius* whereas 30/53 (57%) carried the ~19.5 kb pM19, which encodes for *mtxA* (a virulence factor with activity that appears to be dependent on the genetic background of the carrying strain[42]), and only 8/53 carried a 42.7 kbp plasmid pMP43, which encodes for phage proteins. The consistent presence of pMP19 in our North American collection of bacteria varies from other collections, which reported a lower frequency of the plasmid in just 5/17 strains belonging to CC3, CC12, and CC13 from Europe and Japan.[43,127].

While the distribution of plasmids between sequenced strains varies, the nucleotide sequences of the plasmids are highly conserved with no evidence of ARGs or that they act as significant hotspots for horizontal gene transfer (HGT). Nevertheless, the current number of

sequenced clinical isolates is too low of a sample size to assess the risk of the acquisition of ARGs in plasmids or chromosomal sites via HGT.

### 3.4.4 ASTs in KSBHI predict in vitro efficacy of antibiotics in honey bee larvae.

The use of an animal model is essential to correlating observations of varied AMR phenotypes and the media-dependence thereof to the development of accurate clinical diagnostic tests. Thus, we evaluated the relationship between *in vitro* MICs and the efficacy of antibiotics in a larval infection model. Four *M. plutonius* isolates were investigated: the previously characterized *M. plutonius* 2019BC1[38,39] (MIC OTC: 32 µg/mL) as well as *M. plutonius* 2021BC16 (MIC OTC: 4 µg/mL), 2021AB55 (MIC OTC: 32 µg/mL) and 2021SK40 (MIC OTC: 64 µg/mL). These isolates were selected based on variability in the MICs measured for OTC in kSBHI. The effects of TYL and LMC were also evaluated in the model since (i) they are currently approved for and used to treat AFB in Canada and (ii) the tested isolates were deemed to be sensitive to these antibiotics (MICs TYL: 1-2 µg/mL and LMC: ≤0.5 µg/mL). Survival of larvae infected with *M. plutonius* to a series of antibiotic treatments was measured across a concentration range that was aligned with strain-specific antibiotic MICs (Figure 3.3). In all cases, significant protection was observed when infected larvae were treated with antibiotics at a concentration 4-fold higher than the MIC determined by the microdilution broth method in KSBHI. Dose-dependent survival was evident and generally concordant with the MIC values. Exceptions included the treatments of *M. plutonius* 2019SK40 with TYL and LMC, for which survival did not significantly improve until 4-fold MICs were used and still protection did not confer a level of survival that matched the uninfected control groups. The vast majority (87 ± 8%) of uninfected grafted control (GC) larvae (n = 74 groups of 12 animals; 888 larvae) survived and grew during the 6-day assay period. The survival of the GC group was consistent across all experimental trials. In contrast, only 22 ± 14% of larva in the infected groups survived (n = 74 groups of 12 animals, 888 larvae). Overall, the results suggest that MIC values determined in KSBHI broth can predict the in-animal efficacy of the three antibiotics for *M. plutonius* that belong to CC12.

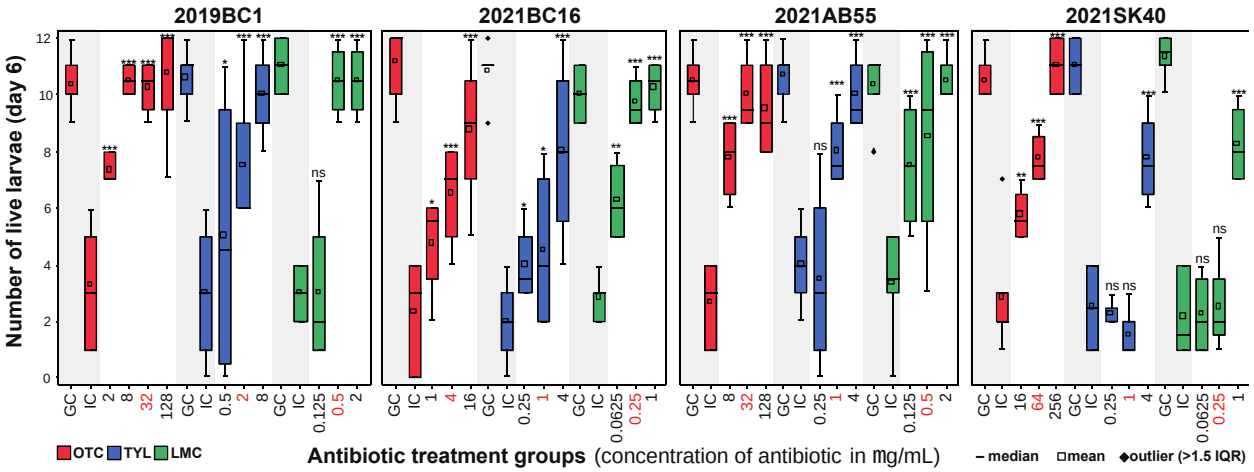


**Figure 3.3** Boxplots showing honey bee larval survival after 6 days of *M. plutonius* infection with or without antibiotic treatment. The antibiotic treatment groups are displayed next to untreated grafting and infection controls (GC and IC; shaded grey) and the MIC values obtained from *in vitro* monocultures are colored red. Statistical analysis of the survival was performed using a chi-square two-way association test comparing IC with treatment groups. Significant differences are denoted by asterisks (* $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$).

The standard amount of OTC used to treat honey bee colonies in North America is 200 mg mixed in icing sugar and applied to colonies every 4 to 5 days for a total of 3 treatments.[55] Experimental estimates of OTC accumulation in larvae of colonies treated with a similar amount of antibiotic (300 mg) were ~10 mg/g and declined to <1 µg/g in 6 days.[124] In our experimental model, OTC is administered daily in the larval diet after infection. For example, larvae that survive treatment weigh ~140 mg (see below) and would have received the equivalent of ~12 mg/g dose when 32 µg/mL was included in the diet on day 6. While daily administration does not replicate field use of OTC (and other antibiotics), it is noteworthy that MICs and sub-MICs typically provided partial protection to individual animals. In the field, even lower concentrations may be effective for EFB treatment due to colony innate and social immunity, including antimicrobial peptides, grooming, and hygienic behaviour[100], though the relationship between survival at a colony level during EFB challenge and antibiotic administration has not been empirically determined.

### 3.4.5 Life-saving antibiotic treatment does not eradicate *M. plutonius in vitro.*

To complement our investigation of the effects of antibiotic treatment on animal survival, we weighed surviving larvae and counted *M. plutonius* that could be recovered across control and treatment groups. Animals were selected at random and either weighed or used for *M. plutonius* counting experiments; however, in cases when only a few larvae survived within a group, the same individuals were both weighed and used to determine *M. plutonius* counts. The

recovery of viable *M. plutonius* on KSBHI were correlated to survival for all four isolates in that survival increased as colony forming units (CFU/mg) decreased (Fig 4A). Interestingly, while treatments at 4-fold MICs of each antibiotic reduced *M. plutonius* population sizes by 3-4 orders of magnitude, substantial counts were recovered regardless of treatment or pathogen identity. Thus, survival of larva in our *in vitro* EFB model was not commensurate with the eradication of *M. plutonius*.

Larval survival and weights were positively correlated. The majority of antibiotic-treated larvae were phenotypically similar to grafting controls on day 6 (Figure 3.4B). Overall, treated larvae had similar weights to the GC group (Treated: 0.12 g ± 0.04 g, GC: 0.14 g ± 0.02 g; Table S3). In light of the observed presence of *M. plutonius* in infected and treated samples, these results suggest that below a certain threshold, larvae are able to tolerate the presence of pathogen. This is in line with previous reports that survival of an infected larva is dependent on both its food supply and bacterial load,[18] as well as cultivation-independent community profiling of larva from an apiary without a history of EFB that showed the presence of *M. plutonius* in animals from the zero to fourth instar.[133]

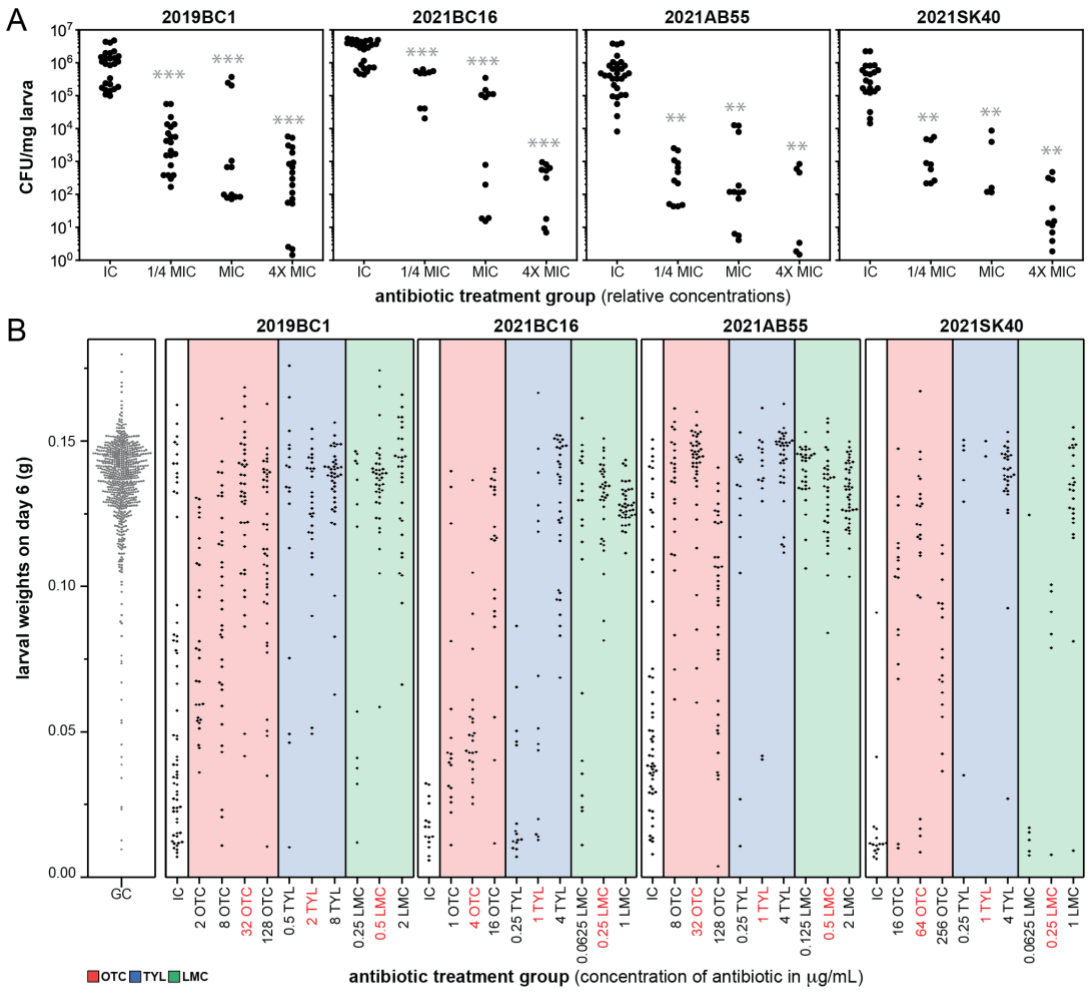


**Figure 3.4 (A)** Beeswarm plot showing the recovery of viable *M. plutonius* from larva surviving infection or treatment on day 6. Treatments are grouped based on MIC values, independent of the identity of the antibiotic. Groups were compared to the IC using a unequal variance t-test with a 95% confidence interval. Significant differences are denoted by asterisks (** $p < 0.001$, *** $p < 0.0001$) **(B)** Beeswarm plots showing the measured weights of uninfected (GC), infected (IC), and antibiotic-treated larvae that survived until day 6.

## 3.5 Conclusion

In summary, we have identified that OTC-resistance is widespread across NA *M. plutonius* isolates. Moreover, all NA isolates remain sensitive to TYL and LMC. Thus, the use of TYL and LMC may be appropriate in the treatment of EFB disease. We provide evidence that all three antibiotics are able to treat this disease in a larval model and that MIC determination of these antibiotics predict the efficacy of these antimicrobials as treatments for CC12 *M. plutonius* isolates. Treatment does not lead to the complete eradication of *M. plutonius* from the larvae, however, it does decrease bacterial loads to tolerable levels allowing the larva to survive

infection and develop to a normal weight. Finally, we demonstrate the lack of any traditional tetracycline and oxytetracycline resistance genes. Resistance in NA isolates is also not explained by strain relatedness. Thus, further research is required to determine how *M. plutonius* isolates are able to resist high OTC levels without the acquisition of resistance genes.

## CHAPTER 4: GENERAL DISCUSSION

### 4.1. Most North American *M. plutonius* isolates are phenotypically resistant to OTC.

OTC is used across North America for the treatment of both AFB and EFB disease. Although TYL and LMC are also approved for the treatment of AFB disease, their use is not as widespread as OTC[55]. Accordingly, OTC resistance is a major concern for the honey industry. This concern is now an existential threat to honey bee health and the economic viability of honey production as OTC resistant *P. larvae*[85] and *M. plutonius* have now been identified. While specific ARGs are responsible for OTC resistance in *P. larvae*, the mechanism by which *M. plutonius* resists the antibiotic is not encoded by a single gene. Instead, *M. plutonius* membrane permeability, which is influenced by its environment (*e.g.* laboratory media type or growth in the larval guy), appears to confer a variable level of intrinsic resistance that is strongly correlated to genotype.

It should be noted that until the first chapter of thesis was published, there were no recent attempts to characterize the antimicrobial susceptibility of *M. plutonius* to OTC and no resistant strains had been reported. In fact, studies dating back ~20 years from the United Kingdom[87] and Australia[86] failed to observe resistance. These studies used a solid agar assay to determine the MIC and yielded results indicating sensitivity to OTC (MICs <4 μg/mL). Moreover, the AMR phenotypes of North American *M. plutonius* isolates to OTC, TYL, and LMC had not been defined. Thus, we aimed to establish these values by using microbroth dilution assays in 96 well plates followed by antibiotic free outgrowth to determine MICs and MBCs for a total of 67 North American isolates. All of the isolates were sensitive to TYL and LMC with MICs ≤4 μg/mL; however, 45 out 67 isolates had OTC MICs ≥16 μg/mL. We defined these strains as being resistant to OTC based on CLSI values that have been determined for *P. larvae*.[118] In contrast to many pathogens of human and agricultural prominence, there has been minimal standardization for defining clinical breakpoints associated with *M. plutonius* sensitivity and resistance to antibiotics. Additionally, there is a lack of clinical data (how successful OTC treatments for EFB are), and thus it is difficult to compare how the distribution of MICs measured correlate to clinical treatments. Future studies are required in order to determine the OTC MIC distribution of *M. plutonius* isolates. While this thesis includes measurements from 67 isolates, typically when setting susceptibility breakpoints, at least 500 strains are used during testing[94]. Furthermore, these studies must correlate the MIC distribution with clinical data as well. While

there is a lack of data, it is possible to determine the possibility of a successful OTC treatment using lab-reared larvae. Our results speak directly to obstacles associated with such standardization, and we ultimately relied on the agreement of MICs observed in KSBHI and protection in an *in vitro* larval model of infection to support claims of resistance and susceptibility.

**4.2 OTC resistance phenotypes differ across medias.**

Initially, KSBHI media was used to determine the MIC of *M. plutonius* isolates, however, a type strain ATCC 35311, which belongs to different clonal complex than our North American collection, does not grow in KSBHI media. Specifically, *M. plutonius* ATCC 35311 belongs to CC3 (instead of CC12), which are typically more fastidious than CC12 and have different growth requirements.[31,139] Thus, in Chapter 3 we chose 4 clinical isolates from western Canada to test how MIC value determination is affected by the use of different media types. Interestingly, while three of the four isolates (2019BC1, 2021AB55, and 2021SK40) were resistant to OTC in KSBHI media, they appeared sensitive when grown in BM. Isolate 2021BC16 remained sensitive in both medias, however, the MIC was two-fold lower in BM. Ultimately, this inconsistency in MIC is likely due to a difference in membrane permeability. This was explicitly tested using a membrane-permeabilizing peptide named STIP3-29[138], which restored the activity of OTC. When STIP3-29 was used to treat *M. plutonius* 2019BC1, the MIC was reduced 8-fold to 2 μg/mL. The implication of the cell membrane is not unique to *M. plutonius*, and a common mechanism that accounts for intrinsic resistance to antibiotics, though it also typically media-independent.[145] Nevertheless, there are examples that demonstrate the impact of bacterial growth media on AMR. A previous study determined the MIC of azithromycin for *Pseudomona aeruginosa* in two medias[140]. In CA-MHB, the MIC was ≥128 μg/mL, while the same MIC determination carried out in RPMI 1640 media resulted in an MIC ≤16 μg/mL. This discrepancy was a result of the downregulation of *oprM* by azithromycin in RPMI 1640 medium, which increased the membrane permeability of the bacterium and ultimately decreased the MIC. Future studies are required to determine the molecular details behind the inconsistency in MICs across medias for *M. plutonius*.

The discrepancies between MIC values determined in media types showed that a large proportion of *M. plutonius* isolates have the potential to resist OTC treatment. In fact, the MIC determined in KSBHI may be more relevant than BM, for example, when determining resistance

or sensitivity of *M. plutonius* isolate in a more realistic environment: the gut of a larva. In Chapters 2 and 3, low OTC concentrations were used to treat larvae infected with 2019BC1. However, these treatments did not significantly protect larva from death by infection. Moreover, recent studies have demonstrated that *Pseudomonas aeruginosa* exhibits antibiotic resistance to azithromycin in media that is physiologically similar to human infection[146]. Demonstrating that the MIC in this media is relevant to the MIC that would be determined in a human. The pH of KSBHI is approximately 7.5-7.8, similar to the pH of a larval gut (7.5)[147]. Thus, the MIC results found in KSBHI, rather than in BM, are most likely more indicative of the OTC dose required during larval infection. Interestingly, low concentrations of OTC often did not perform as well as OTC concentrations corresponding to the MIC and above.

**4.3 OTC resistance does not appear to be associated with a well-established ARG.**

Due to the prevalence of OTC resistance apparent across North American *M. plutonius* noted in Chapter 3, we performed a bioinformatic search of 70 *M. plutonius* isolates for antimicrobial resistance genes (ARGs) using the CARD RGI tool[117]. None of the 70 isolates resulted in a significant hit to any of the known tetracycline resistance genes. This includes >100 genes. Tetracycline resistance is widespread due to its use in human and veterinary medicine[56]. Moreover, resistance to tetracyclines in *P. larvae* is conferred by *tetK* and *tetL,* both of which encode for a tetracycline efflux pump[59,85]. Generally, the *tetL* gene is plasmid-encoded. However, *tetK*, has also been demonstrated to give resistance to *P. larvae* isolates[84]. Currently these genes have not been found any *M. plutonius*, however, the clearly dynamic genomes, reflected in a large accessory genome (1772 non-core genes in CC12 alone) and the presence of plasmids (*e.g.* pMP1, pMP19, and pMP43) indicate a potential for the acquisition through horizontal gene transfer (HGT). Along these lines, honey within hives has been revealed to be a reservoir for tet resistance genes with *Bacillus cereus* strains carrying both *tetL* and *tetK*[92]. These results indicate the prevalence of ARGs within honey bee colonies present a future risk of *M. plutonius* becoming OTC resistant through the acquisition of a specific ARG.

Instead of a conventional resistance gene, the membrane permeability of *M. plutonius* is suspected to play a role in the phenotypic resistance. When adding a membrane permeabilizing peptide to the media (STIP3-29), the OTC MIC was lowered 8-fold in KSBHI. OTC is required to enter the bacteria in order to inhibit the ribosome[56]. For this reason, many double-membraned Gram-negative bacteria are able to resist antibiotics due to decreased permeability[148]. In fact, a

laboratory mutant of *E. coli* was thought to develop cross-resistance to beta lactams and aminoglycosides due to its outer membrane permeability[149]. Alterations to membrane permeability is a common resistance strategy even in Gram-positive bacteria. Vancomycin-intermediate resistant *Staphylococcus aureus* strains, for example, have been demonstrated to produce a thickened cell wall allowing antimicrobial resistance to occur[150]. Thus, decreased membrane permeability in *M. plutonius* may be the cause of resistance. Unfortunately, this intrinsic feature of *M. plutonius* does not appear to be easily predicted by sequence-typing and affects the utility of the only approved method for EFB disease treatment in North America[55]. Due to the apparent resistance seen across North American isolates, OTC may not be an adequate treatment for this disease in an actual colony. However, *in vitro* treatments have demonstrated its efficacy[39,96]. Future studies are required to determine how effective this antibiotic is in a colony model.

**4.4 Phylogenetic and pangenome analysis of *M. plutonius* isolates.**

To analyze the *M. plutonius* pangenome, 53 North American isolates were sequenced, and 17 publicly available were included from Europe and Asia. These genomes were used to assign STs, CCs, and determine the presence of plasmids, in particular the MtxA toxin-encoding pMP19. The major sequence type present in North America was ST19, however 12, 21, 27, and 36 were also present, with 2020SK1 being the first isolate to belong to ST36. Moreover, plasmid pMP1 was present in all isolates, while pMP19 was present only in 58% of isolates. Overall, all North American isolates belonged to clonal complex 12. Previous studies demonstrated that STs were spread globally[33], however CC3 and CC12 were most prevalent in North America[43,52]. ST12 was also identified to be common in North America, in line with the results of Chapter 3. However, the lack of CC diversity in North American isolates may be a result of isolation bias considering that Cc12 growth is less fastidious than CC3 and CC13, which also have different laboratory growth requirements.[31] Nevertheless, CC12 isolates were routinely recovered from samples submitted from apiaries with suspected EFB disease outbreaks.

Pangenome analysis was used to determine the relatedness of sequenced isolates. The results revealed that a core *M.* plutonius genome was made up of 1384 genes, while the pan genome was made up of 1772 genes. These values line up with previous studies that determined the core to be made up of 1304 core genes and 1846 pan genes[43]. The slight variation in numbers may be due to a difference in the manner in which the analysis that was performed, and in

particular how genes were called from the assemblies. In contrast, pan genomic studies of other pathogens, such as *Klebsiella pnuemoniae* revealed a core genome of 4406 genes, and a pan genome of 5809 genes.[151] In this case, the pan genome contained approximately 1400 more genes than the core, whereas there was only a 400 gene difference between the *M. plutonius* core and pan genome. The large pangenome of *K. pneumoniae* indicates genome plasticity[152]. Genome plasticity, which refers to a genome's ability adapt rapidly to changes in their environment, has been demonstrated to contribute to genetic variations. The presence in these distinct environments and alongside other bacteria is also a factor in genomic variation. Another example of a species with a larger accessory genome relative to the core genome is *Escherichia coli*.[153,154] It's core genome is comprised of only 3191 genes, while the pan genome is made up of almost 15000 genes. This variance between the core and accessory genome also lines up with the fact that *E. coli* can be present in various different environments that contain a number of other bacteria that allow for the uptake of new genes. In contrast, *M. plutonius* is typically only found in adult honey bees and larvae[15,33]. The adult honey bee gut microbiome is specialized and considered to be of low complexity[155]. Thus, *M. plutonius* has a limited number of bacteria that it typically interacts with, explaining the almost 50/50 split of the core and pan genome, which is low when compared to other pathogenic bacteria.

Phylogenetic analysis of the 70 isolates showed clustering that reflected the anticipated relatedness. The so-called typical isolates, belonging to CC3 and CC13[31] are more closely related to each other than to CC12. Superposition of this data in the form of a phylogenetic tree based on the 1384 core genes and the OTC MIC revealed little correlation between relatedness and AMR. Additional analysis of the core and pangenomes was carried out by grouping gene ontology (GO) terms. The core and soft-core genes resulted in a majority of genes involved in metabolic and biosynthetic processes. Previous studies demonstrated the abundance of enzymes involved in glycolysis, pentose phosphate pathway, and pyruvate metabolism[43]. The pan genome, however, was comprised of many hypothetical and unannotated genes as well as those typically encoding for phages. Eight isolates were shown to carry a plasmid encoding for phage proteins, pMP43, which was previously demonstrated to be present occasionally in the *M. plutonius* genome[43]. Again, these mobile genetic elements may be a threat to honey bee health and the treatment of EFB as they have been for AFB disease[84,85,119,144].

### 4.5 Approved and alternative methods in the treatment of EFB *in vitro*.

Chapters 2 and 3 include *in vitro* larval infection models to assess the efficacy of approved (OTC) and alternative (TYL and LMC) antibiotics as treatments for EFB disease. In Chapter 2, antibiotics were tested using concentrations that reflected previous experiments[96,103]. Moreover, two treatment approaches were used: co-administration with infection and antibiotic intervention 2 days post infection. Chapter 3 uses concentrations that mirror the MIC determinations for each isolate. Dose-dependent survival was evident and concordant with the MIC values measured. However, exceptions include 2021SK40 when treated with TYL and LMC, which were not able to significantly improve survival until the concentrations were increased to 4X the MIC. Overall, these larval models demonstrated that TYL and LMC are adequate alternatives for treatment of *M. plutonius* infection *in vitro*. TYL and LMC are approved for use in American foulbrood disease[55]. Both antibiotics share a similar mechanism of action where they inhibit protein synthesis by binding to the 23S subunit of the ribosome[63,70]. Due to this similar mechanism of action, bacteria are able to develop cross resistance to these antibiotics as well through macrolides, lincosamides, and streptogramin B ($MLS_B$) resistance[64,109]. To date, no TYL or LMC resistant *M. plutonius* isolates have been identified. However, TYL and LMC-resistant *P. larvae* have been identified[91]. Interestingly, *P. larvae* is able to maintain a plasmid encoding for TYL resistance under non-selective conditions *in vitro*[85]. Additionally, *ermC*, which encodes resistance for TYL, has been demonstrated to exist on mobile genetic elements in bacteria found in honey[85]. Thus, resistance is a possibility, however, their use can still be beneficial in treating for EFB and AFB especially due to current OTC resistance existence[55,59,85].

Chapters 2 and 3 also demonstrated that the use of OTC, despite infection with OTC resistant isolates, was able to treat infected larvae as well. Typically, concentrations correlating to the MIC and 4X the MIC were best at protecting larvae, however, concentrations corresponding to 4X below the MIC were also able to increase larval survival, though this varied by infectious agent. Overall, the results are in line with previous larval infection models demonstrated that concentrations ranging from 1-10 µg/mL reduced larval mortality, however a higher concentration of 20 µg/mL was required to raise the larval survival to that of the uninfected larvae[96].

Antibiotic dosing in these studies are similar to and exceed expected concentrations in field applications to colonies. In a honey bee colony, when treating with 300 mg OTC, approximately 13 µg/g and 9 µg/g OTC is present in honey bee larvae[124]. The dose received by larvae in Chapters 2 and 3 range from 2 µg/g – 260 µg/g (calculated based on an approximate larval weight of 0.14 g ± 0.02 g). The lowest dose of 2 µg/g (corresponding to the 4X below MIC 1 µg/mL OTC treatment of 2021BC16) was able to significantly increase larval survival compared to the IC (* $p < 0.05$). In the field, we anticipate that lower concentrations would be adequate due to the presence of social immunity in honey bees which acts as a defense against pathogens such as antimicrobial peptides, grooming, and hygienic behaviour, whereby infected larvae are detected and removed from the hive[100]. Altogether, the results suggest that MICs obtained from monocultures using a microdilution broth method I KSBHI are predictive of concentrations required to manage infection in individual animals.

**4.6 Life-saving antibiotic treatment does not clear *in vitro* infections.**

To determine the efficacy of OTC, TYL, and LMC at clearing *M. plutonius* infection during the *in vitro* larval infection trials, we measured the number of CFUs that remained in the surviving larvae. In all four isolates tested in Chapter 3, *M. plutonius* was recovered despite using up to 4X the MIC as a treatment. This experiment revealed that even when antibiotic treatments increased larval survival to that of the uninfected control group, *M. plutonius* was still present within the larvae. Additionally, *M. plutonius* was able to be recovered from larvae that looked phenotypically similar to uninfected control larvae, indicating larvae are able to stay healthy in the presence of this pathogen. Previous reports have indicated that infected larval survival is dependent on the bacterial load, which agrees with this data[18]. The lack of complete bacterial clearance from the larva may be indicative of how asymptomatic adult bees arise[15]. The inability of these drugs to fully clear infection may play a part in the persistence of *M. plutonius* in healthy hives. Moreover, despite their social behaviour, antibiotics may be inadequate in treating for EFB disease due to the possibility of the hive undergoing stress and becoming susceptible to recurrent disease. In fact, a study found that when only OTC was used as a treatment for EFB disease, there was a ~20% recurrence, compared to the ~5% recurrence noted when the shook swarm method, where adult honey bees are transferred from the diseased hive into a new one, was used along with OTC treatment[98].

## 4.7 Conclusion

Currently, the only approved treatment for EFB disease in North America is OTC, which is problematic because resistance to OTC is widespread across North American *M. plutonius* isolates. For isolates that belong to C12, this resistance phenotype was demonstrated in KSBHI media, and not *M. plutonius* basal media that is typically used to grow the most fastidious sequence types. In fact, this was the first such report of *M. plutonius* resistance to OTC; a result that has now been replicated by others in Japan[156].

There appears to be no well-established genetic determinant for the OTC resistance seen in these isolates, nor is genetic relatedness of these isolates an explanation for the apparent resistance. However, introduction of a membrane permeabilizing peptide, STIP 3-29, did change the resistant phenotype seen in KSBHI to sensitive for *M. plutonius* 2019BC1. Further studies are required to elucidate the mechanism by which *M. plutonius* isolates appear to be resistant to OTC.

Alternative treatments to OTC, TYL and LMC, were demonstrated to be adequate to protect against EFB disease using an *in vitro* larval model. Specifically, concentrations corresponding to the MIC of a *M. plutonius* isolates, and sometimes, even 4X below this concentration was able to rescue infected larvae from infection. Moreover, larvae that were infected with *M. plutonius* and later treated with 1 of the 3 drugs recovered back to a normal weight (similar to uninfected larvae). Interestingly, *M. plutonius* was able to be recovered from infected larvae that survive due to antibiotic treatments, indicating that suppressing the expansion of *M. plutonius* in the midgut rather than eradication is sufficient to control the disease. This is consistent with the proposed mechanism of disease: starvation through bacterial overgrowth in the midgut[15,35]. While TYL and LMC were shown to be adequate alternatives for EFB disease treatment, additional research is required to determine their efficacy *in vivo.* Moreover, OTC use in honey bees requires further studies as well due to the prevalence of resistance found across North America.

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## APPENDIX

Supplemental materials, including three tables and two figures, for this thesis are provided in this appendix.

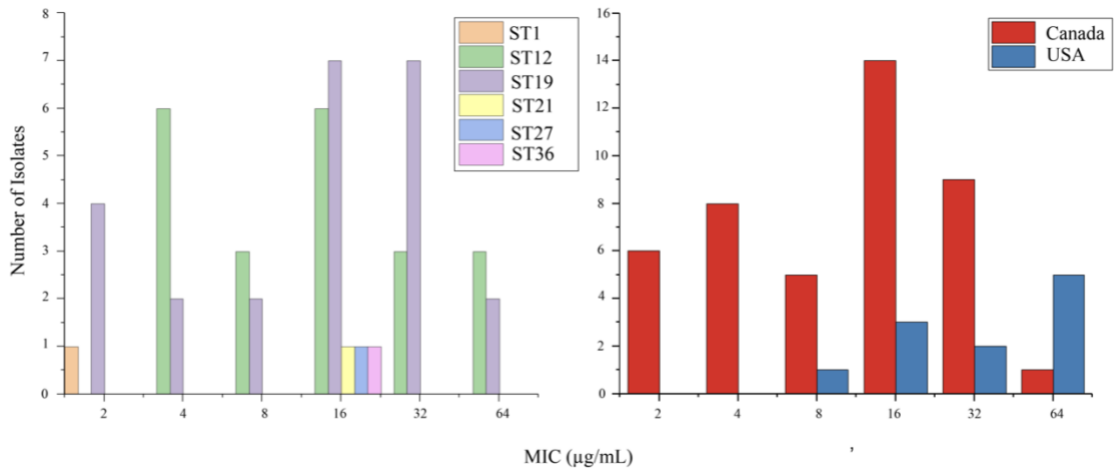


**Figure S1 – Minimum inhibitory concentrations grouped by ST and geography**. **(A)** Bar graph demonstrating the spread of MIC values for OTC when grouped by sequence type. **(B)** Bar graph demonstrating the spread of MIC values for OTC when grouped by country of origin.

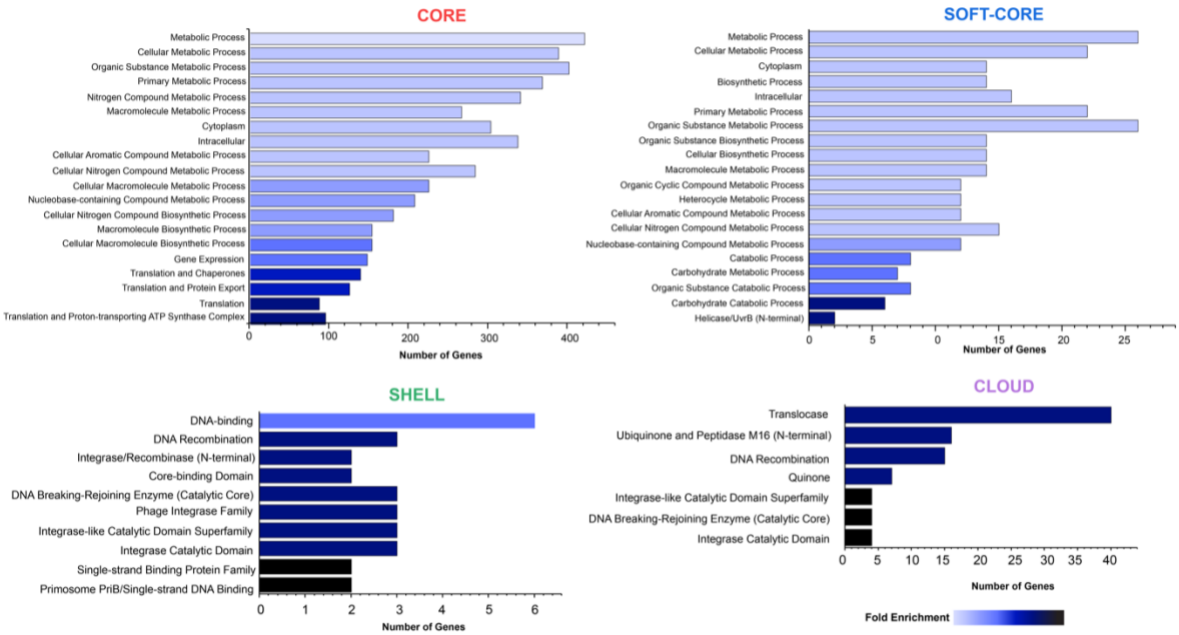


**Figure S2 – GO analysis of *M. plutonius*** Gene ontology results for each section of the genome. Fold enrichment is defined as the percentage of genes belonging to a pathway divided by the corresponding percentage of the background.

## Table S1 – Characterization of North American *M. plutonius* isolates

| Name | pMp1 | pMP19 | Conventional MLST | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | argE | galK | gbpB | purR | ST | CC |
| 2020AB23 | yes | no | 4 | 4 | 10 | 5 | 19 | 12 |
| 2020AB43 | yes | no | 4 | 4 | 10 | 5 | 19 | 12 |
| 2020BC34 | yes | yes | 4 | 4 | 10 | 5 | 19 | 12 |
| 2020BC83 | yes | no | 4 | 4 | 10 | 5 | 19 | 12 |
| 2020BC85 | yes | no | 4 | 4 | 10 | 5 | 19 | 12 |
| 2019BC1 | yes | yes | 4 | 4 | 10 | 5 | 19 | 12 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 2020BC41 | yes | yes | 4 | 4 | 10 | 5 | 19 | 12 |
| 2021BC20 | yes | no | 4 | 4 | 10 | 5 | 19 | 12 |
| 2021BC23 | yes | yes | 4 | 4 | 10 | 5 | 19 | 12 |
| 2021BC16 | yes | yes | 4 | 4 | 10 | 5 | 19 | 12 |
| 2021BC17 | yes | no | 4 | 4 | 10 | 5 | 19 | 12 |
| 2021BC1 | yes | yes | 4 | 4 | 11 | 5 | 21 | 12 |
| 2021BC2 | yes | yes | 4 | 4 | 10 | 5 | 19 | 12 |
| 2021BC3 | yes | yes | 4 | 4 | 10 | 5 | 19 | 12 |
| 2021BC7 | yes | yes | 4 | 4 | 10 | 5 | 19 | 12 |
| 2021BC9 | yes | yes | 4 | 4 | 10 | 5 | 19 | 12 |
| 2021BC13 | yes | yes | 4 | 4 | 10 | 5 | 19 | 12 |
| 2020MI13 | yes | yes | 4 | 4 | 10 | 5 | 19 | 12 |
| 2020MI68 | yes | no | 4 | 4 | 10 | 5 | 19 | 12 |
| 2020OR6 | yes | no | 4 | 4 | 3 | 5 | 12 | 12 |
| 2020OR32 | yes | no | 4 | 4 | 3 | 5 | 12 | 12 |
| 2007QU1 | yes | no | 4 | 4 | 3 | 5 | 12 | 12 |
| 2008QU1 | yes | no | 4 | 4 | 3 | 5 | 12 | 12 |
| 2015QU1 | yes | yes | 4 | 4 | 3 | 5 | 12 | 12 |
| 2016QU1 | yes | no | 4 | 4 | 3 | 5 | 12 | 12 |
| 2016QU2 | yes | yes | 4 | 4 | 3 | 5 | 12 | 12 |
| 2017QU1 | yes | yes | 4 | 4 | 3 | 5 | 12 | 12 |
| 2017QU2 | yes | yes | 4 | 4 | 3 | 5 | 12 | 12 |
| 2018QU1 | yes | no | 4 | 4 | 3 | 5 | 12 | 12 |
| 2018QU2 | yes | no | 4 | 4 | 3 | 5 | 12 | 12 |
| 2018QU3 | yes | yes | 4 | 4 | 3 | 5 | 12 | 12 |
| 2020QU3 | yes | no | 4 | 4 | 3 | 5 | 12 | 12 |
| 2008QU2 | yes | no | 4 | 4 | 3 | 5 | 12 | 12 |
| 2019QU1 | yes | no | 4 | 4 | 3 | 5 | 12 | 12 |
| 2019QU2 | yes | no | 4 | 4 | 3 | 5 | 12 | 12 |
| 2020QU1 | yes | no | 4 | 4 | 3 | 5 | 12 | 12 |
| 2020QU2 | yes | yes | 4 | 4 | 3 | 5 | 12 | 12 |
| 2020SK69 | yes | no | 4 | 4 | 3 | 5 | 12 | 12 |
| 2021SK11 | yes | yes | 4 | 4 | 10 | 5 | 19 | 12 |
| 2021SK36 | yes | yes | 4 | 4 | 3 | 5 | 12 | 12 |
| 2021SK14 | yes | yes | 4 | 4 | 10 | 5 | 19 | 12 |
| 2020TX9 | yes | no | 4 | 4 | 10 | 5 | 19 | 12 |
| 2020TX30 | yes | no | 4 | 4 | 14 | 5 | 27 | 12 |
| 2020UT13 | yes | yes | 4 | 4 | 10 | 5 | 19 | 12 |
| 2020UT9 | yes | no | 4 | 4 | 10 | 5 | 19 | 12 |
| 2021SK40 | Yes | Yes | 4 | 4 | 3 | 5 | 12 | 12 |
| 2021AB55 | Yes | Yes | 4 | 4 | 10 | 5 | 19 | 12 |
| 2020BC9 | Yes | Yes | 4 | 4 | 10 | 5 | 19 | 12 |
| 2020SK1 | Yes | Yes | 4 | 4 | 9 | 5 | 36 | 12 |
| 2020OR4 | Yes | Ues | 4 | 4 | 10 | 5 | 12 | 12 |
| 2020BC6 | Yes | Yes | 4 | 4 | 10 | 5 | 19 | 12 |
| 2020OR23 | Yes | Yes | 4 | 4 | 3 | 5 | 12 | 12 |
| 2020AB19 | yes | Yes | 4 | 4 | 10 | 5 | 19 | 12 |

## Table S2 – Genomic Information about North American *M. plutonius* isolates

| Name | Province / State | Sequence Size (Mbp) | GC Content (%) | Number of Contigs | L50 | N50 | CDS | RNA | tRNA | Accession Number |
|---|---|---|---|---|---|---|---|---|---|---|
| 2020AB23 | AB, CA | 2.11 | 31.1 | 112 | 14 | 49535 | 1903 | 4 | 14 | SAMN35547841 |
| 2020AB43 | AB, CA | 2.11 | 31.1 | 137 | 15 | 46048 | 1917 | 4 | 14 | SAMN35547842 |
| 2020BC34 | BC, CA | 2.12 | 31.1 | 136 | 15 | 46048 | 1940 | 3 | 14 | SAMN35547843 |
| 2020BC83 | BC, CA | 2.09 | 31.1 | 93 | 14 | 49535 | 1877 | 4 | 14 | SAMN35547844 |
| 2020BC85 | BC, CA | 2.06 | 31.1 | 85 | 14 | 47354 | 1835 | 3 | 14 | SAMN35547845 |
| 2019BC1 | BC, CA | 2.17 | 31.3 | 3 | 1 | 1941988 | 1886 | 12 | 61 | |
| 2020BC41 | BC, CA | 2.12 | 31.1 | 143 | 16 | 47076 | 1943 | 4 | 14 | SAMN35547847 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| 2021BC20 | BC, CA | 2.11 | 31.1 | 137 | 14 | 49535 | 1921 | 4 | 14 | SAMN35547848 |
| 2021BC23 | BC, CA | 2.12 | 31.1 | 134 | 15 | 46048 | 1935 | 3 | 14 | SAMN35547849 |
| 2021BC16 | BC, CA | 2.17 | 31.2 | 148 | 14 | 49535 | 2009 | 4 | 14 | SAMN35547850 |
| 2021BC17 | BC, CA | 2.16 | 31.2 | 160 | 15 | 46048 | 2009 | 3 | 17 | SAMN35547851 |
| 2021BC1 | BC, CA | 2.17 | 31.2 | 147 | 14 | 49535 | 2004 | 5 | 14 | SAMN35547852 |
| 2021BC2 | BC, CA | 2.18 | 31.2 | 155 | 14 | 49535 | 2025 | 6 | 14 | SAMN35547853 |
| 2021BC3 | BC, CA | 2.12 | 31.2 | 113 | 15 | 47354 | 1914 | 5 | 14 | SAMN35547854 |
| 2021BC7 | BC, CA | 2.13 | 31.1 | 142 | 15 | 47534 | 1945 | 4 | 14 | SAMN35547855 |
| 2021BC9 | BC, CA | 2.14 | 31.2 | 147 | 15 | 47534 | 1975 | 3 | 14 | SAMN35547856 |
| 2021BC13 | BC, CA | 2.17 | 31.2 | 129 | 16 | 46068 | 2005 | 3 | 14 | SAMN35547857 |
| 2020MI13 | MI, USA | 2.13 | 31.1 | 145 | 15 | 47534 | 1955 | 4 | 14 | SAMN35547858 |
| 2020MI68 | MI, USA | 2.14 | 31.1 | 154 | 14 | 49535 | 1962 | 4 | 14 | SAMN35547859 |
| 2020OR6 | OR, USA | 2.16 | 31.2 | 121 | 13 | 50171 | 1969 | 3 | 22 | SAMN35547860 |
| 2020OR32 | OR, USA | 2.14 | 31.2 | 132 | 13 | 50162 | 1974 | 3 | 23 | SAMN35547861 |
| 2007QU1 | QU, CA | 2.04 | 31.1 | 71 | 13 | 54143 | 1794 | 3 | 22 | SAMN35547862 |
| 2008QU1 | QU, CA | 2.04 | 31.1 | 74 | 12 | 54143 | 1791 | 3 | 22 | SAMN35547863 |
| 2015QU1 | QU, CA | 2.14 | 31.1 | 142 | 14 | 47361 | 1974 | 3 | 22 | SAMN35547864 |
| 2016QU1 | QU, CA | 2.04 | 31.1 | 78 | 13 | 49536 | 1794 | 3 | 22 | SAMN35547865 |
| 2016QU2 | QU, CA | 2.12 | 31.1 | 148 | 15 | 46048 | 1950 | 3 | 22 | SAMN35547866 |
| 2017QU1 | QU, CA | 2.12 | 31.1 | 87 | 13 | 47361 | 1901 | 3 | 23 | SAMN35547867 |
| 2017QU2 | QU, CA | 2.13 | 31.2 | 127 | 14 | 46068 | 1944 | 3 | 22 | SAMN35547868 |
| 2018QU1 | QU, CA | 2.09 | 31.1 | 128 | 15 | 46310 | 1898 | 3 | 22 | SAMN35547869 |
| 2018QU2 | QU, CA | 2.13 | 31.2 | 96 | 14 | 44214 | 1936 | 3 | 22 | SAMN35547870 |
| 2018QU3 | QU, CA | 2.14 | 31.1 | 153 | 16 | 44214 | 1970 | 3 | 22 | SAMN35547871 |
| 2020QU3 | QU, CA | 2.15 | 31.2 | 117 | 14 | 46068 | 1968 | 3 | 22 | SAMN35547872 |
| 2008QU2 | QU, CA | 2.03 | 31.1 | 84 | 14 | 49539 | 1799 | 3 | 21 | SAMN35547873 |
| 2019QU1 | QU, CA | 2.12 | 31.1 | 138 | 14 | 47361 | 1963 | 3 | 22 | SAMN35547874 |
| 2019QU2 | QU, CA | 2.12 | 31.1 | 144 | 16 | 44214 | 1952 | 4 | 22 | SAMN35547875 |
| 2020QU1 | QU, CA | 2.11 | 31.2 | 130 | 15 | 44214 | 1941 | 3 | 23 | SAMN35547876 |
| 2020QU2 | QU, CA | 2.13 | 31.2 | 135 | 15 | 47361 | 1962 | 4 | 22 | SAMN35547877 |
| 2020SK69 | SK, CA | 2.09 | 31.1 | 117 | 14 | 47355 | 1891 | 3 | 22 | SAMN35547878 |
| 2021SK11 | SK, CA | 2.11 | 31.1 | 135 | 14 | 49535 | 1923 | 3 | 14 | SAMN35547879 |
| 2021SK36 | SK, CA | 2.11 | 31.1 | 96 | 14 | 49358 | 1894 | 3 | 14 | SAMN35547880 |
| 2021SK14 | SK, CA | 2.12 | 31.1 | 138 | 13 | 47355 | 1923 | 4 | 22 | SAMN35547881 |
| 2020TX9 | TX, USA | 2.11 | 31.1 | 143 | 15 | 47354 | 1922 | 4 | 14 | SAMN35547882 |
| 2020TX30 | TX, USA | 2.09 | 31.1 | 96 | 15 | 46068 | 1886 | 2 | 14 | SAMN35547883 |
| 2020UT13 | TX, USA | 2.13 | 31.1 | 139 | 14 | 49358 | 1956 | 3 | 14 | SAMN35547884 |
| 2020UT9 | UT, USA | 2.09 | 31.1 | 138 | 15 | 47354 | 1905 | 4 | 14 | SAMN35547885 |
| 2021SK40 | SK, CA | 2.11 | 31.1 | 128 | 13 | 50171 | 1919 | 3 | 22 | SAMN35547886 |
| 2021AB55 | AB, CA | 2.18 | 31.3 | 3 | 1 | 199385 | 1971 | 12 | 61 | SAMN35547887 |
| 2020BC9 | BC, CA | 2.12 | 31.1 | 159 | 16 | 44008 | 2062 | 5 | 30 | SAMN21571795 |
| 2020SK1 | SK, CA | 2.16 | 31.3 | 58 | 4 | 233698 | 1956 | 4 | 31 | SAMN21571799 |
| 2020OR4 | OR, USA | 2.21 | 31.3 | 100 | 5 | 201971 | 2291 | 8 | 50 | SAMN21571801 |
| 2020BC6 | BC, CA | 2.18 | 31.4 | 95 | 4 | 235259 | 2211 | 9 | 45 | SAMN21571796 |
| 2020OR23 | OR, USA | 2.23 | 31.2 | 65 | 5 | 200825 | 2236 | 5 | 45 | SAMN21571800 |
| 2020AB19 | AB, CA | 2.21 | 31.7 | 113 | 4 | 235259 | 2397 | 8 | 47 | SAMN21571798 |

**Table S3 – Summary of larval rearing experiments showing survival and weight on day 6 after grafting and treatment**

| Isolate | Group | Survival (%) | Weight (g) | Infection dose (CFU) |
|---|---|---|---|---|
| 2019BC1 | GC | 89 ± 7 | 0.13 ± 0.02 | 0 |
| | IC | 27 ± 16 | 0.06 ± 0.05 | 30 ± 13 |
| | 2 OTC | 65 ± 8 | 0.08 ± 0.03 | |
| | 8 OTC | 88 ± 5 | 0.09 ± 0.04 | |
| | 32 OTC | 85 ± 8 | 0.13 ± 0.03 | |
| | 128 OTC | 90 ± 21 | 0.11 ± 0.03 | |
| | 0.5 TYL | 42 ± 45 | 0.12 ± 0.05 | |

| | | | | |
|---|---|---|---|---|
| | 2 TYL | 63 ± 25 | 0.12 ± 0.03 | |
| | 8 TYL | 83 ± 14 | 0.13 ± 0.18 | |
| | 0.125 LMC | 25 ± 24 | 0.10 ± 0.05 | |
| | 0.5 LMC | 88 ± 11 | 0.13 ± 0.02 | |
| | 2 LMC | 88 ± 11 | 0.13 ± 0.02 | |
| 2021BC16 | GC | 88 ± 9 | 0.14 ± 0.01 | 0 |
| | IC | 19 ± 11 | 0.02 ± 0.01 | 58 ± 16 |
| | 1 OTC | 40 ± 16 | 0.06 ± 0.04 | |
| | 4 OTC | 54 ± 16 | 0.07 ± 0.08 | |
| | 16 OTC | 72 ± 24 | 0.11 ± 0.04 | |
| | 0.25 TYL | 33 ± 12 | 0.03 ± 0.03 | |
| | 1 TYL | 38 ± 25 | 0.08 ± 0.06 | |
| | 4 TYL | 83 ± 20 | 0.13 ± 0.02 | |
| | 0.0625 LMC | 52 ± 13 | 0.10 ± 0.05 | |
| | 0.25 LMC | 81 ± 8 | 0.13 ± 0.02 | |
| | 1 LMC | 85 ± 8 | 0.13 ± 0.01 | |
| 2021SK40 | GC | 86 ± 8 | 0.13 ± 0.02 | 0 |
| | IC | 13 ± 13 | 0.02 ± 0.02 | 38 ± 8 |
| | 16 OTC | 42 ± 7 | 0.09 ± 0.04 | |
| | 64 OTC | 63 ± 8 | 0.11 ± 0.05 | |
| | 256 OTC | 88 ± 14 | 0.08 ± 0.02 | |
| | 0.25 TYL | 10 ± 4 | 0.12 ± 0.04 | |
| | 1 TYL | 4 ± 8 | 0.15 ± 0.004 | |
| | 4 TYL | 60 ± 16 | 0.13 ± 0.02 | |
| | 0.0625 LMC | 13 ± 14 | 0.03 ± 0.05 | |
| | 0.25 LMC | 13 ± 14 | 0.08 ± 0.03 | |
| | 1 LMC | 64 ± 15 | 0.13 ± 0.03 | |
| 2021AB55 | GC | 87 ± 6 | 0.14 ± 0.02 | 0 |
| | IC | 27 ± 13 | 0.06 ± 0.04 | 30 ± 13 |
| | 8 OTC | 65 ± 13 | 0.13 ± 0.03 | |
| | 32 OTC | 83 ± 12 | 0.14 ± 0.02 | |
| | 128 OTC | 79 ± 16 | 0.09 ± 0.03 | |
| | 0.25 TYL | 29 ± 28 | 0.12 ± 0.04 | |
| | 1 TYL | 67 ± 12 | 0.13 ± 0.04 | |
| | 4 TYL | 83 ± 12 | 0.14 ± 0.01 | |
| | 0.125 LMC | 63 ± 20 | 0.14 ± 0.01 | |
| | 0.5 LMC | 75 ± 26 | 0.13 ± 0.02 | |
| | 2 LMC | 88 ± 8 | 0.13 ± 0.01 | |