Chalkbrood: pathogenesis and the interaction with honeybee defenses

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***Abstract*—** *There are numerous threats that affect bee populations worldwide such as exposure to pesticides; genetic diversity, poor nutrition and the impact of pathogens. Between them, Ascosphaera apis is the etiological agent of chalkbrood disease that affects honeybees brood. To understand the biology of this pathogen, we revised the phylogeny, morphology, and sexual reproduction. The pathogenesis, closely related to the factors that affect the virulence the A. apis and their interactions with the host, are determinant at moment of developing chalkbood. The honeybee develops several strategies to defend themselves from these pathogens. First, the individual immunity mechanisms such us perithrophic membrane, the microbiota of midgut larvae and the humoral and cellular immunity are the first defense barriers against A. apis. Later, other mechanisms would appear, related to the social immunity, such as their social organization, the polyandry, the hygienic behavior and the social fever, that change the environmental conditions in the bee colony reducing A. apis viability. However, other pathogens such as Nosema spp, Varroa destructor, several viruses, and the presence of pesticides affect the sanitary status of the honeybee allowing the fungus to develop easily. Finally, we describe to our knowledge, the best three natural alternatives that could be studied in order to employ them in field trails.*

***Keywords*— chalkbrood, epidemiology, honeybees, host´s resistance, pathogen´s virulence.**

I. INTRODUCTION

Honeybees (*Apis mellifera* L.) not only produce honey, but also ensure the pollination of more than 80% of the crops which provide food for mankind worldwide. The activity of these pollinators becomes crucial for sustaining natural habitats and contributes to local and global economies [1].

Currently, there are numerous threats to bee populations around the world such as exposure to pesticides; stress hives transhumance, genetic diversity, poor nutrition and the impact of large numbers of pathogens. Among the diseases of the honeybee, chalkbrood is a fungal disease that affects only brood honeybees. The disease is caused by *Ascosphaera apis* (Maassen ex Claussen) Olive & Spiltoir [2, 3]. These fungi cause about 14-64% reduction in honey production [4] and 80% of larvae deaths [5].

II. *Ascosphaera apis*, THE PATHOGEN FUNGUS OF HONEYBEE'S BROOD

2.1 Phylogenie of *A. apis*

A. apis was originally described as *Pericistis apis* [6], then was redefined by Claussen [7] and reclassified as *A. apis* by Spiltoir [2]. In the past, the classification of Ascomycota group was based on the description of the spherical spore cyst or ascomata [8]. Thus, *A. apis* belonged to Ascomycotina; Plectomycetes, Ascosphaerales and Ascosphaeceae classification [8]. The phylogeny was frequently discussed because it was based on the phenotypic characteristics of reproductive structures, mostly influenced by the environment. Therefore, the development of molecular methods provides valuable tools to elucidate the phylogeny of these species including Ascosphaerales order in Onygenales [9-13]. The current taxonomic lineage of *A. apis* is: Ascomycota; Pezizomycotina; Eurotiomycetes; Eurotiomycetidae; Onygenales; Ascosphaeraceae; *Ascosphaera apis* [14]. Recently, Klinger [12] developed a phylogenetic study and found that *A. apis* was grouped with the species, *A. flava*, *A. pollenicola, A. variegate, A. larvis* and *A. major*. These species are frequently found in reserves brood nest and pollen. The first three are saprophytic species, however can act as opportunistic pathogens. In the present, most of the 28 species of the genus *Ascosphaera* are associated with social and solitary bees [15].

2.2 Epidemiology

Chalkbrood was identified in the XVIII century by Maassen [6]. In the mid-XIX century it was detected in Russia and in several European countries. Around 1960, other reports of the fungus came from New Zealand and North America. Since then, the pathogen has spread throughout the United States, Alaska and Hawaii. In the 1980s the disease appeared in Argentina, Chile, Central America, Mexico, Japan, Philippines, Israel and Turkey. In 1993 it was identified in Australia [5]. In Africa, chalkbrood disease was reported in Tunisia in 1985, but in the last decade it was also found in Ethiopia, South Africa, Egypt and Nigeria [16].

The predisposing conditions for the development of chalkbrood are more prevalent in dump and cool climate. The disease develops when brood is affected to physiological stress chilling [17]. However, high levels of infection have been documented in Ethiopian alpine areas of dry weather and different climates in the country [4].

2.3 Morphological features, molecular characteristics and sexual reproduction of *A. apis*

The *Ascosphaera* genus is characterized by production of spherical spore cyst, or ascoma, that contains smaller round bodies, called spore balls, and inside them, the ascospores. This type of ascoma is unique for the *Ascosphaeraceae* [18], the species differ in morphological characteristic of the hyphae, spores, spore balls and spore cysts. The range of sizes of the mature ascoma are 47 - 140 μm in diameter, the spore balls, average 12 μm in diameter and the ascospores average 2.9 x 1.4 μm. The ascospores are the unique infective stage [19].

The complete genome of *A. apis* was sequenced [20]. Different studies on the genetic diversity of the pathogen allowed differentiating strains and haplotypes of *A. apis* [21, 22]. Also, it is known that the sexual reproduction occurs between haploid mating type idiomorphs (Mat1-1 and Mat1-2) [23]. On the contrary, the available information about the mechanism of asexual reproduction of *A. apis* is scarce and ambiguous [5].

2.4 Pathogenesis

Ascosphaera infections occur after incoming through the gut of ascosporas which can infect brood of workers, drones, or queens [11]. Even though adult bees are not susceptible to this pathogen, they can transmit the disease within, and between beehives and also apiaries. The infection pathway begins with the consumption of food contaminated with sexual spores of *A. apis* by the bee larvae. Although the larval instars can be infected by *A. apis*, the higher susceptibility was observed in 2^{nd} - 4^{th} instars larvae [24]. Twenty four hours post infection the first signs are evidenced by reduction of feeding; after 48 h the honeybee brood dies, finally, after 72 h the fungal mycelia become visible on the surface of the cadaver [8, 25]. The microaerophilic conditions present in the hind end of the gut could activate and induce the germination of spores that were consumed by the honeybee larvae. Then, the hyphae penetrate the peritrophic membrane and gut wall to finally enter the hemocele [5]. Also, the pressure caused by septated hyphae and the enzymatic activity favor the access into the interstitial space between muscle fibers of infected larvae [26]. The fungal mycelium invades the body cavity, except the brood head [19]. Later, fungal mycelium becomes brown or grey due to the production of spore cyst as resulting of crossing the two idiomorphic types (Mat1 and Mat2) [5]. Each mummy has on the outer surface chalkbrood approximately 10^8–10^9 ascospores [27]. Some authors established that the infective capacity of the ascospores could remain for about 15 years. The exposition to CO_2 to activate the spores is currently discussed [25].

2.4.1 Spread of fungal infection

The first signs of chalkbrood are observed with the appearance of white or black mummies in breeding completely or partially decapped comb of the brood chamber, at the entrance, and on the floor of the hive [5]. To avoid the spread of fungal infection, the mummies are removed from the colony by the house-cleaning. However, fungal spores can be transmitted to healthy brood during the feeding process by nurse. Also, they can enter the hive through foraging bees that bring contaminated food; robbing; drifting and beekeeper management through the exchange of material between hives [27].

2.5 Factors affecting the virulence the *A. apis* in honeybees

2.5.1 Enzyme profile and transcriptome analysis of chalkbrood

The first finding on virulence factors proposed that a β-N-acetylglucosaminidase enzyme allows the fungi to penetrate the peritrophic membrane [28]. However, transcripts of genes were recently identified on honeybee larvae infected with *A. apis*.

They were related with functions of protein degradation, nutritional regulation, RNA processing and immune regulation through the coordinate action of two routes NF-kB signaling, resulting in the production of antimicrobial peptides (AMP) [29]. Other studies identified transcripts involved in the production of *A. apis* chitinase, protease, esterase enzymes and also genes implicated in toxin biosynthesis that could act as virulence factors and assist to pathogen in host invasion [25, 27] found a putative chitinase-encoding glycosyl hydrolase 18 transcripts at 36 h post *A apis* larvae infection. Recent quantitative trait loci analysis indicates that two possible genes may be responsible for resistance, indicating that there exists a level of physiological resistance in the larvae [30].

2.5.2 Evolution of virulence

Factors influencing the evolution of virulence among *A. mellifera* and *A. apis* depend on the specificity host-pathogen; the mechanism of transmission of the pathogen in the host, the competitiveness of *A. apis* with other pathogens in the environment and the host response to the invasion of the pathogen [15]. *A. apis* has the mechanisms to be a successful entomopathogenic fungus because it produces a high number of spores that should germinate and survive digestion after oral uptake. Fungal cells should proliferate within hemocele host tissues and body to collapse the host immune system, causing death. Also, *A. apis* can use the host´s cadaver to optimize production and dispersion of spores in the environment [26]. In addition, the pressure of chalkbrood in the host populations is high and should be successful with a number of spores 5×10^5 ascospores/brood [24], their spores can kill healthy hosts [26].

2.5.3 Mixed infections affecting virulence between the pathogen-host

While the hosts and pathogens populations persist when the virulence of the pathogen is intermediate and pathogen transmission is high, the presence of co-infection with a second pathogen could increase the virulence of the pathogen to overcome primary host´s defenses. In a recent study, it was shown that co- infection of honeybee larvae *A. apis* with *A. atra* increased the percentage of larval mortality [31]. However, the virulence of *A. apis* was not influenced by co - infection of *A. apis* with *A. agregatta* and *A. larvis* [15].

III. HOSTS: HONEYBEES (*A. mellifera, L.*)

3.1 Individual immunity mechanisms of honeybee to *A. apis* pathogens

The honeybee uses different strategies of immunity to limit the impact of the pathogen.

The individual immunity of a honeybee is based on its ability to establish different defense mechanisms to pathogens through the secretion of antimicrobial peptides, phagocytosis, melanization, the enzymatic degradation of pathogens or the biological competition. These mechanisms reduce the likelihood that the pathogen may enter the hemocele and invade the host [32].

3.1.1 Physical barriers to fungal infection

The microorganisms that enter the midgut must be adapted to survive and infect under the environmental conditions present in the insect. The pH, chemical milieu, and resident microbial community, all play a role in which they are able to penetrate into the host´s hemocele [33]. The first barrier to infection is the peritrophic matrix. It is semi-permeable, composed by microfibrils, proteoglycans, proteins and glycoproteins, and acts as a filter for microorganisms seeking access to the epithelial cells of the midgut offering a physical barrier against abrasion by food and microbial invaders, such as fungi [33] (Fig. 1a).

3.1.2 Biological barriers to fungal infection

Intestinal microbiota protects the honeybees of colonization of gut pathogens and enhances nutrient availability [34]. It is known the role the *Lactobacillus* spp. has in the production of honey and beebread and in the preservation of food reserves of the colony [35]. However, their role is discussed because bees prefer eating fresh pollen [36]. Thus, Vojvodic [37] identify Acetobacteraceae *Lactobacillus kunkeei* and *Lactobacillus* sp B (Firm 5) bacteria in european honeybee larvae. Then, these microorganisms could contribute to the immunity of larvae during the early and sensitive age of bee development. In the midgut of the larvae, develop acidophilic bacteria and other fungi as *A. apis* (chalkbrood), adapted to royal jelly. When the nurse bees prepare beebread mixing royal jelly, pollen and honey, the pH (acid to neutral) is modified. *A. apis* is spread with microorganisms associated with adult bees, pollen, and uncapped brood (Fig. 1b).

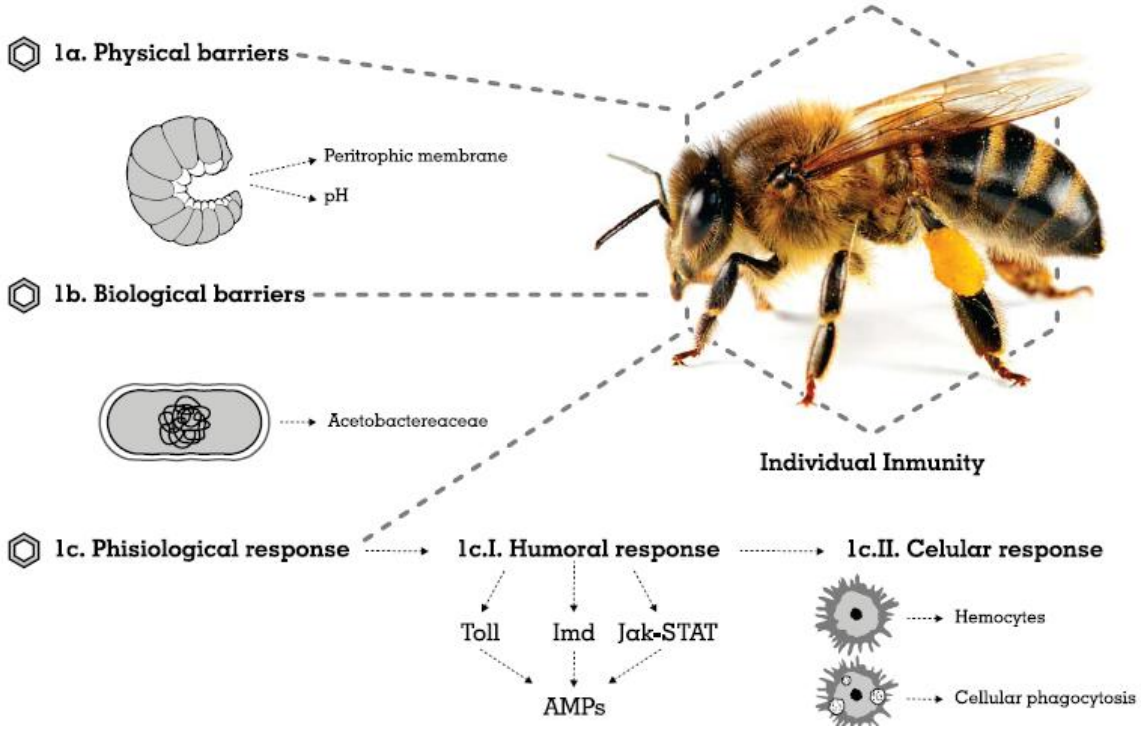

FIGURE 1: RESISTANCE MECHANISM OF HONEYBEE TO *A.apis*

3.1.3 Physiological response to fungi infections

3.1.3.1 Humoral Response System

Honeybees have paths interconnected to act before exposure to the pathogen. These pathways of recognition of the proteins produced by the invasion of the pathogen modulate the infection and produce other effector proteins that inhibit the pathogen. Among the recognition proteins, specificity toward pathogens can be achieved via differential binding properties to Microbe Associated Molecular Patterns (MAMP's).

When the honeybee discovers a foreign target, usually there are two signal transduction pathways (Toll and IMD) that resist different pathogen classes is triggered [38]. The Toll pathway is mobilized in response to Gram-positive and fungal infections, while Immune Deficiency (IMD) is mobilized by Gram-negative bacteria and infections of multiple classes associated with barrier membranes [33]. Toll and IMD have cross-talk between the two complementary pathways and the activation of one of them, can branch to the activation of other immune related signaling pathways, JNK and/or JAK/STAT. Both of these pathways belong to the intracellular nuclear factor-kB (NF-kB) related signal transduction pathway, which ultimately produces the AMPs [25, 29]. However, the proportion of amino-peptide synthesis and gene expression is influenced by various biotic and abiotic factors [39] (Fig. 1c. I).

3.1.3.2 Cellular Response System

Plasmatocytes and granulocytes adhere to foreign molecules and pathogens, and thus are the primary agents involved in phagocytosis and encapsulation process [30]. Oenocytoids produce phenoloxidase components involved in melanization. In honeybees, the fungal infections by *A. apis* are usually followed by host defenses that result in up regulation of antimicrobial peptides, melanization, cellular phagocytosis and encapsulation [33]. Fungal spores are rapidly attacked by the phagocytes. Cellular reactions are typically followed by humoral immune responses (Fig. 1c. II).

3.1.4 Metabolic status

Nutrient deficiency in honeybees can lead to reductions in adult survival and reduced brood development. Furthermore, the connection between nutrition and immunity is already well kwon. In this sense, the pollen´s essential aminoacids are needed for the synthesis of peptides in immune pathways and AMP, while the carbohydrates provide energy for metabolic processes associated with innate humoral and cellular immune reactions [40]. Honeybee larvae infected with *A. apis* activated their

immune system by down-regulation the major storage proteins, leading to depletion of nutritional resources [29]. It has been suggested that there is a trade-off between immune stimulation and expression of storage protein genes [41].

3.2 Social immunity mechanisms of honeybee to *A. apis* infection

Honeybees live in colonies with many individuals which increases the risk of transmission of pathogens. As social insects, honeybees have a number of defense mechanisms arising from assistance between individuals of the colony that help to prevent the spread of the disease. These defenses of the colony together will provide a system of "social immunity".

3.2.1 Social organization

Adult worker honeybees have division of labor correlated with age, called temporal polytheism. Therefore, they are performing multiple activities inside and outside the colony. The process of invasion of a pathogen to the colony implies that the pathogen must be transported actively or passively into the colony, established in the brood nest and distributed to all individuals. Trophallaxis activity, the ability of cleaning, the hygienic behavior and the nurse feeding, influences the speed and frequency of the pathogen and exposes larvae and adult bees to increased risk of disease transmission [42] (Fig. 2a).

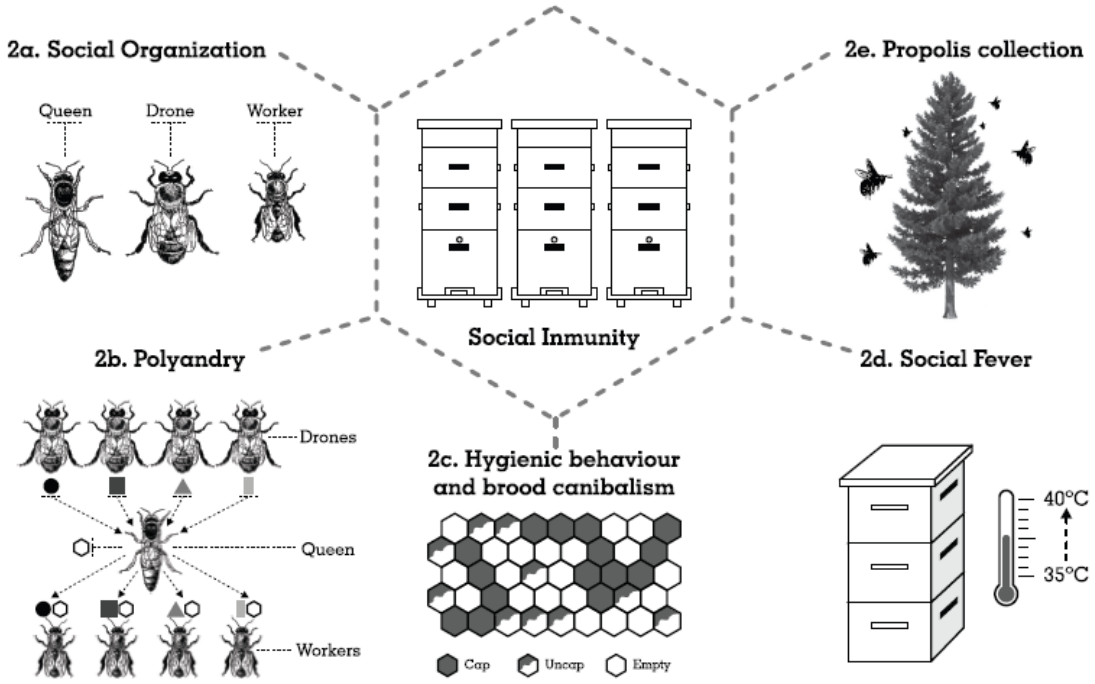

FIGURE 2: RESISTANCE MECHANISM OF BEE COLONIES TO *A.apis*

3.2.2 Polyandry

Polyandry occurs as a result of fertilization of the queen by more than 12 drones. As a result, the workers belong to patrilines or subfamilies [43]. This mechanism generates genetic diversity in honeybee larvae within colonies, produces variation in host immune system, potentially resulting in variation in susceptibility to infections, susceptibility to pathogen replication, or pathogen tolerance [44, 45] supports the hypothesis that queen honeybees cross with several drones, to increase the likelihood of pathogen resistance genes in breeding colony. Thus, the selection pressure of genetic diversity pathogens could have influenced the evolution of polyandry as most genetic diversity among colonies generates greater resistance to disease. In accordance, it was observed that honey bees infected with spores from dead larvae of numerous chalkbrood strains, showed patriline-specific resistance to chalkbrood infections [46] (Fig. 2b).

3.2.3 Hygienic behavior and brood cannibalism behavior

Hygienic behavior is a specific activity defined as to uncap and remove disease brood from combs before the pathogens reaches the infectious stage in the bee host [47]. Is discussed the bees´s age that handle hygienic behavior. Some studies propose that the workers are 15-18 days old. However, Pereira [48] indicated that hygienic behavior is executed similarly, between 2 -13 days old with young ages being more actively involved.

The hygienic behavior and the cannibalism larvae 10-11 days old, observed in brood cells total or partially uncapped by adult bees, are two important defense behaviors. These mechanisms confer resistance to disease, because the infected broods are eliminated before mummification [46]. Also, the detection of chalkbrood infected larvae is based on the honeybee's sensitive olfactory perception of the volatile compounds [49, 50] (Fig. 2c).

3.2.4 Fever social environment of the brood nest

The appropriate development of honeybee larvae and pupae is produced when the colony can maintain stable the temperature within the hive. Starks [51] found that bee colonies raised the temperature of the brood nest preventively when they were exposed to spores of *A. apis*, calling it "social fever behavior". They proposed that the short cooling time of the brood chamber increases the prevalence of infection *A. apis.* In contrast, Simone-Finstrom [43] suggested that the presence of *A. apis* spores alone cannot trigger initiation of fever behavior. It is possible that workers involved in the fever response are able to detect and respond to infected larvae (but not pathological), as seems to be the case in carrying honey bees hygienic behavior (Fig. 2d).

3.2.5 Propolis collection

The honeybees collect and deposit propolis in the nest walls of the hive. Propolis has antimicrobial properties and the colony probably uses it for that purpose. However, it is unclear whether the colony of honeybees collects propolis for physiological demand or because they need to protect themselves from the presence of pathogens. In that case, it would be a process of self-medication, a process that was observed in other insects [52, 53]. In this sense, it was observed that the presence of infected brood chakbrood increases the number of foraging bee's propolis, suggesting that the presence of spores of *A. apis* induce self-medicating of the colony [54]. Nevertheless, the most important function of the propolis envelope may be to act directly on the immune system, reducing the bees' need to activate the physiologically costly production of humoral immune responses [52] (Fig. 2e).

IV. THE INFLUENCE OF HONEY BEE DISEASE AND BIOCIDES IN *A. apis* INFECTIONS

4.1 Parasites and microorganisms

Hedtke [55] demonstrated the association between two pathogens and chalkbrood. *N. ceranae* infection in spring and *V. destructor* infestation in summer caused a significant increase in the outbreak of chalkbrood the following season. *V. destructor* feeds on hemolymph of larvae and adult bees, reducing protein metabolism, while, *N. ceranae* damages the gut epithelial tissue of the adult bee avoiding the absorption of proteins. Therefore, the production of royal jelly and adult bee longevity is reduced and may decrease the number of individuals in the colony, generating a stress cooling for brood that can trigger an outbreak of chalkbrood. Other studies were based on possible co-infections of the pathogenic fungus *A. apis* and other viruses, such as the Deformed Wing Virus (DWV), Black Queen Cell Virus (BQCV) and Acute Paralysis Virus of Israel (IAPV). Because these pathogens use the same route of entry to the bee, through the intestine, could increase the pathogenicity acting as mycoviruses (virus of fungi) [56].

4.2 Pesticides and antimicrobials

Recently was determined the presence of residues of insecticides, fungicides and herbicides in wax and pollen. Between the products were detected high levels of miticides usually employed by the beekeepers as fluvalinate, coumaphos, taufluvalinate, thymol. Other fungicides, herbicides and co-formulants are used in agrochemical formulations and spray tank adjuvants [57, 58]. The pesticides residues affect immune function, colony nutrition, pollen digestion, hemolymph protein levels, and increased virulence of pathogens [59]. The presence of fungicides such as boscalid, captan and myclobutanil could affect the survival of favorable fungi in the hive, suppressing its beneficial effect in the colony, probably favoring outbreaks of chalkbrood [60]. In relation to antibiotics, oxytetracycline does not increase the risk of chalkbrood in bee workers neither in the short nor medium term. However, simultaneous exposure to tau-fluvalinate and oxytetracycline blocks transporters multidrug resistance increasing the toxicity of fluvalinate.

V. CONTROL

5.1 Biological control

Bacillus subtilis, B. megaterium and *B. circulans* [61] have showed *in vitro* some inhibition activity against *A. apis.* In this way, it was demonstrated that *B. subtilis* and *Pseudomonas fluorescens* strains isolated from the gut of the honeybee, showed

the highest antagonistic activity against *A. apis* [62]. Also, Sabaté [63] suggested that *B. subtilis* secreting a fungicide substance. Symbionts can modify the host's immune system to improve efficiency of protection against pathogens. In this sense, probiotic *Lactobacillus* bacteria induced the expression of the antibacterial peptide abaecin in honeybee (*A. mellifera*) larvae [64]. Thus, symbiotic bacteria can activate the immune system of the insect host and thereby increase the efficiency of pathogen defense. Recently, Kwong [65] identified nine bacterial species clusters that are specific to bee gut microbiota.

5.2 Control with essential oils

The essential oils extracted from aromatic plants are an alternative to the use of chemicals, as they are effective for controlling *A. mellifera,* L. diseases. The *in vitro* activity of numerous essential oils was evaluated on *A. apis* strains.

Ansari [66], evaluated the activity of 27 plant essential oils against two isolates of *A. apis* and found that pepper was the most effective essential oil, with a minimal fungicidal concentration value of 50.0 µg/mL. Moreover, Kloucek [67] evaluates *in vitro* inhibitory activity of 70 essential oils (EOs) in the vapor phase for the control of chalkbrood disease. The greatest antifungal action was observed for EO vapors from *Armoracia rusticana*, *Thymus vulgaris*, *Cymbopogon flexosus*, *Origanum vulgare* and *Allium sativum.* Other authors tested essential oils in bee colonies to control ascosferosis [68]. However, although some researchers support the successful use in beehives to control various diseases of the honeybee, some difficulties arise in the colonies of *A. mellifera*, L., such as the replacement of queens [69]. Moreover, some toxicity was observed in studies related to acute oral toxicity of some essential oils on adult honeybees [70].

5.3 Genetic breeding

The first genetic analysis of the hygienic behavior, conducted by Rothenbuhler [71], proposed a two-gene model to explain phenotypic variance of the hygienic behavior. Later, Lapidge [72] detected seven genes associated with hygienic behavior by molecular techniques. These genes increase **genetic diversity** in bees, also can have an important function in reducing the likelihood of outbreaks of the disease [73]. The breeding programs for tolerance/resistance to honeybee diseases based on hygienic behavior began in the 1990s. Investigations in chalkbrood resistance were made by Gilliam [74]. They observed that the colonies of experience exhibited significant hygienic behavior reducing the numbers of fungal spores in stored food and comb wax. Even though, researchers in various countries reported an increase in hygienic behavior due to the constant selection of queens [75], genetic selection programs failed to improve resistance/tolerance to diseases of honeybees and among them chalkbrood.

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