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BACTERIAL CONTAMINATION OF HAEMOLYMPH IN EMERGING WORKER HONEYBEE (*APIS MELLIFERA* L) PARASITIZED BY VARROA DESTRUCTOR

SUMMARY

Varroa destructor is an obligatory ectoparasite of the honeybee (*Apis mellifera* L). The mites use their piercing mouth parts to suck out hemolymph from immature and adult bees caused direct damage (morphological, physiological abnormalities) and indirect damage due to microbial pathogens. The aim of this work was to research the bacterial microflora in hemolymph of emerging healthy and parasitized worker honeybee by *Varroa destructor*. For the isolation and identification of the bacteria, the morphological and biochemical tests were done. The results showed that the haemolymph of the healthy worker (*Apis mellifera* L) is free of bacteria. When the *V.destructor* breaks the cuticle, the microorganisms invade the hemolymph. The infested worker honeybees harbored a total 9 strains belonging to 6 strains of Bacillaceae (66,66%), 1 of Peanibacillaceae (11,11%) and 2 of Enterobacteriaceae (22,22%). Based on the Gallery API 20 E (Bio-Merieux), the genera of Bacillaceae and Peanibacillaceae included *Bacillus licheniformis* (4 strain), *Bacillus mycoide* (1strain), *Bacillus coagulans* (1 strain) and *Brevibacillus chohinensis* (1 strain) respectively. *Bacillus licheniformis* was for probably the most frequent species. The Enterobacteriaceae included *Aeromonas hydrophila* and *Pantoea sp.*

Keywords: Honeybee, *Apis mellifera* L, *Varroa destructor*, bacterial contamination. hemolymph.

INTRODUCTION

Varroa destructor (Anderson and Trueman, 2000) an obligatory ectoparasite of the honeybee (*Apis mellifera* L), has caused severe damage to populations of this species world-wide in recent years (Le Conte *et al.*, 2010). The direct negative effect of the *Varroa* on honeybee has been well documented (Weinberg and Madel, 1985; Daly *et al.*, 1988, Wienands and Madel, 1988; Marcangeli *et al.*, 1992; Bowen-Walker and Gunn, 2001; Contzen *et al.*, 2004; Yang and Cox-Foster, 2005; Belaïd and Doumandji, 2010; Belaïd *et al.*, 2017). However, in the recent years, scientists have diverted their attention towards the indirect effect by virus transmission, the foulbrood diseases and fungal infection (Hrabak, 2003;

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Benoit *et al.*, 2004; Tentcheva *et al.*, 2004, Hamdi *et al.*, 2011). Tentcheva *et al.*, (2004) reported that the infection of bees with the Deformed Wing Virus (DWV) was strongly linked to the presence of *Varroa*. De Rycke *et al.*, (2002) reported that *Varroa destructor* was capable of transporting spores of *Paenibacillus larvae* (the American foulbrood agent) to the surface of its body, thus allowing the parasite to participate in its propagation. The Fungi or spores of fungi are found on the surface of *V. destructor* (*Aspergillus flavus*, *Penicillium multicolor*, *Penicillium simplicissimum*, *Mucor ramosissimus*, *Mucor indicu*, *Mucor hiemalis* and *Ascospaera apis* (Benoit *et al.*, 2004). The cuticle itself constitutes an excellent barrier against parasite invasion. However, the damaging host integument during the feeding behavior of *Varroa* rendered the bees vulnerable to the microbial infections (Kanbar and Engels, 2005). To our knowledge, there are no reports about the bacterial microflora in haemolymph of honeybee parasitized by *Varroa destructor*. In this paper, the bacteria enable to be transmitted into honeybee haemocoel was investigated.

MATERIAL AND METHODS

Healthy and parasitized preemerging honeybees (*Apis mellifera* L) were collected from the brood of the apiary of Tizi Ouzou in early summer 2015. 1 μ l of haemolymph of the samples were diluted in 9 μ l sterile normal saline (1:10 μ l) immediately vortexed, then the haemolymph solution was plating on nutrient agar plates with the help of sterilized loop and incubated at 37° C. The plates were prepared in duplicate. Each different colony was subcultured to obtain pure culture. Selected strains were initially characterized by cell morphology and Gram's, endospore staining using the standard procedures. Primary identification was carried out according to Bergey's Manual of systematic Bacteriology (Holt *et al.*, 1994). Biochemical characteristics were tested with API 20E galleries (Biomerieux).

RESULTS AND DISCUSSION

The preliminary results about the occurrence of bacteria microflora in haemolymph of emerging healthy and parasitized worker honeybee (*Apis mellifera intermissa*) by *Varroa destructor* were shown in table 1, table 2 and Fig 1 (A, B).

The results showed that the haemolymph of the healthy emerging working bee was free of bacteria. As seen in table 1 and Fig 1A, the infested worker honeybees harbored 9 strains belonging to 6 strains (S1, S2, S3, S4, S5 and S6) of Bacillacea (66,66%), 1(S7) Peanibacillacea (11,11%) and 2 (S8 and S9) Enterobacteriaceae (22,22%). The bacteria Gram-positive rods endospore forming aerobes or facultative anaerobic isolated from the haemolymph of parasitized samples were classified according to Bergy in the family Bacillacea (S1, S2, S3, S4, S5 and S6). As seen in table 2 and Fig 1 B, 6 strains were isolated from the

haemolymph. The *Bacillus licheniformis* was for probably the most frequently species (44,44%) with 4 strains (S2, S3, S4 and S5) followed by *Bacillus mycoide* (1strain: S1) and *Bacillus coagulans* (1strain: S6) representing 11,11% of each one. In our study, generally, *Bacillus* sp was positive for the Vogues proskaeur reaction (VP). The isolate bacteria could produce catalase and oxidase. Also, the *Bacillus* sp was capable of using glucose, mannitol, arabinose as carbohydrates sources for growth. In our study, *Bacillus* sp was well represented in our data. One strain of *Brevibacillus chohinensis* (S7) was observed. The isolate was identified as Gram-positive, aerobic, spore-forming *Bacillus*.

According to Bergy's, the strains (S8 and S9) Gram-negative oxidase-positive rods non-sporulating facultative anaerobic capable of fermenting glucose were identified as members of the family Enterobacteriaceae. Based on the Gallery API 20 E (Bio-Merieux), the genera included *Aeromonas hydrophyla* (11,11%) and *Pantoea* sp (11,11%) for strains (S8 and S9) respectively.

Table1. Biochemical characteristics of isolated bacteria from the haemolymph in emerging worker honeybee parasitized by *V. destructor*.

	S1	S2	S3	S4	S5	S6	S7	S8	S9
Ortho-Nitro-phénol-Galactosidase (ONPG)	-	+	+	+	+	-	-	+	-
Arginine Di-Hydroxylase (ADH)	+	+	+	+	+	-	-	+	-
Lysin Di-Carboxylase (LDC)	+	+	+	+	+	-	-	-	-
Ornithine décarboxylase (ODC)	-	+	+	+	+	-	-	-	-
Citrate utilization test (CIT)	+	+	+	+	+	-	+	-	-
H ₂ S Production test	-	-	+	-	-	-	-	-	-
Uréase (URE)	+	+	+	-	-	-	-	-	-
Tryptophane Désaminase (TDA)	+	+	+	+	+	-	-	-	-
Indol production (IND)	-	+	-	+	-	-	-	-	-
Acetoin production (VP)	+	+	+	+	+	+	+	+	+
Gelatinase (GEL)	+	+	+	+	+	-	-	+	-
Glucose (GL)	+	+	+	+	+	+	+	+	+
Mannose (MANE)	-	+	-	+	-	-	+	+	+
Inositol (INO)	+	+	-	-	+	-	-	-	-
Sorbitol (SOR)	-	-	-	+	+	-	+	-	+
Rhamnose (RHA)	-	+	-	+	-	+	-	+	+
Saccharose (SAC)	-	+	-	-	-	+	+	-	+
Melibiose (MEL)	+	+	+	+	-	+	+	-	+
Amydaline (AMY)	-	-	+	-	+	-	+	-	+
Arabinose (ARA)	+	+	+	+	+	+	+	+	+
Mannitol (MAN)	+	+	+	+	+	-	-	+	+
Production NO ₂	+	-	-	+	-	+	-	+	-
N ₂ Nitrate reduction test	-	+	+	-	+	+	+	-	+
Oxidase test (OXY)	+	+	+	+	-	+	+	+	+
Catalase test (CAT)	-	+	+	+	+	-	+	-	+
Motility (MOT)	-	+	+	+	-	+	+	-	-

(+): positive test ;(-): negative test

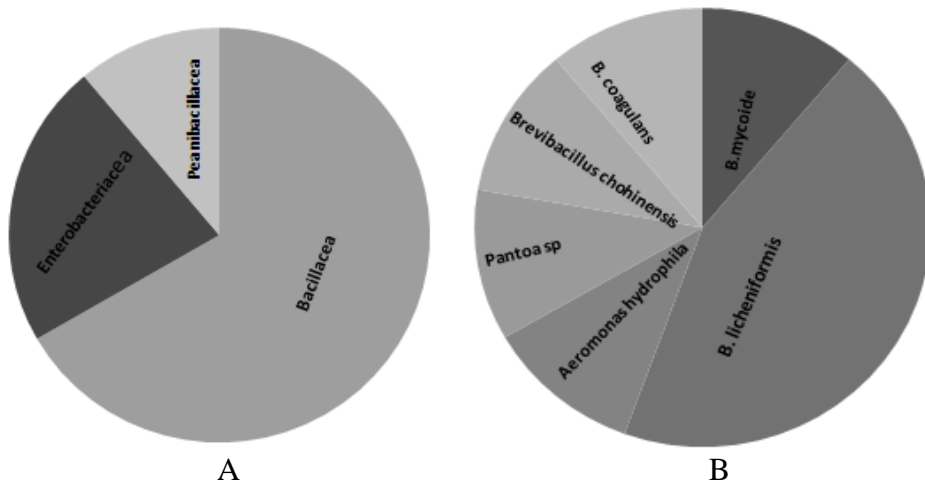


Figure 1: Occurrence of bacteria family (A) and species (B) in haemolymph of emerging worker honeybee infested by *V.destructor*

Table 2: The identification probabilities for isolated Strains

		Probability
S1	<i>Bacillus mycoide</i>	69,43%
S2	<i>Bacillus licheniformis</i>	99,74%
S3	<i>Bacillus licheniformis</i>	66,97%
S4	<i>Bacillus licheniformis</i>	88,65%
S5	<i>Bacillus licheniformis</i>	66,97%
S6	<i>Bacillus coagulans</i>	97,95%
S7	<i>Brevibacillus chohinensis</i>	79,02%
S8	<i>Aeromonas hydrophila</i>	79,31%
S9	<i>Pantoa sp</i>	63,25%

In our work, the haemolymph of the healthy emerging worker honeybee was free of bacteria. According to Tubiash *et al.*, (1975), it was generally assumed that the circulatory system of healthy animals was sterile. The body surface of adult honey bees is relatively free of bacteria, likely due to grooming behavior (Gilliam, 1997). According to Keller *et al.*, (2013), the pre-adult honeybee was almost sterile systems. In invertebrates, the circulating haemocyte has a major role in the protection of the animal against aggressive microorganisms by participating in recognition, melanisation, phagocytosis and cytotoxic activities (Jiravanichpaisal *et al.*, 2006). The presence of bacteria is usually considered to be a sign of disease (Tubiash *et al.*, 1975). *Varroa* bee hive attack is a serious and common problem in beekeeping. The mite gets attached to the body of the bee and brood and weakens the bee by repeated sucking of haemolymph (Vanikova *et al.*, 2015). When the *Varroa destructor* breaks the cuticle, the microorganisms invade the haemolymph (Kanbar and Engels, 2005).

Many scientists increased attention to the microflora of the ectoparasites and their role as vector of viral, fungal and bacterial disease (Ball, 1985; Gliński and Jarosz, 1992; Ball, 1997; Bowen Walker *et al.*, 1999; Chen *et al.*, 2004; Maddaloni and Pascual, 2015; Vanikova *et al.*, 2015).

To our knowledge, this work was the first report studying the role of the mite as vector to bacterial microflora in the haemolymph of honeybees.

In the study, the results showed that the Bacillaceae was the most frequently present in haemolymph of worker honeybees (*Apis mellifera* L) followed by Enterobacteriaceae and Peanibacillaceae. According to De Rycke *et al.*, (2002), *Varroa destructor* could play a role in the transmission of *Paenibacillus larvae* spores, *Bacillus* (formerly known as *Bacillus larvae*) responsible for American foulbrood from infected to healthy bee colonies. Hrabak (2003) identified the genus *Staphylococcus albus* and *Enterobacter cloacae* associated to the ectoparasite mite. Tsagou *et al.*, (2004) isolated bacterial strains from *Varroa destructor* belonging to Bacillaceae (*Bacillus* sp) and Micrococcaceae. Hubert *et al.*, (2015) found *Morganella* sp, *Enterococcus* sp, *Pseudomonas* sp, *Rahnella* sp, *Erwinia* sp and *Arsenophonus* sp. Maddaloni and Pascual (2015) reported the occurrence of *Bacillus subtilis*, *Pseudomonas syringae*, *Pantoea agglomerans*, *Pantoea vagans*, *Paenibacillus wynnii*, *Staphylococcus caprae*, *Bifidobacterium asteroides*, *Staphylococcus caprae* and *Micrococcus luteus* associated to *Varroa destructor*. Vanikova *et al.*, 2015, recorded *Microbacterium* sp and *Bacillus* sp. Because microorganisms are ubiquitous in nature, it is not surprising to find a variety of them associated with insects. Generally, the kinds of microorganisms involved with an insect reflect the microflora of the surrounding environment (Ingraham *et al.*, 1975). The pathogenicity is largely associated with entry to the hemocoel either through a wound in the exoskeleton or more generally through the peritrophic membrane of the gut (Priest, 2000). Bacteria that fall within the category of insect pathogens, families characteristic of entomopathogens, are: Bacillaceae, Enterobacteriaceae, Streptococcaceae (Hrabak, 2003), Pseudomonadaceae, Lactobacillaceae, micrococcaceae (Dhanasekaran and Thangar, 2014). It has been suggested that the presence of bacteria in the haemolymph is indicative of septicemia and a common sequelae to stress (Lightner, 1977, 1988 in Gomez-Gil *et al.*, 1998). According to Hubert *et al.*, (2015), the mite could be reservoirs of the pathogenic bacteria in the apicultures.

CONCLUSIONS

Through our experience, apparently the haemolymph of the healthy emerging worker honeybee (*Apis mellifera* L) is free for bacteria. However, the bacterial contamination enable to be transmitted into honeybee hemocoel are: *Bacillus licheniformis* (4 strains), *Bacillus mycoide* (1strain), *Bacillus coagulans* (1strain), *Brevibacillus chohinensis* (1strain), *Aeromonas hydrophila* (1 strain) and *Pantoea* sp (1 strain). The knowledge of these bacteria microflora opens up a new perspective for integrated control of this parasite, which decimates bee colonies yearly.

REFERENCES

- Anderson D. L., Trueman J. W. H. (2000). *Varroa jacobsoni* is more than one species. *Experim. appl. acarol.*, 24 : 165 - 189.
- Ball B.V. (1985). Acute paralysis virus isolated from honeybee colonies infested with *Varroa jacobsoni*. *J. Apic. Res.* 24: 115–119.
- Ball B.V. (1997). Secondary infections and diseases associated with *Varroa jacobsoni*. *Options Méditerranéennes*, 21 :49–58.
- Belaïd M. et Doumandji S., (2010). Effet du *Varroa destructor* sur la morphométrie alaire et sur les composants du système immunitaire de l'abeille ouvrière *Apis mellifera intermissa*. *Lebanese Science Journal*, 11 (1): 84-90.
- Belaïd M., Acheuk F., Mohand Kaci H., Benzina F., Bennour M. (2017). The effect of *Varroa* mite (*Varroa destructor* Anderson and Trueman, 2000) on morphometry and cuticle component of the worker honeybees (*Apis mellifera* Linnaeus, 1758). 52nd Croatia and 12th international symposium on agriculture; February12-17, Dubrovnik, Croatia, 393-396.
- Benoit J.B., Yoder J.A., Sammataro D., Zettler L.W. (2004). Mycoflora and fungal vector capacity of the parasitic mite *Varroa destructor* (Mesostigmata: Varroidae) in honey bee (Hymenoptera: Apidae) colonies. *Int. J. Acarol.* 30(2): 103-106.
- Bowen-Walker P.L., Martin S.J., Gunn A. (1999). The transmission of deformed wing virus between honeybees (*Apis mellifera* L.) by the ectoparasitic mite *Varroa jacobsoni* Oud. *J. Invertebr. Pathol* 73(1): 101–106.
- Bowen-Walker P.L., Gunn A. (2001). The effect of the ectoparasitic mite, *Varroa destructor* on adult worker honeybee (*Apis mellifera*) emergence weights, water, protein, carbohydrate, and lipid levels. *Entomol. Exp. Appl.*, 101: 207–217.
- Chen Y.P., Pettis J.S., Evans J.D., Kramer M., Feldlaufer M.F. (2004). Transmission of Kashmir bee virus by the ectoparasitic mite *Varroa destructor*. *Apidologie.* 35(4): 441–448.
- Contzen C., Garedeu A., Lamprecht I., Schmalz I. (2004). Calorimetric and biochemical investigations on the influence of the parasitic mite *Varroa destructor* in the development of honeybee. *Thermochimica Acta*, 415 (1-2): 115 – 121
- Daly H.V., De Jong D., Stone N. D. (1988). Effect of parasitism by *Varroa jacobsoni* on morphometrics of Africanised worker honeybees. *J. Apicult. Res.*, 27 (2): 126 - 130.
- De Rycke P.H., Joubert J.J., Hosseinian S.H., Jacobs F.J. (2002). The possible role of *Varroa destructor* in the spreading of American foulbrood among apiaries. *Exp. Appl. Acarol.* 27(4): 313–318.
- Dhanasekaran D., Thangar R. (2014). Microbial secondary metabolites are an alternative approaches against insect vector to prevent zoonotic diseases. *Asian pacific of tropical disease.* 4(4):253-261.
- Gilliam M. (1997). Identification and roles of non pathogenic microflora associated with honeybees. *FEMS Microbiology letters.* 155: 1-10.
- Gliński Z., Jarosz J. (1992). *Varroa jacobsoni* as a carrier of bacterial infections to a recipient bee host. *Apidologie*, 23(1), 25–31.
- Gomez-Gil B., Tron-Mayen, Roque A, Turbull J.F, Inglis V, Guerra-Flores A.L. (1998). Species of *Vibrio* isolated from hepatopancreas, haemolymph and digestive tract of a population of haemolymph juvenile *Penaeus vannamei*. *Aquaculture*, 163, 1-9.
- Hamdi C.A. , Balloi J. , Essanaa E., Crotti E., Gonella N., Raddadi I., Ricci A., Boudabous S., Borin A., Manino C., Bandi A., Alma D., Daffonchio A., Cherif.(2011). Gut microbiome dysbiosis and honeybee health. *J. Appl. Entomol.* 135: 524–533.

- Holt J.G., Kreig N.R., Sneath P.H.A., Staley J.T., Williams S.T. (1994). Bergy's manual of systematic bacteriology. Ninth edition, pp. 151-168.
- Hrabák J. (2003). The microorganisms isolated from the mites *Varroa destructor* and the verification of their pathogenity. Standing Commission of Bee Pathology. Apiacta. XXXVIII Congresso Apimondia. Ljubljana.
- Hubert J., Erban T., Kamler M., Kopecky J., Nesvorna M., Hejdankova S., Titera D., Tyl J., Zurek L. (2015). Bacteria detected in the honeybee parasitic mite *Varroa destructor* collected from beehive winter debris. *J Appl Microbiol*, 119 (3): 640-654.
- Jiravanichpaisal P., Lee B. L., Soderhall K. (2006). Cell- mediated immunity in arthropods: Hematopoiesis, coagulation, melanisation and opsonisation. *Immunobiology*, 211: 213-236.
- Kanbar G., Engels W. (2005). Communal use of integumental wounds in honey bee (*Apis mellifera*) pupae multiply infested by the ectoparasitic mite *Varroa destructor*. *Genet Mol Res.* 4(3):465-72.
- Keller A., Grimmer G., Steffan-Dewenter I. (2013). Diverse microbiota identified in whole intact nest chambers of the red mason bee *Osmia bicornis* (Linnaeus 1758). *Plos One* 8(10): e78296. doi:10.1371
- Le Conte Y., Ellis M., Ritter W. (2010). *Varroa* mites and honey bee health: can *Varroa* explain part of the colony losses?. *Apidologie*, 41(3): 353–363.
- Maddaloni M., Pascual D.W. (2015). Isolation of oxalotrophic bacteria associated with *Varroa destructor* mites. *Lett Appl Microbiol*, 61(5):411-7.
- Marcangeli J., Monetti L., Fernandez N. (1992). Malformations produced by *Varroa jacobsoni* on *Apis mellifera* in the province of Buenos Aires, Argentina. *Apidologie*, 23 (5): 399 - 402.
- Priest F.G. (2000). Biodiversity of the entomopathogenic, endosporeforming bacteria in L.-F. Charles et al. (eds.), *Entomopathogenic Bacteria*, pp1-22.
- Tentcheva D., Gauthier L., Zappula N., Dainat B., Cousserans F., Colin M.E., Bergoin M. (2004). Prevalence and seasonal variations of six bee viruses in *Apis mellifera* L. and *Varroa destructor* mite populations in France. *Appl. Environ. Microbiol.*, 70 (12) : 7 185 - 7 191.
- Tsagou V., Lianou A., Lazarakis D., Emmanoue N., Aggelis G. (2004). Newly isolated bacterial strains belonging to Bacillaceae (*Bacillus* sp.) and Micrococcaceae accelerate death of the honey bee mite, *Varroa destructor* (*V. jacobsoni*), in laboratory assays. *Biotechnology letters*, 26: 529–532.
- Tubiash H.S, Sizemore R.K., Colwell R. (1975). Bacterial flora of the hemolymph of the Blue Crab, *Callinectes sapidus*: Most probable numbers. *Applied Microbiology*, 29(3): 388-392.
- Vanikova S., Noskova A., Pristas P., Jana Judova J., Javorsky P. (2015). Heterotrophic bacteria associated with *Varroa destructor* mite. *Apidologie*, 46 (3): 369-379.
- Wienands A., Madel G. (1988). Haemocytes of the honeybee, *Apis mellifera*, and their changes by varroatosis (Hymenoptera, Apidae). *Entomologia Generalis*, 14 : 81 - 92.
- Weinberg K.P., Madel G. (1985). The influence of the mite *Varroa jacobsoni* Oud on the protein concentration and the haemolymph volume of Brood of worker bees and Drones of the honey bee, *Apis mellifera* L. *Apidologie*, 16 (4): 421 – 436.
- Yang X., Cox-Foster D. (2005). Impact of an ectoparasite on the immunity and pathology of an invertebrate: Evidence for host immunosuppression and viral amplification. *Proceeding. Nati. acad. sci.*, 102 (21): 7470 - 7475.