Review paper

PATHOGENESIS, EPIDEMIOLOGY AND VARIANTS OF *MELISSOCOCCUS PLUTONIUS* (EX WHITE), THE CAUSAL AGENT OF EUROPEAN FOULBROOD

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Received: 17 January 2020; accepted: 27 August 2020

Abstract

The bacterium *Melissococcus plutonius* is the etiologic agent of the European foulbrood (EFB), one of the most harmful bacterial diseases that causes the larvae of bees to have an intestinal infection. Although EFB has been known for more than a century and is practically present in all countries where beekeeping is practiced, the disease has been little studied compared to American foulbrood. Recently, great advances have been made to understand the disease and the interaction between the pathogen and its host. This review summarizes the research and advances to understand the disease. First, the morphological characteristics of *M. plutonius*, the infection process and bacterial development in the gut of the larva are described. Also, the epidemiological distribution of EFB and factors that favor the development of the disease as well as the classification of *M. plutonius* according its genomic and phenotype characteristics are reported. Finally, the new molecular tools for the study of *M. plutonius*, possible virulence factors in its genome, the issue of current EFB control measures and possible alternatives to the use of antibiotics are addressed.

Keywords: *Apis mellifera*, European foulbrood disease, *Melissococcus plutonius*, pathogenesis, virulence

INTRODUCTION

Honey bees (mainly *Apis mellifera*) are the most valuable pollinators in the world. They are inexpensive, versatile, and usually, the only solution to guarantee pollination (Morse & Calderone, 2000). However, their health is impacted by such numerous pathogens as bacteria, viruses, fungi and parasites. The factors involved in bee's health affect sustainable and profitable agriculture, as well as many non-agricultural ecosystems, (Genersch, 2010). One of the

most vital bacterial diseases of honey bees in the world is European Foulbrood (EFB) caused by the bacterium *Melissococcus plutonius*. EFB is generally associated with such secondary bacteria as *Enterococcus faecalis*, *Brevibacillus laterosporus*, *Bacillus pumilis*, *Paenibacillus alvei*, and *Paenibacillus dendritiformis* that may lead to a pathogenic effect, but their role in the development of the disease is unclear (Forsgren, et al., 2018). *M. plutonius* leads to intestinal infection in bee larvae causing death between the fourth and seventh days. Infected larvae

have an abnormal position inside the brood cell and the color of the larvae changes from pearly white to yellow, then brown and finally, when they decompose to grayish-black (Bailey, 1961). Occasionally, the larvae die after the brood cell is sealed. Present *P. alvei* remains and multiplies in the larvae and sunken opercula can be observed, and these symptoms are possibly confused with American foulbrood (Bailey & Ball, 1991; Forsgren, 2010). *M. plutonius* infects European bees *A. mellifera*, Asian bees *A. cerana* (Singh Rana et al., 2012; Takamatsu et al., 2014) and Himalayan bees *A. laboriosa* (Allen, Ball, & Underwood, 1990). The etiologic agent of EFB was identified more than a century ago (White, 1912). To understand and treat a disease, the causative agent, mechanisms of infection, and favorable conditions for its development must be identified. This review summarizes recent research on the epidemiology and pathogenesis of *M. plutonius*, the causative agent of EFB.

Pathogenesis and Epidemiology

Melissococcus plutonius was first classified as *Bacillus pluton* (White, 1912) and later as *Streptococcus pluton* (Bailey, 1957b). However, Bailey & Collins (1982) described *Melissococcus* as a new genus, containing the species *Melissococcus pluton* based on culture, biochemi-

cal and chemical characteristics. The specific epithet 'pluton' was corrected to *plutonius* by Dicks, Endo, & Van Reenen (2014) and Trüper & de'Clari (1998). *M. plutonius* is a Gram-positive cocci lanceolate oval-shaped, non-spore-forming bacteria, with an approximate size of 0.5 x 1.0 µm (Fig. 1), that is found individually, in pairs or chains of various lengths (Forsgren, 2010; Forsgren et al., 2013). The bacteria only affect the larval stages of bees (Bailey & Collins, 1982). The infection begins with the consumption of contaminated food during the early larval stage, when the larvae are exclusively fed with royal jelly (RJ), a mixture of secretions from the hypopharyngeal and mandibular glands of young worker bees (Snodgrass, 1925).

Royal jelly (RJ) has high antimicrobial activity (Melliou & Chinou, 2014) due to proteins like major royal jelly protein 1 (MRJP1) containing jelleins 1, 2 and 3, which inhibit bacterial, yeast and fungus growth (Brudzynski & Sjaarda, 2015; Fontana et al., 2004). Recently Vezetu et al. (2017), showed how MRJP1 can inhibit the development of bacteria associated with EFB, including *M. plutonius*. MRJP2 has an antibacterial effect against *P. larvae* (Bíliková, Wu, & Šimúth, 2001) and the enzyme glucose oxidase, which is essential to inhibiting microbial development in larval foods and honey (Ohashi, Natori, & Kubo,

Fig. 1. Gram staining of *Melissococcus plutonius* strain ATCC 35311, Cultivated under anaerobic conditions in SBK medium for 5 days at 36°C.

1999; Sano et al., 2004). On the other hand, such non-protein components as 10-hydroxy-2-decanoic acid (10-HDA) have demonstrated antipathogenic activity. Šedivá et al. (2018) showed that 10-HDA contributes to the inhibition of *P. larvae*, and Yang, Li, & Wang (2015) proposed that it is a broad-spectrum antimicrobial agent that inhibits multiple pathogenic bacteria. However, despite the RJ antimicrobial activity, *M. plutonius* can survive and infect honey bee larvae through it (Takamatsu et al., 2017).

Studies show that after ingestion, *M. plutonius* reaches the middle intestine of the larvae, where it begins to multiply until it almost occupies the intestinal lumen without crossing the peritrophic membrane (Tarr, 1938). White (1912) determined that *M. plutonius* grows only in the food mass within the peritrophic matrix, killing its host before any bacteria associated with EFB succeeds in invading the larval tissues. Derived from this, Bailey (1983) suggested that the pathogenic effect was due to nutrient competition between the larva and the pathogen, resulting in starvation of the larvae. McKee, Goodman, & Hornitzky (2004) suggested that

the death of the larvae may be the result of such additional pathogenic mechanisms as peritrophic matrix invasion and penetration into other host tissues. However, Takamatsu, Sato, & Yoshiyama (2016) refuted this theory inferring that the presence of substances produced by *M. plutonius* can diffuse into larval tissues during the infection, but these substances and other virulence factors remains unidentified. Only 100 to 200 bacteria cells are required to cause EFB (Bailey, 1960; McKee et al., 2004), and once the infection is established, the larvae can die before the brood cell is covered. In this case, larvae are expelled from the colony or may die after the brood cell is covered where the sunken upper wax layer is observed (Fig. 2-5c). Sometimes, the larvae survive the infection, however, pupation is delayed (McKee et al., 2004), and smaller adults emerge (Bailey, 1959b), which can transmit the bacteria (Fig. 2-6b).

Melissococcus plutonius multiplies only within the larval intestine of the honey bee. The persistence of the pathogen in the hive depends on the infected larvae's survival, which deposits the bacteria along with their feces

Fig. 2. *Melissococcus plutonius* infection cycle 1. egg, 2. Ingestion of *M. plutonius* (Mp) in the royal jelly (RJ). 3. Proliferation of *M. plutonius* in the intestine, 4a. Manifestation of EFB symptoms, 4b Larvae without EFB symptoms, 5a. Death of larvae, scale formation and brood removal by worker bees, 5b. Sealing cell and slow pupae development, 5c. Death of larva after cell was seal, 6a. Cell with *M. plutonius* and secondary agents, 6b. Emergence of smaller adults carrying *M. plutonius* and cells with *M. plutonius* and secondary agents. Adapted from Bailey & Ball (1991).

in the brood cell when they pupate (Fig. 2-6a, 6b). *M. plutonius* stay viable in the brood cell, surviving for several years (Bailey, 1959a). A large number of bacterial cells die during this time, but the remaining bacteria can infect other larvae. If the infected larva dies before it becomes a pupa, the worker bees eliminate the infected larvae (Fig. 2-5a), reducing the number of bacteria that serve as a source of inoculum. Nevertheless, worker bees will subsequently feed new larvae causing the transmission of the bacteria (Fig. 2-2). The infection is not always fatal, and *M. plutonius* can be present in larvae and pupae without any clinical symptoms (Fig. 2-4b) (Forsgren et al., 2005) due to the different degrees of virulence.

Adult worker bees responsible for removing diseased larvae from the hive have higher *M. plutonius* loads than nectar and pollen-collecting bees because workers bees are in direct contact with the infected brood that contains large amounts of the bacteria (Roetschi et al., 2008). Adult bees act as carriers of the bacteria inside the colony, between colonies and between apiaries. More than a third of healthy colonies in an apiary can maintain adult bees carrying the bacteria due to the proximity to other diseased

apiaries (Belloy et al., 2007). In Switzerland, a high density of colonies and hives have been shown to promote the transmission of EFB (von Büren et al., 2019). Additionally, Abrol (2013) and Forsgren (2010) suggested that the robbing of honey and drifting bees contributed to the spread of the bacteria between colonies and apiaries, but in contrast Goodwin, Perry, & Houten (1994) suggested that the drift of bees is not an important to the spread of AFB. However, it is not clear how EFB spreads rapidly, but such beekeeping practices as the exchange of contaminated combs, poor equipment sanitation and poor diet may contribute to it (Fig. 3) (Belloy et al., 2007; Hornitzky & Smith, 1998; McKee et al., 2003).

Bacteria of the genus *Enterococcus* are found in a variety of soil, water, and plants habitats (Forsgren, 2010). Martzy et al. (2017) mentioned the possible presence of *M. plutonius* in water, but the qPCR and loop-mediated isothermal amplification (LAMP) tests which they utilized are not specific, and *M. plutonius* was co-detected with *Tetragenococcus halophilus*, *Vagococcus fluvialis* and *Enterococcus faecalis*. This suggests that water could be a reservoir for *M. plutonius*, but more studies are required.

Fig. 3. Dissemination model of European foulbrood.

Disease outbreaks appear to be related to colony stress conditions - lack of food, water, genetic factors, climate and geography (Budge et al., 2014; Forsgren, 2010). EFB can be manifested for a short period, usually during summer or early spring (Bailey & Ball, 1991). Unexpected outbreaks of the disease have been observed followed by a spontaneous recovery a few weeks later. Severely infected colonies that move from infected endemic areas to disease-free areas can recover spontaneously (Bailey, 1961; Bailey & Locher, 1968).

The incidence of EFB has been reported in five continents and by 2019 only Nicaragua and Mozambique, of the 180 member countries of the World Organization for Animal Health (OIE), had never reported the disease (OIE, 2019). There have been occasional seasonal outbreaks of EFB in many countries (Bailey, 1961; Forsgren et al., 2013). However, in Switzerland, the incidence of EFB has increased dramatically since 1990, in the United Kingdom, it has become the most widespread brood bacterial disease and in Norway an outbreak was reported in 2010 after a thirty-year absence (Forsgren et al., 2018). Geographically, the disease seems to vary in severity, from relatively benign in some areas to very severe in others (Dahle, Sørum, & Weidemann, 2011; Wilkins, Brown, & Cuthbertson, 2007). Virulence tests on individual larvae using exposure bioassays show that *M. plutonius* strains collected in different European geographic locations vary in their morbidity (Charriere, Kilchenmann, & Roetschi, 2011).

Several studies mention the molecular detection of *M. plutonius* in such countries as Mexico, Turkey, India, Saudi Arabia, Spain and Colombia (Ansari et al., 2017; Borum et al., 2015; de León-Door et al., 2018; Garrido-Bailón et al., 2013; Singh Rana et al., 2012; Tibatá et al., 2018). For an epidemiological study, isolated strains should be classified. The chosen method to classify a specific pathogenic species must meet several criteria. All strains of the species must be classified with the chosen method, which must have a high power of differentiation and the results must be reproducible within

a laboratory and between laboratories for the construction of reliable databases. Classification methods based on the phenotype have several deficiencies, especially with the applicability to all members of a species. Tenover et al. (1995) established classification methods based on the microbial genotype or a particular DNA sequence. For the epidemiological study of EFB, Haynes et al. (2013) designed a classification system of Multi-locus sequence typing (MLST), based on the locus used for typing of *Enterococcus faecalis* (Ruiz-Garbajosa et al., 2006) and *Enterococcus faecium* (Homan et al., 2002). This scheme allowed the analysis of 381 *M. plutonius* isolates from different countries, achieving the identification of thirty-two sequences types, which were grouped into three genetically distinct groups, called clonal complexes (CCs). Budge et al. (2014), created the public database, <https://pubmlst.org/mplutonius/>, where the epidemiological information of *M. plutonius* has been uploaded.

Genotypes and virulence

Melissococcus plutonius was initially described by White (1912) and characterized by Bailey (1957b) as a demanding organism, which requires anaerobic or microaerophilic conditions for growth. Furthermore, its isolation and support requires the addition of potassium phosphate to the culture medium (Bailey & Collins, 1982). Early morphological, physiological, immunological and genetic studies suggested that the *M. plutonius* species was homogeneous (Allen & Ball, 1993; Djordjevic et al., 1999), and several reports mention that *M. plutonius* loses its virulence rapidly when it is subcultured *in vitro*. Due to this situation, the infection of bee larvae with artificially cultivated *M. plutonius* was challenging, and Giersch, Barchia, & Hornitzky (2010) reported that a high dose of it in addition to a secondary inoculation with *Paenibacillus alvei* caused high larval mortality. However, high doses of *M. plutonius* used, since Lewkowski & Erler (2019) showed that additional infections with possible secondary invaders do not increase larval mortality. On the other hand, McKee et al. (2004) and Bailey (1957a) failed to produce EFB

in artificially bred larvae with pure *M. plutonius* cultures. Consequently, studies to determine the etiology of EFB were complicated.

Arai et al. (2012) called *M. plutonius* variants present in Japan “atypical” because they did not match the phenotypic characteristics originally described for this species. The atypical strains do not require high concentrations of potassium phosphate, grow even in the presence of oxygen, are positive for β -glucosidase activity and produce acid from L-arabinose, D-cellobiose, and salicin. The analysis of atypical strains by pulsed-field gel electrophoresis showed genetic differences. Additionally, these strains showed a high virulence, killing all infected larvae in a period of 5 d, while the typical strains did not show mortality.

Haynes et al. (2013) used the sequences of *argE*, *galK*, *purR*, and *gbpB* genes to develop a higher resolution classification scheme using MLST. This allowed the identification of thirty-two sequence types (STs), grouped into three clonal complexes (CCs), in which Japanese atypical isolates belonged to CC12 and typical strains to CC3 and CC13. The studies conducted by Budge et al. (2014), Takamatsu et al. (2014) and de León-Door et al. (2018), showed that these CCs were widely distributed throughout the world, even in Asian bees *Apis ceranae*. Some isolates from Brazil, United Kingdom, USA and the Netherlands have been classified inside the CC12 where atypical strains are found. An analysis of the database designed by Budge et al. (2014) (<https://pubmlst.org/mplutonius/>) showed that from the 381 isolates reported 66% correspond to CC3, 20% to CC12 and the remaining 14% to CC13 (Consulted on April 12, 2020).

In the study conducted by Nakamura et al. (2016), the virulence of the different CCs was tested. CC12 showed the highest virulence, killing more than 90% of infected larvae before pupation, similar to the report by Arai et al. (2012). However, CC3 belonged to the strains reported as typical and showed a higher virulence than reported by Arai et al. (2012), killing approximately 60% of infected larvae. For its part, CC13 showed no virulence, being similar to the mortality in the control larvae.

Similar results were shown by Lewkowski and Erler (2019), where the CC3 strain caused greater mortality than the CC13 strains, and all cases of CC13 lethality are similar to the control. Additionally, different genetic backgrounds contribute significantly to the course of the disease. Although not all CCs caused the death of infected larvae, an increase in the number of *M. plutonius* bacteria from the three CCs was observed during the six days post-infection (Nakamura et al., 2016), which indicates that even when CC13 does not cause death, it can colonize the larva's intestine. However, its effect on adult bees remains unknown.

The CC12 strains show the highest virulence *in vitro* at the individual level, but this is inconsistent with the number of isolates reported. Budge et al. (2014) claimed that CC3 is the most virulent at the colony level, followed by CC12 and CC13. These results suggest the physiological advantage of the CC3 strains for their survival and pathogenic potential. Takamatsu et al. (2017) mentioned that CC3 strains can survive and even proliferate in a concentration of 50% RJ. On the other hand, CC13 was the most susceptible to RJ components, because its number of viable bacterial cells decreased by 99% while the number of CC12 strains' viable bacterial cells decreased by only 80% at the same concentration of RJ. Arai et al. (2014) concluded that larvae infected with strains of CC12 develop EFB rapidly and are expelled from the colony by worker bees before the disease is evident. For this reason, fewer isolates of CC12 have been reported.

Genomic analysis

The lack of molecular tools challenges the studies on *M. plutonius*. To improve this situation, Takamatsu et al. (2013) developed a gene expression vector, (pMX2) for gene complementation and an electroporation protocol for *M. plutonius*. They observed how mutations in genes that encode for the Na^+/H^+ antiporter and cation transport ATPase are involved in the potassium requirement for the growth of typical strains of *M. plutonius*. Based on these results, Arai et al. (2014) developed a duplex

PCR, selecting Na⁺ /H⁺ antiporter gene and Fur family transcriptional regulator gene as targets for the detection of typical and atypical strains, respectively.

Takamatsu et al. (2015) designed a gene inactivation system in *M. plutonius*, through the use of a pSET6s thermosensitive plasmid vector, originally used for the construction of deletion mutants in *Streptococcus suis* (Takamatsu, Osaki, & Sekizaki, 2001). A non-functional *srtA* mutated gene was added to the pSET6, and after recombination, sortase A activity in *M. plutonius* was suppressed. However, to verify if this gene inactivation system is specific, it is necessary to determine if the phenotypic changes observed in the mutant strains are caused by the elimination of the target genes, by carefully genetic analyses. The combination of pSET6s and pMX2 could be a useful genetic tool for future molecular analyzes in *M. plutonius*.

Djukic et al. (2018) conducted a comparative genome analysis of fourteen strains of *M. plutonius*, including the already reported genome of the reference strain *M. plutonius* ATCC 35311 (Okumura et al., 2011), belonging to CC13 (Non-virulent), and the genome of the Japanese atypical strain *M. plutonius* DAT561, (Okumura et al., 2012; Okumura, Takamatsu, & Okura, 2018) belonging to CC12 (highly virulent). Their observations show that genomes exhibited sizes ranging from 2.021 to 2.101 Mbp and included 1,595 to 1,686 genes encoding proteins. Bioinformatics analysis revealed the following genes and proteins that could play a role in the pathogenesis of EFB: putative bacteriocins, cell surface-associated proteins, adhesion, and enterococcal polysaccharide antigen, an epsilon toxin, proteolytic enzymes and capsule associated proteins.

Typical strains belonging to CC13 lack such important virulence factors as tyrosine decarboxylase, endo-alpha-N-acetylgalactosaminidase and a toxin as compared to CC3 and CC12 (Djukic et al., 2018). In addition, plasmid pMP19 carried by the strains of CC12 and CC3 (Okumura, Takamatsu, & Okura, 2019), can confer additional advantages as compared to strains without the plasmid. However, this plasmid does not remain

stable during *in vitro* propagation and the association of pMP19 with virulence is not clear since this plasmid has also been reported in strains of the three CCs (Takamatsu et al., 2017).

The advantage of atypical CC12 strains could be due to rapid nutrient consumption. The DAT561 strain uses several carbohydrates as a source of energy through glycolysis, the pentose phosphate pathway, ED-pathway and sugar interconversions. Also, the production of virulence-related molecules could favor the rapid death of larvae infected with CC12 strains (Djukic et al., 2018). However, for the verification of these virulence factors, infection studies with fully characterized strains are required to predict a difference in virulence between strains of the different CCs of *M. plutonius*. Another factor is the genetic variability of the host, whose origin has been observed to strongly influence the mortality of bees infected with *M. plutonius* (Lewkowski & Erler, 2019). Additionally, the results presented by Tibatá et al. (2018) are consistent with the appreciation that Africanized hybrids are more resistant to diseases.

EFB control

Before focusing on the treatment of EFB, it is worth mentioning that the best form of disease control is prevention. Beekeepers must maintain proper sanitation practices to prevent the spread of *M. plutonius*. The equipment must be disinfected correctly before being stored, reused or moved from one colony to another (Arbia & Babbay, B., 2011). All combs should be inspected for signs of diseased brood when new nucs or full-size colonies are purchased. Upon detection of diseased colonies, a quarantine strategy should be followed (Locke, Low, & Forsgren, 2019). Frequently replacing queens with those that promote hygienic behavior results in more offspring of workers that remove diseased larvae from the hive more efficiently. Additionally, racks should be replaced annually and exchange between hives avoided (Flores, Spivak, & Gutiérrez, 2005; Spivak et al., 2003). In addition, providing enough food prevents the robbing of honey and stimulates the queen's posture to produce a greater number of bees

for diseased brood removal (Bailey & Ball, 1991; Gochnauer, Furgala, & Shimanuki, 1975). Weak colonies with less prolific queens are more susceptible to EFB. The death of many larvae result in more contaminated food for the remaining larvae, causing colony collapse (Bailey & Ball, 1991).

There have been few studies on control measures for EFB. Oxytetracycline hydrochloride (OTC), a bacteriostatic antibiotic used in many countries, inhibits the multiplication of *M. plutonius*. Since the 1950s, beekeepers have used this antibiotic for the prevention of EFB and American foulbrood (AFB) (Moeller, 1978). However, in AFB, it has caused the emergence of antibiotic resistant *Paenibacillus larvae* strains in different regions of the world (Genersch, 2010). The most recent reports in the United Kingdom and Australia evaluated the resistance and susceptibility of *M. plutonius* to OTC (Hornitzky & Smith, 1999; Waite et al., 2003), but new large-scale studies are needed to determine if the bacteria are still susceptible to the antibiotic.

As an alternative to OTC treatment, the shook swarm method is proposed, in which adult bees are changed to new hives and the combs with sick brood are eliminated. Budge et al. (2010) mention that this control method has an efficacy similar to the use of antibiotics. On the other hand, Waite et al. (2003) suggest that the combination of the shook swarm method and the use of antibiotics reduces the recurrence of the disease at the colony level. However, the shook swarm method was subsequently recommended without the use of antibiotics for the control of EFB, since the disease's clinical signs are only suppressed with bacteriostatic antibiotics and beekeepers unconsciously spread the infection between the colonies (Thompson et al., 2005). Nevertheless, adult bees present in infected hives, especially those in contact with the young, have high loads of *M. plutonius*, and therefore the shook swarm method is probably not as effective, (Belloy et al., 2007; Roetschi et al., 2008), since adult bees act as a vector for the dissemination of *M. plutonius*. In Switzerland, the sanitation procedure is based only on swarm shaking, due to EU antibiotic restrictions.

Therefore colonies with clinical symptoms are destroyed, but the sanitation procedure is not enough to prevent new outbreaks of EFB in the same hives the following year (Roetschi et al., 2008).

Doughty, Luck & Goodman (2004) identified ampicillin and amoxicillin, beta-lactam antibiotics, as candidates for the control of EFB. However, these antibiotics are currently used to fight different infections in humans, and the routine use of antibiotics in animal feed, as documented, contributes to the generation of resistant bacteria, a concern to human health leading to a greater public health concern (Cota-Rubio et al., 2014). In addition, bacteriostatic antibiotics only suppress clinical symptoms but do not cure the disease, leading to chemical residues persisting in honey, affecting their quality and safety for human consumption (Martel et al., 2006). Next, antibiotics can affect the vitality and longevity of bees (Peng et al., 1992). Bacterial resistance in *M. plutonius* has not been reported yet, but in such beekeeping pathogens as *P. larvae* it has become widespread (Evans, 2003), and therefore, alternatives to the use of chemical treatments must be found.

Antagonistic microorganisms have been proposed as a potential alternative to the treatment of EFB. Lactic acid bacteria (LAB) added to the food of the larvae exposed to *M. plutonius*, was noted to decrease the number of larvae that succumb to the infection (Vásquez et al., 2012). Wu et al. (2014) showed how *Bacillus subtilis* exhibited inhibitory activity *in vitro* against *M. plutonius*. In addition, *in vivo* feeding trials revealed that the mortality of infected larvae decreased. On the other hand, Killer et al. (2014) reported a new taxon called *Lactobacillus apis* sp. with the ability to inhibit the growth of *M. plutonius*. However, the use of these microorganisms presents challenges, since dosing, timing, duration and the microbiological safety of hive products require optimization for the application of these treatments.

Tea tree oil nanoparticles (Santos et al., 2014), *Malva sylvestris* oil (Cecotti et al., 2016), aqueous and ethanolic extracts of *Cinnamomum* spp. and *Persian salvadora* (Hashish et al.,

2016), macelignan and corosolic acid (Kim et al., 2018), terpenes of *Thymus vulgaris* (Wiese et al., 2018), among others, have been shown to have significant antibacterial effects against *M. plutonius*. The use of extracts and essential oils for integrated EFB treatment is promising and stimulates the search for other bioactive plant phytochemicals with bactericidal capacity and low toxicity for bees. Currently, the use of bacteriophages for the treatment of EFB shows promising results in many fields (Vandamme & Mortelmans, 2019). In beekeeping, it has been used successfully for the control of AFB (Brady et al., 2017; Yost, Tsourkas, & Amy, 2016). However, the isolation of bacteriophages of *M. plutonius* could be challenging since the cultivation of *M. plutonius* has been described as difficult (Allen & Ball, 1993).

CONCLUSIONS

The objective of this review was to present the new advances and pathogenesis of *M. plutonius*. An essential step has been the identification of variants of *M. plutonius* that maintain their virulence. The MLST has enabled the differentiation of strains and the database will augment our understanding of the global epidemiology of EFB. It is understood how *M. plutonius* behaves in the early stages of infection, but most infectious process stages are still not clearly explained. The complete genomic sequences allowed the identification of putative virulence factors, probably used by *M. plutonius* during pathogenesis. Additionally, the new genomic tools available for the manipulation of *M. plutonius* will allow a better understanding of these processes at the molecular level. However, more studies are vital to understanding the complex interactions between *M. plutonius* and honey bee larvae. Although multiple efforts have been conducted to develop sustainable treatment strategies, progress is limited. The treatment of infected hives with new antibiotics will remain problematic, due to the health risks associated with antibiotic use and the increased awareness to limit antibiotic use in animal production. Recently, this is revealed in the implementation

of stricter regulations on the prophylactic use of antibiotics and the presence of residues in bee products. Overall, for the increased sustainable development of the beekeeping industry, new strategies are required to control such bacterial infections as EFB.

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