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Inhibition of *Paenibacillus larvae* by an extracellular protein fraction from a honeybee-borne *Brevibacillus laterosporus* strain

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ABSTRACT

The inhibitory action that a *Brevibacillus laterosporus* strain isolated from the honeybee body causes against the American Foulbrood (AFB) etiological agent *Paenibacillus larvae* was studied by *in-vitro* experiments. A protein fraction isolated from *B. laterosporus* culture supernatant was involved in the observed inhibition of *P. larvae* vegetative growth and spore germination. As a result of LC–MS/MS proteomic analyses, the bacteriocin laterosporulin was found to be the major component of this fraction, followed by other antimicrobial proteins and substances including lectins, chaperonins, various enzymes and a number of putative uncharacterized proteins. The results obtained in this study highlight the potential of *B. laterosporus* as a biological control agent for preserving and improving honeybee health.

1. Introduction

A variety of bacterial species have developed unique and host-specific relationships with the honeybee *Apis mellifera* (Moran, 2015). These include the Gammaproteobacteria *Gilliamella apicola* and *Frischella perrara* (Orbaceae), the Betaproteobacterium *Snodgrassella alvi* (Neisseriaceae), *Lactobacillus* (Firmicutes) and *Bifidobacterium* (Actinobacteria) species, residing in the bee gut and representing its core bacterial community (Kwong and Moran, 2016). Such bacterial species are believed to play a significant role in maintaining a good health status counteracting against stress factors like insect pathogens and parasites (Engel and Moran, 2013). Other bacterial species found in the bee hive ecosystem have instead specifically evolved as pathogens. This is the case of *Paenibacillus larvae* (Firmicutes), the causal agent of the American Foulbrood (AFB), a destructive disease of worldwide importance, affecting the colony (Genersch, 2010). The management of this bacterium is a main issue for the beekeeper, especially because of the limitations associated with the use of antibiotics in the beehive ecosystem and the resistance development in diverse *P. larvae* strains (Evans, 2003; Murray et al., 2007). Hence, research studies are being carried out to find alternative and effective natural antimicrobial substances (Isidorov et al., 2018). This pathogen, typically enter the body of a neonate larva by the ingestion of food contaminated by its spores. Once in the midgut, spores germinate producing new vegetative cells releasing several toxins, enzymes and virulence factors supporting the infection process. After the degradation of the peritrophic matrix, probably by the action of proteases and chitinases (Garcia-Gonzalez et al., 2014), bacterial cells interact with the midgut epithelial cells causing damages and invading the haemocoel where they proliferate causing a widespread septicaemia. At this infection phase, dead larvae appear flaccid, with glue-like consistency (ropy stage), which will contribute to further spread of new bacterial spores within the colony and outside the hive (Ebeling et al., 2016).

The honeybee defense mechanisms against this pathogen are primarily based on social immunity (hygienic behavior) and on the innate humoral and cellular response (Chan et al., 2009), but may also rely on additional immune-related functions performed by components of its core bacterial community (Zheng et al., 2018). Among the bacterial species commonly inhabiting the honeybee body, there is the spore former *Brevibacillus laterosporus* (Marche et al., 2016), whose antimicrobial potential is well documented (Miljkovic et al., 2019). Despite this bacterium was found as a secondary invader in bee colonies affected by the European foulbrood caused by *Melissococcus pluton* (Firmicutes) (White, 1912), it has more recently been reported to have beneficial effects on bees, showing a specific inhibitory action against *P. larvae* (Alippi and Reynaldi, 2006; Hamdi and Daffonchio, 2011). However, the mechanism leading to such inhibition has not yet been clarified (Khaled et al., 2018).

The purpose of this study was to investigate the *in-vitro* inhibition that a *B. laterosporus* strain isolated from the honeybee body causes against *P. larvae*. The proteomic characterization of an extracellular bacterial fraction involved in such effects is for the first time presented.

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2. Materials and methods

2.1. Bacterial strains and growth conditions

Paenibacillus larvae reference strain DMS 7030 (=ATCC 9045) corresponding to genotype ERIC I, gently provided by Istituto Zooprofilattico Sperimentale delle Venezie (Italy), was used in this study. Solid and liquid cultures were routinely conducted at 35 °C on agar plates or liquid broth containing J medium: Tryptone (Fluka) (5 g/l), K2HPO4 (Sigma Aldrich) (3 g/l), yeast extract (Sigma Aldrich) (15 g/l), agar (Sigma Aldrich) (20 g/l) (only for plates), 10% glucose (Sigma Aldrich) (20 ml/l), pH adjusted to 7.3 (Hornitzky and Nicholls, 1993). This medium was preliminarily tested for its suitability for our *in-vitro* bioassay model (De Graaf et al., 2013).

Vegetative cells or sporulated cultures were obtained by inoculating liquid J medium with fresh *P. larvae* cells and the presence of different stages of growth was checked under phase-contrast microscopy (Forsgren et al., 2008).

Brevibacillus laterosporus strain F5 maintained in glycerol at −80 °C at the University of Sassari, was employed in this study. This honeybee-borne strain was isolated and identified in a previous work (Marche et al., 2016). *Brevibacillus laterosporus* was routinely cultivated on LB agar, while bacterial cell culture for bioassays and analyses was conducted on J broth shaking at 180 rpm at 30 °C for 72 h. Culture supernatant of a whole sporulated culture was collected by centrifugation at 15,000x*g* at 4 °C for 15 min (Marche et al., 2017). Supernatant used in bioassays or processed for protein precipitation was preliminarily sterilized through 0.2 μm pore size filter (Minisart®).

2.2. Protein precipitation and analyses

For protein precipitation, filter-sterilized culture supernatant of *B. laterosporus* was mixed with ammonium sulfate up to 85% saturation (w/v) before being incubated overnight at 4 °C with gentle stirring. The solution was centrifuged at 15,000x*g* for 20 min and the protein pellet obtained was resuspended in phosphate-buffered saline (PBS) and dialyzed against the same buffer to remove ammonium sulfate residues. Protein concentration of different samples was routinely measured by Bio-Rad Protein Assay according to Bradford dye-binding method (Bradford, 1976).

Aliquots of the same protein solutions used in bioassay were supplied to the proteomic facility of Porto Conte Ricerche Srl (Tramariglio, Alghero, Italy) for LC–MS/MS analyses. The protein fraction was subjected to on-filter reduction, alkylation, and trypsin digestion according to the filter-aided sample preparation (FASP) protocol, with slight modifications (Addis et al., 2009). Peptide mixtures were analyzed through a LTQ Orbitrap Velos mass spectrometer (Thermo Scientific) interfaced with an UltiMate 3000 RSLCnano LC system (Thermo Scientific) (Tanca et al., 2013). Mass spectrometry output data were analyzed through Proteome Discoverer (version 1.4; Thermo Scientific), using Sequest-HT (Eng et al., 1994), the database UniprotKB and Mascot (Matrix Science, Boston, MA) as search engine for protein identification. Protein relative abundance was expressed by means of the normalized spectral abundance factor (NSAF) (McIlwain et al., 2012). Peptide sequence data were further processed against the NCBI database (http://www.ncbi.nlm.nih.gov).

2.3. Antimicrobial bioassays

The inhibitory effects of *B. laterosporus* on *P. larvae* were preliminarily studied by agar well diffusion tests and subsequently by liquid culture bioassays. Culture supernatant of *B. laterosporus* to be employed in bioassays was fresh prepared and filter-sterilized as described above.

Preliminary experiments were conducted on agar plates according to the agar well diffusion method (Valgas et al., 2007). Briefly, the whole agar plate surface was inoculated by spreading 500 μl of a *P. larvae* liquid cell culture with a concentration of at least 10^6 cells/ml. After leaving plates to dry inside a laminar flow cabinet, a hole (8 mm diameter) was made aseptically using a tip, and *B. laterosporus* supernatant (100 μl) or sterile J broth (control) was introduced into the well. After incubation at 35 °C for 48 h, the diameter of the *P. larvae* growth inhibition halo crossing the well was measured (Balouiri et al., 2016).

Secondly, three different liquid culture bioassays were conducted to study the inhibitory action of *B. laterosporus* on *P. larvae* vegetative growth. For this purpose, *P. larvae* liquid cultures (5 ml) at the exponential phase, with a concentration of 10^6 cells/ml, were inoculated into flasks containing 20 ml J broth incubated at 35 °C shaking at 120 rpm. Bacterial growth was monitored by measuring optical density (OD) at 600 nm every hour. In the first experiment, when *P. larvae* exponential vegetative growth reached 0.5–0.6 OD in the flask, an aliquot (5 ml) of *B. laterosporus* supernatant (treated) or sterile J broth (control) was added to the culture, and bacterial growth was monitored during the next hours. In the second experiment conducted under the same conditions, the following three bacterial preparations were used to treat *P. larvae* culture at the exponential phase: heat-treated (100 °C for 10 min) and untreated *B. laterosporus* culture supernatant (at the same proportion as in the previous experiment) and the protein fraction (at a concentration of 1 μg/μl) obtained by precipitation from the supernatant. Bacterial growth was assessed for 6 h after treatment. In the third experiment conducted under the same experimental conditions, the concentration–response effect of the protein fraction was determined assaying progressive protein concentrations within the range 0.1–1 μg/μl. Growth inhibition percentage in treated flasks was calculated against growth in the control.

A fourth experiment in liquid culture was conducted to assay the possible inhibitory properties of *B. laterosporus* preparations on *P. larvae* spore germination. For this purpose, aliquots (500 μl) of sporulated cultures of *P. larvae* with a concentration of 10^6 spores/ml were inoculated into tubes containing fresh J broth (2 ml) and 500 μl of *B. laterosporus* culture supernatant or protein fraction at a concentration of 1 μg/μl, according to the above described protocol conditions. During the next 12 h, spore germination was checked under phase microscopy and vegetative cell growth was monitored by OD measurements.

The design of all the above experiments involved 3–5 replicates and each experiment was repeated at least three times.

2.4. Statistical analysis

Statistical analyses were performed with SAS software (version 9.1) with significance level set at $\alpha = 0.05$ (SAS, 2004).

Data on *P. larvae* growth inhibition and spore germination in liquid cultures, involving different *B. laterosporus* preparations (*i.e.*, supernatant and protein fraction) were subjected to analysis of variance (one factor design: bacterial preparation) followed by multiple comparison of means (adjust = Tukey).

Overtime *P. larvae* growth in liquid medium was analyzed using repeated measures ANOVA (PROC MIXED), and means were separated using LSMEANS comparison (adjust = Tukey).

Linear regression analyses were used for analyzing the relationship between protein concentration and bacterial growth (OD) in liquid culture.

3. Results

3.1. Antimicrobial bioassays

Preliminary experiments on agar plates inoculated with *P. larvae* showed a clear inhibition zone around wells filled up with *B. laterosporus* supernatant (Fig. S1). An average inhibition halo of 13.30 ± 3.78 mm was obtained employing supernatants from different preparations of *B. laterosporus* cultures, showing a clear difference in

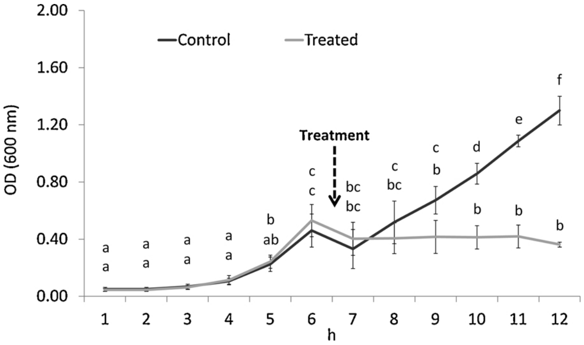

Fig. 1. Comparison of progressive *P. larvae* growth (mean OD ± SE) in *J* broth treated *vs* non-treated with *B. laterosporus* culture supernatant. Treatment was performed after 6 h growth. Different letters indicate significant differences among means (ANOVA PROC MIXED, followed by Tukey test).

comparison with controls in which no inhibition halo was detected.

The growth of *P. larvae* in J broth treated with *B. laterosporus* culture supernatant in the first experiment in liquid culture is shown in Fig. 1. Growth was significantly affected by treatment ($F_{1,8} = 32.96$, $P = 0.0004$) and time ($F_{11,88} = 209.99$, $P < 0.0001$). A significant interaction treatment x time was also observed ($F_{11,88} = 72.57$, $P < 0.0001$). Treatment caused a significant slowing or interruption of bacterial growth, while *P. larvae* cellular replication continued exponentially in the control.

Inhibition of *P. larvae* growth in the second experiment in liquid cultures treated with *B. laterosporus* culture supernatant or protein fraction at a concentration of 1 μg/μl is shown in Table 1. A high inhibition, over 80% in respect to growth in the control, was achieved by both culture supernatant and the protein fraction obtained from the same supernatant. On the other hand, heat treatments caused a significant reduction of their inhibitory properties ($F_{3,8} = 63.58$, $P < 0.0001$).

The inhibition effect caused by the protein fraction as determined in the third experiment was concentration dependent (Fig. 2). As a result of linear regression analysis, the percentage of *P. larvae* growth inhibition was shown to be positively correlated with the concentration of the *B. laterosporus* protein fraction in the culture broth (adjusted $R^2 = 0.8801$, $F = 610.1$, $P < 0.0001$).

Germination of *P. larvae* spores in liquid cultures treated with *B. laterosporus* culture supernatant or protein fraction at a concentration of 1 μg/μl (fourth experiment) was significantly inhibited or slowed down in comparison with post-germination growth in the control. No significant *P. larvae* growth was observed in the 12 h following spore inoculation in J medium treated with *B. laterosporus* culture supernatant or protein fraction ($F_{2,8} = 39.48$, $P = 0.0004$) (Fig. S2).

Table 1
Inhibition of *Paenibacillus larvae* growth by heat-treated and untreated *Brevibacillus laterosporus* culture supernatant and protein fraction.

Preparation[a]	Inhibition %[b] (means ± S.E.)
Culture supernatant	81.1 ± 12.0 a[c]
Protein fraction	86.9 ± 7.6[a]
Heat-treated supernatant	25.7 ± 11.5[b]
Heat-treated protein fraction	0.57 ± 1.0[c]

[a] Culture supernatant was collected from a 48 h *B. laterosporus* liquid culture in J broth. Protein fraction was obtained by protein precipitation from supernatant. Heat treatments were performed at 100 °C for 10 min.

[b] *Paenibacillus larvae* growth inhibition percentages were calculated against growth in the control.

[c] Means followed by different letters, are significantly different (ANOVA, followed by Tukey's test).

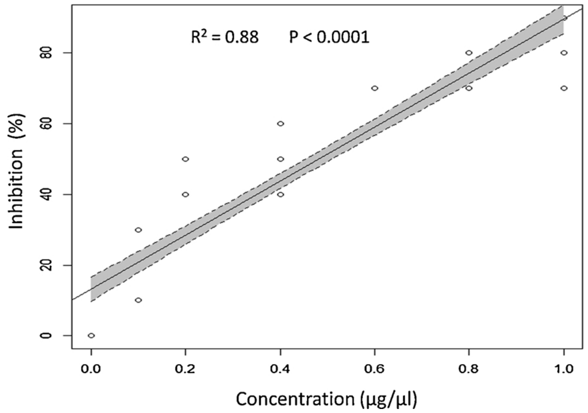

Fig. 2. Linear regression plot with 95% confidence intervals (shaded areas) showing the predicted relationship between concentration of *Brevibacillus laterosporus* proteins isolated from culture supernatant and *Paenibacillus larvae* growth inhibition.

3.2. Proteomic analysis

The proteome of the *B. laterosporus* culture supernatant showing inhibitory properties against *P. larvae* appeared to be a complex protein mixture. As a result of mass spectrometry, including the analysis of 4502 internal peptides, a variety of proteins were identified in this fraction. Major proteins were found within the 5–121 kDa range (Table 2). The antimicrobial peptide laterosporulin appeared to be the relatively most abundant, based on the normalized spectral abundance factor (NSAF) determination. Proteins involved in the bacterial cell structure, including surface layer and wall proteins were also well represented. Several enzymes and stress related proteins were detected. In addition, a lectin domain protein and a 60 kDa chaperonin were significantly represented. Among others, a number of putative uncharacterized proteins were found.

4. Discussion

Among the variety of bacteria found in the beehive ecosystem, the family Paenibacillaceae include the pathogenic species *P. larvae* (Genersch, 2010) and the honey bee body resident *B. laterosporus* (Marche et al., 2016). While they are likely to have developed strategies to compete through evolution in the same environment, the inhibitory properties of *B. laterosporus* against the American Foulbrood etiological agent have only recently been outlined (Alippi and Reynaldi, 2006). Such antagonistic potential is in line with the reported production of several antimicrobial peptides and antibiotics by different *B. laterosporus* strains (Ruiu, 2013). In this regard, the availability of genes related to the antimicrobial activity in the genome of *B. laterosporus* was found to be highly conserved in this species (Djukic et al., 2011; Camiolo et al., 2017). Most of the deriving bioactive compounds have been isolated from the *B. laterosporus* culture supernatant (Zhao et al., 2012; Chawawisit and Lertcanawanichakul, 2014; Yang et al., 2016; Yang and Yousef, 2018). Accordingly, the inhibitory properties of the honeybee-borne strain F5 of *B. laterosporus* against *P. larvae* were associated with the culture supernatant, and this finding is in line with previous observations conducted employing other hive-isolated bacterial strains (Alippi and Reynaldi, 2006; Bartel et al., 2018). Inhibition of both *P. larvae* vegetative growth and spore germination was demonstrated in our experiments. These effects were analogously observed when ammonium sulfate precipitated proteins from the culture supernatant of *B. laterosporus* were employed in bioassays, thus demonstrating a main role of the proteins released during bacterial growth. Mass spectrometry analyses revealed a protein complex in which the 5.7 kDa bacteriocin, laterosporulin, was the major

Table 2
Mass spectrometry identification of major proteins of the *Brevibacillus laterosporus* culture supernatant.

UniprotKB Acc. No.	Protein description[a]	Coverage (%)	PSMs	Score	PI	MW (kDa)	NSAF
A0A075R4 × 6	Putative laterosporulin	50	43	70.39	6.71	5.7	272.87
A0A075RBQ6	Hexagonal wall protein	67.22	485	586.32	5.45	121.2	142.28
H0UAR9	Surface layer protein	56.68	368	445.09	5.40	121.2	108.19
A0A075RA20	Uncharacterized protein	63.11	142	362.87	5.86	49.9	100.00
A0A075R7D3	Thioredoxin OS	61.54	30	36.67	4.88	11.5	91.36
H0U9 × 0	Putative uncharacterized protein	66.67	18	22.11	8.37	8.3	81.83
A0A075RAM3	Alkyl hydroperoxide reductase subunit C	55.25	44	49.98	4.97	19.8	77.00
A0A075R7C5	Jacalin-like lectin domain protein OS	48.55	33	50.86	6.54	15.3	75.71
A0A0F7C0H0	General stress protein OS	44.59	35	36.8	4.92	16.8	74.92
H0UCY7	Peptidylprolyl isomerase OS	50.42	81	109.77	7.39	38.7	72.71
A0A075R7T2	Outer cell wall protein	44.15	254	301.34	4.86	115.8	72.37
A0A075R7N0	Cold shock-like protein CspD	60.61	14	15.96	4.94	7.4	67.13
A0A075QVU7	50S ribosomal protein L7/L12	32.77	25	27.51	4.70	12.4	66.50
A0A075RCX3	Uncharacterized protein	60	24	33.34	8.94	12.8	66.14
H0UH15	Putative uncharacterized protein	47.33	31	41.22	5.58	16.8	65.43
A0A075R7P5	Major cold shock protein CspB	61.54	13	13.09	4.72	7.2	63.33
A0A075R3Y6	Ribosomal protein S1	51.65	77	113.59	5.19	43.5	62.07
A0A0F7C0U1	Polyamine aminopropyltransferase	46.21	52	69.94	5.52	31.2	59.45
A0A075RGL0	Fe/S biogenesis protein NfuA	43.84	13	23.00	4.61	8.0	56.39
A0A075R8T7	4-hydroxyphenylpyruvate dioxygenase [A0A075R8T7_BRELA]	39.67	64	85.78	5.08	41.1	55.89
A0A075QYC0	30S ribosomal protein S13	44.26	21	29.6	11.31	14.0	54.58
A0A075QZ29	60 kDa chaperonin	43.93	92	125.68	4.98	57.6	53.55
A0A075R0L2	Alanine dehydrogenase	87.17	63	79.71	5.82	39.2	53.41

[a] Proteins with a concentration > 0.5 ng/ml based on NSAF determination are indicated.

component, followed by higher molecular weight proteins representing the different layers that make up the cell wall, and other functional (*i.e.*, enzymes) and stress-related peptides.

While the presence of cell wall and other cell proteins in the culture supernatant is substantially due to the normal events characterizing the stationary phase of the bacterial growth such as lysis of old cells and sporangia, the detection of laterosporulin and other bioactive compounds like a lectin domain protein and a 60 kDa chaperonin among major proteins, might be related to the observed antimicrobial activity. In addition, several putative uncharacterized proteins detected in this fraction, may also represent important factors, whose specific role would however have to be specifically evaluated.

Laterosporulin was initially discovered in the soil-borne *B. laterosporus* strain GI-9 (Sharma et al., 2012) and was shown to be produced and released in the culture supernatant during the stationary phase of growth (Singh et al., 2012). In the same study, this bacteriocin exhibited significant inhibitory activity against a wide range of Gram-positive and Gram-negative bacteria including *Bacillus subtilis* and *Staphylococcus aureus*. However, its potential against insect pathogens is not known. Laterosporulin produced by the honey-bee borne *B. laterosporus* strain F5, showed 100% homology with lateroporulin produced by strain GI-9 and strain LMG 15441 (Djukic et al., 2011), confirming that this bacteriocin is highly conserved in this broad-spectrum antimicrobial species. Crystallography studies showed that cysteines are disulfide-bonded, so that laterosporulin keeps its peptide structure in solution, revealing a human defensin-like structural module (Singh et al., 2015; Baindara et al., 2016). These studies suggested that bacterial growth inhibition is determined by membrane permeability increase, which if confirmed would be in line with the inhibitory activity against *P. larvae* vegetative cells we observed in liquid cultures. On the other hand, different mechanisms or components of the protein mixture may regulate the spore germination inhibition we observed. Among other putative inhibition factors, we may speculate that a jackalin-like lectin found in this mixture, could also be involved in the observed antagonism against *P. larvae*. Accordingly, this class of carbohydrate binding proteins is counted among potential virulence factors and antimicrobial compounds (Sharon, 1987; Ziółkowska et al., 2006; Breitenbach Barroso Coelho et al., 2018). Another protein suspected of being involved in the action against *P. larvae* is the *B. laterosporus* 60 kDa chaperonin found in the bio-active culture supernatant protein precipitate. Consistently, in addition to their primary protein folding function, bacterial chaperones have recently been associated with either mutualistic and pathogenic interactions between bacteria and their host (Kupper et al., 2014). Besides a possible direct action of all these proteins against *P. larvae*, we cannot exclude that *B. laterosporus* proteome arsenal could at the same time interact with honeybees stimulating their innate immune system, but such a hypothesis should be confirmed by specific experiments.

The exploitation of *B. laterosporus* antagonistic potential in biological control programs against *P. larvae* has previously been suggested (Hamdi and Daffonchio, 2011). More recently, the production of antimicrobial products by naturally occurring *B. laterosporus* strains was successfully induced in the hive through the application of selected bacteriophages (Brady et al., 2018). As a result, a significant action against *P. larvae* in AFB infected hives was obtained. Advances in the knowledge of *B. laterosporus* factors specifically inhibiting *P. larvae* development in the hive, is expected to contribute to further improvement of such biological control methods, ensuring a more targeted action against this honeybee pathogen.

Another aspect arising from this study and deserving further investigation is the actual *B. laterosporus* ecological significance in the beehive ecosystem and within the honeybee body (Berg et al., 2018). Recent microbiome studies revealed that a natural selection have led to the establishment of a core honeybee bacterial community, mostly residing in the gut, represented by few and beneficial species (Kwong and Moran, 2016). Beyond evolutionary origin, such bacterial community is thought to take part in the complex of defense mechanisms against pathogens and stress factors (Engel et al., 2016). Similarly, *B. laterosporus* might be naturally involved in defense mechanisms of the hive, exploiting its own competitive potential (Marche et al., 2019). The promising results obtained in experiments with *B. laterosporus* against *P. larvae*, promote further studies to clarify the role of each putative antimicrobial component and to evaluate its actual potential contribution to honeybee health preservation and improvement within the complex hive ecosystem.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.micres.2019.126303.

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