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European foulbrood in Czechia after 40 years: application of next-generation sequencing to analyze *Melissococcus plutonius* transmission and influence on the bacteriome of *Apis mellifera*

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Worker honeybees (*Apis mellifera*) transmit *Melissococcus plutonius* between colonies. However, the transmission of *M. plutonius*, which causes European foulbrood (EFB), is poorly understood. To analyze the first EFB outbreak in 40 years in Czechia, we collected 49 hive worker samples from 18 beehives in two diseased apiaries for bacteriome analysis of the V1-V3 portion of the 16S rRNA gene. When we compared control samples obtained outside of the EFB zone, bees from an EFB apiaries containing colonies without clinical symptoms and bees from colonies with EFB clinical symptoms, there was a 100-fold higher occurrence of *M. plutonius* in colonies with EFB symptoms. The presence of *M. plutonius* in controls indicated that this pathogen exists in an enzootic state. EFB influenced the core bacteria in the worker bacteriome because the number of *Snodgrassella alvi*, *Lactobacillus mellis*, *Lactobacillus melliventris*, and *Fructobacillus fructosus* sequences increased, while *Bartonella apis*, *Frischella perrara*, and *Commensalibacter intestine* sequences decreased. Together, the results of this study suggest worker bees from EFB-diseased apiaries serve as vectors of *M. plutonius*, and eliminating such colonies is an appropriate method to overcome disease outbreaks. Because *M. plutonius* exists in honeybee colonies in an enzootic state, there may be similar abundances in control colonies outside the EFB zone to those in asymptomatic colonies from EFB apiaries. High-throughput Illumina next-generation sequencing permitted the quantitative interpretation of *M. plutonius* within the honeybee worker bacteriome. Future studies focusing on honeybee diseases, colony losses, detection of bacterial pathogens and interactions of bacteriome with pathogenic bacteria will benefit of this study.
European foulbrood in Czechia after 40 years: application of next-generation sequencing to analyze *Melissococcus plutonius* transmission and influence on the bacteriome of *Apis mellifera*

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ABSTRACT

Worker honeybees (*Apis mellifera*) transmit *Melissococcus plutonius* between colonies. However, the transmission of *M. plutonius*, which causes European foulbrood (EFB), is poorly understood. To analyze the first EFB outbreak in 40 years in Czechia, we collected 49 hive worker samples from 18 beehives in two diseased apiaries for bacteriome analysis of the V1-V3 portion of the 16S rRNA gene. When we compared control samples obtained outside of the EFB zone, bees from an EFB apiary containing colonies without clinical symptoms and bees from colonies with EFB clinical symptoms, there was a 100-fold higher occurrence of *M. plutonius* in colonies with EFB symptoms. The presence of *M. plutonius* in controls indicated that this pathogen exists in an enzootic state. EFB influenced the core bacteria in the worker bacteriome because the number of *Snodgrassella alvi*, *Lactobacillus mellis*, *Lactobacillus melliventris*, and *Fructobacillus fructosus* sequences increased, while *Bartonella apis*, *Frischella perrara*, and *Commensalibacter intestine* sequences decreased. Together, the results of this study suggest worker bees from EFB-diseased apiaries serve as vectors of *M. plutonius*, and eliminating such colonies is an appropriate method to overcome disease outbreaks. Because *M. plutonius* exists in honeybee colonies in an enzootic state, there may be similar abundances in control colonies outside the EFB zone to those in asymptomatic colonies from EFB apiaries. High-throughput Illumina next-generation sequencing permitted the quantitative interpretation of *M. plutonius* within the honeybee worker bacteriome. Future studies focusing on honeybee diseases, colony losses, detection of bacterial pathogens and interactions of bacteriome with pathogenic bacteria will benefit of this study.

Keywords  *Apis mellifera* / European foulbrood / *Melissococcus plutonius* / bacteriome / *Snodgrassella alvi* / *Lactobacillus* / *Fructobacillus fructosus* / *Bartonella apis* / *Frischella perrara* / *Commensalibacter intestine*
European foulbrood (EFB) is caused by the Gram-positive lanceolate coccus *Melissococcus plutonius* and is one of the most important diseases of the European honeybee, *Apis mellifera* L. This emerging honeybee disease is listed in the Terrestrial Animal Health Code of The World Organisation for Animal Health (*OIE*, 2016b). Although EFB spread is worldwide and the clinical signs are similar to American foulbrood (AFB), EFB is not notifiable in all countries (*Forsgren et al.*, 2013). Indeed, there is a need to understand how this honeybee disease contributes to colony losses.

EFB weakens a honeybee colony through brood loss and results in colony collapse (*Forsgren et al.*, 2013). Due to the presence of *M. plutonius* forms demonstrating differential virulence (*Arai, et al.* 2012; *Budge et al.*, 2014), disease development differs between countries (*Forsgren et al.*, 2013). In the past few years, EFB has increased in some European countries; in particular, it has increased consistently in Switzerland since 1997 (*Belloy et al.*, 2007; *Roetschi et al.*, 2008). In the UK, EFB has become the most common brood disease (*Budge et al.*, 2011; *Wilkins, Brown & Cuthbertson*, 2007), and there was a regional outbreak in Norway in 2010 after 30 years of absence (*Dahle, Sorum & Weideman*, 2011). In addition, EFB has historically occurred in Czechia. During the 1970s, it was found in Kralupy nad Vltavou in Central Bohemia (personal communication from Dr. Frantisek Kamler). EFB was also recently found in 2015, and additional signs of the disease emerged in 2016 in Krkonose Mountains National Park in Eastern Bohemia (*KVSH*, 2015), representing the first cases after 40 years of no EFB in Czechia (*Kamler et al.*, 2016).

Methods used to detect EFB were reviewed by *Forsgren et al.* (2013) and are included in the COLOSS BeeBook (*COLOSS 2013*) and in the OIE Terrestrial Manual (*OIE, 2016a*). Signs of EFB disease include bacteremia of honeybee larvae, which usually die between 4 and 5 days of age or sometimes when they are older, after sealing (*Forsgren, 2010*). The first step in EFB infection is asymptomatic colonization of the larval gut after food transmission by nurse bees. Infection can cause fever than 100 *M. plutonius* cells which rapidly reproduce in the larval gut (*Bailey, 1960*). Compared to *Paenibacillus larvae*, which forms spores, non-sporulating *M. plutonius* is more difficult to identify utilizing cultivation techniques because less than 0.2% of cells are detectable (*Djordjevic et al.*, 1998; *Hornitzky & Smith*, 1999). Freshly dead larvae are preferred for diagnostics (*OIE 2016a*). Useful methods for detecting *M. plutonius* include
immunochemical methods such as enzyme-linked immunosorbent assay (ELISA) or lateral flow immunoassay (LFIA) (Pinnock & Featherstone, 1984; Tomkies et al., 2009) and molecular genetics techniques such as polymerase chain reaction (PCR) (Govan et al. 1998), hemi-nested PCR (Belloy et al., 2007; Djordjevic et al., 1998) or real-time PCR (Budge et al., 2010; Roetschi et al., 2008).

Based on the analysis of colonies exhibiting clinical signs using real-time PCR, bees collected from brood frames contain approximately 20-fold more *M. plutonius* than bees from frames containing worker bees (Roetschi et al., 2008). However, this result, obtained using 100 bees per sample, has not been repeated (Forsgren et al., 2013). Using hemi-nested PCR, Belloy et al. (2007) demonstrated honeybee carriers of *M. plutonius* in more than 90% of colonies without EFB symptoms located in apiaries with EFB symptoms. In apiaries without EFB symptoms but located in proximity to apiaries with EFB symptoms, bees carrying *M. plutonius* were detected in approximately 30% of colonies (Belloy et al., 2007). The number of *M. plutonius* cells in adult bees varies, but bees from asymptomatic colonies in diseased apiaries are at higher risk for disease development (Budge et al., 2010). A metagenomics approach to analyze honeybee RNA is capable of identifying *M. plutonius* (Tozkar et al., 2015). However, this approach has not been tested with honeybee worker samples from EFB outbreaks.

In this study, we investigated EFB in the context of the disease outbreak in Krkonose Mountains National Park in Czechia by performing Illumina next-generation sequencing (NGS). We followed the experimental design of (Belloy et al., 2007; Roetschi et al., 2008), analyzing honeybees obtained from both symptomatic and asymptomatic colonies in EFB diseased apiaries. For comparison, the study also employed NGS to analyze worker honeybees from control colonies located far from the outbreak occurrence. The worker bacteriome was described based on the V1-V3 portion of the 16S rRNA gene analyzed using the Illumina MiSeq platform. In addition to determining the relative numbers of *M. plutonius* sequences correlating with different sample types, we showed the effects of the presence of *M. plutonius* on honeybee gut symbiotic bacterial community composition.
MATERIAL & METHODS

Apiaries and sampling

Samples of the managed European honeybee *Apis mellifera carnica* were collected from two EFB diseased apiaries (Table S1), both located in the EFB outbreak region in Eastern Bohemia, Czechia. Control samples were obtained in 2014 from six apiaries that were geographically isolated from the outbreak zone and analyzed previously (*Hubert et al., 2016a; Hubert et al., 2016b*). One control sample was newly analyzed in 2015. Honeybee samples comprised colonies with and without visible clinical symptoms obtained from a Pec pod Snezkou apiary, Trutnov District, and four colonies with clinical symptoms and two colonies without clinical symptoms obtained from a Horni Marsov apiary, Trutnov District. The State Veterinary Administration of the Czech Republic declared these two apiaries to be the epicenter of the outbreak zone, and plans to move any colonies in protective zone (radius 5 km from each disease outbreak) were abandoned. The samples in our study were similar to those analyzed in a study from Switzerland (*Belloy et al., 2007*). Worker honeybees from the nest were more suitable for EFB detection than bees obtained from the beehive entrance (*Roetschi et al., 2008*). The honeybees were shaken off the brood comb into plastic bags and placed in a box with dry ice for transport, followed by storage at -80 °C in a deep freezer (Thermo, Waltham, MA, USA).

For our analyses, we coded (EFB factor) the samples in the following manner: (i) EFB0 – control outside EFB zone with no EFB signs; (ii) EFB1 – bees from an EFB apiary without clinical symptoms; and (iii) EFB2 – bees from colonies with clinical symptoms of EFB.

DNA extraction from honeybees

Each honeybee sample included 10 hive worker bees, and analyses were performed in triplicate per colony from the EFB outbreak apiaries. To process the samples, we followed a previously described procedure (*Hubert et al., 2016a; Hubert et al., 2016b*). Prior to DNA extraction, samples were surface-sterilized using bleach and ethanol washes followed by a phosphate-buffered saline (PBS)-T wash. Then, the bees were transferred to polypropylene vials (Cat No. 3205, BioSpec Products, Bartlesville, OK, USA). Each vial was sterilized prior to use and contained a 0.6-g mixture of glass and garnet beads that were 0.1–1 mm in diameter (Cat. Nos. 11079101, 11079103gar, 11079105, 11079110gar. Biospec) (1/1/1 wt/wt/wt). Next, 2 mL of PBS-T and
4 mL of phenol/chloroform/isopropanol (Roti-Phenol®, Cat No. A156.2, Carl Roth, Karlsruhe, Germany) were added and homogenized for 2 min using a Mini-Beadbeater-16 (Biospec). The homogenates were transferred to sterile 15-mL centrifuge tubes (Orange Scientific, Braine-l'Alleud, Belgium) and centrifuged (4,508 g for 5 min). The supernatants were mixed with 6 mL of sterilized ddH$_2$O containing Tween 20 (Cat. No. P2287, Sigma-Aldrich, St. Louis, MO, USA) and centrifuged at 4,508 g for 5 min. The upper aqueous phases were extracted twice with chloroform/isopropanol (ratio: 24/1) and centrifuged. Then, the upper aqueous phases were transferred into Eppendorf tubes and precipitated with 0.7 mL of 3 M ammonium acetate (Cat No. S7899, Sigma-Aldrich, St. Louis, MO, USA) and isopropanol mixed at a volume ratio of 0.1/0.74. For precipitation, the mixture was incubated at -40 °C for 15 min. The tubes were subsequently centrifuged (13,845 g, 15 min), and the pellets were washed twice with 70% ethanol. The dried pellets were resuspended in 200 µL of ddH$_2$O tempered at 56 °C by pipetting. Finally, the DNA was cleaned using a GeneClean® Turbo kit (Cat No. 1102-600, MP Biomedicals, Santa Ana, CA, USA) and stored in a deep freezer (-40 °C) until use.

**Amplification, sequencing and bioinformatics**

The quality and presence of bacterial DNA in every sample was tested by performing PCR amplification using eubacterial primers and EFB primers (Govan et al., 1998; Lane, 1991). If an amplicon was not obtained, the sample was substituted with a new one that was amplicon-positive. DNA samples were sent to MR DNA (http://mrdnalab.com, Shallowater, TX, USA) for sequencing of the V1-V3 portion of the 16S rRNA gene using the Illumina MiSeq platform according to the manufacturer's guidelines. The universal eubacterial primers 27Fmod and 519Rmod were sequenced on the Illumina MiSeq platform utilizing methods based on the bTEFAP® process (Chiodini et al., 2015) by MR DNA (Shallowater, TX, USA). The read length was 300 bp, and both forward and reverse reads were obtained. Sequences were processed as described previously (Hubert et al., 2016b) using MOTHUR v.1.36.1 software (Schloss et al., 2009) according to the MiSeq standard operating procedure (MiSeq SOP) (Kozich et al., 2013) and UPARSE (Edgar, 2013). The actual MOTHUR commands used are available at http://www.mothur.org/wiki/MiSeq_SOP (accession date - 3/22/2016). Two sets of reads were aligned and the barcodes and primers were trimmed using MOTHUR. Then, the fastq file was processed using UPARSE. Individual operational taxonomic units (OTUs) were constructed by
binning sequences into clusters of 97% similarity and discarding singletons and putative chimeric OTUs in the process. The OTUs were identified according to the Ribosomal Database Project (http://rdp.cme.msu.edu) using training set No. 16 (Cole et al., 2014). Abundance data were then reincorporated into the dataset by mapping the initial sequences against the representative OTUs. A representative sequence of each OTU was further tested for chimeric artifacts using the SILVA reference database (Quast et al., 2013) and UCHIME (Edgar et al., 2011). Then, the representative sequences were processed using the blastn program on the NCBI platform (https://blast.ncbi.nlm.nih.gov/) (Altschul et al., 1997). OTUs exhibiting similarity to chloroplasts and Archaea were removed. The best search hits were chosen based on the highest bit score. The data were deposited in GenBank as SRA project No. SRP093440 (The microbiome of Apis mellifera associated to European foulbrood), and the list of samples is given in Table 1. The taxonomic features of the samples were visualized via KRONA projection (Ondov, Bergman & Phillippy, 2011). Abundance data were then transformed into a shared file and processed in MOTHUR.

Data analyses

Using MOTHUR, α-diversity was assessed by calculating the inverse Simpson index; additionally, the number of OTUs (sOTU) was calculated and analyzed for a subsample of 17,759 sequences per sample. Note that the subsample dataset consisted of 49 worker samples. The inverse Simpson index and number of OTUs were compared by performing a nonparametric Kruskal–Wallis test and the Dunn post-hoc procedure using XLSTAT software (http://www.xlstat.com/en/, Addinsoft, New York, NY, USA). β-diversity was assessed by transforming OTU data into a Bray–Curtis dissimilarity matrix. OTU variance in the honeybee bacteriome was analyzed by determining the homogeneity of molecular variance (HOMOVA) followed by the analysis of molecular variance (AMOVA); both were calculated using MOTHUR and employing the subsample data and 100,000 permutations. Moreover, a distance-based redundancy analysis (db-RDA) was performed using the “vegan” R package (Oksanen et al., 2016). In particular, a partial version of the db-RDA was carried out in which the influences of geographic coordinates and the time of bee collection (in terms of Julian days) were suppressed. The environmental variables included geographical position, sampling time expressed as Julian days, EFB factor and the results of PCR carried out with EFB primers (conventional PCR confirmation). Because the primary focus here was the
identification of explanatory variables from environmental variables correlated to OTU4 was of
the primary focus here, also the logarithmic transformation (LOG2), as previously recommended
(Anderson, Ellingsen & McArdle, 2006), was applied only to the column representing this OTU,
and then another partial RDA model was constructed using this column and all possible
explanatory variables. In both cases, including the RDA with all OTUs and the RDA with OTU4
alone, the significance of explanatory variables was also studied by performing a forward selection
procedure using the “packfor” R package (Dray, Legendre & Blanchet, 2013). The redundancy of
explanatory variables was controlled using variance inflation factors (VIFs) (Kutner et al., 2005).
An attempt was made to find the best partial RDA model in terms of the smallest P value that
would explain the variance in OTUs. Population-level analyses were carried out by comparing the
effects of the EFB zone on OTUs in honeybee samples. Relative OTU abundance in the samples
was tested using METASTATS in MOTHUR with 100,000 permutations and with random forest
algorithms using 1,500 trees. Using the relative OTU abundance data, a heatmap was constructed
to determine whether OTUs clustered across samples with the “gplots” R package (Warnes et al.,
2016). In addition, dendrogram nodes were tested for significance utilizing the similarity profile
(SIMPROF) procedure (Clarke, Somerfield & Gorley, 2008), which was implemented with the

RESULTS

Bacteriome analyses

The worker bee bacteriome was formed from 2,737,138 sequences in 94 OTUs (Table S2). The
composition of the bacteriome was characterized using Krona projections for different situations
according to the EFB factor (Fig. S1). The inverse Simpson diversity index was not influenced by
the coded EFB factor (Kruskal–Wallis test; K = 3.183, P = 0.208).

The effects of the coded EFB factor on OTU distribution in the worker bee bacteriome were
tested using the Bray–Curtis dissimilarity measure. The HOMOVA was not influenced by the EFB
factor (BValue = 2.747, P = 0.087). AMOVA indicated differences in the bacteriome based on the
coded EFB factor (Fs = 5.48, P < 0.001). Pairwise comparison after the Bonferroni correction (P
≤ 0.05) indicated pairwise differences in the bee bacteriome, specifically between colonies with
(EFB2) and without clinical symptoms (EFB1) (Fs = 3.565, P = 0.023), colonies with (EFB2) clinical symptoms and control colonies (EFB0) (Fs = 7.211, P < 0.001), and colonies without clinical symptoms (EFB1) and control colonies (EFB0) (Fs = 4.256, P < 0.001). Sample position and the Pearson correlation for selected OTUs to the axes were visualized using non-metric multidimensional scaling functions (Fig. 1).

During RDA analyses, one of the VIFs slightly crossed the limit of 10, which is considered an indication of multicollinearity. Specifically, the VIF connected to the third level of the EFB factor (EFB2) reached a value of 10.6. However, this value was not too high, and therefore, all explanatory variables were included in the RDA models (i.e., EFB0-2 plus PCR detection). Julian days, which represent the sample collection times, and geographic coordinates in terms of latitude and longitude conditioned the influence of the EFB factor and PCR detection for all models. These conditioning proved to be good choices because longitude had a significant negative effect on OTU abundance. Thus, lower abundance may be expected in locations further to the east. Based on the forward selection procedure, Julian days also had a certain effect. Moreover, only the first axis of the RDA was significant (P < 0.01) if all explanatory variables were added to the model with all OTUs. The situation was not very different when M. plutonius (OTU4) (or its logarithm) was studied as the only dependent variable. However, taking into account the partial RDA counterparts, none of the models (either with all OTUs or with OTU4 only) were significant at the level of 0.05. Furthermore, none of the terms added as explanatory variables (excluding those acting as conditions) appeared to be significant, and newly obtained axes were not significant either.

Population level analyses indicated differences in the relative numbers of OTUs according to EFB factor, and random forest tree algorithms revealed OTUs that significantly contributed to differences in the bacteriome (Error Rate = 0.12) (Table 1). For symbiotic bacteria, there were significantly higher relative numbers of Snodgrassella alvi (OTU6), Lactobacillus mellis (OTU8), Lactobacillus melliventris (OTU25), and Fructobacillus fructosus (OTU5) in samples with clinical symptoms based on METASTATS analyses (Table 1). In contrast, Bartonella apis (OTU2), Frischella perrara (OTU18), and Commensalibacter intestini (OTU15) numbers decreased in samples from colonies with EFB clinical symptoms.

The heatmap (Fig. 2) shows the relative abundance of 24 OTUs selected based on total abundance equal to or greater than 2,000 in the cluster analyses shown in Figure S2. For example,
the OTU distribution appeared to be random relative to the band on the left-hand side that captured three levels of EFB factors. However, this trend might have occurred because a subsample was used to construct the heatmap; with a greater number of samples, the pattern may differ, as suggested by the population level analyses described above. Indeed, an association between greater numbers of *M. plutonius* (OTU4) and clinical symptoms (EFB2) is apparent. However, in the subsample, a random distribution, as suggested by the RDA analyses, appears more likely.

**Population level analyses of *M. plutonius***

Based on random forest algorithms, *M. plutonius* (OTU4) had the highest mean accuracy of all OTUs analyzed using the EFB factor (Table 1). The relative numbers of sequences were highest in the outbreak colonies with clinical signs (EFB2), followed by highly decreased numbers in colonies from EFB apiaries without clinical symptoms (EFB1) and the lowest numbers or the absence of sequences in control apiaries outside the outbreak zone (EFB0). METASTATS analyses (Table 1) indicated the coded effects of the EFB factor were significant in terms of relative numbers at the P = 0.05 level; specifically, EFB2 was different from EFB1 and EFB0, and EFB1 was different from EFB0. However, when sequence numbers were compared in the subsample dataset, there were three apiaries from control colonies outside the EFB zone with numbers of sequences ranging from 10 to 15 (Fig. 3), although *M. plutonius* (OTU4) was absent in the other control colonies (Table S1).

**Comparison of *M. plutonius* detection via NGS bacteriome analysis and conventional PCR**

Based on the conventional PCR results (Table 1), *M. plutonius* was detected in 26 of 29 tested samples from EFB2 colonies with clinical symptoms and in 0 of 7 tested samples from EFB1 colonies without clinical symptoms but located in outbreak apiaries, while no samples from the 16 tested control colonies were positive for *M. plutonius*. In contrast, the use of Illumina NGS to investigate the honeybee bacteriome indicated all samples from outbreak sites (including both EFB1 and EFB2) were positive for *M. plutonius*, and 3 of 16 tested samples from EFB0 control colonies outside the outbreak zone were positive for *M. plutonius*. 


Comparison of *M. plutonius* detected in larvae and pupae from outbreak apiaries

The bacteriomes of 4 larvae and 2 pupae samples were comparatively analyzed (Fig. S3). Relative numbers of *M. plutonius* sequences represented ca. 50% of total sequences in larvae from EFB2 colonies with clinical symptoms, while 0-1% of total sequences were isolated from larvae in EFB1 colonies without clinical symptoms. The relative sequence numbers isolated from pupae obtained from colonies with clinical symptoms were 11% and 42%.
DISCUSSION

In this study, we analyzed EFB occurrence in apiaries for the first time using an NGS approach. The experimental sampling design was similar to previous studies (Belloy et al., 2007; Roetschi et al., 2008), that is, the distribution of *M. plutonius* was investigated in apiaries and colonies with and without clinical signs of EFB. Previous studies (i.e., Belloy et al. (2007)) employing conventional (hemi-nested) PCR techniques were not able to demonstrate quantitative comparisons. However, our approach enabled us to express the prevalence of *M. plutonius* in diseased and asymptomatic colonies and apiaries. Due to the quantitative advantage of real-time PCR, Roetschi et al. (2008) were able to show the increased load of *M. plutonius* in workers isolated from brood combs compared to bees near hive entrances. In this study, according to our NGS analysis, all colonies, including asymptomatic ones, in two tested apiaries exhibiting clinical EFB symptoms were positive for *M. plutonius*. The comparison of our NGS results with conventional PCR analysis revealed 7 false negative results from the diseased apiaries. Overall, the NGS approach better matched the *M. plutonius* distribution in the tested apiaries. Moreover, the evaluation of NGS results permitted the quantitative interpretation of *M. plutonius* within the honeybee worker bacteriome. Thus, this advanced high-throughput approach offers benefits when studying the spread of this pathogen, even in clinical or latent phases.

Our investigation of the worker honeybee bacteriome revealed a 100-fold higher load of *M. plutonius* in worker bees from colonies exhibiting clinical symptoms compared to asymptomatic colonies at diseased sites. These results support the previous suspicion that workers in brood combs from EFB asymptomatic colonies contain high levels of *M. plutonius*; furthermore, colonies exhibiting clinical symptoms demonstrated *M. plutonius* loads above 50,000 CFU per honeybee, and bees from colonies with less than 10 visibly diseased larvae had bacterial loads up to 100-fold or lower (Roetschi et al., 2008). Notably, the pathogen load in worker bees was likely influenced by their hygienic behavior (Waite, Brown & Thompson, 2003). According to Roetschi et al. (2008), the quantification threshold serves as a tool to screen colony health status. When considering molecular tools other than qPCR (Roetschi et al., 2008), NGS may be useful for analyzing worker bees to quantify EFB levels in a colony.

One important finding of this study was the detection of *M. plutonius* in 3 of 16 control colonies located far from the EFB zone. Given these results, *M. plutonius* potentially spontaneously occurs in healthy honeybee colonies in low numbers. These results also support the
data obtained in our NGS experiments (unpublished data). This trend was previously observed using ELISA, and it was confirmed by hemacytometer and plate counts (Pinnock & Featherstone, 1984) that confirmed the occurrence of *M. plutonius* in the larvae of certain healthy colonies. Support for the ordinary occurrence of *M. plutonius* in colonies was provided by a study conducted in Spain, in which the prevalence of *M. plutonius* in both broods and workers was determined by PCR to be lower than 1% (Garrido-Bailon et al., 2013). In summary, we believe the honeybee bacteriome will allow us to study how *M. plutonius* occurs in honeybee colonies in an enzootic state (Pinnock & Featherstone, 1984).

Compared to asymptomatic colonies in EFB apiaries, the proportion of *M. plutonius* in the bacteriome was considerably lower in control colonies. Therefore, asymptomatic colonies from diseased sites are at a high risk of disease development. Although sanitation was performed as part of a study in Switzerland, this measure was not sufficient to prevent EFB outbreaks the following year in the same apiaries (Roetschi et al., 2008). Thus, even after symptomatic colonies were removed from a diseased site, there was still a danger of EFB outbreak. Therefore, elimination of the entire site should be strongly considered. According to regulations in Czechia, apiaries currently showing evidence of the EFB outbreak have been eliminated, which was the right choice in our opinion.

Cultivation experiments have revealed the suppressive effects of certain cultivable bacteria from the gut of *A. cerana japonica* worker bees on the growth of *M. plutonius* (Wu et al., 2014). None of the observed bacteria belonged to the core symbiotic bacteria found in honeybees. However, among *Lactobacillus kunkeei* isolates, studies have identified strains exhibiting antibacterial activity against *M. plutonius* (Endo & Salminen, 2013; Vasquez et al., 2012). *L. kunkeei* was observed to be dominant in honeybee larvae (Endo & Salminen 2013), but it also forms biofilms in adult bees (Vasquez et al., 2012). Suppression was suggested to occur through the production of anti-*M. plutonius* peptides (Endo & Salminen, 2013). Despite that mechanism, we did not observe any effects of the presence of *M. plutonius* on the alpha diversity of the bacterial community in honeybee workers, and although AMOVA showed a significant effect of EFB zones on bacterial distribution, RDA did not confirm the significant effects of selected environmental variables. The presence of *M. plutonius* in asymptomatic larvae and pupae in the outbreak zone was confirmed, but very high variability, ranging from 0-50% of total sequences, was observed. Due to low sample numbers, the presence of *M. plutonius* in juvenile stadia was for orientation
purposes only, and a more detailed study of those stadia is necessary. Nevertheless, our results indicated no significant interactions between *M. plutonius* and core bacteria in the worker bacteriome based on β-diversity statistical analyses. *M. plutonius* potentially passes through the gut in a passive manner or is present in the glands of bees, or the number of samples was too low to observe any correlations.

METASTATS analyses of the honeybee worker bacteriome revealed differences between core bacteria and non-core bacteria (*Engel et al., 2016*). The relative abundance of *Snodgrassella alvi*, *Lactobacillus mellis*, *L. melliventris* and *Fructobacillus fructosus* was increased in bee samples from EFB colonies exhibiting clinical symptoms, while the relative abundance of *Bartonella apis*, *Frischella perrara* and *Commensalibacter intestini* decreased. Based on the results of our population-level statistical analyses, EFB indirectly influenced the bacteriome; specifically, colony weakness corresponded to nutrient changes in the honeybee bacteriome, which resulted in the observed statistical differences.

Based on 16S rRNA analyses, we cannot rule out the existence of bacterial strains with low pathogenic effects (*Arai et al., 2012*); strains exhibiting differing pathogenic effects due to geographical and time isolation are well-documented (*Budge et al., 2014; Haynes et al., 2013; Takamatsu et al., 2014*). However, as observed in this study, the detection limit of conventional PCR did not permit us to identify the presence of bacteria, and a similar situation is likely the case for the cultivation limit.

**ACKNOWLEDGEMENTS**

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**ADDITIONAL INFORMATION AND DECLARATIONS**

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**Competing interests**

The authors declare that they have no competing interests.
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FIGURES AND TABLES
Table 1  Relative proportion of selected OTUs in the honeybee bacteriome. Coding for the sample types: EFB0 – control outside the EFB zone without signs of EFB; (ii) EFB1 – bees from an EFB apiary but from colonies without clinical symptoms; and (iii) EFB2 – bees from colonies with clinical symptoms of EFB. Sequences were analyzed using METASTATS, and P values are presented. The OTUs are described according to the closest match in GenBank. For a detailed description, see Table S2.

<table>
<thead>
<tr>
<th>OTU&lt;sub&gt;27&lt;/sub&gt;</th>
<th>GenBank identification</th>
<th>aOTU</th>
<th>Mean decrease accuracy</th>
<th>EFB2</th>
<th>EFB1</th>
<th>EFB0</th>
<th>EFB factor</th>
<th>2/1</th>
<th>2/0</th>
<th>1/0</th>
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<tbody>
<tr>
<td>OTU4</td>
<td>Melissococcus plutonius (99)</td>
<td>13,112</td>
<td>1.361</td>
<td>0.03055</td>
<td>0.00705</td>
<td>0.00046</td>
<td>0.00009</td>
<td>0.00012</td>
<td>0.00007</td>
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<td>OTU5</td>
<td>Fructobacillus fructosus (99)</td>
<td>9,845</td>
<td>0.737</td>
<td>0.02272</td>
<td>0.00817</td>
<td>0.00133</td>
<td>0.00066</td>
<td>0.00006</td>
<td>0.00003</td>
<td>0.028</td>
</tr>
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<td>OTU109</td>
<td>Lactobacillus kimbladii (99)</td>
<td>2,668</td>
<td>0.313</td>
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<td>0.00021</td>
<td>0.00487</td>
<td>0.00100</td>
<td>0.00264</td>
<td>0.00057</td>
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</tr>
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<td>OTU9</td>
<td>Lactobacillus kunkeei (99)</td>
<td>5,679</td>
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<td>0.01260</td>
<td>0.00500</td>
<td>0.00045</td>
<td>0.00010</td>
<td>0.00077</td>
<td>0.00063</td>
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<tr>
<td>OTU8</td>
<td>Lactobacillus mellis (99)</td>
<td>44,528</td>
<td>0.273</td>
<td>0.06059</td>
<td>0.00527</td>
<td>0.05986</td>
<td>0.00988</td>
<td>0.03653</td>
<td>0.00707</td>
<td>0.962</td>
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<td>OTU6</td>
<td>Snodgrassella alvi (99)</td>
<td>101,925</td>
<td>0.269</td>
<td>0.12245</td>
<td>0.01005</td>
<td>0.22007</td>
<td>0.02506</td>
<td>0.07790</td>
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<td>OTU7</td>
<td>Lactobacillus helsingborgensis (99)</td>
<td>61,676</td>
<td>0.245</td>
<td>0.07324</td>
<td>0.00906</td>
<td>0.10213</td>
<td>0.00599</td>
<td>0.05802</td>
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<td>OTU12</td>
<td>Lactobacillus kullberogensis (99)</td>
<td>26,246</td>
<td>0.243</td>
<td>0.02732</td>
<td>0.00246</td>
<td>0.04641</td>
<td>0.00354</td>
<td>0.02862</td>
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<td>0.000</td>
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<tr>
<td>OTU28</td>
<td>Pseudomonas indica (88)</td>
<td>2,147</td>
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<td>0.00031</td>
<td>0.00023</td>
<td>0.00008</td>
<td>0.00004</td>
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<tr>
<td>OTU16</td>
<td>Gilliamella apicola (97)</td>
<td>7,951</td>
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<td>0.00033</td>
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<td>OTU3</td>
<td>Gilliamella apicola (99)</td>
<td>203,595</td>
<td>0.222</td>
<td>0.26670</td>
<td>0.01680</td>
<td>0.18932</td>
<td>0.02695</td>
<td>0.20672</td>
<td>0.02193</td>
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<tr>
<td>OTU15</td>
<td>Commensalibacter intestini (95)</td>
<td>9,021</td>
<td>0.222</td>
<td>0.00318</td>
<td>0.00082</td>
<td>0.00313</td>
<td>0.00117</td>
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<td>0.01430</td>
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<td>OTU25</td>
<td>Lactobacillus melliventris (97)</td>
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<td>0.205</td>
<td>0.01301</td>
<td>0.00161</td>
<td>0.02103</td>
<td>0.00266</td>
<td>0.00863</td>
<td>0.00115</td>
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<td>OTU18</td>
<td>Frischella perrara (99)</td>
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<td>0.02100</td>
<td>0.00269</td>
<td>0.01231</td>
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<tr>
<td>OTU33</td>
<td>Bifidobacterium asteroides (98)</td>
<td>18,563</td>
<td>0.200</td>
<td>0.02090</td>
<td>0.00259</td>
<td>0.02553</td>
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<td>0.02056</td>
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<td>OTU13</td>
<td>Lactobacillus mellifer (99)</td>
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<td>0.198</td>
<td>0.00849</td>
<td>0.00095</td>
<td>0.00991</td>
<td>0.00186</td>
<td>0.01197</td>
<td>0.00197</td>
<td>0.634</td>
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<td>OTU2</td>
<td>Bartonella bacilliformis (96)</td>
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<td>0.05323</td>
<td>0.01818</td>
<td>0.05017</td>
<td>0.02327</td>
<td>0.14713</td>
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<tr>
<td>OTU14</td>
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<td>0.04562</td>
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<td>0.02072</td>
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<tr>
<td>OTU10</td>
<td>Enterococcus faecalis (99)</td>
<td>16,167</td>
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<td>0.00029</td>
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<td>0.00020</td>
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<tr>
<td>OTU17</td>
<td>Bifidobacterium coryneforme (99)</td>
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<td>0.189</td>
<td>0.00969</td>
<td>0.00169</td>
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<td>0.00913</td>
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<tr>
<td>OTU23</td>
<td>Ornithobacterium rhinotraceale (84)</td>
<td>2,974</td>
<td>0.183</td>
<td>0.00290</td>
<td>0.00104</td>
<td>0.00294</td>
<td>0.00077</td>
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<td>OTU55</td>
<td>Gilliamella apicola (98)</td>
<td>38,619</td>
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<td>0.00292</td>
<td>0.03045</td>
<td>0.00246</td>
<td>0.05264</td>
<td>0.01110</td>
<td>0.017</td>
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<tr>
<td>OTU21</td>
<td>Dysgonomonas carcinophagoides (92)</td>
<td>3,944</td>
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<td>0.00231</td>
<td>0.00100</td>
<td>0.00541</td>
<td>0.00204</td>
<td>0.00706</td>
<td>0.00402</td>
<td>0.228</td>
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<tr>
<td>OTU1</td>
<td>Lactobacillus apis (99)</td>
<td>131,977</td>
<td>0.163</td>
<td>0.13239</td>
<td>0.01461</td>
<td>0.15581</td>
<td>0.01973</td>
<td>0.17471</td>
<td>0.02587</td>
<td>0.486</td>
</tr>
</tbody>
</table>
Non-metric multidimensional scaling visualization of the distribution of honeybee samples and selected OTUs.

OTUs were correlated with axes (Pearson), and those demonstrating significant correlations were selected. Coding for the sample types: EFB0 – control outside the EFB zone without signs of EFB; (ii) EFB1 – bees from an EFB apiary but from colonies without clinical symptoms; and (iii) EFB2 – bees from colonies with clinical symptoms of EFB. For the list of samples and OTUs, see Table S2.
Stress = 0.160
R² = 0.84

Legend:
EFB0  
EFB1  
EFB2
Figure 2 (on next page)

Heatmap describing the distribution of samples and OTUs in the *Apis mellifera* bacteriome.

Coding for the sample types: EFB0 – control outside the EFB zone without signs of EFB; (ii) EFB1 – bees from an EFB apiary but from colonies without clinical symptoms; and (iii) EFB2 – bees from colonies with clinical symptoms of EFB. For the legend of samples and OTUs, see Table S2.
Figure 3 (on next page)

Scatterplots describing the mean numbers of *Melissococcus plutonius* (OTU 4) sequences in the subsample for different situations according to EFB occurrence.

Coding for the sample types: EFB0 - control outside the EFB zone without signs of EFB; (ii) EFB1 - bees from an EFB apiary but from colonies without clinical symptoms; and (iii) EFB2 - bees from colonies with clinical symptoms of EFB.
Control EFB with clinical symptoms

EFB without clinical symptoms

EFB with clinical symptoms