

**A peer-reviewed version of this preprint was published in PeerJ on 25 September 2017.**

[View the peer-reviewed version](https://doi.org/10.7717/peerj.3816) (peerj.com/articles/3816), which is the preferred citable publication unless you specifically need to cite this preprint.

Erban T, Ledvinka O, Kamler M, Hortova B, Nesvorna M, Tyl J, Titera D, Markovic M, Hubert J. 2017. Bacterial community associated with worker honeybees (*Apis mellifera*) affected by European foulbrood. PeerJ 5:e3816 <https://doi.org/10.7717/peerj.3816>

# 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*

Tomas Erban <sup>Corresp., 1</sup>, Ondrej Ledvinka <sup>2</sup>, Martin Kamler <sup>3</sup>, Bronislava Hortova <sup>1</sup>, Marta Nesvorna <sup>1</sup>, Jan Tyl <sup>3</sup>, Dalibor Titera <sup>4,5</sup>, Martin Markovic <sup>1</sup>, Jan Hubert <sup>1</sup>

<sup>1</sup> Crop Research Institute, Prague, Czech Republic

<sup>2</sup> Hydrological Database and Water Budget Department, Czech Hydrometeorological Institute, Prague, Czechia

<sup>3</sup> Bee Research Institute at Dol, Libcice nad Vltavou, Czech Republic

<sup>4</sup> Research, Bee Research Institute at Dol, Libcice nad Vltavou, Czech Republic

<sup>5</sup> Faculty of Agrobiological Sciences, Department of Zoology and Fisheries, Czech University of Life Sciences, 165 21 Praha 6, Czech Republic

Corresponding Author: Tomas Erban

Email address: arachnid@centrum.cz

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***

Tomas Erban<sup>1,\*</sup>, Ondrej Ledvinka<sup>2</sup>, Martin Kamler<sup>3</sup>, Bronislava Hortova<sup>1</sup>, Marta Nesvorna<sup>1</sup>, Jan  
Tyl<sup>3</sup>, Dalibor Titera<sup>3</sup>, Martin Markovic<sup>1</sup> and Jan Hubert<sup>1</sup>

<sup>1</sup>Crop Research Institute, Drnovska 507/73, Prague 6-Ruzyne, CZ-161 06, Czechia

<sup>2</sup>Czech Hydrometeorological Institute, Na Sabatce 2050/17, Prague 412, CZ-143 06, Czechia

<sup>3</sup>Bee Research Institute at Dol, Maslovice-Dol 94, Libcice nad Vltavou, CZ-252 66, Czechia

Running title: *Melissococcus plutonius* and honeybee bacteriome

Journal: PeerJ

Journal link: <https://peerj.com>

No. of tables: 1

No. of figures: 3

No. of supplementary figures: 3

No. of supplementary tables: 2

\*Corresponding author:

Tomas Erban; Crop Research Institute; Laboratory of Proteomics; Drnovska 507/73, Praha 6-  
Ruzyne; CZ-16106; Czechia

E-mail: [arachnid@centrum.cz](mailto:arachnid@centrum.cz)

## 24 ABSTRACT

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

44

45 **Keywords** *Apis mellifera* / European foulbrood / *Melissococcus plutonius* / bacteriome /  
 46 *Snodgrassella alvi* / *Lactobacillus* / *Fructobacillus fructosus* / *Bartonella apis* / *Frischella*  
 47 *perrara* / *Commensalibacter intestine*

## 48 INTRODUCTION

49 European foulbrood (EFB) is caused by the Gram-positive lanceolate coccus *Melissococcus*  
50 *plutonius* and is one of the most important diseases of the European honeybee, *Apis mellifera* L.  
51 This emerging honeybee disease is listed in the Terrestrial Animal Health Code of The World  
52 Organisation for Animal Health (OIE) (OIE, 2016b). Although EFB spread is worldwide and the  
53 clinical signs are similar to American foulbrood (AFB), EFB is not notifiable in all countries  
54 (Forsgren et al., 2013). Indeed, there is a need to understand how this honeybee disease contributes  
55 to colony losses.

56 EFB weakens a honeybee colony through brood loss and results in colony collapse (Forsgren  
57 et al., 2013). Due to the presence of *M. plutonius* forms demonstrating differential virulence (Arai,  
58 et al. 2012; Budge et al., 2014), disease development differs between countries (Forsgren et al.,  
59 2013). In the past few years, EFB has increased in some European countries; in particular, it has  
60 increased consistently in Switzerland since 1997 (Belloy et al., 2007; Roetschi et al., 2008). In the  
61 UK, EFB has become the most common brood disease (Budge et al., 2011; Wilkins, Brown &  
62 Cuthbertson, 2007), and there was a regional outbreak in Norway in 2010 after 30 years of absence  
63 (Dahle, Sorum & Weideman, 2011). In addition, EFB has historically occurred in Czechia. During  
64 the 1970s, it was found in Kralupy nad Vltavou in Central Bohemia (personal communication from  
65 Dr. Frantisek Kamler). EFB was also recently found in 2015, and additional signs of the disease  
66 emerged in 2016 in Krkonose Mountains National Park in Eastern Bohemia (KVSH, 2015),  
67 representing the first cases after 40 years of no EFB in Czechia (Kamler et al., 2016).

68 Methods used to detect EFB were reviewed by Forsgren et al. (2013) and are included in the  
69 COLOSS BeeBook (COLOSS 2013) and in the OIE Terrestrial Manual (OIE, 2016a). Signs of  
70 EFB disease include bacteremia of honeybee larvae, which usually die between 4 and 5 days of  
71 age or sometimes when they are older, after sealing (Forsgren, 2010). The first step in EFB  
72 infection is asymptomatic colonization of the larval gut after food transmission by nurse bees.  
73 Infection can cause fewer than 100 *M. plutonius* cells which rapidly reproduce in the larval gut  
74 (Bailey, 1960). Compared to *Paenibacillus larvae*, which forms spores, non-sporulating *M.*  
75 *plutonius* is more difficult to identify utilizing cultivation techniques because less than 0.2% of  
76 cells are detectable (Djordjevic et al., 1998; Hornitzky & Smith, 1999). Freshly dead larvae are  
77 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) (1/1/1/1 wt/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<sub>2</sub>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<sub>2</sub>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](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,  $\alpha$ -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).  $\beta$ -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 “clustsig” R package (Whitaker & Christman, 2014).

## 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 ( $F_s = 5.48$ ,  $P < 0.001$ ). Pairwise comparison after the Bonferroni correction ( $P \leq 0.05$ ) indicated pairwise differences in the bee bacteriome, specifically between colonies with

(EFB2) and without clinical symptoms (EFB1) ( $F_s = 3.565$ ,  $P = 0.023$ ), colonies with (EFB2) clinical symptoms and control colonies (EFB0) ( $F_s = 7.211$ ,  $P < 0.001$ ), and colonies without clinical symptoms (EFB1) and control colonies (EFB0) ( $F_s = 4.256$ ,  $P < 0.001$ ). 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  $\beta$ -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

The authors are grateful to the beekeepers and the State Veterinary Administration of the Czech Republic (<http://eagri.cz/public/web/en/svs/portal/>) for allowing us to collect bee samples during the outbreak. We thank Marie Bostlova and Jan Hubert Jr. for technical help.

## ADDITIONAL INFORMATION AND DECLARATIONS

### Funding

The study was supported by project No. QJ1310085 of the Ministry of Agriculture of the Czech Republic (<http://www.eagri.cz>).

### Competing interests

The authors declare that they have no competing interests.

# REFERENCES

- Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* **25**(17):3389–3402 DOI 10.1093/nar/25.17.3389.
- Anderson MJ, Ellingsen KE, McArdle BH. 2006. Multivariate dispersion as a measure of beta diversity. *Ecology Letters* **9**(6):683–693 DOI 10.1111/j.1461-0248.2006.00926.x.
- Arai R, Tominaga K, Wu M, Okura M, Ito K, Okamura N, Onishi H, Osaki M, Sugimura Y, Yoshiyama M, Takamatsu D. 2012. Diversity of *Melissococcus plutonius* from honeybee larvae in Japan and experimental reproduction of European foulbrood with cultured atypical isolates. *PLoS One* **7**(3):e33708 DOI 10.1371/journal.pone.0033708.
- Bailey L. 1960. The epizootiology of European foulbrood of the larval honey bee, *Apis mellifera* Linnaeus. *Journal of Insect Pathology* **2**(2):67–83.
- Belloy L, Imdorf A, Fries I, Forsgren E, Berthoud H, Kuhn R, Charriere J-D. 2007. Spatial distribution of *Melissococcus plutonius* in adult honey bees collected from apiaries and colonies with and without symptoms of European foulbrood. *Apidologie* **38**:136–140 DOI 10.1051/apido:2006069.
- Budge GE, Barrett B, Jones B, Pietravalle S, Marris G, Chantawannakul P, Thwaites R, Hall J, Cuthbertson AGS, Brown MA. 2010. The occurrence of *Melissococcus plutonius* in healthy colonies of *Apis mellifera* and the efficacy of European foulbrood control measures. *Journal of Invertebrate Pathology* **105**(2):164–170 DOI 10.1016/j.jip.2010.06.004.
- Budge GE, Jones B, Powell M, Anderson L, Laurenson L, Pietravalle S, Marris G, Haynes E, Thwaites R, Bew J, Wilkins S, Brown M. 2011. Recent advances in our understanding

of European foulbrood in England and Wales. In: Jensen AB, Forsgren E, Genersch E, eds. *Proceedings of the COLOSS workshop: The future of brood disease research – guidelines, methods and development, Copenhagen, Denmark, April 10–12, 2011*. Bern, Switzerland: COLOSS (Prevention of honey bee Colony LOSSes), 7–7. Available at <http://www.coloss.org/publications/annex-5-a-copenhagen-april-2011-proceedings> (accessed 6 August 2016).

**Budge GE, Shirley MD, Jones B, Quill E, Tomkies V, Feil EJ, Brown MA, Haynes EG.**

**2014.** Molecular epidemiology and population structure of the honey bee brood pathogen *Melissococcus plutonius*. *ISME Journal* **8(8)**:1588–1597 DOI 10.1038/ismej.2014.20.

**Chiodini RJ, Dowd SE, Chamberlin WM, Galandiuk S, Davis B, Glassing A. 2015.**

Microbial population differentials between mucosal and submucosal intestinal tissues in advanced Crohn's disease of the ileum. *PLoS One* **10(7)**:e0134382, DOI 10.1371/journal.pone.0134382.

**Clarke KR, Somerfield PJ, Gorley RN. 2008.** Testing of null hypotheses in exploratory

community analyses: similarity profiles and biota-environment linkage. *Journal of Experimental Marine Biology and Ecology* 366:56–69 DOI 10.1016/j.jembe.2008.07.009.

**Cole JR, Wang Q, Fish JA, Chai B, McGarrell DM, Sun Y, Brown CT, Porras-Alfaro A,**

**Kuske CR, Tiedje JM. 2014.** Ribosomal Database Project: data and tools for high throughput rRNA analysis. *Nucleic Acids Research* **42(Database issue)**:D633–D642 DOI 10.1093/nar/gkt1244.

**COLOSS. 2013.** The COLOSS BEEBOOK – Standard Methods for *Apis mellifera* research.

Bern, Switzerland: COLOSS (Prevention of honey bee Colony LOSSes). Available at <http://www.coloss.org/beebook> (accessed 6 August 2016).

- 429 **Dahle B, Sorum H, Weideman JE. 2011.** European foulbrood in Norway: how to deal with a  
430 major outbreak after 30 years absence. In: Jensen AB, Forsgren E, Genersch E, eds.  
431 *Proceedings of the COLOSS workshop: The future of brood disease research – guidelines,*  
432 *methods and development, Copenhagen, Denmark, April 10–12, 2011.* Bern, Switzerland:  
433 COLOSS (Prevention of honey bee COlony LOSSes), 9–9. Available at  
434 <http://www.coloss.org/publications/annex-5-a-copenhagen-april-2011-proceedings>  
435 (accessed 6 August 2016).
- 436 **Djordjevic SP, Noone K, Smith L, Hornitzky MAZ. 1998.** Development of a hemi-nested  
437 PCR assay for the specific detection of *Melissococcus pluton*. *Journal of Apicultural*  
438 *Research* **37(3)**:165–174 DOI 10.1080/00218839.1998.11100968.
- 439 **Dray S, Legendre P, Blanchet G. 2013.** packfor: Forward selection with permutation (Canoco  
440 p. 46). R-Forge, The R Project for Statistical Computing. Available at [http://R-Forge.R-](http://R-Forge.R-project.org/projects/sedar/)  
441 [project.org/projects/sedar/](http://R-Forge.R-project.org/projects/sedar/) (accessed 6 August 2016).
- 442 **Edgar RC. 2013.** UPARSE: highly accurate OTU sequences from microbial amplicon reads.  
443 *Nature Methods* **10(10)**:996–998 DOI 10.1038/nmeth.2604.
- 444 **Edgar RC, Haas BJ, Clemente JC, Quince C, Knight R. 2011.** UCHIME improves sensitivity  
445 and speed of chimera detection. *Bioinformatics* **27(16)**:2194–2200 DOI  
446 10.1093/bioinformatics/btr381.
- 447 **Endo A, Salminen S. 2013.** Honeybees and beehives are rich sources for fructophilic lactic acid  
448 bacteria. *Systematic and Applied Microbiology* **36(6)**:444–448 DOI  
449 10.1016/j.syapm.2013.06.002.
- 450 **Engel P, Kwong WK, McFrederick Q, Anderson KE, Barribeau SM, Chandler JA,**  
451 **Cornman RS, Dainat J, de Miranda JR, Doublet V, Emery O, Evans JD, Farinelli L,**

- 452 **Flenniken ML, Granberg F, Grasis JA, Gauthier L, Hayer J, Koch H, Kocher S,**
- 453 **Martinson VG, Moran N, Munoz-Torres M, Newton I, Paxton RJ, Powell E, Sadd**
- 454 **BM, Schmid-Hempel P, Schmid-Hempel R, Song SJ, Schwarz RS, vanEngelsdorp D,**
- 455 **Dainat B. 2016.** The bee microbiome: impact on bee health and model for evolution and
- 456 ecology of host-microbe interactions. *mBio* **7(2):**e02164-15 DOI 10.1128/mBio.02164-15.
- 457 **Forsgren E. 2010.** European foulbrood in honey bees. *Journal of Invertebrate Pathology*
- 458 **103(Suppl. 1):**S5–S9 DOI 10.1016/j.jip.2009.06.016.
- 459 **Forsgren E, Budge GE, Charriere J-D, Hornitzky MAZ. 2013.** Standard methods for
- 460 European foulbrood research. *Journal of Apicultural Research* **52(1):**52.1.12 DOI
- 461 10.3896/IBRA.1.52.1.12.
- 462 **Garrido-Bailon E, Higes M, Martinez-Salvador A, Antunez K, Botias C, Meana A, Prieto**
- 463 **L, Martin-Hernandez R. 2013.** The prevalence of the honeybee brood pathogens
- 464 *Ascosphaera apis*, *Paenibacillus larvae* and *Melissococcus plutonius* in Spanish apiaries
- 465 determined with a new multiplex PCR assay. *Microbial Biotechnology* **6(6):**731–739 DOI
- 466 10.1111/1751-7915.12070.
- 467 **Govan VA, Brozel V, Allsopp MH, Davison S. 1998.** A PCR detection method for rapid
- 468 identification of *Melissococcus pluton* in honeybee larvae. *Applied and Environmental*
- 469 *Microbiology* **64(5):**1983–1985.
- 470 **Haynes E, Helgason T, Young JPW, Thwaites R, Budge GE. 2013.** A typing scheme for the
- 471 honeybee pathogen *Melissococcus plutonius* allows detection of disease transmission events
- 472 and a study of the distribution of variants. *Environ Microbiol Rep* **5(4):**525–529 DOI
- 473 10.1111/1758-2229.12057.
- 474 **Hornitzky MAZ, Smith LA. 1999.** Sensitivity of Australian *Melissococcus pluton* isolates to

oxytetracycline hydrochloride. *Australian Journal of Experimental Agriculture* **39(7)**:881–883 DOI 10.1071/EA99064.

**Hubert J, Bicianova M, Ledvinka O, Kamler M, Lester PJ, Nesvorna M, Kopecky J, Erban T. 2016a.** Changes in the bacteriome of honey bees associated with the parasite *Varroa destructor*, and pathogens *Nosema* and *Lotmaria passim*. *Microb Ecol* (in press) DOI 10.1007/s00248-016-0869-7.

**Hubert J, Kamler M, Nesvorna M, Ledvinka O, Kopecky J, Erban T. 2016b.** Comparison of *Varroa destructor* and worker honeybee microbiota within hives indicates shared bacteria. *Microbial Ecology* **72(2)**:448–459 DOI 10.1007/s00248-016-0776-y.

**Kamler M, Tyl J, Nesvorna M, Hubert J, Merta J, Karesova B, Titera D. 2016.** The European foulbrood - rediscovered infection of honeybee in the Czech Republic. *Veterinarstvi* **66(6)**:435–438. (in Czech with English summary)

**Kozich JJ, Westcott SL, Baxter NT, Highlander SK, Schloss PD. 2013.** Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq Illumina sequencing platform. *Applied and Environmental Microbiology* **79(17)**:5112–5120 DOI 10.1128/AEM.01043-13.

**Kutner MH, Nachtsheim CJ, Neter J, Li W. 2005.** *Applied linear statistical models*, 5<sup>th</sup> edn. Boston, MA, USA: McGraw-Hill Irwin.

**KVSH. 2015.** Specific veterinary precautions in the course of incidence of the dangerous European foulbrood disease in breeding of honeybees on the territory of the Hradec Kralove Region, to prevent spread and overcome the outbreak. Regulation of the State Veterinary Administration of the Czech Republic, ref. no. SVS/2015/084740-H, 19<sup>th</sup> August 2015. [Mimoradna veterinarni opatreni pri vyskytu nebezpecne nakazy hniloba vceliho plodu v

- chovech vcel v regionu Kralovehradeckeho kraje, k zamezení jejího sireni a k jejimu  
zdolani. Narizení Statni veterinární spravy, c. j. SVS/2015/084740-H, 19. 8. 2015.] Hradec  
Kralove, Czechia: Krajska veterinární sprava Statni veterinární spravy pro Kralovehradecky  
kraj (KVSH). Available at  
[http://eagri.cz/public/web/file/417238/Narizeni\\_MVO\\_hniloba\\_vceliho\\_plodu\\_c.j.\\_SVS\\_2015\\_084740\\_H.pdf](http://eagri.cz/public/web/file/417238/Narizeni_MVO_hniloba_vceliho_plodu_c.j._SVS_2015_084740_H.pdf) (accessed 6 August 2016). (in Czech)
- Lane DJ. 1991.** 16S/23S rRNA sequencing. In: Stackebrandt E, Goodfellow M, eds. *Nucleic acid techniques in bacterial systematics*. Chichester, UK & New York, NY, USA: John Wiley and Sons, 115–175.
- OIE. 2016a.** Chapter 2.2.3.: European foulbrood of honey bees (infection of honey bees with *Melissococcus plutonius*). In: Manual of diagnostic tests and vaccines for terrestrial animals 2016. Paris France: OIE - World Organisation for Animal Health. Available at  
[http://www.oie.int/fileadmin/Home/eng/Health\\_standards/tahm/2.02.03\\_EUROPEAN\\_FOULBROOD.pdf](http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.02.03_EUROPEAN_FOULBROOD.pdf) (accessed 6 August 2016).
- OIE. 2016b.** OIE-Listed diseases, infections and infestations in force in 2016. Paris France: OIE - World Organisation for Animal Health. Available at <http://www.oie.int/en/animal-health-in-the-world/oie-listed-diseases-2016/> (accessed 6 August 2016).
- Oksanen J, Blanchet FG, Kindt R, Legendre P, Minchin PR, O'Hara RB, Simpson GL, Solymos P, Stevens MHH, Wagner H. 2016.** vegan: Community Ecology Package. CRAN - The Comprehensive R Archive Network. Available at <http://CRAN.R-project.org/package=vegan> (accessed 6 August 2016).
- Ondov BD, Bergman NH, Phillippy AM. 2011.** Interactive metagenomic visualization in a Web browser. *BMC Bioinformatics* **12**:385 DOI 10.1186/1471-2105-12-385.

- 521 **Pinnock DE, Featherstone NE. 1984.** Detection and quantification of *Melissococcus pluton*  
522 infection in honeybee colonies by means of enzyme-linked immunosorbent assay. *Journal*  
523 *of Apicultural Research* **23(3)**:168–170 DOI 10.1080/00218839.1984.11100627.
- 524 **Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, Peplies J, Glockner FO.**  
525 **2013.** The SILVA ribosomal RNA gene database project: improved data processing and  
526 web-based tools. *Nucleic Acids Research* **41(Database issue)**:D590–D596 DOI  
527 10.1093/nar/gks1219.
- 528 **Roetschi A, Berthoud H, Kuhn R, Imdorf A. 2008.** Infection rate based on quantitative real-  
529 time PCR of *Melissococcus plutonius*, the causal agent of European foulbrood, in honeybee  
530 colonies before and after apiary sanitation. *Apidologie* **39(3)**:362–371 DOI  
531 10.1051/apido:200819.
- 532 **Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, Lesniewski RA,**  
533 **Oakley BB, Parks DH, Robinson CJ, Sahl JW, Stres B, Thallinger GG, Van Horn DJ,**  
534 **Weber CF. 2009.** Introducing mothur: open-source, platform-independent, community-  
535 supported software for describing and comparing microbial communities. *Applied and*  
536 *Environmental Microbiology* **75(23)**:7537–7541 DOI 10.1128/AEM.01541-09.
- 537 **Takamatsu D, Morinishi K, Arai R, Sakamoto A, Okura M, Osaki M. 2014.** Typing of  
538 *Melissococcus plutonius* isolated from European and Japanese honeybees suggests spread of  
539 sequence types across borders and between different *Apis* species. *Veterinary Microbiology*  
540 **171(1–2)**:221–226 DOI 10.1016/j.vetmic.2014.03.036.
- 541 **Tomkies V, Flint J, Johnson G, Waite R, Wilkins S, Danks C, Watkins M, Cuthbertson**  
542 **AGS, Carpana E, Marris G, Budge G, Brown MA. 2009.** Development and validation of  
543 a novel field test kit for European foulbrood. *Apidologie* **40(1)**:63–72 DOI

10.1051/apido:2008060.

**Tozkar CO, Kence M, Kence A, Huang Q, Evans JD. 2015.** Metatranscriptomic analyses of honey bee colonies. *Frontiers in Genetics* **6**:100, DOI 10.3389/fgene.2015.00100.

**Vasquez A, Forsgren E, Fries I, Paxton RJ, Flaberg E, Szekely L, Olofsson TC. 2012.** Symbionts as major modulators of insect health: lactic acid bacteria and honeybees. *PLoS One* **7(3)**:e33188, DOI 10.1371/journal.pone.0033188.

**Waite R, Brown M, Thompson H. 2003.** Hygienic behaviour in honey bees in the UK: a preliminary study. *Bee World* **84(1)**:19–26 DOI 10.1080/0005772X.2003.11099567.

**Warnes GR, Bolker B, Bonebakker L, Gentleman R, Huber W, Liaw A, Lumley T, Maechler M, Magnusson A, Moeller S, Schwartz M, Venables B. 2016.** gplots: Various R programming tools for plotting data. CRAN - The Comprehensive R Archive Network. Available at <https://CRAN.R-project.org/package=gplots> (accessed 6 August 2016).

**Whitaker D, Christman M. 2014.** clustsig: Significant Cluster Analysis. CRAN - The Comprehensive R Archive Network. Available at <https://CRAN.R-project.org/package=clustsig> (accessed 6 August 2016).

**Wilkins S, Brown MA, Cuthbertson AGS. 2007.** The incidence of honey bee pests and diseases in England and Wales. *Pest Management Science* **63(11)**:1062–1068 DOI 10.1002/ps.1461.

**Wu M, Sugimura Y, Iwata K, Takaya N, Takamatsu D, Kobayashi M, Taylor D, Kimura K, Yoshiyama M. 2014.** Inhibitory effect of gut bacteria from the Japanese honey bee, *Apis cerana japonica*, against *Melissococcus plutonius*, the causal agent of European foulbrood disease. *Journal of Insect Science* **14**:129 DOI 10.1093/jis/14.1.129.

567 **FIGURES AND TABLES**

568

**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.

| OTU <sub>97</sub> | GenBank identification                      | aOTU    | Mean<br>decrease<br>accuracy | EFB factor |         |         |         |         |         |       |       | P -values |  |  |
|-------------------|---|---------|------------------------------|------------|---------|---------|---------|---------|---------|-------|-------|-----------|--|--|
|                   |   |         |                              | EFB2       |         | EFB1    |         | EFB0    |         |       |       |           |  |  |
|                   |   |         |                              | mean       | stderr  | mean    | stderr  | mean    | stderr  | 2/1   | 2/0   | 1/0       |  |  |
| OTU4              | <i>Melissococcus plutonius</i> (99)         | 13,112  | 1.361                        | 0.03055    | 0.00705 | 0.00046 | 0.00009 | 0.00012 | 0.00007 | 0.001 | 0.000 | 0.011     |  |  |
| OTU5              | <i>Fructobacillus fructosus</i> (99)        | 9,845   | 0.737                        | 0.02272    | 0.00817 | 0.00133 | 0.00066 | 0.00006 | 0.00003 | 0.028 | 0.000 | 0.079     |  |  |
| OTU109            | <i>Lactobacillus kimbladii</i> (99)         | 2,668   | 0.313                        | 0.00296    | 0.00021 | 0.00487 | 0.00100 | 0.00264 | 0.00057 | 0.096 | 0.598 | 0.079     |  |  |
| OTU9              | <i>Lactobacillus kunkeei</i> (99)           | 5,679   | 0.275                        | 0.01260    | 0.00500 | 0.00045 | 0.00010 | 0.00077 | 0.00063 | 0.040 | 0.002 | 0.714     |  |  |
| OTU8              | <i>Lactobacillus mellis</i> (99)            | 44,528  | 0.273                        | 0.06059    | 0.00527 | 0.05986 | 0.00988 | 0.03653 | 0.00707 | 0.962 | 0.010 | 0.083     |  |  |
| OTU6              | <i>Snodgrassella alvi</i> (99)              | 101,925 | 0.269                        | 0.12245    | 0.01005 | 0.22007 | 0.02506 | 0.07790 | 0.00995 | 0.003 | 0.003 | 0.000     |  |  |
| OTU7              | <i>Lactobacillus helsingborgensis</i> (99)  | 61,676  | 0.245                        | 0.07324    | 0.00906 | 0.10213 | 0.00599 | 0.05802 | 0.00625 | 0.025 | 0.183 | 0.000     |  |  |
| OTU12             | <i>Lactobacillus kullabergensis</i> (99)    | 26,246  | 0.243                        | 0.02732    | 0.00246 | 0.04641 | 0.00354 | 0.02862 | 0.00368 | 0.000 | 0.772 | 0.004     |  |  |
| OTU28             | <i>Pseudomonas indica</i> (88)              | 2,147   | 0.235                        | 0.00031    | 0.00023 | 0.00008 | 0.00004 | 0.00595 | 0.00362 | 0.474 | 0.058 | 0.143     |  |  |
| OTU16             | <i>Gilliamella apicola</i> (97)             | 7,951   | 0.225                        | 0.00209    | 0.00062 | 0.00033 | 0.00016 | 0.02082 | 0.01113 | 0.020 | 0.088 | 0.097     |  |  |
| OTU3              | <i>Gilliamella apicola</i> (99)             | 203,595 | 0.222                        | 0.26670    | 0.01680 | 0.18932 | 0.02695 | 0.20672 | 0.02193 | 0.040 | 0.036 | 0.710     |  |  |
| OTU15             | <i>Commensalibacter intestini</i> (95)      | 9,021   | 0.222                        | 0.00318    | 0.00082 | 0.00313 | 0.00117 | 0.02174 | 0.01430 | 0.975 | 0.011 | 0.241     |  |  |
| OTU25             | <i>Lactobacillus melliventris</i> (97)      | 10,699  | 0.205                        | 0.01301    | 0.00161 | 0.02103 | 0.00266 | 0.00863 | 0.00115 | 0.031 | 0.024 | 0.001     |  |  |
| OTU18             | <i>Frischella perrara</i> (99)              | 26,368  | 0.201                        | 0.02100    | 0.00269 | 0.01231 | 0.00174 | 0.04773 | 0.00948 | 0.021 | 0.005 | 0.002     |  |  |
| OTU33             | <i>Bifidobacterium asteroides</i> (98)      | 18,563  | 0.200                        | 0.02090    | 0.00259 | 0.02553 | 0.00380 | 0.02056 | 0.00524 | 0.391 | 0.956 | 0.564     |  |  |
| OTU13             | <i>Lactobacillus mellifer</i> (99)          | 8,712   | 0.198                        | 0.00849    | 0.00095 | 0.00991 | 0.00186 | 0.01197 | 0.00197 | 0.634 | 0.122 | 0.569     |  |  |
| OTU2              | <i>Bartonella bacilliformis</i> (96)        | 77,679  | 0.197                        | 0.05323    | 0.01818 | 0.05017 | 0.02327 | 0.14713 | 0.02547 | 0.942 | 0.005 | 0.015     |  |  |
| OTU14             | <i>Bifidobacterium asteroides</i> (99)      | 22,375  | 0.193                        | 0.02504    | 0.00275 | 0.04562 | 0.01461 | 0.02027 | 0.00428 | 0.219 | 0.354 | 0.133     |  |  |
| OTU10             | <i>Enterococcus faecalis</i> (99)           | 16,167  | 0.193                        | 0.03665    | 0.02014 | 0.00029 | 0.00006 | 0.00153 | 0.00020 | 0.107 | 0.240 | 0.000     |  |  |
| OTU17             | <i>Bifidobacterium coryneforme</i> (99)     | 7,768   | 0.189                        | 0.00969    | 0.00169 | 0.00523 | 0.00090 | 0.00913 | 0.00205 | 0.045 | 0.839 | 0.121     |  |  |
| OTU23             | <i>Ornithobacterium rhinotracheale</i> (84) | 2,974   | 0.183                        | 0.00290    | 0.00104 | 0.00294 | 0.00077 | 0.00422 | 0.00140 | 0.978 | 0.461 | 0.545     |  |  |
| OTU55             | <i>Gilliamella apicola</i> (98)             | 38,619  | 0.176                        | 0.04133    | 0.00292 | 0.03045 | 0.00246 | 0.05264 | 0.01110 | 0.017 | 0.356 | 0.078     |  |  |
| OTU21             | <i>Dysgonomonas capnocytophagoides</i> (92) | 3,944   | 0.173                        | 0.00231    | 0.00100 | 0.00541 | 0.00204 | 0.00706 | 0.00402 | 0.228 | 0.318 | 0.791     |  |  |

|      |                                |         |       |         |         |         |         |         |         |       |       |       |
|------|--------------------------------|---------|-------|---------|---------|---------|---------|---------|---------|-------|-------|-------|
| OTU1 | <i>Lactobacillus apis</i> (99) | 131,977 | 0.163 | 0.13239 | 0.01461 | 0.15581 | 0.01973 | 0.17471 | 0.02587 | 0.486 | 0.162 | 0.670 |
|------|--------------------------------|---------|-------|---------|---------|---------|---------|---------|---------|-------|-------|-------|



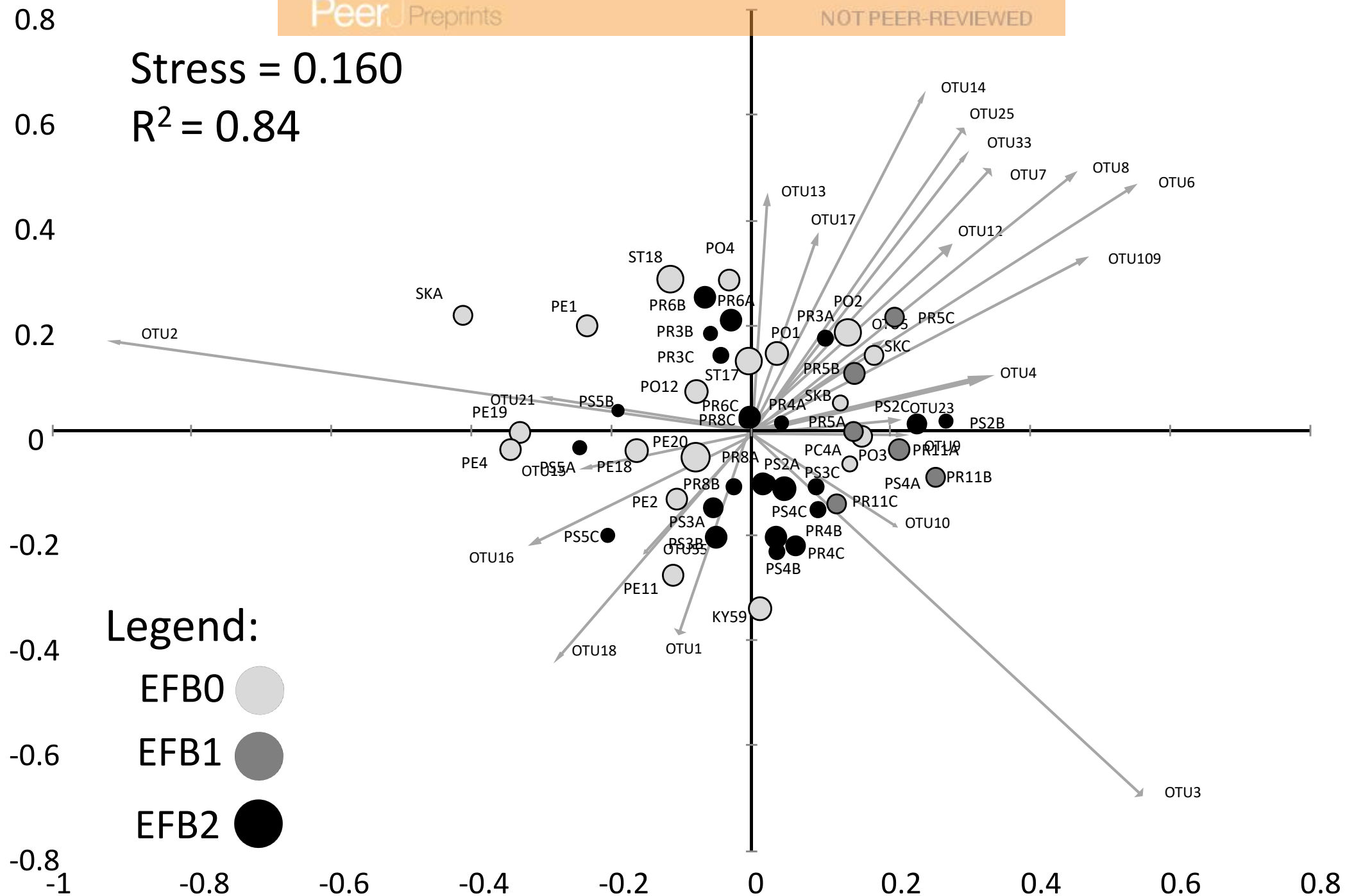
# Figure 1(on next page)

Non-metric multidimensional scaling visualization of the distribution of honeybee samples and selected OTUs<sub>97</sub>.

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

$R^2 = 0.84$

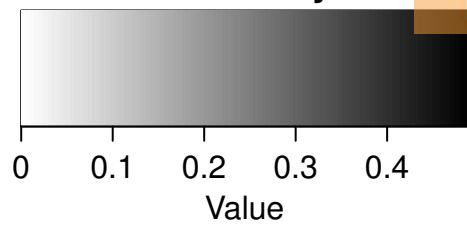


## Figure 2 (on next page)

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.

# Color Key



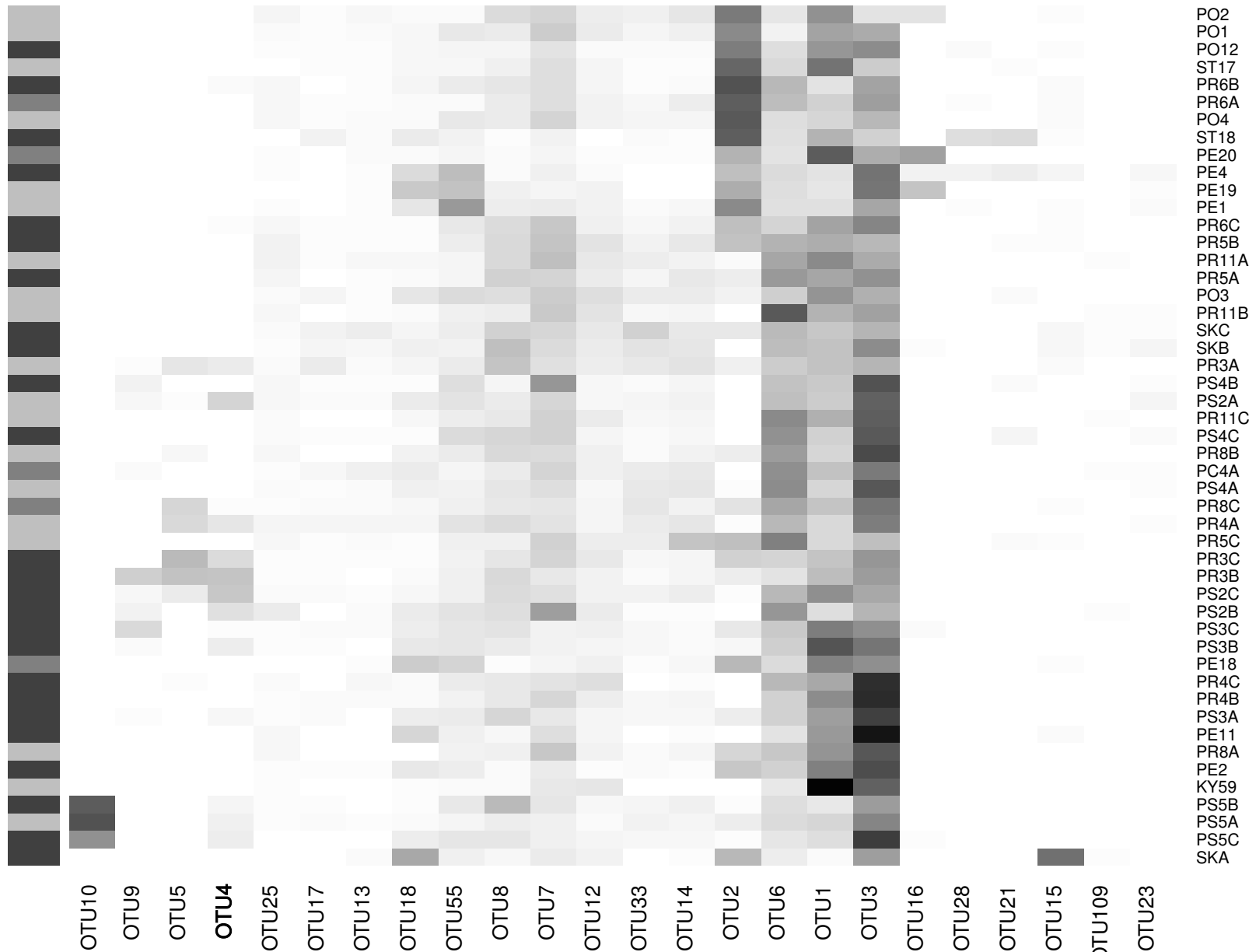
PeerJ

Preprints

NOT PEER-REVIEWED

EFB factor:

EFB0 EFB1 EFB2



### 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. oms of EFB.

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.

