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SHORT COMMUNICATION

Identifying bacterial predictors of honey bee health

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18 **Abstract**

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

27

28 **Keywords**

29 probiotic; symbiont; microbiome

30

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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 and Initiative, 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 amended with lactic
42 acid bacteria can protect honey bees against American (Forsgren et al., 2010) and European
43 foulbrood (Vasquez et al., 2012) whilst members of the gut microbiota have putative roles in
44 the metabolism of carbohydrates (Lee et al., 2015). Microbiota of the honey bee may
45 therefore contribute to pathogen defence, nutrition and protection against environmental
46 compounds.

47

48 Here we used pyrosequencing of the 16S 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.

51

52

53 2. Materials and Methods

54 2.1. Sampling

55 A recent study collected adult honey bee samples from healthy and unhealthy UK colonies
56 to investigate known pathogens as predictors of poor honey bee colony health (Budge et al.,
57 2015). We identified two case studies within these samples where professional beekeepers
58 managed apiaries experiencing persistently poor colony health as well as apiaries showing
59 consistently good colony health, despite using similar beekeeping practices. Beekeeper A
60 had one healthy apiary (AH; 6 colonies) and two unhealthy apiaries (AU1; 5 colonies and
61 AU2; 6 colonies). Beekeeper B had one healthy apiary (BH; 3 colonies) and one unhealthy
62 apiary (BU; 3 colonies). DNA was extracted from 30 adult honey bees from each colony as
63 described previously (Budge et al., 2015).

64

65 2.2. Pyrosequencing 16S amplicons

66 16S amplicons were produced using composite primers (Hamady et al., 2008) with Multiplex
67 Identifiers (MIDs) from Roche using a different MID tagged reverse primer for each sample
68 (Table S1). The forward primer comprised the Roche 454 Primer B (underlined) and 'TC'
69 linker (italics) concatenated to the conserved bacterial primer 27F (bold) (5'-
70 GCCTTGCCAGCCCGCTCAG *TCAGAGTTTGATCCTGGCTCAG*-3'). The reverse primer
71 comprised the Roche 454 Primer A (underlined) followed by the 10 nt MID, a 'CA' linker
72 (italics) and the conserved bacterial primer 338R (bold) (5'-GCCTCCCTCGCGCCATCAG-
73 MID-CATGCTGCCTCCCGTAGGAGT-3').

74

75 16S PCR reactions were set up using Advantage 2 Reagents (Clontech, USA) comprising 5
76 µL 50x SA buffer, 1 µL Advantage 2 polymerase mix, 0.2 mM dNTPs, 1 µL of template 400
77 nM forward and reverse primers and 40 µL water. Reactions were carried out in a Biometra
78 T3 thermocycler PCR machine (Biometra, Germany) beginning with 94°C for 10 min followed
79 by 30 cycles of 95°C for 30 s (denaturing), 55°C for 30 s (annealing) and 72°C for 1 min
80 (extension). PCR products were visualised on a 1% gel and quantified using the Quant-iT

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

86

87 *2.3. Screening colonies with known health status*

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

98

99 *2.4. Arsenophonus PCR sequencing*

100 To further characterise *Arsenophonus* spp. detected in *A. mellifera* adults, we generated
101 sequence from two genes; the house keeping gene fructose-bisphosphate aldolase class II
102 (*fbaA*) and 16S rRNA for two colony samples using established protocols. *FbaA* sequences
103 were amplified using the primer pair *fbaAF* (5'-GCCGCTAAGGTTGGTTCTCC) and *fbaAR*
104 (5'-CCTGAACCACCATGGAAAACAAAA; 658 bp amplicon) adapted from a previous study
105 (Duron et al., 2010). 16S rRNA sequences were amplified using established primers (Duron
106 et al., 2008) generating a 804 bp amplicon. Products were purified and Sanger sequenced
107 through both strands using the original primers. Data were used to infer the relatedness of
108 the *A. mellifera Arsenophonus* strain to others in the genus. . Model selection was made

109 using the best-fit nucleotide substitution test in MEGA6 (Tamura et al., 2013), and maximum
110 likelihood tree estimated using the Tamura 3-parameter model (Tamura, 1992) for fbaA
111 sequence, and the Kimura 2-parameter model (Kimura, 1980) for 16S rRNA.. The
112 evolutionary rate differences between sites was modelled using Gamma distribution (fbaA)
113 or uniform rates (16S rRNA). Accession numbers and references for sequences from the
114 related species used in phylogenetic reconstruction are provided (Tables S3, S4)
115

116 3. Results and Discussion

117 3.1. Pyrosequencing 16S amplicons

118 In total, 15,633 16S amplicon sequences (NCBI Bioproject PRJNA315609) were identified
119 by MID and classified with 95% confidence using the RDP webtools. Bacteria from 17
120 identifiable genera generated at least 1% of the sequence reads in samples from either
121 healthy or unhealthy honey bee colonies (Table 1).

122 [Table 1]

123

124 Sequences of *Arsenophonus* were more frequently found in adult bee samples from
125 unhealthy apiaries whilst *Lactobacillus* and *Leuconostoc* were more frequently found in
126 healthy apiaries (Table 1). These bacterial genera were selected for further study and real-
127 time PCR primers designed to confirm species presence (Table S2).

128

129 3.2. Screening colonies with known health status

130 PCR-based rescreening of DNA samples from adult honey bees for the remaining three
131 bacterial genera revealed positive results for *Arsenophonus* (62/129), *Lactobacillus* (20/129)
132 and *Leuconostoc* (18/129). The multiple linear regression suggested the established parasite
133 DWV and newly associated bacterial species *Arsenophonus*, *Lactobacillus* and *Leuconostoc*
134 were significant predictors of honey bee colony size ($F=20.81$; $df=4,124$; $P<0.001$). DWV
135 ($F=18.68$; $df=1,124$; $P<0.001$) and *Arsenophonus* ($F=9.4$; $df=1,124$; $P=0.003$) presence were
136 negatively correlated and *Lactobacillus* ($F=4.14$; $df=1,124$; $P=0.044$) and *Leuconostoc*
137 ($F=51.01$; $df=1,124$; $P<0.001$) were positively correlated to the number of combs of bees
138 (Figure 1A).

139 [Figure 1]

140 3.3. *Arsenophonus* PCR sequencing

141 *Apis mellifera* *Arsenophonus* grouped with *Arsenophonus* strains previously identified in
142 *Colletes* using 16S Sequence (Figure 2A), a result congruent with results from Switzerland
143 (Yañez et al., 2016). FbaA sequences suggested *Apis mellifera* *Arsenophonus* formed a

144 monophyletic group with *Arsenophonus nasoniae* from the parasitoid wasp (*Nasonia*
145 *vitripennis*) and *Arsenophonus* isolated from the raspberry aphid (*Aphis idaei*; Figures 1C).
146

147 **4. Discussion**

148 Our results provide the first evidence that members of the genus *Arsenophonus* are
149 associated with poor health in UK honey bee colonies. In total, 48% of adult bee samples
150 tested positive from eleven counties demonstrating *Arsenophonus* is well distributed
151 geographically, and more common in the UK than Switzerland where only 24% of colonies
152 tested positive (Yañez et al., 2016). Increased abundance of bacteria with 90% sequence
153 identity to *Arsenophonus* has been reported in honey bee colonies suffering from Colony
154 Collapse Disorder (CCD) in the United States, indicating a potential association with poor
155 bee health (Cornman et al., 2012). There are two competing and equally significant
156 hypotheses for the correlation between *Arsenophonus* presence and the poor health of
157 honey bee colonies. Firstly, *Arsenophonus* could increase host susceptibility to infection.
158 This might occur, for instance, if the symbiont modulated host immune pathways are
159 affected to reduce pathogen clearance. Alternatively, *Arsenophonus* may protect its host
160 against parasites, and thus reaches high prevalence in areas where parasite pressure is
161 high. *Arsenophonus* has been associated with foraging honey bees in Israel (Aizenberg-
162 Gershtein et al., 2013), Switzerland (Babendreier et al., 2007) and The United States
163 (Corby-Harris et al., 2014) and was associated with hive debris from the Czech republic
164 (Hubert et al., 2015), so whilst we do not know which of our hypotheses is correct,
165 elucidation of the association is of clear importance to international apiculture and merits
166 future experimental studies.

167
168 We also report the novel finding that lactic acid bacteria (LAB) from the genera *Lactobacillus*
169 and *Leuconostoc* were predictors of increased colony size in UK honey bee colonies.
170 *Leuconostoc* spp. have rarely been associated with aculeate pollinators, the only previous
171 reports being presence in fresh pollen collected by foraging honey bees in Algeria (Belhadj
172 et al., 2010) and a finding in the gut of *Bombus terrestris* in Belgium (Praet et al., 2015).
173 *Lactobacillus* is better studied, becoming associated with adult bees soon after eclosure
174 (Vasquez et al., 2012) and thought to be important to honey production (Olofsson and

175 Vasquez, 2008) and the maturation of pollen (Vasquez and Olofsson, 2009). LABs have
176 long been associated with good health in humans and although they have recently been
177 shown to inhibit bacterial honey bee pathogens (Forsgren et al., 2010; Vasquez et al., 2012)
178 our data are the first to link their presence with good colony health. Several commercial
179 feeds contain blends of LAB (including *Lactobacillus*) to offer the promise of improved honey
180 bee colony vigour, however none of these products are known to contain *Leuconostoc* spp..
181 Future experiments should determine whether the inclusion of *Leuconostoc* spp. could
182 improve the health of honey bee colonies as part of a novel probiotic.

183

184 Our results contribute to the growing body of evidence that the honey bee microbiota,
185 outwith known pathogens, may offer an important contribution to honey bee colony health.
186 Non-targeted sequencing methods are a useful tool to highlight previously unknown
187 microbes and other genera, such as *Microbacterium*, *Proteus* and *Staphylococcus*,
188 represent additional possible candidates for further study.

189

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193

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290

292 **Table and figure legends**

293 **Table 1** Frequency of 16S amplicon sequences detected in adult honey bee samples for all
294 17 identifiable genera with greater than 1% read abundance in either healthy or unhealthy
295 groups.

296

297 **Figure 1** Estimated number of combs of adult bees as predicted by presence or absence of
298 deformed wing virus (DWV), *Arsenophonus*, *Lactobacillus* and *Leuconostoc* using a multiple
299 linear regression (A). Error bars represent 95% CI. Maximum likelihood inference of the
300 relatedness of *Arsenophonus* spp. isolated from *Apis mellifera* to other *Arsenophonus*
301 strains using sequence from 16S rRNA (B) and *fbaA* (C). Branch length denotes the number
302 of substitutions per site and bootstrap values from 1000 replications are shown at nodes.
303 Strains that have not been formally taxonomised are labelled following their host species.

304