Unique antimicrobial activity in honey from the Australian Honeypot Ant (*Camponotus inflatus*)

Andrew Dong [1] , **Nural Cokcetin** [2] , **Dee A Carter** [Corresp., 1, 3] , **Kenya Fernandes** [1]

[1] School of Life and Environmental Sciences, University of Sydney, Camperdown, NSW, Australia

[2] Australian Institute for Microbiology and Infection, , University of Technology,,,, Sydney, NSW, Australia

[3] Sydney Institute for Infectious Diseases, University of Sydney, Camperdown, NSW, Australia

Corresponding Author: Dee A Carter
Email address: dee.carter@sydney.edu.au

Honey produced by the Australian honeypot ant (*Camponotus inflatus*) is valued nutritionally and medicinally by Indigenous peoples, but its antimicrobial activity has never been formally studied. Here, we determine the activity of honeypot ant honey (HPAH) against a panel of bacterial and fungal pathogens, investigate its chemical properties, and profile the bacterial and fungal microbiome of the honeypot ant for the first time. We found HPAH to have strong total activity against *Staphylococcus aureus* but not against other bacteria, and strong non-peroxide activity against *Cryptococcus* and *Aspergillus* sp. Comparing its antimicrobial activity and chemical properties with currently marketed therapeutic-grade jarrah and manuka honey bee honeys, we found HPAH to be markedly different, suggesting it has unique properties and mechanisms of action. We found the bacterial microbiome of honeypot ants to be dominated by the known endosymbiont genus *Candidatus Blochmannia* (99.75%), and the fungal microbiome to be dominated by the plant-associated genus *Neocelosporium* (92.77%). Our results indicate that HPAH has a unique non-peroxide-based mode of antimicrobial action and could provide a lead for the discovery of novel antimicrobial compounds such as antimicrobial peptides.

1 **Unique antimicrobial activity in honey from the**

2 **Australian Honeypot Ant (*Camponotus inflatus*)**

3 Andrew Z. Dong[1], Nural N. Cokcetin[2], Dee A. Carter[1,3] & Kenya E. Fernandes[1]

4 [1]School of Life and Environmental Sciences, University of Sydney, Sydney, NSW, Australia

5 [2]Australian Institute for Microbiology and Infection, University of Technology, Sydney, NSW,

6 Australia

7 [3]Sydney Institute for Infectious Diseases, University of Sydney, Sydney, NSW, Australia

8

9 Corresponding Author:

10 Kenya E. Fernandes

11 F22 Life, Earth and Environmental Sciences (LEES) Building, City Rd & Eastern Ave,

12 Camperdown, NSW, 2006, Australia

13 Email address: kenya.fernandes@sydney.edu.au

14

15

16

17

18

19

20

21

22

23

Abstract

Honey produced by the Australian honeypot ant (*Camponotus inflatus*) is valued nutritionally and medicinally by Indigenous peoples, but its antimicrobial activity has never been formally studied. Here, we determine the activity of honeypot ant honey (HPAH) against a panel of bacterial and fungal pathogens, investigate its chemical properties, and profile the bacterial and fungal microbiome of the honeypot ant for the first time. We found HPAH to have strong total activity against *Staphylococcus aureus* but not against other bacteria, and strong non-peroxide activity against *Cryptococcus* and *Aspergillus* sp. Comparing its antimicrobial activity and chemical properties with currently marketed therapeutic-grade jarrah and manuka honey bee honeys, we found HPAH to be markedly different, suggesting it has unique properties and mechanisms of action. We found the bacterial microbiome of honeypot ants to be dominated by the known endosymbiont genus *Candidatus Blochmannia* (99.75%), and the fungal microbiome to be dominated by the plant-associated genus *Neocelosporium* (92.77%). Our results indicate that HPAH has a unique non-peroxide-based mode of antimicrobial action and could provide a lead for the discovery of novel antimicrobial compounds such as antimicrobial peptides.

Introduction

Honey has been utilised since ancient times as a traditional remedy against various ailments. In recent years, there has been a resurgence of interest in the use of natural products such as honey as antimicrobials, in large part due to the growing crisis of antimicrobial resistance. While the vast majority of honey in the world is produced by the European honey bee *Apis mellifera*, many other insects produce honey including stingless bees, wasps (*Brachygastra* sp.), bumblebees (*Bombus* sp.) and ants. One such example is the Australian honeypot ant, *Camponotus inflatus*. As a rare source of natural sugar in an arid environment, honeypot ants are highly prized as a bush food by Indigenous Australians and have a long history of nutritional and cultural significance (O'Dea et al., 1991). The Honey Ant Dreaming site is located in Central Australia and is shared by all Indigenous groups in the area (Jurra 2000). For these groups, the honeypot ant represents their Dreaming or *Tjukurpa*, the Aboriginal philosophy based on the spiritual

52   interrelation of people and things. In addition to their use as a food source, there are records of

53   honeypot ant honey being used to treat sore throats and colds (Faast & Weinstein 2020).

54   Honeypot ants are found only in environments that have an arid, dry, or desert-chaparral terrain.

55   There are at least six different genera that live around the world and these have undergone

56   convergent evolution and independently developed the same adaptation for novel nectar storage

57   (Conway 1986). Designated worker ants of the sterile helper caste that store food for the colony

58   are known as "repletes". These repletes are fed by other workers until their abdomens become

59   engorged and semi-transparent (Froggatt 1896). Becoming largely immobile, repletes take up a

60   sacrificial role as a "living pantry" hanging off the roofs of their nests. Through antennae

61   communication, repletes regurgitate this stored food during times of scarcity which is then

62   distributed via worker ants to the rest of the colony (Duncan & Lighton 1994).

63   The antimicrobial activity of honeypot ant honey has not been studied, unlike that of honey bee

64   honey where activity is attributed to physical characteristics such as high osmolarity and low pH,

65   as well as other chemical factors. These chemical factors are highly variable and include plant-

66   derived components such as flavonoids, amino acids, minerals, and phenolic acids (Almasaudi

67   2021), and entomological additions such as glucose oxidase, which catalyses the production of

68   hydrogen peroxide (H_2O_2), and antimicrobial peptides including defensin-1 and jellein-1, 2, and

69   4 (Brudzynski & Sjaarda 2015). The total activity (TA) of a honey refers to the broad-spectrum

70   activity that results from the synergistic efforts of these factors combined. Honey from different

71   sources can have vastly differing activity levels and mechanisms of action but are broadly

72   categorised into having either peroxide-activity (PA) or non-peroxide activity (NPA). For

73   example, jarrah (*Eucalyptus marginata*) honey typically possesses high levels of H_2O_2 making it

74   a PA honey, while manuka (*Leptospermum scoparium*) honey typically contains high levels of

75   methylglyoxal (MGO) and retains its bioactivity even when H_2O_2 is removed, making it a NPA

76   honey.

77   Unlike honey bee honey, the composition and bioactivity of HPAH is not well characterised,

78   including the processes between nectar collection and storage. Honeypot ants worldwide are

79   reported to source nectar from a variety of floral sources depending on availability and

80   seasonality (Hölldobler 1981). In Australia, *Camponotus inflatus* is thought to have a preferential

81   association with mulga trees and the aphids that live on them, though they are reported to gather

82   nectar from a variety of other floral sources at different times of the year including black

83   corkwood and native fuchsia flowers (Conway 1991; Islam et al. 2022). Mulga trees possess

84   nectar-secreting plant glands known as extrafloral nectaries that attract honeypot ants, who in

85   turn protect the plant against herbivores (Buckley 1982). Aphids feed on the sugary mulga sap,

86   metabolising its amino acids and honeypot ants stroke the aphids with their antennae, coaxing

87   them to excrete excess honeydew from their anuses, which they then collect (Blüthgen &

88   Feldhaar 2010). In return, the ants provide hygienic services and protect the aphids from

89   predators and parasitoids (Ness et al. 2010).

90   Given the resurgence of interest in the medicinal value of honey bee honey, it is of interest to

91   investigate the bioactivity of honey from other species, particularly one that has been utilised

92   medicinally by Indigenous peoples for thousands of years. In this study, we determine the

93   antimicrobial activity of Australian HPAH against a variety of bacterial and fungal pathogens,

94   compare its physical and chemical properties with therapeutic-grade honey bee honey in order to

95   determine potential mechanisms of action, and investigate the honeypot ant microbiome through

96   metagenomic analysis. We report here for the first time the bacterial and fungal microbiome of

97   Australian honeypot ants and the antimicrobial activity profile of their honey.

98

Materials & Methods

Sample Collection

101   Honeypot ants and honeypot ant honey (HPAH) samples were collected from Kurnalpi, located

102   in the Goldfields-Esperance region of Western Australia, on 22nd May 2022 with the help of

103   local Indigenous guides. A *Camponotus inflatus* nest was located by searching for Mulga

104   (*Acacia aneura*) trees in the area and then identifying a worker ant that would lead to the

105   entrance of the nest. Careful excavation of the nest took place from 1pm to 3pm, exposing

106   underground galleries containing repletes. These were surface sterilised and subsequently

107   euthanised using 80% ethanol. HPAH was harvested by pricking the abdomen of a replete with a

108   sterile needle and squeezing its contents. Ant bodies and honey samples were stored in the dark

109   at 4 ℃ until use.

Honey Sample Preparation

111 HPAH was mixed thoroughly by pipetting, diluted to the target concentration in sterile water,

112 and vortexed thoroughly prior to use. Honeys with known activity levels and marketed as

113 therapeutically active were Barnes TA 10+ Jarrah honey (hereafter referred to as jarrah honey),

114 which has peroxide-based activity, and Comvita UMF 18+ Manuka honey (hereafter referred to

115 as manuka honey), which has methylglyoxal (MGO)-based non-peroxide activity. Artificial

116 honey (1.5 g sucrose, 7.5 g maltose, 40.5 g fructose, 33.5 g glucose, 17 mL sterile water), was

117 included as an inactive, non-floral, non-bee control. These honey samples were mixed

118 thoroughly with a spatula, incubated at 35 °C for 30 mins to dissolve crystals, diluted to the

119 target concentration in sterile water, and vortexed thoroughly before use.

120 **Antimicrobial Susceptibility Testing**

121 Bacterial strains, yeast strains, and mould strains excluding *M. gypseum* were maintained as

122 glycerol stocks at −80 °C. Bacterial strains were grown on Nutrient Agar (NA; Oxoid) and

123 incubated at 30 °C for 24 hours before use. Yeast strains and mould strains excluding *M.*

124 *gypseum* were grown on Potato Dextrose Agar (PDA; Oxoid) and incubated at 30 °C for 24 − 48

125 hours. *M. gypseum* was maintained on an agar slope, grown on Oatmeal Agar (Sigma Aldrich),

126 and incubated at 30 °C for up to 7 days until good sporulation was obtained. The phenol

127 equivalence (PE) assay was performed according to the method outlined in Irish et al. (2011).

128 This assay is the current industry standard for quantifying antimicrobial activity in honey and

129 determines the activity of honey against *Staphylococcus aureus* in relation to phenol standards

130 (% PE), with a greater number indicating more active honey. Antimicrobial susceptibility testing

131 by broth microdilution in 96-well plates was performed in accordance with Clinical and

132 Laboratory Standards Institute (CLSI) guidelines for aerobic bacteria M07-A10 (CLSI 2002b),

133 yeasts M27-A4 (CLSI 2017), and filamentous fungi M38-A3 (CLSI 2002a). Broth microdilution

134 assays determine the minimum inhibitory concentration (MIC), the lowest percentage of honey

135 diluted in water which inhibits a certain amount of growth, with a smaller number indicating

136 more active honey. Honeys were assayed at doubling dilutions beginning at 32% (w/v) and were

137 diluted in either sterile water for total activity or freshly prepared 5600 U/mL catalase solution

138 for non-peroxide activity. Absorbance values at 600 nm relative to a growth control were used to

139 determine the MIC_{100} (100% growth inhibition), MIC_{80} (80% growth inhibition) and MIC_{50}

140 (50% growth inhibition). For heat treatments, honey was heated to 90 °C for 10 min using a heat

141   block, before being allowed to return to room temperature naturally. Raw data is presented in

142   Supplementary Table S1.

Assessment of Honey Colour, pH, Water Content, and Water Activity

144   The optical density of honey samples at 50% (w/v) was measured at 450, 635, and 720 nm using

145   a UV/Vis spectrophotometer (UV-1600PC, VWR International, Radnor, PA, USA) with sterile

146   water as a blank. Colour intensity was calculated using the equation $(A_{720} - A_{450}) \times 1000$ and

147   expressed in milli-absorbance units (mAU). Pfund value was calculated using the equation

148   $-38.70 + 371.39(A_{635})$ and expressed in mm. For pH measurements, 1 g of honey was diluted

149   in 7.5 mL of sterile water and pH was determined using a pH meter (Seven Compact S220,

150   Mettler Toledo, Greifensee, Switzerland). Brix value and moisture content were measured at 20

151   °C using a refractometer (HI96801, Hanna Instruments, Smithfield, RI, USA) according to the

152   AOAC Official Method 969.38 (AOAC 1999). Water activity (a_w) was assessed using a water

153   activity analyser (PRE, Aqualab Scientific, Pullman, WA, USA) at 25 °C with a correction of ±

154   0.005 a_w made per 0.1 °C deviation (Stoloff 1978). Raw data is presented in Supplementary

155   Table S1.

FC and FBBB Phenolics Assays

157   For the Folin-Ciocalteu (FC) assay, 20 μL aliquots of 20% (w/v) honey samples were added to

158   the wells of a 96-well plate in triplicate. To each well, 100 μL of FC reagent (1 mL Folin-

159   Ciocalteu reagent in 30 mL sterile water) was added and the plate was incubated at room

160   temperature for 5 min in the dark. Next, 80 μL of Na_2CO_3 solution was added with incubation at

161   room temperature for 2 hours in the dark. Absorbance was measured at 760 nm using a

162   microplate reader (CLARIOstar Plus, BMG Labtech, Ortenberg, Germany). For the Fast Blue

163   BB (FBBB) assay, 200 μL aliquots of 20% (w/v) honey samples were added to the wells of a 96-

164   well plate in triplicate. To each well, 20 μL of 0.1% Fast Blue BB reagent was added and

165   thoroughly mixed by pipetting up and down 50 times. Next, 20 μL of 5% NaOH solution was

166   added, and the plate was incubated at room temperature for 45 min in the dark. Absorbance was

167   measured at 420 nm using a microplate reader. For both assays, gallic acid standards ranging

168   from 0.06 – 0.18 mg/mL were used to generate a standard curve and the resulting equation for

169   the line of best fit was used to calculate the phenolics content of honey samples, expressed as mg

170   of gallic acid equivalent per kg of honey (mg GAE/kg). Raw data is presented in Supplementary

171   Table S1.

FRAP and DPPH Antioxidant Assays

173   For the ferric-reducing antioxidant power (FRAP) assay, FRAP reagent consisting of 1:1:10

174   (v/v/v) of 10 mM TPTZ in 40 mM HCl, 20 mM $FeCl_3$, and 300 mM pH 3.6 acetate buffer was

175   prepared fresh and incubated at 37 ºC prior to use. Honey samples (20 µl of 20% (w/v)) were

176   added to the wells of a 96-well plate in triplicate. Next, 180 µL of FRAP reagent was added and

177   plates were incubated at 37 ºC for 30 min. Absorbance was measured at 594 nm using a

178   microplate reader. Iron (II) sulfate ($FeSO_4$) standards ranging from 200 – 1200 µM, made freshly

179   and stored on ice until use, were used to generate a standard curve and the resulting equation

180   from the line of best fit was used to calculate FRAP value, expressed as µmol Fe^{2+}/kg. For the

181   2,2-diphenyl-1-picrylhyrazyl (DPPH) assay, 10 µL aliquots of 20% (w/v) honey samples were

182   added to the wells of a 96-well plate in triplicate. Next, 100 µL of 100 mM pH 5.5 sodium

183   acetate buffer and 250 µL of DPPH reagent (130 µM DPPH in methanol) were added with

184   incubation at room temperature for 2 hours in the dark. Absorbance was measured at 520 nm

185   using a microplate reader using methanol as a blank. Trolox standards at pH 7 ranging from 100

186   – 600 µM were used to generate a standard curve and the resulting equation from the line of best

187   fit was used to calculate radical scavenging activity, expressed as µmol Trolox equivalent per kg

188   of honey (µmol TE/kg). Raw data is presented in Supplementary Table S1.

HRP Hydrogen Peroxide Assay

190   The horseradish peroxidase (HRP) assay was performed according to the method outlined in

191   (Lehmann et al. 2019) with minor modifications. Briefly, honey samples were diluted to 50%

192   (w/v) with sterile water, passed through a 0.22 µm pore filter, and 1 mL was aliquoted in 6 well-

193   plates to allow for adequate overhead aeration. Samples were further diluted to 25% (w/v) with

194   either sterile water or 5600 U/mL catalase solution and incubated at 35 ºC with 180 rpm shaking

195   in the dark. At each timepoint, 40 µL aliquots of each sample were taken, 135 µL of freshly

196   prepared HRP reagent (50 µg/mL *o*-dianisidine and 20 µg/mL HRP in 10 mM pH 6.5 sodium

197   phosphate butter) was added, and samples were incubated at room temperature for 5 min in the

198   dark before the reaction was terminated by the addition of 120 µL of 6 M H_2SO_4. Absorbance

199   was measured at 550 nm using a microplate reader (ELx800, BioTek Instruments, Winooski,

200   VT, USA). Honey blanks were taken at each timepoint by adding 135 µL of sodium phosphate

201   buffer in place of HRP reagent. H_2O_2 standards ranging from $0.5 – 1024$ µM were used to

202   generate a standard curve and the resulting equation from the line of best fit was used to

203   calculate the amount of H_2O_2 in each sample. Raw data is presented in Supplementary Table S1.

204   **DNA Preparation**

205   Seven ants ranging in size from 0.1 to 1.66 g were individually processed and analysed. Ant

206   bodies were surface sterilised with 1% (v/v) bleach for 3 min and thoroughly rinsed with sterile

207   water. Whole ants were placed individually in tubes containing 500 mg of 2 mm glass beads in

208   500 µL of PBS and homogenised in a beat beater (PowerLyzer 24, Qiagen, Hilden, Germany)

209   using 6 cycles of 30 sec at 3000 rpm with 30 sec rests between. The mixture was briefly

210   centrifuged, and the supernatant transferred into fresh tubes. DNA was extracted using a DNeasy

211   Blood and Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions for

212   animal tissue.

213   **PCR & Gel Electrophoresis**

214   PCR and gel electrophoresis were conducted to confirm the presence of sufficient bacterial and

215   fungal DNA in samples. The V3-V4 region of the 16S rRNA gene was amplified using the

216   primer pair 341F/805R. The internal transcribed spacer 1 (ITS1) region was amplified using the

217   primer pair ITS1F/ITS2. PCR conditions were as follows: initial denaturation at 94 °C for 30 sec,

218   followed by 25 cycles of denaturation at 94 °C for 30 sec, annealing at 60 °C for 30 sec, and

219   extension at 68 °C for 90 sec, then a final extension at 68 °C for 5 min. PCR products were

220   analysed by electrophoresis on a 0.8% agarose gel in TAE buffer run at 90 V for 1 hour.

221   **Amplicon Sequencing & Analysis**

222   DNA was sent to Ramaciotti Centre for Genomics at the University of New South Wales,

223   Sydney for 16S V3-V4 amplicon sequencing with the 341F-805R primer set using the Illumina

224   Miseq v3 2x300 bp platform, and to BGI Genomics, Hong Kong for ITS1 amplicon sequencing

225   with the ITS1F-ITS2 primer set using the DNBSEQ PE300 platform. Raw sequence reads were

226   processed in R v4.2.2 using the DADA2 pipeline. Default parameters were used to filter and

227   trim, learn error rates, merge paired reads, and remove chimeras with the following adjustments:

228   the truncLen parameter was adjusted to c(260, 220) to allow for sufficient overlap of forward and

229 reverse reads for merging of the V3-V4 amplicons, and this step was not performed for the

230 variable length ITS1 amplicons. Taxonomy was assigned using the SILVA database release

231 138.1 for 16S, the SILVA species database release v132 for species-level assignment, and the

232 UNITE database release 27.10.2022 for ITS. Non-bacteria, mitochondria and chloroplast were

233 filtered out from 16S taxonomic tables and non-fungi from ITS taxonomic tables. Taxonomic

234 relative abundances were calculated using the phyloseq R package. Raw metagenomic data

235 obtained during this study is publicly available in the NCBI Sequence Read Archive under

236 Bioproject ID PRJNA957126.

237

238 ## Results

239 ### Honeypot ant honey has activity against bacteria, yeasts, and moulds

240 The antimicrobial activity of honeypot ant honey (HPAH) was tested and compared against

241 active peroxide (jarrah) and non-peroxide (manuka) based bee honeys. Using the PE assay, the

242 total activity of HPAH against *S. aureus* was found to be 8.3 % PE (Table 1). This is in the low

243 activity range (5-10 %), and was lower than the 11.6 % PE total activity of the jarrah honey,

244 which is in the 'potentially beneficial for therapeutics' range (10-20 %), and the 19.7% PE total

245 activity of the manuka honey which is in the high activity (>20 %) range (Irish et al. 2011).

246 HPAH and the jarrah honey had no detectable non-peroxide activity, while the manuka honey

247 had non-peroxide activity of 20.1% PE. The artificial honey control had no detectable total or

248 non-peroxide activity.

249 Although it is the current industry standard, the PE assay only tests activity against a single

250 organism, and as a diffusion-based assay can sometimes underestimate the activity of honey

251 samples with unique properties (Hossain et al. 2022). To address these issues, broth

252 microdilution assays were used to assess the total activity of HPAH against a range of

253 pathogenic microbes including bacteria, yeasts, and moulds (Table 2). Artificial honey produced

254 a MIC_{100} of >32% for all species tested, except *P. aeruginosa* which is more susceptible to

255 osmolarity, with an MIC_{100} of 32%. Unlike the PE assay, HPAH was found to be more active

256 against *S. aureus* (MIC_{100} 8%) than the jarrah honey (MIC_{100} 16%) and was on par with the

257 manuka honey (MIC_{100} 8%). HPAH had very low detectable activity against the three other

258   bacterial species tested, *E. faecalis*, *P. aeruginosa*, and *E. coli*, with an MIC_{100} of >32% and an

259   MIC_{50} of 32% for all three. The jarrah honey had the same MIC_{100} of 16% for all four bacterial

260   species. The manuka honey had an MIC_{100} of 16% for *E. faecalis*, *P. aeruginosa*, and *E. coli*.

261   For the yeast species, HPAH had no detectable activity against either *Candida* species (MIC_{50}

262   >32%), but was active against both *Cryptococcus* species with an MIC_{100} of 16% for *C.*

263   *neoformans* and 32% for *C. deuterogattii*. The jarrah and manuka honeys had the same MIC_{100}

264   for all yeast species at 16%. For moulds, HPAH was active against *Aspergillus* species with an

265   MIC_{100} of 16% for both *A. fumigatus* and *A. flavus*, but was less active against *F. oxysporum* and

266   *M. gypseum*, with an MIC_{100} of 32 %. The inverse was seen for the jarrah and manuka honeys

267   which were more effective against *F. oxysporum* (MIC_{100} 8 and 16%, respectively) and *M.*

268   *gypseum* (MIC_{100} 8%) than *A. fumigatus* and *A. flavus* (MIC_{100} 16%).

Non-peroxide components contribute to the activity of honeypot ant honey

270   To investigate potential active components, HPAH was subjected to catalase and heat treatment.

271   Catalase degrades H_2O_2, while heat denatures glucose oxidase, the enzyme that catalyses the

272   production of H_2O_2 from glucose and water. Both catalase and heat treated HPAH was tested via

273   broth microdilution for bacterial and fungal species producing a $MIC_{100} \leq 32\%$ (Table 3). For *S.*

274   *aureus*, catalase treatment decreased the activity of HPAH, raising all MIC values from 8% to

275   32%. The MIC_{100} of heat-treated HPAH was the same as catalase-treated HPAH at 32%, while

276   the MIC_{50-80} increased from 16% to 32% indicating a potential role of other non-glucose oxidase

277   heat-labile components in the antimicrobial activity of HPAH. For the fungi, catalase-treatment

278   increased the MIC_{100} of HPAH for *C. neoformans* from 16% to 32% but had no effect on the

279   MIC_{100} of *C. deuterogattii* (32%), *A. fumigatus* (16%), or *A. flavus* (16%) indicating that the

280   inhibition of these pathogens by HPAH is likely due to non-peroxide mechanisms alone. The

281   limited volume of HPAH available meant heat treatment could not be assessed for the fungal

282   species.

283   The horseradish peroxidase (HRP) assay was used to determine the amount and kinetics of H_2O_2

284   production in the different honey samples over 4 hours. HPAH had low levels of H_2O_2, with a

285   maximum of 2.1 μM detected 1 hour into the assay (Fig 1; Table 4). This was lower than the

286   manuka honey, with a maximum of 4.5 μM detected 1.5 hours into the assay, and much lower

287 than the jarrah honey that exhibited a typical 'inverted U-shape' curve with H_2O_2 peaking at 9.4

288 μM at 1 hour into the assay. The artificial honey control had no detectable H_2O_2 production.

Honeypot ant honey has very different properties to jarrah and manuka honey
bee honeys

291 Various chemical properties of HPAH that may contribute to antimicrobial activity were

292 measured and compared to the manuka and jarrah honeys (Table 4). The moisture content of

293 HPAH at 36.5% was considerably higher than the bee honeys (16.5 – 20.2%), and the sugar

294 content 63.2 º Brix was considerably lower (79.4 – 83.1 º Brix). Water activity (a_w), which is the

295 measure of unbound or biologically available water, was also substantially higher in HPAH at

296 0.80 than in the bee honeys (0.54 – 0.60). The pH of HPAH at 3.4 was lower than bee honeys

297 (3.8 – 4.5), but within what is considered a normal range for honeybee honey (~3.2 to 4.5). The

298 colour intensity of HPAH was 1844 mAU with a Pfund value of 165 mm, placing it in Dark

299 Amber, the darkest colour category along with the jarrah and manuka honeys.

300 Total phenolics content was assessed using the Folin-Ciocalteu (FC) assay, which works via a

301 redox reaction and is thus affected by non-phenolic reducing compounds, and the Fast Blue BB

302 (FBBB) assay, which is more specific and based on a direct reaction with active hydroxyl groups

303 in the phenolic compounds. Total phenolics detected by FBBB (range 159 – 434 mg GAE/kg)

304 were consistently lower than detected by FC (range 437 – 568 mg GAE/kg), confirming an

305 interference by non-phenolic compounds in the FC assay. Nonetheless, the trends of each

306 sample remained the same, with HPAH (FC = 437 mg GAE/kg; FBBB = 159 mg GAE/kg) lower

307 than the jarrah honey (FC = 471 mg GAE/kg; FBBB = 295 mg GAE/kg), which was in turn

308 lower than the manuka honey (FC = 558 GAE/kg; FBBB = 434 mg GAE/kg).

309 Antioxidant activity was assessed using the Ferric Reducing Antioxidant Power (FRAP) assay,

310 which measures the capacity of samples to reduce Fe^{3+} to Fe^{2+}, and the 2, 2-diphenyl-1-

311 picrylhydrazyl (DPPH) assay, which measures the ability of samples to scavenge the DPPH free

312 radical. Antioxidant activity detected by FRAP (range 0 – 4468 μmol Fe^{2+}/kg) was consistently

313 lower than measured by DPPH (range 1246 – 5476 μmol TE/kg) although the trends of each

314 sample remained the same, showing good correlation between assays. The antioxidant activity of

315 HPAH was 3268 μmol Fe^{2+}/kg via FRAP and 4498 μmol TE/kg via DPPH, placing it below

316 jarrah (4158 and 5098, respectively) and manuka (4468 and 5476, respectively) honey.

The bacterial and fungal microbiomes of honeypot ants are each dominated by a single genus

16S and ITS rRNA gene sequencing were used to assess the bacterial and fungal composition of the honeypot ant microbiome, respectively. Seven repletes were chosen, with various levels of honey engorgement, from the smallest weighing 0.1 g to the largest weighing more than 16x greater at 1.66 g (Fig 2). DNA was extracted from individual whole honeypot ant bodies. The bacterial microbiome of the honeypot ant samples was almost exclusively *Candidatus Blochmannia* (99.75 %), a known endosymbiont of the *Camponotus* genus. Within the remaining 0.14 % of ASVs that were identifiable, the next 10 most abundant genera were as follows: *Gilliamella*, *Pseudomonas*, *Enterobacter*, *Bacillus*, *Apilactobacillus*, *Erwinia*, *Cutibacterium*, *Gaiella*, *Actinomyces*, and *Bombella*. The fungal microbiome was also dominated almost entirely by a single taxon, *Neocelosporium* (92.77 %), with the second most abundant genera being *Endosporium* (5.51 %). Within the remaining 0.21 % of ASVs that were identifiable, the genera were as follows: *Penicillium*, *Spirographa*, *Aureobasidium*, and *Metapochonia*. Phylogenetic trees showing all taxa identified at the level of genus are presented in Fig 3.

To investigate whether HPAH contained a microbial composition itself, honey was spread onto nutrient agar, potato dextrose, and oatmeal agar plates and incubated at 20 or 35 °C to provide conditions that would be suitable for growth of a variety of bacteria and fungi (Fig 4). HPAH was found to be relatively sterile compared to raw bee honey which can harbour a wide variety of microbes (Sereia et al. 2017). After 7 days of incubation, a single colony was observed on potato dextrose agar at 20 ºC and on oatmeal agar at 35 ºC. After 21 days of incubation, further growth was seen on nutrient and potato dextrose agar at 20 ºC, and oatmeal agar at 35 ºC. Plates with visible growth were scraped and DNA extracted for 16S and ITS rRNA gene sequencing to identify colonies. Analysis of the plates identified the following bacterial genera: *Stenotrophomonas* (32.09%), *Rummeliibacillus* (29.71%), *Bacillus* (26.86%), *Paenibacillus* (9.11%), and *Streptomyces* (2.16%). Fungal genera identified were *Neocelosporium* (93.75%), and *Endosporium* (6.25%).

Discussion

Medicinal honey has gained much attention as an effective broad-spectrum antimicrobial, though little investigation has been undertaken on honey produced by insects other than the honeybee. In this study, we investigate the honey produced by the honeypot ant *Camponotus inflatus*, which has a long history of cultural importance to the Indigenous people of Australia as a delicacy and a bush medicine. The honeypot ant, like the honeybee, is a eusocial hymenopteran, and both collect nectar to produce honey for long-term storage in their respective colonies. However, differing sources of nectar, the unique form of storage and chemical composition, and unknown entomological additions in HPAH make the properties of the end-product quite different from honeybee honey (summarised in Figure 5).

Honeypot ant honey has unique species-specific and non-peroxide antimicrobial activity that may reflect evolutionary pressures

Comparing the activity of HPAH against therapeutic-grade active jarrah and manuka honeys, we found a markedly different activity profile, with HPAH outperforming these honeys against some pathogens but exhibiting low or no activity against others. Additionally, we found strong evidence for non-peroxide mechanisms of action, with HPAH producing low levels of H_2O_2 and retaining activity against *S. aureus*, *Cryptococcus sp.*, and *Aspergillus sp.* after catalase or heat treatment. While the bee honeys had similar activity against all bacterial species tested, HPAH had high activity against *S. aureus* (MIC_{100} 8%) and low activity against the others (MIC_{50} 32%). These results suggest that the activity of HPAH against *S. aureus* is most likely due to a non-peroxide component, with the low level of activity against the other bacteria due to H_2O_2. The large and species-specific differences in activity against fungal organisms, and the broader range of antimicrobial activity of HPAH (MIC_{100} 8 – >32%) compared to the bee honeys (MIC_{100} 8 – 16%) further suggest the presence of non-peroxide compounds. While peroxide clearly plays a role in the activity against certain species, with activity substantially diminished after the addition of catalase, the relatively small amounts of H_2O_2 produced by HPAH suggests that it would not be sufficient on its own to account for the activity observed. It is thus likely that one or more non-peroxide components in HPAH synergise with H_2O_2 to exert antimicrobial activity but are less or non-functional without it. Overall, these results indicate that HPAH has unique underlying mechanisms of action that are derived from the honeypot ant.

375 A notable difference of HPAH compared to the bee honeys was the substantial variation in

376 susceptibility across different bacterial and fungal species. Evolutionary pressure exerted by the

377 honeypot ant environment may be responsible for this variable activity, particularly toward the

378 different fungal species. Honeypot ants live exclusively in dry, arid, or desert-like environments.

379 Both *A. fumigatus* and *A. flavus* are ubiquitous thermotolerant fungi with an ecological niche in

380 soil debris (Bhabhra & Askew 2005; Latgé 1999). A study surveying various plots of Australian

381 soil found *A. fumigatus* to be present in 79% of all plots across different climate regions, and in

382 100% of plots in hot, arid, desert climates (Ellis & Keane 1981). This makes it likely that

383 honeypot ants have evolved to be resistant to *Aspergillus* sp. for their survival, reflected in the

384 strong activity (MIC_{100} 16%) of HPAH against *Aspergillus* sp. HPAH had similarly strong

385 activity (MIC_{100} 16%) against *Cryptococcus* sp., which are environmental saprotrophs that thrive

386 on decaying wood and soil. *Cryptococcus* sp. are spread among vegetation by a variety of animal

387 species and insects including ants (Edwards et al. 2021), making it likely that worker ants come

388 into contact with this pathogen as they venture through trees and other plants in search for food.

389 Conversely, HPAH was not very active (MIC_{100} 32%) against *Microsporum gypseum*, a member

390 of the dermatophyte group of fungi, which tend to be highly susceptible to active bee honeys.

391 Although *M. gypseum* is geophilic and found in soils worldwide (Souza et al. 2016), soil with

392 less than 5% moisture does not support its growth making it mostly found in gardens, parks and

393 soils protected by shade (Ranganathan & Balajee 2000). This makes it unlikely that *M. gypseum*

394 would be present in the honeypot ant environment.

395 **Few taxa dominate the bacterial and fungal microbiome of the honeypot ant**

396 Over 99% of the bacterial microbiome of the honeypot ant was comprised of a single genus,

397 *Candidatus Blochmannia* (*Blochmannia*). This is a mutualist that has been found in all

398 *Camponotus* species screened to date (de Souza et al. 2009; Degnan et al. 2004; Feldhaar et al.

399 2007; Sauer et al. 2002; Schröder et al. 1996). *Blochmannia* generally live in specialised

400 bacteriocyte cells located in the midgut, however they have also been found in the crop and the

401 hindgut suggesting that they may have the capacity to invade other gut tissues (He et al. 2014).

402 The vertical maternal transmission of *Blochmannia* to ant offspring suggests that it is engaged in

403 a long-term stable relationship with its host; the hallmark of a primary endosymbiont (Degnan et

404 al. 2004). These highly developed symbiotic systems are often found in insects that specialise on

405   unbalanced diets (Sauer et al. 2000). With honeypot ants feeding largely on honeydew and

406   sugary secretions, *Blochmannia* is likely involved in essential metabolic processes, such as

407   nitrogenous compound recycling, that allow the honeypot ant to occupy its ecological niche

408   (Zientz et al. 2004). Endosymbionts are often also involved in modulation of the host immune

409   system, priming it for more efficient protection against pathogens. However, studies

410   investigating this possibility in other *Camponotus* species have reported mixed findings, ranging

411   from positive effects with increased *Blochmannia* numbers in *C. fellah* (de Souza et al. 2009), to

412   neutral (Sauer et al. 2002) or negative (Sinotte et al. 2018) effects in *C. floridanus*. Other

413   bacterial taxa in the honeypot ant microbiome, although comprising a very small percentage,

414   included several genera identified in the core honey bee microbiome including *Gilliamella*,

415   *Apilactobacillus*, and *Bombella*. These are thought play a role in digestion by secreting

416   substances that aid in the metabolism of certain toxic carbohydrates in the nectar diet (Ahmad et

417   al. 2022; Härer et al. 2022; Zheng et al. 2016).

418   The fungal microbiome has not previously been profiled in any *Camponotus* species.

419   *Neocelosporium*, the dominant fungal genus, is an environmental saprotroph involved in nutrient

420   recycling and produces spreading mycelia on leaves. This genus was first identified in 2018 and

421   to date contains only two species: *Neocelosporium eucalypti* isolated from *Eucalyptus*

422   *cyanophylla* trees in southern Australia (Crous et al. 2018), and *Neocelosporium corymbiae*

423   isolated from *Corymbia variegata* trees in eastern Australia (Crous et al. 2021). It is not known if

424   *Neocelosporium* is associated with leaves of the mulga tree or with other plants likely to be in the

425   vicinity of honeypot ant nests, or if it plays a role in the biology or nutrition of *Camponotus*, and

426   these would be interesting areas for further study. The five remaining genera identified in the

427   fungal microbiome all also have environmental niches in plants, water, and/or the soil suggesting

428   that the honeypot ant fungal microbiome is largely obtained from foraging and environmental

429   exposure (Bizarria Jr. et al. 2022; Flakus et al. 2019; Siedlecki et al. 2021; Tsuneda et al. 2008).

430   Unsurprisingly, the microbiome of HPAH was largely sterile and did not contain any yeasts. As

431   the honey is stored within the ant's body, any fermentation or spoilage by transient microbes

432   would likely kill the ant. The mechanism by which HPAH is rendered sterile is unknown but

433   may include physical filtration of small particles by the infrabuccal pocket, which has been

434   identified in other ant species but never studied in *C. inflatus* (Zheng et al. 2022), or acidopore

435   grooming by which acidic poison gland secretions are swallowed for microbial control, which

436   has likewise been identified in other *Camponotus* species but not studied in *C. inflatus* (Tragust
437   et al. 2020).

438   **Potential sources of active compounds in honeypot ant honey**

439   In medicinal bee honey, non-peroxide activity is valued in a clinical setting due to its
440   comparative resistance to heat, light and catalase (Cooper et al. 1999). The known suite of non-
441   peroxide factors in honey includes phenolic compounds and phytochemicals that are heavily
442   influenced by nectar source (Johnston et al. 2018), and proteins and antimicrobial peptides that
443   are derived from the bee. The bee honey with the closest nectar source to HPAH may be
444   honeydew honey, derived from the excretions of plant sucking insects such as aphids, rather than
445   from plant nectar (Codex Alimentarius 2001). Honeydew honey has a different sugar profile
446   from most other honeys, and is usually darker in colour, with greater phenolic and antioxidant
447   content than blossom honeys (Pita-Calvo & Vázquez 2017). In a study looking at the
448   antimicrobial activity of honeydew honey, Bucekova et al. (2018) suggested there may be non-
449   peroxide components that synergise with H_2O_2, similar to what we propose for HPAH. However,
450   they reported a much higher accumulation of H_2O_2 and inhibition of *P. aeruginosa*, which was
451   not seen in our study. Entomological differences as well as differences in honeydew foraged by
452   bees compared to mulga-derived honeydew collected by the ants may underlie differences in the
453   resulting property of HPAH, suggesting it is unique among honey types.

454   Likely entomological candidates for the non-peroxide components of HPAH are antimicrobial
455   peptides (AMPs). These are short proteins that form part of the hymenopteran innate immune
456   system and play a role in defence, including killing pathogens, binding to and neutralising
457   endotoxins and modulating immune responses (Brogden 2005). In honeybees, the most notable
458   AMP is bee defensin-1, which has been identified in both the hemolymph and hypopharyngeal
459   glands and is secreted into honey. Bee defensin-1 has activity against fungi and bacteria through
460   disruption of the cell membrane (Nolan et al. 2019) and likely plays a role in protecting honey
461   from microbial spoilage (Kwakman et al. 2010). The evolution of AMPs in insects is driven by
462   gene duplication, loss, and divergence along with positive selection by organisms adapting to
463   their unique environments (Bulmer & Crozier 2004; Viljakainen & Pamilo 2008). The
464   *Camponotus* genus differs from other ants in that it lacks the metapleural gland that produces
465   antimicrobial compounds (Schluns & Crozier 2009), and harbours the endosymbiont *Candidatus*

466 *Blochmannia*, which requires the immune system to recognise and tolerate it while

467 simultaneously fighting off other pathogenic microbes (Gupta et al. 2015). This means that

468 honeypot ants are likely to possess unique antimicrobial peptides, distinct both from honey bees

469 and other ant species. Although there have been no studies to date investigating AMPs in *C.*

470 *inflatus*, a study on an ant of the same genus, *Camponotus floridanus*, found unique defensin

471 AMPs, and a hymenoptaecin AMP expressed by genes that are evolutionarily conserved in ants,

472 suggesting the importance of this AMP in the immunity of multiple ant species (Ratzka et al.

473 2012).

474

Conclusions

476 Our research has shown that honeypot ants produce antimicrobial honey with unique species-

477 specific activity that we propose may be linked to their unique evolution and ecology. We tested

478 HPAH against a suite of clinical and environmental pathogens including some commonly used to

479 evaluate therapeutic bee honey, and found that a significant portion of activity likely stems from

480 unique non-peroxide mechanisms. This discovery highlights the potential for the isolation of key

481 compounds or peptides contained within HPAH, which may provide useful leads for therapeutic

482 applications. Our profile of the bacterial and fungal microbiome of *Camponotus inflatus*

483 demonstrated extreme dominance by single bacterial and fungal species, with additional minor

484 microbial species present that could be linked to foraging behaviour or environmental exposure.

485 These results suggest a potential relationship between microbiota and insect health, which may in

486 turn influence the characteristics of honey. Overall, our study shows that broadening the scope of

487 therapeutic honey research to include other honey-producing hymenopterans can yield valuable

488 insights, and should be encouraged in order to better understand this medically and economically

489 significant commodity.

490

494   Yen Phan-Thien, Svetlana Ryazanova, Iona Gyorgy, Adriana Hoxha, Bridie Stanfield, and

495   Daniel Susantio for their technical assistance.

496

497   **References**

498   Ahmad W, Khaliq S, Akhtar N, El Arab J, Akhtar K, Prakash S, Anwar MA, and Munawar N.
499   2022. Whole Genome Sequence Analysis of a Novel Apilactobacillus Species from Giant
500   Honeybee (Apis dorsata) Gut Reveals Occurrence of Genetic Elements Coding Prebiotic
501   and Probiotic Traits. *Microorganisms* 10. 10.3390/microorganisms10050904

502   Almasaudi S. 2021. The antibacterial activities of honey. *Saudi Journal of Biological Sciences*
503   28:2188-2196. 10.1016/j.sjbs.2020.10.017

504   AOAC. 1999. Official Methods of Analysis. 96938: Association of Analytical Chemists.

505   Bhabhra R, and Askew DS. 2005. Thermotolerance and virulence of Aspergillus fumigatus: role
506   of the fungal nucleolus. *Med Mycol* 43 Suppl 1:S87-93. 10.1080/13693780400029486

507   Bizarria Jr. R, Pagnocca FC, and Rodrigues A. 2022. Yeasts in the attine ant–fungus mutualism:
508   Diversity, functional roles, and putative biotechnological applications. *Yeast* 39:25-39.
509   10.1002/yea.3667

510   Blüthgen N, and Feldhaar H. 2010. Food and shelter: how resources influence ant ecology. *Ant*
511   *Ecology* (Lach L. Parr C.L. and Abbott K.L. Eds), Oxford University Press, Oxford.
512   UK:115-136.

513   Brogden KA. 2005. Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria?
514   *Nature Reviews Microbiology* 3:238-250. 10.1038/nrmicro1098

515   Brudzynski K, and Sjaarda C. 2015. Honey glycoproteins containing antimicrobial peptides,
516   jelleins of the major royal jelly protein 1, are responsible for the cell wall lytic and
517   bactericidal activities of honey. *PLoS ONE* 10:e0120238. 10.1371/journal.pone.0120238

518   Bucekova M, Buriova M, Pekarik L, Majtan V, and Majtan J. 2018. Phytochemicals-mediated
519   production of hydrogen peroxide is crucial for high antibacterial activity of honeydew
520   honey. *Scientific Reports* 8:9061. 10.1038/s41598-018-27449-3

521 Buckley R. 1982. Ant-Plant interactions: a world review. In: Buckley, R (eds) Ant-Plant
522   interactions in Australia. *Geobotany* 111-141.

523 Bulmer MS, and Crozier RH. 2004. Duplication and diversifying selection among termite
524   antifungal peptides. *Mol Biol Evol* 21:2256-2264. 10.1093/molbev/msh236

525 Clinical and Laboratory Standards Institute. 2002a. Reference method for broth dilution
526   antifungal susceptibility testing of filamentous fungi, 3rd ed. M38-A3. Wayne, PA, USA.

527 Clinical and Laboratory Standards Institute. 2002b. Reference method for dilution antimicrobial
528   susceptibility tests for bacteria that grow aerobically; Approved standard 10th ed. M07-
529   A10. Wayne, PA, USA.

530 Clinical and Laboratory Standards Institute. 2017. Reference method for broth dilution
531   antifungal susceptibility testing of yeasts, 4th ed. M27-A4. Wayne, PA, USA.

532 Codex Alimentarius. 2001. Codex standard for honey. FAO, Rome. 19-26.

533 Conway JR. 1986. The Biology of Honey Ants. *The American Biology Teacher* 48:335-343.
534   10.2307/4448321

535 Conway JR. 1991. The biology and aboriginal use of the honeypot ant, "Camponotus inflatus"
536   Lubbock, in Northern Territory, Australia. *Australian Entomologist* 18:49-56.

537 Cooper RA, Molan PC, and Harding K. 1999. Antibacterial activity of honey against strains of
538   Staphylococcus aureus from infected wounds. *Journal of the royal society of medicine*
539   92:283-285.

540 Crous PW, Cowan DA, Maggs-Kölling G, Yilmaz N, Thangavel R, Wingfield MJ, Noordeloos
541   M, Dima B, Brandrud TE, Jansen G, Morozova O, Vila J, Shivas R, Tan YP, Bishop-
542   Hurley S, Lacey E, Marney TS, Larsson E, Floch G, and Reschke K. 2021. Fungal Planet
543   description sheets: 1182–1283. *Persoonia - Molecular Phylogeny and Evolution of Fungi*
544   46:313-528. 10.3767/persoonia.2021.46.11

545 Crous PW, Luangsa-Ard JJ, Wingfield MJ, Carnegie AJ, Hernández-Restrepo M, Lombard L,
546   Roux J, Barreto RW, Baseia IG, Cano-Lira JF, Martín MP, Morozova OV, Stchigel AM,
547   Summerell BA, Brandrud TE, Dima B, García D, Giraldo A, Guarro J, Gusmão LFP,

548   Khamsuntorn P, Noordeloos ME, Nuankaew S, Pinruan U, Rodríguez-Andrade E, Souza-
549   Motta CM, Thangavel R, van Iperen AL, Abreu VP, Accioly T, Alves JL, Andrade JP,
550   Bahram M, Baral HO, Barbier E, Barnes CW, Bendiksen E, Bernard E, Bezerra JDP,
551   Bezerra JL, Bizio E, Blair JE, Bulyonkova TM, Cabral TS, Caiafa MV, Cantillo T,
552   Colmán AA, Conceição LB, Cruz S, Cunha AOB, Darveaux BA, da Silva AL, da Silva
553   GA, da Silva GM, da Silva RMF, de Oliveira RJV, Oliveira RL, De Souza JT, Dueñas M,
554   Evans HC, Epifani F, Felipe MTC, Fernández-López J, Ferreira BW, Figueiredo CN,
555   Filippova NV, Flores JA, Gené J, Ghorbani G, Gibertoni TB, Glushakova AM, Healy R,
556   Huhndorf SM, Iturrieta-González I, Javan-Nikkhah M, Juciano RF, Jurjević Ž, Kachalkin
557   AV, Keochanpheng K, Krisai-Greilhuber I, Li YC, Lima AA, Machado AR, Madrid H,
558   Magalhães OMC, Marbach PAS, Melanda GCS, Miller AN, Mongkolsamrit S,
559   Nascimento RP, Oliveira TGL, Ordoñez ME, Orzes R, Palma MA, Pearce CJ, Pereira
560   OL, Perrone G, Peterson SW, Pham THG, Piontelli E, Pordel A, Quijada L, Raja HA,
561   Rosas de Paz E, Ryvarden L, Saitta A, Salcedo SS, Sandoval-Denis M, Santos TAB,
562   Seifert KA, Silva BDB, Smith ME, Soares AM, Sommai S, Sousa JO, Suetrong S, Susca
563   A, Tedersoo L, Telleria MT, Thanakitpipattana D, Valenzuela-Lopez N, Visagie CM,
564   Zapata M, and Groenewald JZ. 2018. Fungal Planet description sheets: 785-867.
565   *Persoonia* 41:238-417. 10.3767/persoonia.2018.41.12

566 de Souza DJ, Bézier A, Depoix D, Drezen J-M, and Lenoir A. 2009. Blochmannia
567   endosymbionts improve colony growth and immune defence in the ant Camponotus
568   fellah. *BMC Microbiology* 9:29. 10.1186/1471-2180-9-29

569 Degnan PH, Lazarus AB, Brock CD, and Wernegreen JJ. 2004. Host–Symbiont Stability and
570   Fast Evolutionary Rates in an Ant–Bacterium Association: Cospeciation of Camponotus
571   Species and Their Endosymbionts, Candidatus Blochmannia. *Systematic Biology* 53:95-
572   110. 10.1080/10635150490264842

573 Duncan FD, and Lighton JRB. 1994. The Burden within: The Energy Cost of Load Carriage in
574   the Honeypot Ant, Myrmecocystus. *Physiological Zoology* 67:190-203.

575 Edwards HM, Cogliati M, Kwenda G, and Fisher MC. 2021. The need for environmental
576   surveillance to understand the ecology, epidemiology and impact of Cryptococcus
577   infection in Africa. *FEMS Microbiology Ecology* 97. 10.1093/femsec/fiab093

578   Ellis D, and Keane P. 1981. Thermophilic Fungi Isolated From Some Australian Soils.
579   *Australian Journal of Botany* 29:689-704. 10.1071/BT9810689

580   Faast R, and Weinstein P. 2020. Plant-derived medicinal entomochemicals: an integrated
581   approach to biodiscovery in Australia. *Austral Entomology* 59:3-15. 10.1111/aen.12433

582   Feldhaar H, Straka J, Krischke M, Berthold K, Stoll S, Mueller MJ, and Gross R. 2007.
583   Nutritional upgrading for omnivorous carpenter ants by the endosymbiont Blochmannia.
584   *BMC Biology* 5:48. 10.1186/1741-7007-5-48

585   Flakus A, Etayo J, Miadlikowska J, Lutzoni F, Kukwa M, Matura N, and Rodriguez-Flakus P.
586   2019. Biodiversity assessment of ascomycetes inhabiting Lobariella lichens in Andean
587   cloud forests led to one new family, three new genera and 13 new species of
588   lichenicolous fungi. *Plant and Fungal Systematics* 64:283-344. 10.2478/pfs-2019-0022

589   Froggatt WW. 1896. Honey ants. *Report on the Work of the Horn Scientific Expedition to
590   Central Australia II*:385-392.

591   Gupta SK, Kupper M, Ratzka C, Feldhaar H, Vilcinskas A, Gross R, Dandekar T, and Förster F.
592   2015. Scrutinizing the immune defence inventory of Camponotus floridanus applying
593   total transcriptome sequencing. *BMC Genomics* 16:540. 10.1186/s12864-015-1748-1

594   Härer L, Hilgarth M, and Ehrmann MA. 2022. Comparative Genomics of Acetic Acid Bacteria
595   within the Genus Bombella in Light of Beehive Habitat Adaptation. *Microorganisms*
596   10:1058.

597   He H, Wei C, and Wheeler DE. 2014. The gut bacterial communities associated with lab-raised
598   and field-collected ants of Camponotus fragilis (Formicidae: Formicinae). *Curr
599   Microbiol* 69:292-302. 10.1007/s00284-014-0586-8

600   Hölldobler B. 1981. Foraging and spatiotemporal territories in the honey ant Myrmecocystus
601   mimicus wheeler (Hymenoptera: Formicidae). *Behavioral Ecology and Sociobiology*
602   9:301-314. 10.1007/BF00299887

603   Hossain ML, Lim LY, Hammer K, Hettiarachchi D, and Locher C. 2022. A Review of
604   Commonly Used Methodologies for Assessing the Antibacterial Activity of Honey and
605   Honey Products. *Antibiotics* 11:975.

606 Irish J, Blair S, and Carter DA. 2011. The antibacterial activity of honey derived from Australian
607   flora. *PLoS ONE* 6:e18229. 10.1371/journal.pone.0018229

608 Islam MK, Lawag IL, Sostaric T, Ulrich E, Ulrich D, Dewar T, Lim LY, and Locher C. 2022.
609   Australian Honeypot Ant (Camponotus inflatus) Honey-A Comprehensive Analysis of
610   the Physiochemical Characteristics, Bioactivity, and HPTLC Profile of a Traditional
611   Indigenous Australian Food. *Molecules* 27. 10.3390/molecules27072154

612 Johnston M, McBride M, Dahiya D, Owusu-Apenten R, and Nigam PS. 2018. Antibacterial
613   activity of Manuka honey and its components: An overview. *AIMS microbiology* 4:655.

614 Jurra KJ. 2000. The historical development of Papunya community. *Ngoonjook*.

615 Kwakman PHS, Velde AAt, de Boer L, Speijer D, Christina Vandenbroucke-Grauls MJ, and
616   Zaat SAJ. 2010. How honey kills bacteria. *The FASEB Journal* 24:2576-2582.
617   10.1096/fj.09-150789

618 Latgé JP. 1999. Aspergillus fumigatus and aspergillosis. *Clin Microbiol Rev* 12:310-350.
619   10.1128/cmr.12.2.310

620 Lehmann DM, Krishnakumar K, Batres MA, Hakola-Parry A, Cokcetin N, Harry E, and Carter
621   DA. 2019. A cost-effective colourimetric assay for quantifying hydrogen peroxide in
622   honey. *Access Microbiol* 1:e000065. 10.1099/acmi.0.000065

623 Ness JH, Mooney K, and Lach L. 2010. Ants as Mutualists. *Ant Ecology*, 97-114.

624 Nolan VC, Harrison J, and Cox JAG. 2019. Dissecting the Antimicrobial Composition of Honey.
625   *Antibiotics* 8:251.

626 Pita-Calvo C, and Vázquez M. 2017. Differences between honeydew and blossom honeys: A
627   review. *Trends in Food Science & Technology* 59:79-87. 10.1016/j.tifs.2016.11.015

628 Ranganathan, and Balajee. 2000. Microsporum gypseum complex in Madras, India. *Mycoses*
629   43:177-180. 10.1046/j.1439-0507.2000.00557.x

630 Ratzka C, Förster F, Liang C, Kupper M, Dandekar T, Feldhaar H, and Gross R. 2012. Molecular
631   characterization of antimicrobial peptide genes of the carpenter ant Camponotus
632   floridanus. *PLoS ONE* 7:e43036. 10.1371/journal.pone.0043036

633 Sauer C, Dudaczek D, Hölldobler B, and Gross R. 2002. Tissue localization of the
634   endosymbiotic bacterium "Candidatus Blochmannia floridanus" in adults and larvae of
635   the carpenter ant Camponotus floridanus. *Appl Environ Microbiol* 68:4187-4193.
636   10.1128/aem.68.9.4187-4193.2002

637 Sauer C, Stackebrandt E, Gadau J, Hölldobler B, and Gross R. 2000. Systematic relationships
638   and cospeciation of bacterial endosymbionts and their carpenter ant host species:
639   proposal of the new taxon Candidatus Blochmannia gen. nov. *Int J Syst Evol Microbiol*
640   50 Pt 5:1877-1886. 10.1099/00207713-50-5-1877

641 Schluns H, and Crozier RH. 2009. Molecular and chemical immune defenses in ants
642   (Hymenoptera: Formicidae). *Myrmecological news* 12:237-249.

643 Schröder D, Deppisch H, Obermayer M, Krohne G, Stackebrandt E, Hôlldobler B, Goebel W,
644   and Gross R. 1996. Intracellular endosymbiotic bacteria of Camponotus species
645   (carpenter ants): systematics, evolution and ultrastructural characterization. *Mol*
646   *Microbiol* 21:479-489. 10.1111/j.1365-2958.1996.tb02557.x

647 Sereia MJ, Perdoncini MRFG, Março PH, Parpinelli RS, Lima EGd, and Anjo FA. 2017.
648   Techniques for the Evaluation of Microbiological Quality in Honey. In: Vagner de
649   Alencar Arnaut de T, ed. *Honey Analysis*. Rijeka: IntechOpen, Ch. 12.

650 Siedlecki I, Gorczak M, Okrasińska A, and Wrzosek M. 2021. Chance or Necessity—The Fungi
651   Co−Occurring with Formica polyctena Ants. *Insects* 12:204.

652 Sinotte VM, Freedman SN, Ugelvig LV, and Seid MA. 2018. Camponotus floridanus Ants Incur
653   a Trade-Off between Phenotypic Development and Pathogen Susceptibility from Their
654   Mutualistic Endosymbiont Blochmannia. *Insects* 9:58.

655 Souza BD, Sartori DS, Andrade C, Weisheimer E, and Kiszewski AE. 2016. Dermatophytosis
656   caused by Microsporum gypseum in infants: report of four cases and review of the
657   literature. *An Bras Dermatol* 91:823-825. 10.1590/abd1806-4841.20165044

658 Stoloff L. 1978. Calibration of water activity measuring instruments and devices: Collaborative
659   study. *Association of Official Analytical Chemists* 61:1166-1178.

660 Tragust S, Herrmann C, Häfner J, Braasch R, Tilgen C, Hoock M, Milidakis MA, Gross R, and
661   Feldhaar H. 2020. Formicine ants swallow their highly acidic poison for gut microbial
662   selection and control. *eLife* 9. 10.7554/eLife.60287

663 Tsuneda A, Davey M, Hambleton S, and Currah R. 2008. Endosporium, a new endoconidial
664   genus allied to the Myriangiales. *Botany* 86:1020-1033. 10.1139/B08-054

665 Viljakainen L, and Pamilo P. 2008. Selection on an antimicrobial peptide defensin in ants. *J Mol*
666   *Evol* 67:643-652. 10.1007/s00239-008-9173-6

667 Zheng H, Nishida A, Kwong WK, Koch H, Engel P, Steele MI, and Moran NA. 2016.
668   Metabolism of Toxic Sugars by Strains of the Bee Gut Symbiont Gilliamella apicola.
669   *mBio* 7:e01326-01316. 10.1128/mBio.01326-16

670 Zheng Z, Zhao M, Zhang Z, Hu X, Xu Y, Wei C, and He H. 2022. Lactic Acid Bacteria Are
671   Prevalent in the Infrabuccal Pockets and Crops of Ants That Prefer Aphid Honeydew.
672   *Frontiers in Microbiology* 12. 10.3389/fmicb.2021.785016

673 Zientz E, Dandekar T, and Gross R. 2004. Metabolic interdependence of obligate intracellular
674 bacteria and their insect hosts. *Microbiol Mol Biol Rev* 68:745-770. 10.1128/mmbr.68.4.745-
675 770.2004

Table 1(on next page)

Total and non-peroxide activity of honeypot ant and active bee honeys against *Staphylococcus aureus* determined by the phenol equivalence assay

1 **Table 1.** Total and non-peroxide activity of honeypot ant and active bee honeys against

2 *Staphylococcus aureus* determined by the phenol equivalence assay.

Honey Sample	Phenol Equivalence (%)	
	Total Activity	**Non-Peroxide Activity**
Honeypot	8.3	<5
Artificial	<5	<5
Jarrah	11.6	<5
Manuka	19.7	20.1

3

Table 2(on next page)

Total activity of honeypot ant and active bee honeys (% w/v) against various bacterial and fungal pathogens determined by broth microdilution

1 **Table 2.** Total activity of honeypot ant and active bee honeys (% w/v) against various bacterial

2 and fungal pathogens determined by broth microdilution

Group	Species	Honeypot[1]			Artificial	Jarrah	Manuka
		MIC_{100}	MIC_{80}	MIC_{50}	MIC_{100}		
Bacteria	*Staphylococcus aureus*	8	8	8	>32	16	8
	Enterococcus faecalis	>32	32	32	>32	16	16
	Pseudomonas aeruginosa	>32	>32	32	32	16	16
	Escherichia coli	>32	32	32	>32	16	16
Yeasts	*Candida albicans*	>32	>32	>32	>32	16	16
	Candida glabrata	>32	>32	>32	>32	16	16
	Cryptococcus neoformans	16	8	8	>32	16	16
	Cryptococcus deuterogattii	32	16	16	>32	16	16
Moulds	*Aspergillus fumigatus*	16	16	16	>32	16	16
	Aspergillus flavus	16	16	8	>32	16	16
	Fusarium oxysporum	32	32	32	>32	16	8
	Microsporum gypseum	32	32	32	>32	8	8

3 [1]MIC_{100} = 100% inhibition, MIC_{80} = 80% inhibition, MIC_{50} = 50% inhibition

4

Table 3(on next page)

Non-peroxide activity of honeypot ant honey (% w/v) after heat or catalase treatment determined by broth microdilution

Table 3. Non-peroxide activity of honeypot ant honey (% w/v) after heat or catalase treatment determined by broth microdilution

Species	No Treatment[1]			Catalase Treatment[2]			Heat Treatment[3]		
	MIC_{100}	MIC_{80}	MIC_{50}	MIC_{100}	MIC_{80}	MIC_{50}	MIC_{100}	MIC_{80}	MIC_{50}
Staphylococcus aureus	8	8	8	32	16	16	32	32	32
Cryptococcus neoformans	16	8	8	32	16	16	-	-	-
Cryptococcus deuterogattii	32	16	16	32	16	8	-	-	-
Aspergillus fumigatus	16	16	16	16	16	16	-	-	-
Aspergillus flavus	16	16	8	16	16	8	-	-	-

[1]MIC_{100} = 100% inhibition, MIC_{80} = 80% inhibition, MIC_{50} = 50% inhibition

[2]For catalase treatment, samples were diluted with a 5600 U/ mL catalase solution

[3]For heat treatment, samples were heated at 90º C for 10 mins

Table 4(on next page)

Chemical properties of honeypot ant, jarrah, and manuka honeys

1 **Table 4.** Chemical properties of honeypot ant, jarrah, and manuka honeys

Property	Honeypot	Artificial	Jarrah	Manuka
Maximum H_2O_2 (μM)	2.1	0	9.4	4.5
Time at Maximum H_2O_2 (hours)	1	N/A	1	1.5
Sugar Content (˚Brix)	63.2	78.1	83.1	79.4
Moisture Content (%)	36.5	21.5	16.5	20.2
Water Activity (a_w)	0.80	0.58	0.54	0.60
pH	3.4	4.5	4.5	3.8
Colour Intensity (mAU)	1844	50	1657	2433
Colour (Pfund Value)	165	0	203	248
Colour (Pfund Colour)	Dark Amber	Water White	Dark Amber	Dark Amber
Phenolics via FC (mg GAE/kg)	437	38	471	558
Phenolics via FBBB (mg GAE/kg)	159	0	295	434
Antioxidants via FRAP (μmol Fe^{2+}/kg)	3268	0	4158	4468
Antioxidants via DPPH (μmol TE/kg)	4498	1246	5098	5476

2

Figure 1

Honeypot ant honey produces low amounts of hydrogen peroxide.

Hydrogen peroxide (H_2O_2) production by honeypot ant and active bee honeys measured using the horseradish-peroxidase assay over the course of 4 hours.
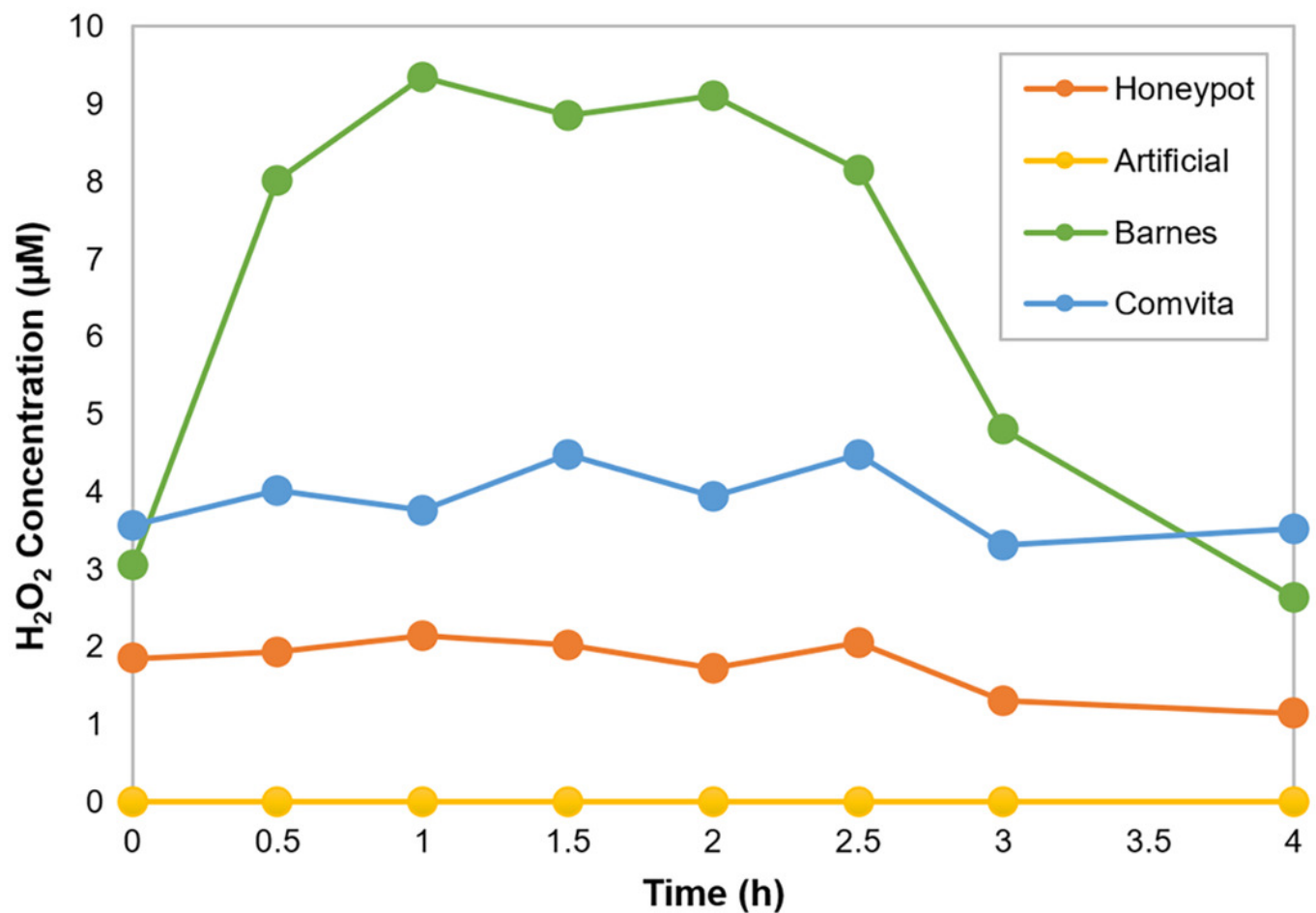
Figure 2

The bacterial and fungal microbiomes of the honeypot ant are both dominated by individual species

The appearance and weights of honeypot ant repletes selected for microbiome analysis at various levels of honey engorgement (top). Relative abundance of bacterial and fungal genera in the honeypot ant microbiome, averaged across seven ants for bacteria and four ants for fungi (bottom). Charts on the right show relative abundance of genera within the "Other" category shown on left charts.
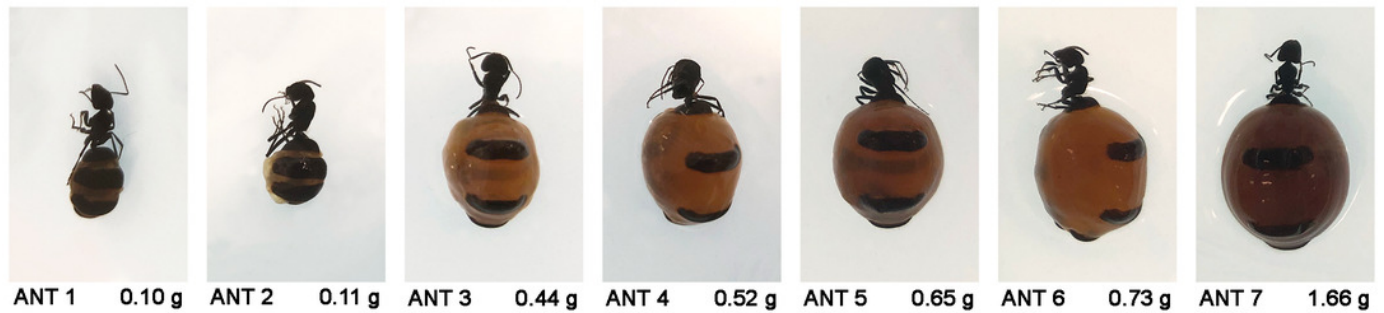
Figure 3

Phylogenetic trees of the bacterial and fungal honeypot ant microbiome at genus level.

Trees were generated using Geneious (6.0.6) and visualised with iTOL (v6). Genera are colour-coded at phylum level.
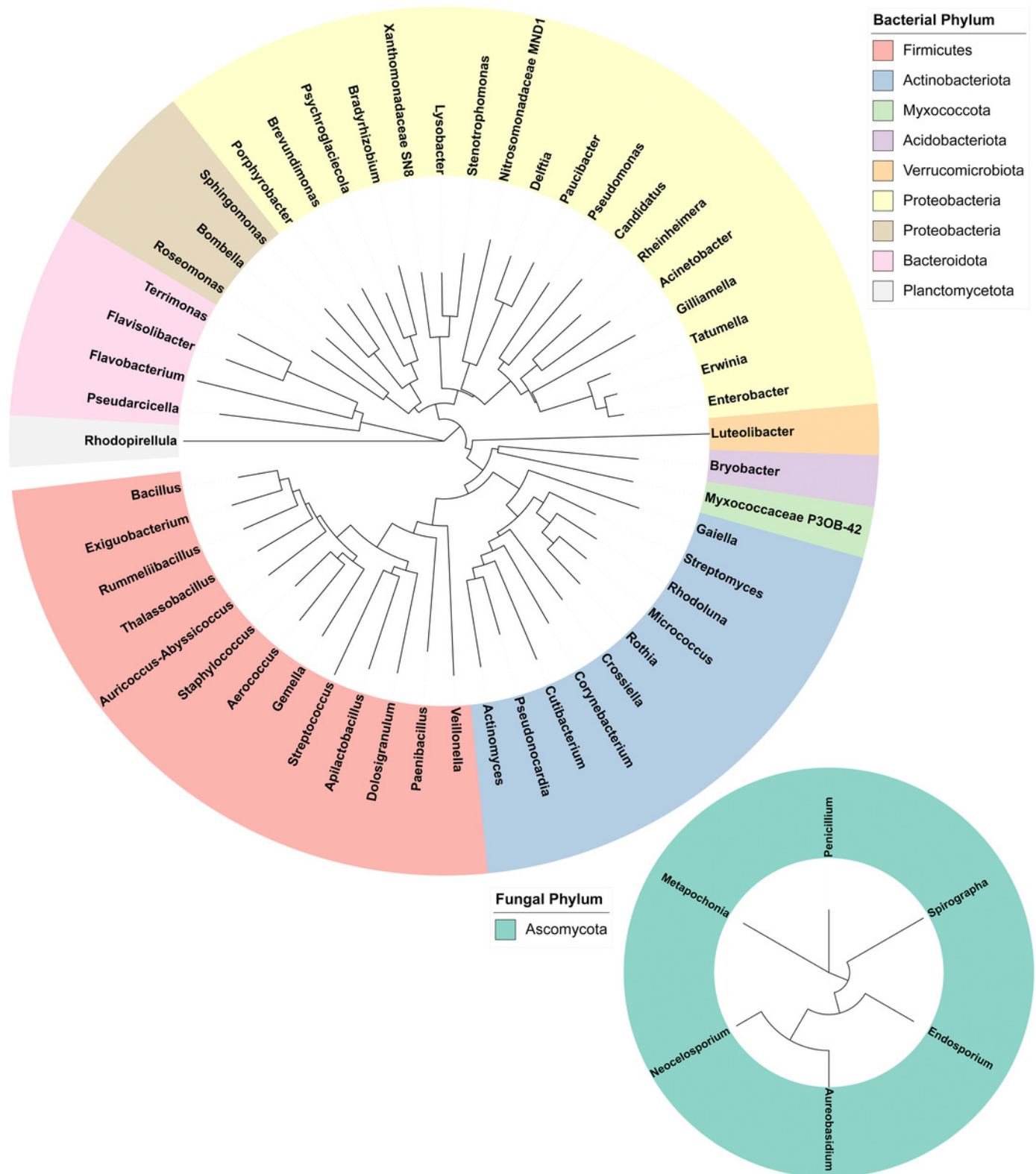
Figure 4

Honeypot ant honey contains few microbes

Spread plates of honeypot ant honey on nutrient, potato dextrose, and oatmeal agar incubated at 20 or 35 °C for 7 and 21 days (top). After 21 days, plates with visible growth were scraped, and DNA was extracted and analysed via 16S or ITS1 rRNA gene sequencing to determine the relative abundance of bacterial and fungal genera present (bottom).
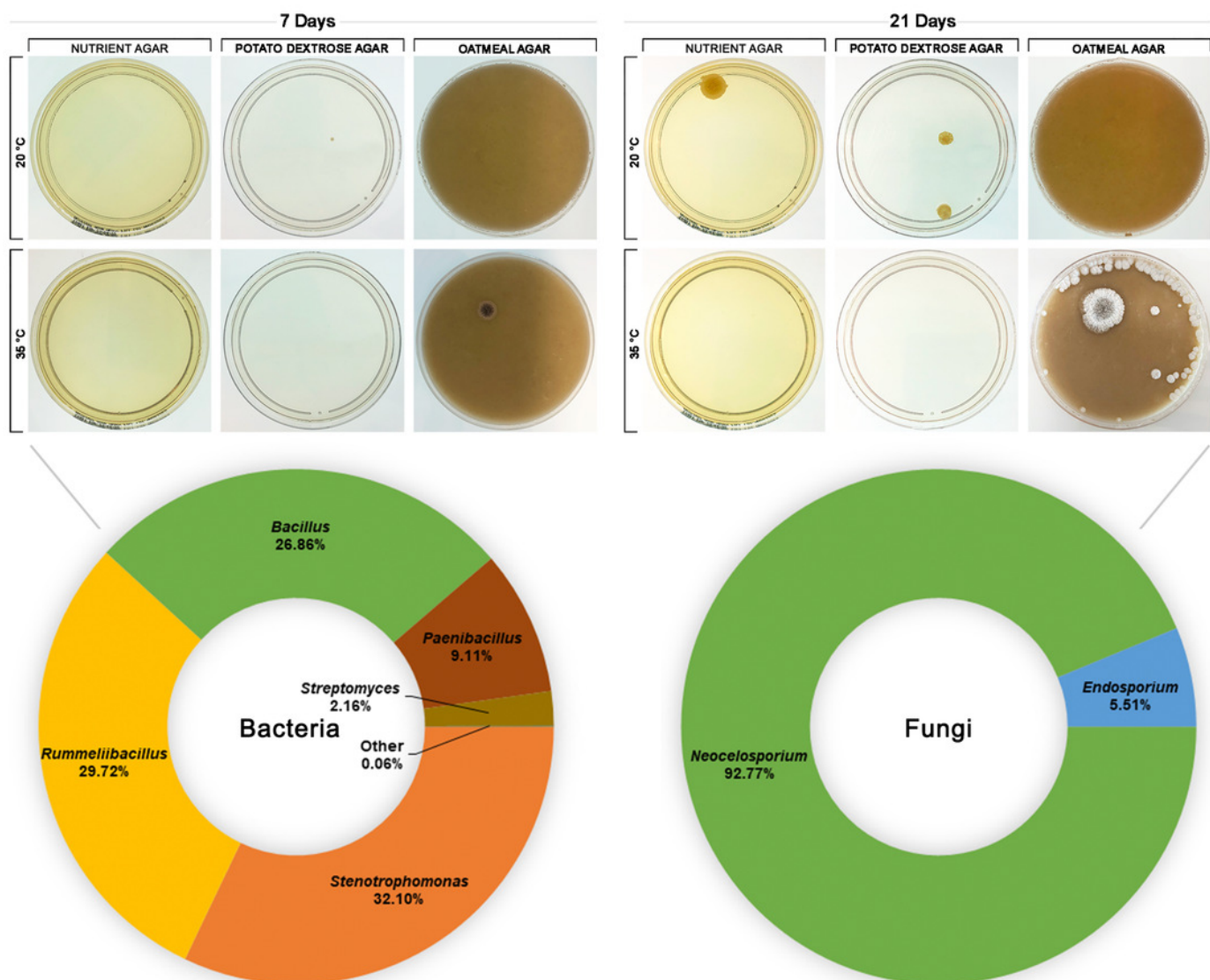
Figure 5

Characteristics of honey bee (*Apis mellifera*) honey and honeypot ant (*Camponotus inflatus*) honey that contribute to their antimicrobial properties.

Honeys produced by honey bees and honeypot ants are influenced by their specific forage sources, entomological additions and storage types, resulting in particular, unique characteristics.