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Microbial investigation on honey bee larvae showing atypical symptoms of European foulbrood

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Abstract

Culture-dependent and culture-independent techniques were applied to investigate the bacterial communities occurring in 5-6 days-old honey bee larvae (*Apis mellifera* L.), with evident symptoms of disease and healthy-looking ones. Samples were collected by the Bee Emergency Service Team (BeeNet Project) from two apiaries located in different geographical sites (North and South Italy). Observed symptoms were atypical, but very close to those attributed to the European foulbrood (EFB) and to the American foulbrood (AFB), which are severe diseases affecting honey bee larvae worldwide. Isolates from diseased larvae were identified as *Enterococcus faecalis* Schleifer et Kilpper-Balz and *Paenibacillus dendritiformis* Tcherpakov *et al*.. *Melissococcus plutonius* (ex White) Bailey et Collins corrig. Truper et de Clari, the causative agent of EFB, was detected by polymerase chain reaction in both diseased and healthy-looking larval samples, whereas *Paenibacillus larvae* White, the causative agent of the AFB, failed to be detected. Microbial profiles obtained by denaturing gradient gel electrophoresis did not show relevant differences among samples, thus evidencing that the healthy-looking samples were partially affected. Besides confirming the presence of *E. faecalis* and *P. dendritiformis,* as found by plate count, the technique confirmed the presence of *M. plutonius* in all samples. The study has evidenced that honey bee larvae were affected by the EFB, with the presence of an atypical *Paenibacillus* species as second invader, which presumably confers a different symptomatology to the diseased brood.

Key words: European foulbrood, American foulbrood, honey bee larvae, atypical symptoms, *Paenibacillus dendritiformis*, denaturing gradient gel electrophoresis (DGGE).

Introduction

Honey bees (*Apis mellifera* L.) are one of the insect species more relevant for humans as pollinator for the production of many fruits, vegetables and stimulant crops (Abrol, 2011). In recent years, larvae and adult honey bees are subjected to different kind of biotic and abiotic stresses, leading to a severe colony loss and decrease in hive products with large economic damages. The parasites and pathogens, affecting larvae and bee health, include mites (*Varroa destructor* Anderson et Truemann), microsporidia (*Nosema* spp.), fungi [*Ascosphaera apis* (Maassen ex Claussen) Olive et Spiltoir], bacteria (*Paenibacillus larvae* White, *Melissococcus plutonius* (ex White) Bailey et Collins corrig. Truper et de Clari, and viruses (Genersch, 2010).

The most known diseases affecting the bee larval stage are the European foulbrood (EFB) and the American foulbrood (AFB). EFB is a severe bacterial brood disease, caused by the Gram positive bacterium *M. plutonius*. The disease is widely distributed, leading to brood losses and the consequent colony collapse (Bailey and Ball, 1991). The ingestion of contaminated food induces the proliferation of the pathogen in the larval midgut and its consumption of the larval food. Larvae are susceptible at any stage before cell capping and usually death occurs during the 4^{th}-5^{th} day of life. Infected larvae die from starvation (Bailey, 1983), twisted around the cell wall or stretched out, and are then decomposed by secondary invaders like *Paenibacillus alvei* Cheshire et Cheyne and *Enterococcus faecalis* Schleifer et Kilpper-Balz, two saprophytic bacteria frequently associated with EFB (Forsgren, 2010). Diseased larvae are easily identifiable, since larvae move in the brood cell leaving off the normal coiled position. Moreover, their colour changes from pearly white to yellow, then brown and finally, when they decompose, greyish black (Bailey, 1960). Affected brood may have a very stale or sour odour, sometimes acidic, like vinegar, but often there is no smell (OIE, 2008).

The causative agent of AFB is the Gram positive, spore-forming bacterium *P. larvae* (Genersch *et al*., 2006), which contaminates the first instar larvae leading them to death after cell capping. *P. larvae* passes from the midgut throughout the epithelium and invades the hemocoel (Davidson, 1973; Bailey and Ball, 1991). It is reported that during the vegetative growth and infection it secretes highly active extracellular proteases (Hrabak and Martinek, 2007), which probably cause the degradation of the epithelial barrier, thereby allowing the hemocoel invasion. In a second stage the larvae become a brownish, semi-fluid, glue-like colloid (ropy stage) releasing a putrid smell. The ropy aspect (dead larvae adhere and form a thread span when touched with a wooden stick) confirmed the presence of AFB. Finally, the larval remains dry out to a hard scale (foulbrood scale), which tightly adheres to the lower cell wall. The scales contain millions of spores, which could distribute the infection for many years within and between colonies (Bailey and Ball, 1991).

The current work was performed in spring 2012, following the warning of beekeepers from all Italy, denunciating, to the Bee Emergency Service Team (BeeNet Project) (BEST, 2014), misleading symptoms very close to both EFB and AFB, affecting honey bee larvae. Suspected diseased brood looked like a mosaic with open cells (typical symptoms of EFB) and pierced cells (typical symptoms of AFB). It was possible to observe si-

multaneously in the same frame, dead larvae before and after the cell capping. Compared with the classic EFB symptoms, the larvae often died 2-3 days later, closer to AFB death timing. Uncapped diseased larvae quickly lost their shape and then liquefied in the bottom of the cells. Larvae had white-ivory colour without any characteristic smell. Partially capped cells showed diseased larvae (6^{th}-7^{th} day) softly adhering to the lower cell wall; if touched with a wooden stick the larvae explode realising a non-colloid, not smelling, white-brown liquid. Dried scales (similar to those observed in the AFB) were easily removable. To understand deeply the observed symptomatology, available material of diseased and healthy-looking larvae was sampled from two apiaries, one in North Italy and one in South Italy. Culture dependent (plate-count, 16S rDNA sequencing) and independent techniques (PCR-DGGE) were applied to allow the most accurate microbiological investigation.

Materials and methods

Larvae sampling

Honey bee larvae (5^{th}-6^{th} day of life) were collected from infected hives in two apiaries located one at North and one at South Italy. Based on their aspect and position, the larval material was pooled and samples were classified as follows: NHHL (healthy-looking honey bee larvae from North Italy) NDHL (diseased honey bee larvae from North Italy), SHHL (healthy-looking honey bee larvae from South Italy), SDHL (diseased honey bee larvae from South Italy).

Bacteria isolation and enumeration from diseased honey bee larvae

One gram of pooled diseased honey bee larvae, previously washed in sterile water and homogenised with a plastic pestle, was dissolved in 9 ml of phosphate-buffered saline (PBS) and tenfold serial dilution prepared. For the presumable detection of *M. plutonius*, the resulting suspension was streaked out onto SYPG agar (Bailey and Ball, 1991) and anaerobically incubated at 35 ± 1 °C for 3-5 days. Isolation and enumeration of *P. larvae* was attempted on MYPGP agar (Dingman and Stahly, 1983) after a pasteurization treatment on NDHL and SDHL samples (NDHL-P and SDHL-P) at 80 °C for 10 min (aerobic incubation at 35 ± 1 °C, 24 h). Experiments were performed in triplicate and results were expressed as mean $\log_{10}$ of colony-forming units (cfu) per gram of triplicates ± standard deviation (SD). Between 20 and 40 colonies from each sample and from both media (SYPG and MYPGP agar), randomly selected, were re-streaked and purified. For long-term storage, purified isolates were stored at −80 °C.

DNA extraction

DNA was extracted from isolated bacterial colonies, from *M. plutonius* ATCC 35311, *P. larvae* ATCC 9545 and from *P. alvei* ATCC 6344 using the InstaGene Matrix DNA extraction kit following the manufacturer's instructions (Bio-Rad, Hercules, CA, USA). Total genomic DNA from pooled samples of diseased and healthy-looking larvae was extracted in duplicate with the QIAamp DNA stool kit (Qiagen, West Sussex, UK). Following extraction, the purity and concentration of DNA were determined by measuring the absorbance at 260 and 280 nm (Infinite® 200 PRO NanoQuant, Tecan, Männedorf, Switzerland). The DNA was stored at −20 °C.

BOX-PCR analysis

Bacterial isolates were fingerprinted by BOX-PCR. The reaction was carried out in a 30 µl volume containing 1 U Taq DNA polymerase (AmpliTaq Gold, Applied Biosystems, Foster City, CA, USA), 3 µl 10X PCR Gold Buffer (Applied Biosystems), 200 µM of each dNTPs (Fermentas International Inc., Thermo Fisher Scientific Inc., Waltham, MA, USA), 2 mM $MgCl_2$ (Applied Biosystems), 0.4 µM of primer BOXA1R (5'-CTACGGCAAGGCGACGCTGACG-3') (Eurofins Genomics, Ebersberg, Germany), 0.1% (wt/vol) Bovine Serum Albumin (BSA, Fermentas), 2 µl of DNA template, and sterile MilliQ water. The PCR reaction was performed on a TGradient Biometra thermocycler (Biotron, Göttingen, Germany) under the following thermocycling program: 7 min of initial denaturation at 95 °C, 30 cycles of 94 °C for 1 min, 53 °C for 1 min, 72 °C for 3 min followed by a final elongation step of 72 °C for 10 min. After electrophoresis (2% w/v agarose gel at 75 V for four hours), gels were stained with ethidium bromide and visualized with the gel documentation system Gel DocTM X^{+}R (Bio-Rad). A dendrogram was constructed using the Dice similarity coefficient and the UPGMA (unweighted pair group method with arithmetic mean) algorithm with GelCompar II software, version 6.6 (Applied Maths, Sint-Martens-Latem, Belgium).

16S rDNA amplification

Representative isolates (SDHL-5 and NDHL-P1) were selected after BOX-PCR analysis and the 16S rDNA amplification was performed with universal primers 27f and 1492r, according to Gaggìa *et al*. (2013). The 50 µl reaction mixture contained 1X PCR Gold Buffer (Applied Biosystems), 200 µM of each dNTPs (Fermentas) 0.2 µM of each primer (Eurofins Genomics), 1.5 mM $MgCl_2$ (Fermentas), 1 U Taq polymerase (AmpliTaq Gold, Applied Biosystems), 4 µl of DNA template (20-50 ng/µl) and sterile MilliQ water. The PCR reaction was performed on a TGradient Biometra thermocycler (Biotron, Göttingen, Germany) under the following thermocycling program: 7 min of initial denaturation at 95 °C, 30 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min and a final extension step at 72 °C for 7 min. After electrophoresis (1.5% w/v agarose gel at 75 V), gels were stained with ethidium bromide and visualized with the gel documentation system Gel DocTM XR (Bio-Rad).

Detection of *M. plutonius* and *P. larvae* by species-specific PCR

A PCR method was used to selectively amplify the 16S rDNA of *M. plutonius* from a pure culture (ATCC 35311) and from DNA samples (NDHL, SDHL, NHHL, SHHL), according to Govan *et al*. (1998). Detection of

P. larvae from the same samples was performed according to Bakonyi *et al*. (2003) with a modified annealing temperature, which was set at 55 °C. *P. larvae* ATCC 9545 was used as positive control. The molecular weights of the PCR products were determined by electrophoresis in a 1.5% agarose, stained with ethidium bromide and visualized with the gel documentation system Gel DocTM XR (Bio-Rad).

PCR-DGGE analysis

The V3 region of the 16S rRNA gene (rDNA) of the four samples in duplicate (NDHL, SDHL, NHHL, SHHL,) was amplified by PCR with the universal primer set HDA1-GC (5′-**CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG G**AC TCC TAC GGG AGG CAG CAG T-3′) and HDA2 (5′-GTA TTA CCG CGG CTG CTG GCA-3′) (Walter *et al.*, 2000). An identification ladder was also prepared with the DNA of the strains *M. plutonius* ATCC 35311, *E. faecalis* SDHL-5, and *Paenibacillus dendritiformis* Tcherpakov *et al*. NDHL-P1. The reaction was carried out in a 50 µl volume containing 1.5 U AmpliTaq Gold DNA polymerase (Applied Biosystems), 5 µl of 10X PCR Gold Buffer (Applied Biosystem), 200 µM of each deoxynucleotide triphosphate (Fermentas), 1.50 mM $MgCl_2$ (Fermentas), 0.45 µM of each primer (Eurofins Operon), 2.5% (w/v) bovine serum albumin (BSA; Fermentas), 4 µl DNA template, and sterile MilliQ water for adjustment of the volume to 50 µl. The PCR reaction was performed on a TGradient Biometra thermocycler (Biotron, Göttingen, Germany) under the following thermocycling program: 7 min initial denaturation at 95 °C; 35 cycles of 95 °C for 30 s, 54 °C for 60 s, 72 °C for 40 s; followed by a final elongation step of 72 °C for 7 min. The size and amount of the PCR products were estimated by analysing 5 µl of samples by agarose gel (1.5% w/v) electrophoresis and ethidium bromide staining.

The DGGE analysis was performed as first described by Muyzer *et al*. (1993), using a DCode System apparatus (Bio-Rad). Polyacrylamide gels [8% (w/v) acrylamide:bisacrylamide (37.5 : 1) (Bio-Rad)] in 1X Tris-Acetate-EDTA (TAE) buffer were prepared using a Bio-Rad Gradient Delivery System (Model 475, Bio-Rad), using solutions containing 40-60% denaturant [100% denaturant corresponds to 7 M urea (Sigma-Aldrich, Milan, Italy) and 40% (v/v) formamide (Sigma-Aldrich)]. The electrophoresis was run at 55 V for 16 h at 60 °C. Gels were stained in a solution of 1X SYBR-Green I (Sigma-Aldrich) in 1X TAE for 20 min and their images captured in UV transillumination with Gel Doc™ XR apparatus (Bio-Rad). Cluster analysis was performed with the software GelCompar II version 6.6 (Applied Maths), by the UPGMA algorithm based on the Pearson correlation coefficient with an optimization coefficient of 1%.

Selected bands, particularly those migrated at the same distance of the reference species, were cut from the gel with a sterile scalpel and DNA was eluted by incubating overnight the gel fragments in 50 µl of sterile deionised water at 4 °C. 2 µl of the solution were then used as template to re-amplify the band fragments with the same PCR condition describe above. After amplification and repeated DGGE, purity and co-mobility with amplified DNA obtained directly from larvae samples were assured. Bands were excised again and after overnight elution in sterile deionized water, an amplification without GC-clamp was performed.

Sequence analysis of 16S rDNA of bacterial isolates and DGGE bands

The amplified 16S rDNA from the isolated strains and the obtained amplicons from DGGE bands were then purified (PCR clean-up; Macherey-Nagel GmbH & Co. KG, Germany) and sequenced (Eurofins Operon) with primers 27f and 1492r and HDA-2 respectively. Sequence chromatograms were edited and analysed by using Finch TV software version 1.4.0 (Geospiza Inc., Seattle, WA, USA) and percentage of similarity was determined searching against the NCBI GenBank database using megablast algorithm (http://www.ncbi.nlm.nih.gov/BLAST/).

Results

Plate count, BOX-PCR and sequence analysis of 16S rDNA

The results of viable counts obtained in SYPG and MYPGP agar from diseased larvae samples are reported below, indicating the average of triplicate expressed as $\log_{10}$ (cfu/g of sample) ± standard deviation. High colony counts were obtained from SYPG agar in SDHL (8.75 ± 0.05), whereas less colonies were obtained in plates referred to NDHL samples (3.37 ± 0.04). Enumeration of sporogenic bacteria (NDHL-P and SDHL-P samples) was comparable in both geographical areas (4.57 ± 0.08 vs 5.71 ± 0.02). The cluster analysis of BOX-PCR of all isolates (NDHL, NDHL-P, SDHL and SDHL-P) evidenced two clusters (similarity of 30%) (figure 1). All of the isolates on SYPG agar, belonging to NDHL and SDHL samples, displayed a unique fingerprinting (100% similarity), as well as isolates from MYPGP agar NDHL-P and SDHL-P, whose profiles were 100% similar. The strains SDHL-5 and NDHL-P1 (one from each cluster) were identified by sequence analysis of the 16S rRNA gene, based on the closest match on GenBank database. SDHL-5 sequence was ascribed to *E. faecalis* (100% similarity; accession number KR073926) while the strain NHDL-P1 was identified as *P. dendritiformis* (100% similarity; accession number KR073927).

M. plutonius and *P. larvae* detection by species-specific PCR

Results of the amplification reaction for the detection of *M. plutonius* are shown in figure 2a.

All the analysed samples (in duplicate), including the positive control *M. plutonius* ATCC 35311, produced a band of 812 bp. On the other hand, the amplified product was absent in the negative control (*E. faecalis* SDHL-5). With regard to *P. larvae*, all samples were negative except for the positive control *P. larvae* ATCC 9545 (figure 2b).

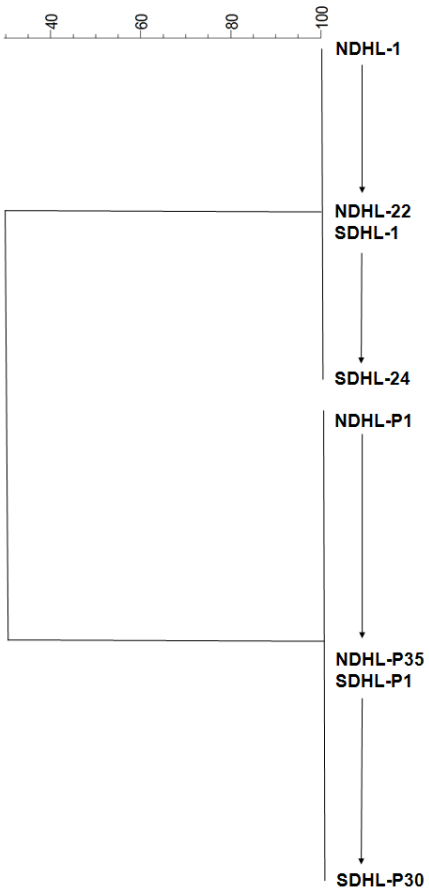

Figure 1. UPGMA dendrogram of bacterial isolates from SYPG and MYPGP agar. NDHL: diseased honey bee larvae from North Italy; SDHL: diseased honey bee larvae from South Italy; NDHL-P: diseased honey bee larvae from North Italy after pasteurization; SDHL-P: diseased honey bee larvae from South Italy after pasteurization.

PCR-DGGE and band sequencing

The PCR-DGGE analysis on diseased and healthy larval duplicate samples showed profiles with a few bands. UPGMA dendrogram and bacterial communities fingerprinting are shown in figure 3a and 3b, respectively. Overall, the analysis revealed high similarity of the DGGE patterns obtained from each of the two replicates. The cluster analysis showed a distinct division between samples of South Italy and North Italy. The diseased samples in both geographical area clustered separately from healthy samples (similarity less than 60% and 85% respectively).

Sequencing results of excised bands (figure 3b) are shown in table 1. Bands 1, 3, 4 and 5 belonged to different species of *Lactobacillus*, detectable in all samples, mainly in SHHL and SDHL. Interestingly, bands 6, 8 and 10 showed a migration distance comparable to the reference strains in the ladder profile (*E. faecalis* SDHL-5, *M. plutonius* ATCC 35311 and *P. dendritiformis* NDHL-P1). Band 6, common to all profiles, was identified as *M. plutonius*. Band 8, excised from sample SDHL was identified as *E. faecalis*. Bands migrating at the same level, although showing a decreased intensity, were also detected in SHHL. Finally, band 10 excised from SDHL, was identified as *Paenibacillus* spp.. A weak band migrating at the same distance is also present in NDHL. Band 7 (NDHL) was identified as *Gilliamella apicola* Kwong et Moran.

Discussion

The microbial investigation by plate count on diseased honey bee larvae, exhibiting atypical clinical symptoms closely related to both EFB and AFB, allowed the massive isolation of two microorganisms further identified as *E. faecalis* and *P. dendritiformis* respectively. Plates

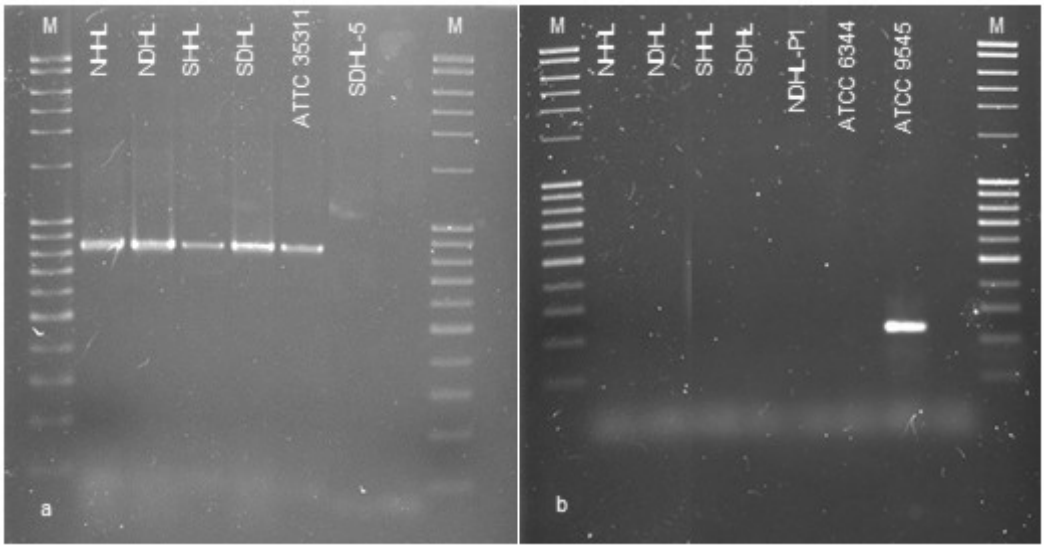

Figure 2. Species-specific PCR for the detection of *M. plutonius* (a) and *P. larvae* (b) from one replicate of DNA samples. M: 100 bp DNA Ladder: NHHL: healthy-looking honey bee larvae from North Italy; NDHL: diseased honey bee larvae from North Italy; SHHL: healthy-looking honey bee larvae from South Italy; SDHL: diseased honey bee larvae from South Italy; ATCC 35311: *M. plutonius*; SDHL-5: *E. faecalis*; NDHL-5: *P. dendritiformis*; ATCC 6344: *P. alvei*; ATCC 9545: *P. larvae*.

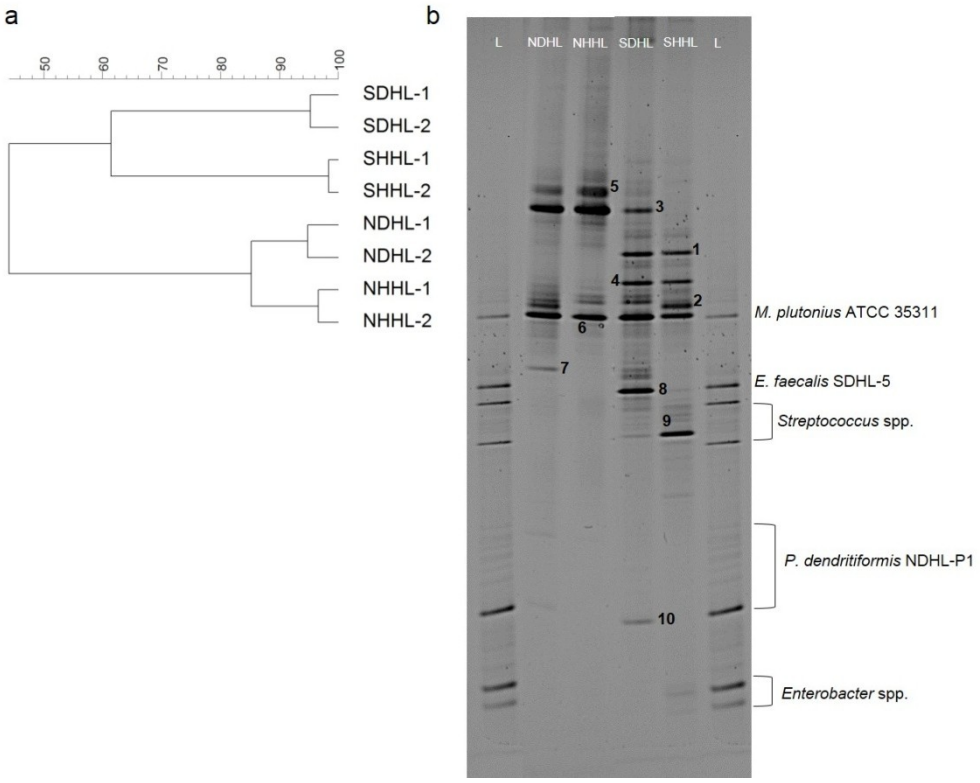

Figure 3. UPGMA dendrogram (a) and DGGE profiles of eubacteria (b) from the amplified V2-V3 region of 16S rRNA obtained from one replicate of DNA samples. The bands indicated by the numbers were excised, re-amplified and subjected to sequencing. L: ladder with reference strains; NDHL: diseased honey bee larvae from North Italy; NHHL: healthy-looking honey bee larvae from North Italy; SDHL: diseased honey bee larvae from South Italy; SHHL: healthy-looking honey bee larvae from South Italy.

Table 1. Eubacteria sequence alignment with the megablast algorithm in the GenBank database.

Band	Closest match	% similarity*	GenBank accession number
1	*Lactobacillus helsingborgensis*	99	KR073912
2	nd	-	-
3	*Lactobacillus kunkeei*	99	KR073913
4	*Lactobacillus kimbladii*	98	KR073914
5	*Lactobacillus kunkeei*	100	KR073919
6	*Melissococcus plutonius*	100	KR073920
7	*Gilliamella apicola*	97	KR073925
8	*Enterococcus faecalis*	100	KR073918
9	nd	-	-
10	*Paenibacillus* spp.	100	KR073924

* similarity represents the % similarity shared with the sequences in the GenBank database. nd: not determined (scarce quality of the obtained sequencing).

did not evidence neither *M. plutonius*, nor *P. larvae*. *E. faecalis* is one of the typical second invaders often described in the EFB (Forsgren, 2010). It does not multiply in bee larvae in the absence of *M. plutonius*, and its presence is a presumptive evidence of EFB (OIE, 2008). *E. faecalis* is morphologically similar to *M. plutonius* and it has frequently been confused as being the causative agent of EFB (Bailey and Gibbs, 1962; Hornitzky and Wilson, 1989). Interestingly, pasteurized samples allowed the isolation of *P. dendritiformis* and the unsuccessful recovery of *P. larvae* on MYPGP plates. *P. dendritiformis* is a soil-borne bacterium, which can be found in various plant-related niches (Campos-Herrera *et al*., 2011); nowadays, no data on its presence in honey bee larvae are available. Its genome encodes various genes for the production of offensive compounds (toxins, lytic enzymes, antibiotics) to regulate its population size and compete with other bacteria (Sirota-Madi *et al.*, 2012). It could be hypothesized that, in low nutrient availability as in larval remains, this antimicrobial arsenal could provide a competitive advantage, replacing neighbouring species as the EFB second invader *P. alvei*, conferring atypical symptoms to the disease. Indeed, *P. alvei* usually confers a characteristic stale or

sour odour to the EFB (OIE, 2008), which was not perceived in the current study. Moreover, Australian researchers suggested that *in vitro* reared larvae only developed EFB symptoms when both *M. plutonius* and *P. alvei* were present (Hornitzky and Giersch, 2008).

These findings let us suppose that analysed honey bee larvae in both geographical areas exhibited an advanced disease status, presumably ascribed to the EFB. The confirmation came out from molecular techniques (both qualitative PCR and PCR-DGGE), which evidenced the presence of *M. plutonius* in all samples, including larvae without symptoms.

Obtained molecular data are in contrast with plate counts. However, it is always reported that *M. plutonius* is a fastidious microorganism and culture methods could be very insensitive, detecting less than 0.2% of microscopically counted cells (Djordjevic *et al*., 1998; Hornitzky and Smith, 1998). Moreover, *E. faecalis*, if present in high quantity as resulted in this study, could overgrow SYPG plates, thus avoiding *M. plutonius* detection (Bailey and Ball, 1991; OIE, 2008). Detection of *M. plutonius* in healthy colonies has been already reported (Pinnock and Featherstone, 1984; Mckee *et al*., 2003; Forsgren *et al.*, 2005). It could be supposed that healthy-looking larvae (not affected in the aspect and in the morphological position) were in an early stage of infection and their microbiota was changing; the DGGE profile revealed, indeed, a high similarity to the diseased samples. However, in some cases, infected larvae without apparent symptoms, could survive and successfully pupate and emerge as adults, thus withstanding the infection (OIE, 2008). Contrarily to *M. plutonius*, *P. larvae* was not detected, neither by species-specific PCR nor by DGGE, thus excluding any implication of the AFB.

Overall, the combination of traditional and molecular techniques was successful in evidencing the disease origin. Besides confirming plate count results and species-specific PCR, PCR-DGGE analysis and band sequencing provided a more complete picture of the bacterial communities shift on the analysed samples. Interestingly, DGGE bands ascribed to *Lactobacillus* spp. were detected in all samples; as reported by Vojvodic *et al*. (2013), *Lactobacillus* spp. is the most representative genus of honey bee larvae gut microbiota.

The absence of the band ascribed to *E. faecalis* in NDHL profile probably reflects the low colony detection observed by plate count. It should be emphasized that DGGE shares with the other PCR-based molecular biology techniques some limitations in terms of detection limits and of quantitative comparison of detected populations (Marzorati *et al*., 2008). Sequencing of band 10, identified as *Paenibacillus* spp. in DNHL and SDHL, was not able to discriminate at species level, since the 100% similarity was referred to *P. dendritiformis* but also to *Paenibacillus popiliae* Dutky and *Paenibacillus thiaminolyticus* Nakamura. However, considering that from plate count the only strain isolated and identified by 16S rDNA sequencing was *P. dentritiformis*, band 10 presumably refers to this bacterial species. A further confirmation derives from the presence in the ladder of the isolated strain *P. dendritiformis* NDHL-P1, whose migration distance is comparable to the excised band.

The current work represent a preliminary investigation on the microbiology of the honey bee larvae and put in evidence the importance to combine culture dependent and independent techniques. With the above-described approach, a different bacterial species has been detected in honey bee larvae affected by the EFB. Further research are envisaged (*e.g.* infection trial in healthy larvae) to better understand the role of *P. dendritiformis* in the development of the disease.

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