SHORT COMMUNICATION

Identifying bacterial predictors of honey bee health

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Abstract

Non-targeted approaches are useful tools to identify new or emerging issues in bee health. Here, we utilise next generation sequencing to highlight bacteria associated with healthy and unhealthy honey bee colonies, and then use targeted methods to screen a wider pool of colonies with known health status. Our results provide the first evidence that bacteria from the genus *Arsenophonus* are associated with poor health in honey bee colonies. We also discovered *Lactobacillus* and *Leuconostoc* spp. were associated with healthier honey bee colonies. Our results highlight the importance of understanding how the wider microbial population relates to honey bee colony health.

Keywords

probiotic; symbiont; microbiome

1. Introduction

The economic contribution of insect pollination to crop production (Gallai et al., 2009) and human nutrition security (Ellis et al., 2015) is significant. Managed honey bees are often singled out as a substantial global supplier of pollination services (Kleijn et al., 2015) but are exposed to a range of pressures that contribute to poor health, including parasites (Budge et al., 2015; Higes et al., 2008), pesticides (Henry et al., 2012) and climate change; for review see (Vanbergen et al, 2013).

As pollinators are placed under increasing pressures, the microbiome of bees is emerging as an important and understudied factor in the maintenance of health. Food supplemented with lactic acid bacteria can protect honey bees against American (Forsgren et al., 2010) and European foulbrood (Vasquez et al., 2012), whilst members of the gut microbiota have putative roles in the metabolism of carbohydrates (Lee et al., 2015). Microbiota of the honey bee may therefore contribute to pathogen defence, nutrition and protection against environmental compounds; for review see (Kwong and Moran, 2016).

Here, we used pyrosequencing of the 16S rRNA amplicon to highlight bacteria differentially associated with healthy and unhealthy honey bee colonies, and then developed targeted real-time PCR methods to explore microbial relationships with colony health.

2. Materials and Methods

2.1. Sampling

A recent study collected adult honey bee samples from healthy and unhealthy UK colonies to investigate known pathogens as predictors of poor honey bee colony health (Budge et al., 2015). We identified two case studies within these samples where professional beekeepers managed apiaries experiencing persistently poor colony health as well as apiaries showing consistently good colony health, despite using similar beekeeping practices. Beekeeper A had one healthy apiary (AH; 6 colonies) and two unhealthy apiaries (AU1; 5 colonies and AU2; 6 colonies). Beekeeper B had one healthy apiary (BH; 3 colonies) and one unhealthy apiary (BU; 3 colonies). DNA was extracted from 30 adult honey bees from each colony as described previously (Budge et al., 2015).

2.2. Pyrosequencing 16S amplicons to detect bacteria present

16S rRNA sequences were produced using composite primers (Hamady et al., 2008) with Multiplex Identifiers (MIDs) from Roche using a different MID tagged reverse primer for each sample (Table S1). The forward primer comprised the Roche 454 Primer B (underlined) and 'TC' linker (italics) concatenated to the conserved bacterial primer 27F (bold) (5'-GCCTTGCCAGCCCGCTCAG*TC***AGAGTTTGATCCTGGCTCAG**-3'). The reverse primer comprised the Roche 454 Primer A (underlined) followed by the 10 nt MID, a 'CA' linker (italics) and the conserved bacterial primer 338R (bold) (5'-GCCTCCCTCGCGCCATCAG-MID-*CA***TGCTGCCTCCCGTAGGAGT-3'**).

16S rRNA PCR reactions were set up using Advantage 2 Reagents (Clontech, USA) comprising 5 µL 50x SA buffer, 1 µL Advantage 2 polymerase mix, 0·2 mM dNTPs, 1 µL of template, 400 nM forward and reverse primers and 40 µL water. Reactions were carried out in a Biometra T3 thermocyler PCR machine (Biometra, Germany) beginning with 94˚C for 10 min followed by 30 cycles of 95˚C for 30 s (denaturing), 55˚C for 30 s (annealing) and 72˚C for 1 min (extension). PCR products were visualised on a 1% gel and quantified using the

Quant-iT dsDNA BR assay kit (Invitrogen). Amplicons were sequenced on two sixteenths of a plate from a GS-FLX Genome Sequencer (University of Newcastle, Institute of Human Genetics) and sequences analysed using the Ribosomal Database Project (RDP) pyrosequencing pipeline (Cole et al., 2009). Sequences were trimmed and identified based on MID using the initial processing feature and each read assigned to a taxon using the RDP classifier.

2.3. Association of microbes with colony heath status

Three bacterial species with differential expression between healthy and unhealthy colonies were selected for the development of targeted real-time PCR tests following previously published protocols (Budge et al., 2010) (Table S2). Targeted real-time PCR tests were used to rescreen DNA extracts from 129 adult honey bee samples reported previously (Budge et al., 2015). To investigate the relationship between the presence of the newly identified bacteria and honey bee colony health, the square root of the number of combs of adult bees was used as the response variable in a multiple linear regression model with the detection of established parasites (*N. apis*, *N. ceranae*, *M. plutonius*, KBV, DWV, BQCV, SBV, CBPV, APBV, IV, IAPV) and newly associated bacterial species (*Arsenophonus*, *Lactobacillus, Leuconostoc*) as potential explanatory variables (GenStat version 17.1).

2.4. Relationship of Apis mellifera Arsenophonus to other isolates

To further characterise *Arsenophonus* spp. detected in *A. mellifera* adult workers, we generated sequence from two bacterial housekeeping genes; fructose-bisphosphate aldolase class II (fbaA) and 16S rRNA for two colony samples using established protocols. FbaA sequences were amplified using the primer pair fbaAF (5′-GCCGCTAAGGTTGGTTCTCC) and fbaAR (5′-CCTGAACCACCATGGAAAACAAAA; 658 bp amplicon) adapted from a previous study (Duron et al., 2010). 16S rRNA sequences were amplified using established primers (Duron et al., 2008) generating a 804 bp amplicon. Products were purified and Sanger sequenced through both strands using the original

primers. Data were used to infer the relatedness of the *A. mellifera Arsenophonus* strain to others in the genus. Model selection was made using the best-fit nucleotide substitution test in MEGA6 (Tamura et al., 2013), and maximum likelihood tree estimated using the Tamura 3-parameter model (Tamura, 1992) for fbaA sequence, and the Kimura 2-parameter model (Kimura, 1980) for 16S rRNA. Accession numbers and references for sequences from the related species used in phylogenetic reconstruction are provided (Tables S3, S4)

3. Results and Discussion

In total, 15,633 16S amplicon sequences (NBCI Bioproject PRJNA315609) were identified by MID and classified with 95% confidence using the RDP webtools. Bacteria from 17 identifiable genera, each generated at least 1% of the sequence reads in samples from either healthy or unhealthy honey bee colonies (Table 1). Sequences of *Arsenophonus* were more frequently found in adult bee samples from unhealthy apiaries whilst *Lactobacillus* and *Leuconostoc* were more frequently found in healthy apiaries (Table 1).

[Table 1]

We examined the association of *Arsenophonus*, *Lactobacillus* and *Leuconostoc* with colony health more widely by targeted real-time PCR to diagnose infection and then evaluated associations with colony health. PCR-based rescreening of DNA from adult honey bees revealed the presence of *Arsenophonus* in 48%, *Lactobacillus* in 16% and *Leuconostoc* in 14% of samples (n=129). *Arsenophonus* prevalence was higher than previously recorded in Swiss samples, where only 24% of colonies tested positive (Yañez et al., 2016), and was well distributed geographically, being observed in samples from over 11 counties. The multiple linear regression suggested the established parasite DWV and newly associated bacterial species *Arsenophonus*, *Lactobacillus* and *Leuconostoc* were significant predictors of honey bee colony size ($F=20.81$; $df=4,124$; $P<0.001$). DWV ($F=18.68$; $df=1,124$; $P<0.001$) and *Arsenophonus* ($F=9.4$; $df=1,124$; $P=0.003$) presence were related negatively and *Lactobacillus* ($F=4.14$; $df=1,124$; $P=0.044$) and *Leuconostoc* ($F=51.01$; $df=1,124$; $P<0.001$) were related positively to the number of combs of bees (Figure 1A).

[Figure 1]

We further examined the relatedness of *Apis mellifera Arsenophonus* to previously identified strains. Analysis of 16S rRNA sequence grouped *Apis Arsenophonus* with strains previously

identified in *Colletes* bees (Figure 2A), a result congruent with data from Switzerland (Yañez et al., 2016). FbaA sequences suggested *Apis mellifera Arsenophonus* formed a monophyletic group with *Arsenophonus nasoniae* from the parasitoid wasp (*Nasonia vitripennis*) and *Arsenophonus* isolated from the raspberry aphid (*Aphis idaei*; Figures 1C).

Overall, our results provide the first evidence that members of the genus *Arsenophonus* are associated with poor health in UK honey bee colonies. Increased abundance of bacteria with 90% sequence identity to *Arsenophonus* has also been reported in honey bee colonies suffering from Colony Collapse Disorder (CCD) in the United States, indicating a potential association with poor bee health (Cornman et al., 2012). There are two competing and equally plausible hypotheses for the correlation between *Arsenophonus* presence and the poor health of honey bee colonies. Firstly, *Arsenophonus* could increase host susceptibility to infection. This might occur, for instance, if the symbiont modulated host immune pathways that affect pathogen clearance. Alternatively, *Arsenophonus* may represent a secondary infection that occurs following a decline to poor health. *Arsenophonus* has been associated with foraging honey bees in Israel (Aizenberg-Gershtein et al., 2013), Switzerland (Babendreier et al., 2007) and The United States (Corby-Harris et al., 2014) and was associated with hive debris from the Czech republic (Hubert et al., 2015). Whilst we do not know which of our hypotheses is correct, elucidation of the association is of clear importance to international apiculture and merits future experimental studies.

We also report the novel finding that lactic acid bacteria (LAB) from the genera *Lactobacillus* and *Leuconostoc* were predictors of increased colony size in UK honey bee colonies. *Leuconostoc* spp. have rarely been associated with aculeate pollinators, the only previous reports being presence in fresh pollen collected by foraging honey bees in Algeria (Belhadj et al., 2010) and a finding in the gut of *Bombus terrestris* in Belgium (Praet et al., 2015). *Lactobacillus* is better studied, becoming associated with adult bees soon after eclosure (Vasquez et al., 2012) and thought to be important to honey production (Olofsson and

172 Vasquez, 2008) and the preservation (Anderson et al., 2014) or fermentation (Vasquez and
173 Olofsson, 2009) of pollen. LABs have long been associated with good health in humans and,
174 although they have recently been shown to inhibit bacterial honey bee pathogens (Forsgren
175 et al., 2010; Vasquez et al., 2012), our data are the first to link their presence with good
176 colony health. Several commercial feeds contain blends of LAB (including *Lactobacillus*) to
177 offer the promise of improved honey bee colony vigour, however none of these products are
178 known to contain *Leuconostoc* spp.. Future experiments should determine whether the
179 inclusion of *Leuconostoc* spp. could improve the health of honey bee colonies as part of a
180 novel probiotic.
181
182 Our results contribute to the growing body of evidence that the honey bee microbiota, in
183 addition to known pathogens, may represent important determinants of honey bee colony
184 health (Kwong and Moran, 2016). Non-targeted sequencing methods are a useful culture
185 independent tool to highlight previously unknown microbes and other genera, such as
186 *Microbacterium*, *Proteus* and *Staphylococcus* (Table 1). Future studies should combine non-
187 targeted sequencing methods to describe the microbiome with other important determinates
188 such as diet, environment and host genotype (Engel et al., 2016) to further understand the
189 role of microbes in honey bee colony health.
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306 **Table and figure legends**

307 **Table 1** Frequency of 16S amplicon sequences detected in adult honey bee samples for all
308 17 identifiable genera with greater than 1% read abundance in either healthy or unhealthy
309 groups.

310

	Number of sequence reads for each apiary				
Genus designation	**AH**	**AU1**	**AU2**	**BH**	**BU**
Acinetobacter	12	2	1	7	1
*Arsenophonus**	2	971	1551	0	208
Bifidobacterium	87	249	93	42	43
Brenneria	0	12	22	0	0
Brevundimonas	15	9	4	5	1
Carnobacterium	17	0	0	0	0
*Lactobacillus**	3	1	2	94	29
*Leuconostoc**	51	2	5	2	0
Microbacterium	69	25	8	43	7
Propionibacterium	10	0	0	9	1
Proteus	1	11	25	204	0
Pseudomonas	3	0	87	0	2
Rhodococcus	12	5	0	7	1
Staphylococcus	3	1	1035	5	2
Streptophyta	108	66	24	7	2
Yersinia	3	9	17	1	0
Zymobacter	0	9	7	0	0
Total # reads	1483	1737	3311	554	465

311 * Selected for further investigation

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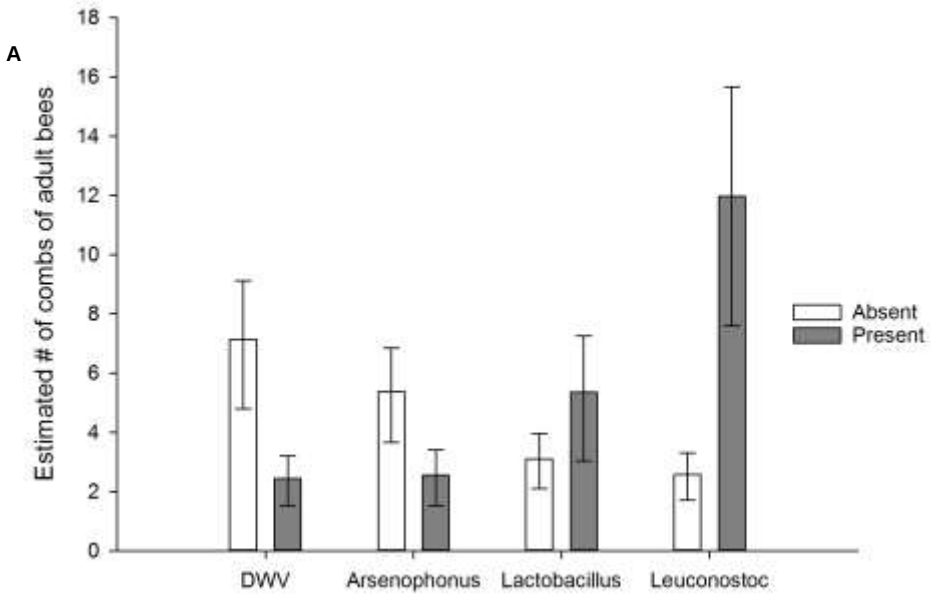

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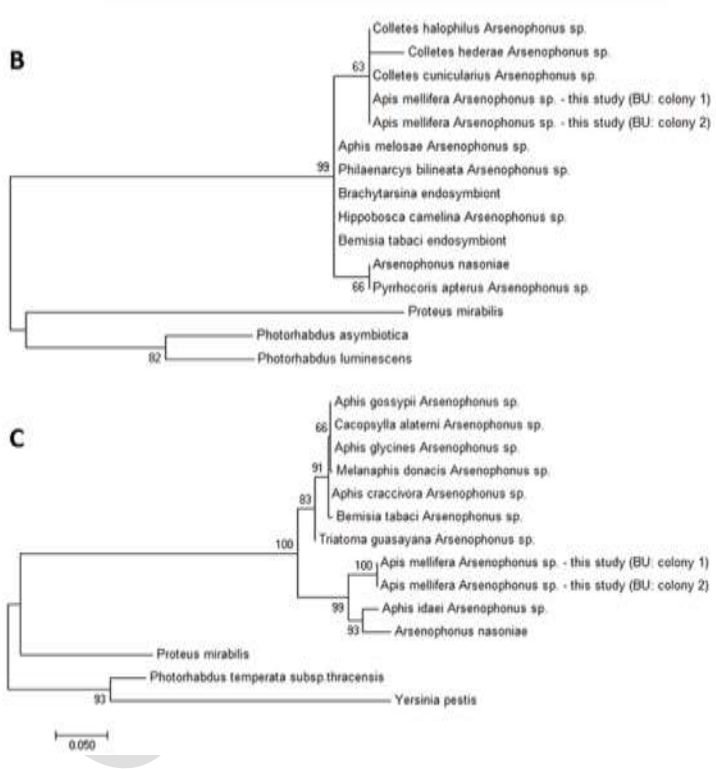

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316 **Figure 1** Estimated number of combs of adult bees as predicted by presence or absence of
317 deformed wing virus (DWV), *Arsenophonus*, *Lactobacillus* and *Leuconostoc* using a multiple
318 linear regression (A). Error bars represent 95% CI. Maximum likelihood inference of the
319 relatedness of *Arsenophonus* spp. isolated from *Apis mellifera* to other *Arsenophonus*
320 strains using sequence from 16S rRNA (B) and fbaA (C). Branch length denotes the number

of substitutions per site and bootstrap values from 1000 replications are shown at nodes. Strains that have not been formally identified are labelled following their host species.