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2 **SHORT COMMUNICATION**

3 # Identifying bacterial predictors of honey bee health

4

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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

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32 **1. Introduction**

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

39

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

47

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

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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

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81 Quant-iT dsDNA BR assay kit (Invitrogen). Amplicons were sequenced on two sixteenths of
82 a plate from a GS-FLX Genome Sequencer (University of Newcastle, Institute of Human
83 Genetics) and sequences analysed using the Ribosomal Database Project (RDP)
84 pyrosequencing pipeline (Cole et al., 2009). Sequences were trimmed and identified based
85 on MID using the initial processing feature and each read assigned to a taxon using the RDP
86 classifier.
87
88 *2.3. Association of microbes with colony heath status*
89 Three bacterial species with differential expression between healthy and unhealthy colonies
90 were selected for the development of targeted real-time PCR tests following previously
91 published protocols (Budge et al., 2010) (Table S2). Targeted real-time PCR tests were used
92 to rescreen DNA extracts from 129 adult honey bee samples reported previously (Budge et
93 al., 2015). To investigate the relationship between the presence of the newly identified
94 bacteria and honey bee colony health, the square root of the number of combs of adult bees
95 was used as the response variable in a multiple linear regression model with the detection of
96 established parasites (*N. apis*, *N. ceranae*, *M. plutonius*, KBV, DWV, BQCV, SBV, CBPV,
97 APBV, IV, IAPV) and newly associated bacterial species (*Arsenophonus*, *Lactobacillus,*
98 *Leuconostoc*) as potential explanatory variables (GenStat version 17.1).
99
100 *2.4. Relationship of Apis mellifera Arsenophonus to other isolates*
101 To further characterise *Arsenophonus* spp. detected in *A. mellifera* adult workers, we
102 generated sequence from two bacterial housekeeping genes; fructose-bisphosphate
103 aldolase class II (fbaA) and 16S rRNA for two colony samples using established protocols.
104 FbaA sequences were amplified using the primer pair fbaAF (5′-
105 GCCGCTAAGGTTGGTTCTCC) and fbaAR (5′-CCTGAACCACCATGGAAAACAAAA; 658
106 bp amplicon) adapted from a previous study (Duron et al., 2010). 16S rRNA sequences were
107 amplified using established primers (Duron et al., 2008) generating a 804 bp amplicon.
108 Products were purified and Sanger sequenced through both strands using the original

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109 primers. Data were used to infer the relatedness of the *A. mellifera Arsenophonus* strain to
110 others in the genus. Model selection was made using the best-fit nucleotide substitution test
111 in MEGA6 (Tamura et al., 2013), and maximum likelihood tree estimated using the Tamura
112 3-parameter model (Tamura, 1992) for fbaA sequence, and the Kimura 2-parameter model
113 (Kimura, 1980) for 16S rRNA. Accession numbers and references for sequences from the
114 related species used in phylogenetic reconstruction are provided (Tables S3, S4)
115

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116 **3. Results and Discussion**

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

123

124 **[Table 1]**

125

126 We examined the association of *Arsenophonus*, *Lactobacillus* and *Leuconostoc* with colony
127 health more widely by targeted real-time PCR to diagnose infection and then evaluated
128 associations with colony health. PCR-based rescreening of DNA from adult honey bees
129 revealed the presence of *Arsenophonus* in 48%, *Lactobacillus* in 16% and *Leuconostoc* in
130 14% of samples (n=129). *Arsenophonus* prevalence was higher than previously recorded in
131 Swiss samples, where only 24% of colonies tested positive (Yañez et al., 2016), and was
132 well distributed geographically, being observed in samples from over 11 counties. The
133 multiple linear regression suggested the established parasite DWV and newly associated
134 bacterial species *Arsenophonus*, *Lactobacillus* and *Leuconostoc* were significant predictors
135 of honey bee colony size ($F=20.81$; $df=4,124$; $P<0.001$). DWV ($F=18.68$; $df=1,124$; $P<0.001$)
136 and *Arsenophonus* ($F=9.4$; $df=1,124$; $P=0.003$) presence were related negatively and
137 *Lactobacillus* ($F=4.14$; $df=1,124$; $P=0.044$) and *Leuconostoc* ($F=51.01$; $df=1,124$; $P<0.001$)
138 were related positively to the number of combs of bees (Figure 1A).

139

140 **[Figure 1]**

141

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

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144 identified in *Colletes* bees (Figure 2A), a result congruent with data from Switzerland (Yañez
145 et al., 2016). FbaA sequences suggested *Apis mellifera Arsenophonus* formed a
146 monophyletic group with *Arsenophonus nasoniae* from the parasitoid wasp (*Nasonia*
147 *vitripennis*) and *Arsenophonus* isolated from the raspberry aphid (*Aphis idaei*; Figures 1C).
148
149 Overall, our results provide the first evidence that members of the genus *Arsenophonus* are
150 associated with poor health in UK honey bee colonies. Increased abundance of bacteria with
151 90% sequence identity to *Arsenophonus* has also been reported in honey bee colonies
152 suffering from Colony Collapse Disorder (CCD) in the United States, indicating a potential
153 association with poor bee health (Cornman et al., 2012). There are two competing and
154 equally plausible hypotheses for the correlation between *Arsenophonus* presence and the
155 poor health of honey bee colonies. Firstly, *Arsenophonus* could increase host susceptibility
156 to infection. This might occur, for instance, if the symbiont modulated host immune pathways
157 that affect pathogen clearance. Alternatively, *Arsenophonus* may represent a secondary
158 infection that occurs following a decline to poor health. *Arsenophonus* has been associated
159 with foraging honey bees in Israel (Aizenberg-Gershtein et al., 2013), Switzerland
160 (Babendreier et al., 2007) and The United States (Corby-Harris et al., 2014) and was
161 associated with hive debris from the Czech republic (Hubert et al., 2015). Whilst we do not
162 know which of our hypotheses is correct, elucidation of the association is of clear importance
163 to international apiculture and merits future experimental studies.
164
165 We also report the novel finding that lactic acid bacteria (LAB) from the genera *Lactobacillus*
166 and *Leuconostoc* were predictors of increased colony size in UK honey bee colonies.
167 *Leuconostoc* spp. have rarely been associated with aculeate pollinators, the only previous
168 reports being presence in fresh pollen collected by foraging honey bees in Algeria (Belhadj
169 et al., 2010) and a finding in the gut of *Bombus terrestris* in Belgium (Praet et al., 2015).
170 *Lactobacillus* is better studied, becoming associated with adult bees soon after eclosure
171 (Vasquez et al., 2012) and thought to be important to honey production (Olofsson and

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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.
190

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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

312

313

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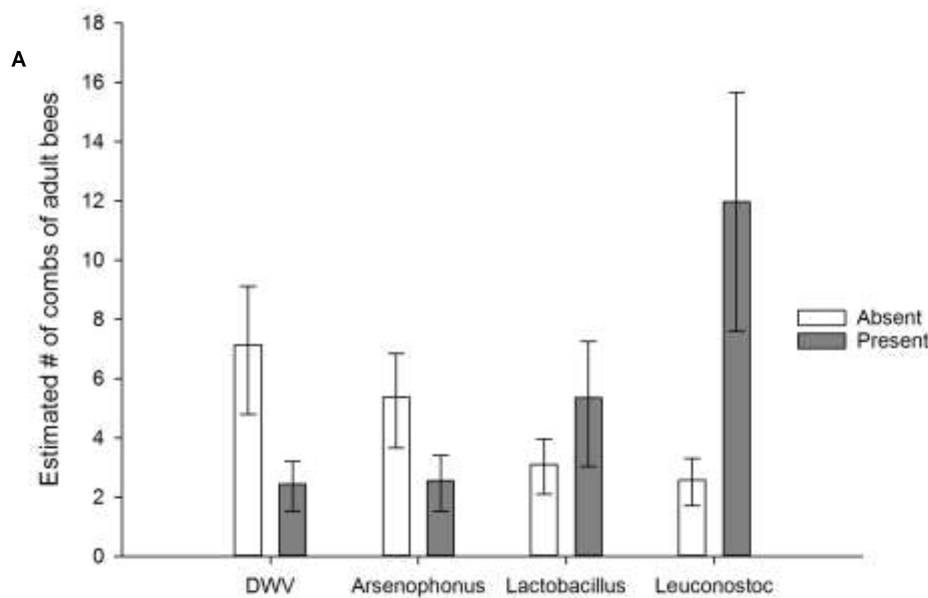
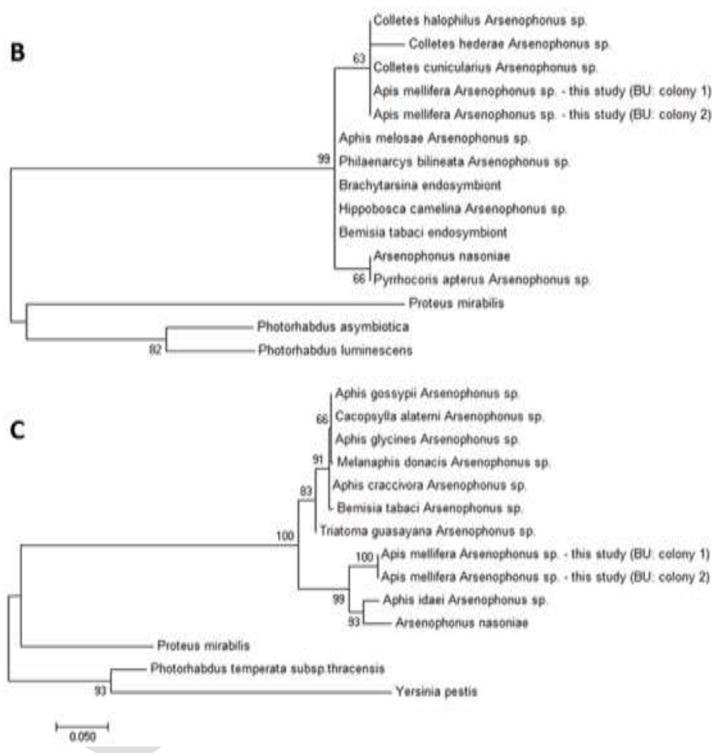


314



315

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

321 of substitutions per site and bootstrap values from 1000 replications are shown at nodes.

322 Strains that have not been formally identified are labelled following their host species.

323

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