Evolution of Honey Resistance in Experimental Populations of Bacteria Depends on the Type of Honey, and Has no Major Side Effects for Antibiotic Susceptibility

Running title: Honey resistance evolution in *E. coli*

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Abstract:

With rising antibiotic resistance, alternative treatments for communicable diseases are increasingly relevant. One possible alternative for some types of infections is honey, used in wound care since before 2000 BCE and more recently in licensed, medical-grade products. However, it is unclear whether medical application of honey results in the evolution of bacterial honey resistance, and whether this has collateral effects on other bacterial traits such as antibiotic resistance. Here, we used single-step screening assays and serial transfer at increasing concentrations to isolate honey-resistant mutants of *Escherichia coli*. We only detected bacteria with consistently increased resistance to the honey they evolved in with two of the four tested honey products, and the observed increases were small (maximum two-fold increase in IC_{90}). Genomic sequencing and experiments with single-gene knockouts showed a key mechanism by which bacteria increased their honey resistance was by mutating genes involved in detoxifying methylglyoxal, which contributes to the antibacterial activity of *Leptospermum* honeys. Crucially, we found no evidence that honey adaptation conferred cross-resistance or collateral sensitivity against nine antibiotics from six different classes. These results reveal constraints on

bacterial adaptation to different types of honey, improving our ability to predict downstream consequences of wider honey application in medicine.

Keywords:

honey, *Escherichia coli*, anti-bacterial agents, microbial drug resistance, bacteria, communicable diseases, leptospermum

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41 **Introduction**

42 Antimicrobial resistance is one of the biggest challenges facing global public health (WHO, 2018). To
43 preserve the effectiveness of antibiotics and to treat infections caused by resistant bacteria, alternative
44 approaches are required that can be used instead of antibiotics or after they have failed. One possible
45 alternative currently being investigated for some applications is honey (Descottes, 2009; Knipping *et*
46 *al.*, 2012; Vandamme *et al.*, 2013). Produced by the honey bee, *Apis mellifera*, honey has a long history
47 in human medicine (Breasted, 1948) and has remained a staple treatment in traditional medicine. More
48 recently, medically certified honeys and honey-containing products have been licensed in various part
49 of the world, primarily for topical application, such as in wound healing (Molan and Betts, 2004; Cooke
50 *et al.*, 2015), and treatment of otorhinolaryngological diseases (Werner and Laccourreye, 2011). The
51 idea is appealing: honey is generally cheap and non-harmful to patients (Dunford and Hanano, 2004;
52 Knottenbelt, 2014), inhibits bacterial growth (Basualdo *et al.*, 2007; Carter *et al.*, 2016) and can promote
53 wound closure and healing (Molan, 1999). If honey could be used instead of antibiotics for some
54 applications, this could contribute to managing antibiotic resistance. However, several open questions
55 remain about whether bacteria exposed to inhibitory concentrations of honey evolve resistance to it,
56 which genes or pathways are involved, and whether any such evolutionary responses have downstream
57 effects on other properties relevant for treatment, in particular antibiotic resistance.

58 Despite open questions about how bacteria evolve honey resistance, the physiological causes of
59 honey's antibacterial activity have been investigated in various species (Kwakman *et al.*, 2008, 2011;
60 Badet and Quero, 2011; Camplin and Maddocks, 2014). Multiple mechanisms have been implicated,
61 including high sugar/low water content, acidity, hydrogen peroxide and non-peroxide molecules such
62 as methylglyoxal (White *et al.*, 1963; Allen *et al.*, 1991; Molan, 1992; Mavric *et al.*, 2008). At the
63 phenotypic level, various effects of honey have been reported across different species, including
64 changes in cell-wall integrity and cell shape (Henriques *et al.*, 2011; Brudzynski and Sjaarda, 2014;
65 Wasfi *et al.*, 2016), quorum sensing (Truchado *et al.*, 2009; Lee *et al.*, 2011; Wang *et al.*, 2012), iron
66 acquisition (Kronda *et al.*, 2013; Ankley *et al.*, 2020) and biofilm formation (Merckoll *et al.*, 2009;
67 Badet and Quero, 2011; Halstead *et al.*, 2016). Perhaps unsurprisingly given this complex picture of
68 inhibitory mechanisms, research to date on the evolution of honey resistance in bacteria has reached no

69 clear consensus. For example, Cooper *et al.* (2010) reported no stable increase in resistance after
70 exposure of various species to medical-grade *Leptospermum* honey *in vitro*, while Camplin and
71 Maddocks (2014) and Lu *et al* (2019) detected increased resistance in *Pseudomonas aeruginosa* cells
72 exposed to honey in biofilms *in vitro*. Thus, it remains unclear how rapidly bacteria exposed to honey
73 can evolve reduced susceptibility to it. Another important question is whether adaptation to honey has
74 side effects for antibiotic susceptibility. This is important for assessing the risk that honey application
75 could contribute to the spread of antibiotic resistance, affecting the success of antibiotic treatments used
76 in combination or later against the same bacteria. There is some evidence that honey resistance affects
77 susceptibility to rifampicin and imipenem in *P. aeruginosa* (Camplin and Maddocks, 2014). To
78 understand the general picture of how honey resistance affects antibiotic susceptibility, we need to test
79 a wider range of bacteria and antibiotics, and characterise the genetic pathways by which bacteria
80 become resistant.

81 We chose to study *E. coli* because it is a common pathogen in humans and animals (Dowd *et al.*,
82 2008; Suojala *et al.*, 2013), frequently associated with surface wounds that might be suitable for honey
83 treatment. Antibacterial resistance has increased in *E. coli* (Tadesse *et al.*, 2012; O'Neill, 2014), and
84 many of the resistance mechanisms found in *E. coli* can also be found in other species (Philippon *et al.*,
85 1989; Paulsen *et al.*, 1996), suggesting it as a good model for studying resistance evolution. To find out
86 how bacteria respond to honey exposure phenotypically and genotypically, we experimentally evolved
87 *E. coli* in the presence of four different honeys (two medical-grade honeys, two commercially available
88 honeys) by gradually exposing bacteria to increased honey concentrations during serial passage. We
89 then measured honey susceptibility of evolved bacteria from this experiment, as well as population
90 growth in the absence of honey. To identify genes involved in honey resistance, we used whole-genome
91 sequencing of these evolved isolates, as well as single-gene knockout variants. We also used a second
92 screen for honey-resistant mutants, by exposing many replicate populations to high honey
93 concentrations in a single-step (selective plating of overnight cultures). Lastly, we tested for collateral
94 effects of honey adaptation on antibiotic resistance by determining the phenotypic resistance of honey-
95 adapted isolates to antibiotics of different classes. Our results show that, even upon gradually increasing
96 exposure, large changes in honey resistance in *E. coli* populations growing *in vitro* are rare. However,

97 for some honey products we identified mechanisms driving moderate increases in honey resistance, and
98 we find no indication for cross-resistance between honey and antibiotics.
99
100 **Materials and Methods**
101 **Organisms and Growth Conditions**
102 We used *E. coli* K-12 MG1655 as parental strain for the evolution experiment and isolation of single-
103 step mutants. We used *E. coli* K-12 BW25113 and single-gene knockout variants derived from it (Keio
104 Knockout Collection (Baba *et al.*, 2006)) for knockout experiments. We stored all isolates in 25%
105 glycerol at -80°C. We performed routine culturing in lysogeny broth (LB) at 37°C with shaking at
106 180rpm.
107
108 **Honeys and Antibiotics**
109 The different honey products we used are listed in Table 1. We purchased SurgihoneyRO™ in 20g and
110 50g tubes from H&R Healthcare, Hull, UK; Medihoney™ Medical Honey in 20g tubes from Puras AG,
111 Bern, Switzerland, and in 50g tubes from The Honeydoctor, The Littledart Company Ltd, Tiverton,
112 UK; *MGO*™ Manuka Honey MGO™550+ in 250g jars from nu3 GmbH, Berlin, Germany; and
113 Fairtrade Liquid Blossom Honey in 550g jars from Coop, Basel, Switzerland. Honeys were stored in a
114 cool, dark place and, in the case of medical-grade honeys, opened tubes were only used as long as
115 recommended by the manufacturer. Non-medical-grade honeys were used for a maximum of six months
116 after opening. After plating honey samples on agar plates, we observed colony-forming units with the
117 commercial honey and Manuka honey. This was no longer the case after filtering honey solutions with
118 a Filtropur S 0.45 filter (Sarstedt, Germany) (Wasfi *et al.*, 2016). Accordingly, for all four honeys,
119 honey-containing growth media were prepared immediately before the start of each experiment by
120 diluting honey in LB (pre-heated to 55°C) and filter sterilizing. We purchased amoxicillin (product
121 number A8523), chloramphenicol (product number 23275), ciprofloxacin (product number 17850),
122 gentamicin (product number 48760), kanamycin (product number 60615), neomycin trisulfate salt
123 hydrate (product number N1876), polymyxin B (product number 5291), tobramycin (product number
124 T4014), and trimethoprim (product number 92131) from Sigma-Aldrich (Merck KGaA, Germany). We

125 prepared antibiotic stock solutions at the outset of the experiments and filter sterilized (Filtropur S 0.2
126 (Sarstedt, Germany)) and stored them according to the manufacturers' instructions (stock solutions:
127 amoxicillin 25mg/mL in sterile distilled water (dH_2O); chloramphenicol 50mg/mL in 70% ethanol;
128 ciprofloxacin 20mg/mL in 0.1M HCl; gentamicin 50mg/mL in dH_2O; kanamycin 40mg/mL in dH_2O;
129 neomycin 40mg/mL in dH_2O; polymyxin B 20mg/mL in dH_2O; tobramycin 40mg/mL in dH_2O;
130 trimethoprim 25mg/mL in DMSO).
131
132 **Measuring Susceptibility of Isolates to Different Honeys**
133 We used the 90% inhibitory concentration (IC_{90}) of each antibacterial compound as an indicator of
134 resistance. We defined the IC_{90} as the lowest concentration tested above which bacterial growth did not
135 exceed 10% of growth of the same isolate in the absence of antibacterials. We also tried an alternative
136 definition, taking the IC_{90} as the lowest concentration tested where growth did not exceed 10% of the
137 same isolate's growth in the absence of antibacterials (even if there was a score above this cut-off at
138 one of the higher concentrations, which we observed in a minority of dose-response curves); this did
139 not alter our overall conclusions. For each assay, we transferred independent LB-overnight cultures
140 (cultured in flat-based 96-well microplates (Sarstedt, Germany)) into microplates filled with various
141 honey concentrations and plain LB as a control, using a pin replicator (1/100 dilution, 2µL in 200µL).
142 We used slightly different concentration ranges in different sets of assays (as described below), which
143 were conducted with independent controls on different days. We incubated microplates overnight at
144 37°C and quantified bacterial growth by measuring optical density at 600nm (OD_{600}) with a microplate
145 reader (Infinite® 200 PRO, Tecan Trading AG, Switzerland) at the beginning and end of the experiment
146 (0h and 24h). We corrected OD scores for the optical density of the media. In each assay, we made 3-5
147 replicates of all tested strains in all tested compounds.
148
149 **Experimental Evolution (Fig. S1A)**
150 We serially passaged multiple selection lines of *E. coli* K-12 MG1655 in filtered solutions of each of
151 the four honey products and in the absence of honey for 22 days, transferring daily (4 honey treatments
152 + 1 control treatment = 5 evolution environments). In summary: at each transfer, each selection line

153 was inoculated into multiple wells containing various honey concentrations. After overnight incubation,
154 we transferred from the well with the highest concentration supporting viable growth (Fig. S1A). In
155 more detail: to begin the experiment, we inoculated six selection lines in each evolution environment,
156 each with an independent colony (5 evolution environments x 6 colonies = 30 selection lines). In this
157 first step, we cultured each selection line over night in 5mL of LB at 37°C with shaking at 180rpm.
158 Then, for every selection line, we inoculated seven microplate wells filled with: 200µL of
159 unsupplemented LB ("rescue well"), LB supplemented with one of five concentrations of the respective
160 honey product, or honey stock solution (30 or 50% (w/v)). We incubated microplates overnight at 37°C
161 and quantified bacterial growth by measuring OD_{600} after 0h and 24h. For every selection line, we
162 transferred 5µL from one of the seven inoculated wells to seven wells in a new microplate. Each time,
163 we transferred from the well at the highest honey concentration where ΔOD_{600} (OD_{600} 24h – OD_{600} 0h)
164 > 0.1 (an arbitrary cut-off we took as an indication of viable growth). In cases where ΔOD_{600} in all
165 honey-supplemented wells was < 0.1, we used the rescue-well culture to inoculate the fresh microplate.
166 To gradually expose selection lines to higher concentrations of honey, we adjusted the range of
167 concentrations tested over time, according to the performance of individual selection lines
168 (concentrations and OD scores over time are given in Fig. S2. In the control treatment (no honey), we
169 used a single well for each selection line at each transfer. We did this for 22 days, freezing microplates
170 from days 3, 6, 9, 12, 15, 18, 21 and 22.

171 At the end of the experiment, we isolated a single colony isolate at random from each selection line.
172 We streaked out a sample from the well with the highest honey concentration where ΔOD_{600} > 0.1 onto
173 LB agar. After overnight incubation, we picked one colony per selection line, grew it overnight in 5mL
174 LB and stored it at -80°C. We used colony PCR (primer sequences: forward: 5′-AGA CGA CCA ATA
175 GCC GCT TT-3′; reverse: 5′-TTG ATG TTC CGC TGA CGT CT-3′) to ensure that all colony isolates were
176 *E. coli* K-12 MG1655. For five selection lines (Medihoney_01, Commercial_02, Commercial_03,
177 Commercial_04, Commercial_05), we found no colonies when streaking out samples at day 22. We
178 initially screened for honey-resistant phenotypes of colony isolates from the other selection lines by
179 culturing each colony isolate in honey-supplemented medium at a concentration in which the parental

180 strain was not able to grow. This led us to exclude five isolates that did not grow at honey concentrations
181 above that of the parental strain (Surgihoney_01, Surgihoney_02, Surgihoney_03, Surgihoney_06,
182 Commercial_01). We then proceeded with the remaining 14 serially-passaged putative honey-resistant
183 mutants, plus six control isolates serially passaged in LB, sequencing their genomes and quantifying
184 their honey susceptibility as described below/above respectively.

185 To test whether isolates from different evolution environments had different honey-resistance
186 profiles, we used a linear-mixed effects model (lmer function in R's lmerTest package (R version: 4.0;
187 package version: 3.1-2)), with evolution environment and assay compound as fixed effects and
188 genotype (isolate) as a random effect. We compared models with and without the interaction between
189 fixed effects, using the anova function of the stats package to test significance. When looking at
190 individual assay compounds, we used evolution environment as a fixed effect and genotype as a random
191 effect. We used maximum likelihood estimation (*REML = F*). Wild-type data was excluded from this
192 analysis; we tested for differences in IC_{90} between evolved isolates and the wild type separately, with
193 *t*-tests (*p*-values adjusted for multiple testing using the Holm-Bonferroni method).

194

195 **Genetic Analysis of Serially-Passaged Mutants**

196 We sequenced the 14 serially-passaged, putative resistant mutants and the six LB-adapted control
197 isolates. We used the QIAGEN Genomic-tip 20/G (Cat. No. 10223, Qiagen, the Netherlands) according
198 to the manufacturer's instructions for genomic DNA (gDNA) extraction. Overnight cultures inoculated
199 with single colonies were centrifuged at 5000xg at room temperature for 10min. After removal of the
200 supernatant, we stored cell pellets at -20°C until further processing. We quantified the obtained gDNA
201 using the Quant-iTTM dsDNA BR (Broad Range) Assay Kit (Thermo Fisher Scientific, USA) in the
202 QubitTM Fluorometer (Thermo Fisher Scientific). We used a Nanodrop (Thermo Fisher Scientific) to
203 control the purity of gDNA (ratios A_{260}/A_{280}). We sequenced at the Functional Genomic Center, Zurich,
204 Switzerland, using the Illumina Hiseq 4000 platform after library preparation with the Nextera XT DNA
205 Library Prep kit (Illumina, USA).

206 We trimmed and quality-filtered all sequences with trimmomatic (Bolger *et al.*, 2014). We mapped
207 the sequence of the ancestral strain of our resistance isolation experiment against the reference sequence

208 of *E. coli* K-12 MG1655 (NCBI accession number: U00096) using breseq 0.33.1 (Deatherage and
209 Barrick, 2014). We used gdtools implemented in breseq to integrate the identified mutations into the
210 reference genome. For variant calling, we mapped all sequences of the serially-passaged and single-
211 step putative resistant mutants against the refined reference genome using breseq. The sequencing data
212 has been deposited in the European Nucleotide Archive under the study accession number PRJEB35347
213 (https://www.ebi.ac.uk/ena).
214
215 **Experiments with Single-Gene Knockout Variants**
216 After identifying genes that potentially contribute to honey adaptation (Table S1), we tested for further
217 evidence of the role of these genes in honey resistance using single-gene knockout variants from the
218 Keio Knockout Collection (Baba *et al.*, 2006) for *E. coli* K-12. We tested for a change in the resistance
219 phenotype of these knockout variants relative to the ancestral strain of the knockout collection, *E. coli*
220 K-12 BW25113, using the same resistance phenotyping assay as described above. When choosing genes
221 to investigate, we concentrated on those (1) which were affected by independent mutations in at least
222 two selection lines, (2) where mutations were not detected in isolates from the control treatment, (3)
223 which were not annotated as "pseudogene", "intergenic" nor "non-coding", and (4) for which there is
224 an available knockout variant in the Keio Collection (limited to non-essential genes).
225
226 **Measuring Population Growth of Serially-Passaged Mutants in the Absence of Honey**
227 We tested whether our serially passaged isolates showed altered population growth in the absence of
228 honey relative to the ancestral strain. To do this, we grew four independent cultures (each inoculated
229 from a different colony) of each serially-passaged isolate (including control-evolved isolates, $n = 20$)
230 and the ancestral isolate, each in 150µL LB in a microplate in a randomized layout. After overnight
231 incubation, we used a pin replicator to inoculate a fresh microplate (all wells filled with 150µL LB).
232 We then measured OD_{600} every 15min for 24h (shaking before each measurement). We used R's nls
233 and SSlogis functions in the stats package to estimate the growth rate and yield for each culture, with
234 yield corresponding to the *Asym* parameter of the models (= asymptote) and growth rate corresponding

235 to the inverse of the *scal* parameter (= numeric scale parameter on input axis). We tested for a difference
236 in growth parameters between each evolved isolate and the parental strain with a *t*-test, adjusting *p*-
237 values using sequential Bonferroni correction. We also tested for an average difference in growth rate
238 or yield among evolved isolates from different evolution environments. We did this using a linear
239 mixed-effects model, with evolution environment as a fixed effect and genotype as random effect, and
240 excluding data from the wild type. We used maximum likelihood estimation (*REML* = *F*) with the lmer
241 function in the lmerTest package.
242
243 **Measuring Susceptibility of Serially-Passaged Mutants to Antibiotics**
244 We measured the phenotypic resistance (IC_{90}) of the 20 serially-passaged isolates (14 putative resistant
245 mutants, six LB-adapted control isolates) and of the parental strain (*E. coli* K-12 MG1655) for nine
246 antibiotics representing six different classes: amoxicillin (penicillin), ciprofloxacin (fluoroquinolone),
247 chloramphenicol (chloramphenicol), gentamicin (aminoglycoside), kanamycin (aminoglycoside),
248 neomycin (aminoglycoside), polymyxin B (polymyxin), tobramycin (aminoglycoside), and
249 trimethoprim (dihydrofolate reductase inhibitor). We decided to test several aminoglycoside drugs
250 (gentamicin, kanamycin, neomycin, tobramycin) because previous studies have found that bacteria
251 exposed to honey reduce the expression of genes involved in the TCA cycle (Lee *et al.*, 2011; Jenkins
252 *et al.*, 2014), while others report a link between aminoglycoside susceptibility and defects or down-
253 regulated gene expression in the bacterial respiratory chain, including the TCA cycle (Magnet and
254 Blanchard, 2005; Chittezham Thomas *et al.*, 2013; Shan *et al.*, 2015; Su *et al.*, 2018; Zhou *et al.*, 2019).
255 We conducted the assays using a similar protocol as described above for honey. In brief, we first
256 incubated independent replicate populations of each isolate in a randomized layout in microplates
257 overnight. From these microplates we inoculated assay plates filled with LB supplemented with
258 antibiotics at various concentrations in two-fold broth dilution series, measuring bacterial growth by
259 the change in OD_{600} (0h, 24h) as described above.
260
261 **Single-Step Isolation of Honey-Resistant Mutants (Fig. S1B)**

262 As a second screen for mutants of *E. coli* K12 MG1655 with increased honey-resistance, we plated
263 aliquots of multiple independent overnight cultures, grown in the absence of antibiotics, on agar
264 supplemented with each honey product.

265 For each honey type, we first grew 54 independent overnight cultures (250µL per culture in LB in a
266 96-well microplate; Fig. S1B). We then plated each culture onto a separate agar plate (prepared in 6-
267 well culture plates filled with 4mL honey-supplemented agar per well (Sarstedt, Germany)), plating
268 100µL of 18 separate cultures at each of three concentrations per honey product. We chose
269 concentrations 1.25-, 1.5-, 2- or 3-times higher than previously determined IC_{90}s of the wild-type strain
270 in liquid. We then incubated the plates overnight at 37°C before checking for resistant mutants. For
271 SurgihoneyRO™, Medihoney™ and Manuka honey, we picked six putative honey-resistant colonies,
272 each from a separate well. When isolating colonies, we prioritized those from wells with higher
273 concentrations of honey. For commercial honey, we observed no viable colonies on honey-
274 supplemented agar despite four attempts on different days (total of 216 cultures). We cultured the
275 selected putative resistant colonies overnight in 5mL LB and suspended them in 25% glycerol for
276 storage at -80°C. We then tested these 18 single-step putative honey-resistant mutants for phenotypic
277 resistance as described above. We also used a second test to see if putative honey resistance phenotypes
278 were robust, by streaking out frozen stocks of each colony isolate on honey-supplemented agar at a
279 concentration inhibitory to the parental strain. Only three of 18 single-step putative honey-resistant
280 mutants were able to form colonies under these conditions (Medihoney_10, Surgihoney_09,
281 Surgihoney_10).

282

283
284 **Results**
285

286 **Experimental Evolution of Manuka Honey-Resistant Bacteria by Serial Passaging**

287 During serial passage at gradually increasing honey concentrations (Fig. S1A), most selection lines
288 reached higher population densities at concentrations that were initially inhibitory to the ancestral strain
289 (Fig. S2 & S3). Of 14 putative honey-resistant mutants and six control isolates, each isolated from a

290 different selection line after 22 days, several showed increased resistance compared to the ancestral
291 strain against one or more honey products (Fig. 1 & S4). The average change in resistance varied among
292 evolved isolates passaged with different honey compounds (effect of evolution environment: $F_{4, 20}$ =
293 1.799, $p > 0.1$). These differences also depended on which honey compound was used in the assay
294 (evolution environment × assay environment interaction: $\chi^2(12) = 234.99$, $p < 0.001$). We observed the
295 largest change in resistance for mutants selected with Manuka honey or Medihoney™ when they were
296 assayed with Manuka honey (mean change relative to the ancestor of 2.13-fold (s.d. = 0.22) and 2.07-
297 fold, respectively). Manuka honey resistance of Manuka honey- and Medihoney™-evolved isolates was
298 also significantly higher than for control-evolved isolates from the honey-free LB-medium treatment
299 ($t_{9.7} = 7.73$, $p < 0.001$ and $t_{8.9} = 6.77$, $p < 0.001$, respectively). On average, Manuka honey-evolved and
300 Medihoney™-evolved isolates also had moderately increased resistance to Medihoney™ (mean change
301 relative to the ancestor of 1.15-fold (s.d. = ±0.16) and 1.17-fold (s.d. = ±0.1), respectively), but this was
302 not significantly different from that of control-evolved isolates on average (Manuka: $t_2 = -0.30$, $p >$
303 0.05; Medihoney™: $t_2 = 0$, $p > 0.05$). Some other individual isolates had consistently increased Manuka
304 honey or SurgihoneyRO™ resistance (all replicates for a given isolate higher than all replicates for the
305 ancestor; Fig. 1 & S4), but these changes were small compared to those for Manuka honey- and
306 Medihoney™-evolved isolates tested with Manuka honey. Thus, after experimental evolution we
307 observed the strongest evidence of honey adaptation with Manuka honey.
308

309 **Honey Adaptation Is Linked to Mutations in *nemAR* and *clpP***

310 Genomic sequencing of our serially passaged isolates revealed changes at several loci (Fig. 2, Table
311 S1). Some loci were mutated multiple times independently in honey-evolved colony isolates, but not in
312 control-evolved isolates (Table S1, Fig. 2), indicating a possible role in adaptation to honey. Two such
313 genes were *clpP* (serine protease, mutated in two Manuka honey-evolved isolates) and *nemR* (DNA-
314 binding transcriptional repressor, mutated in four Manuka honey-evolved isolates and three
315 Medihoney™-evolved isolates; Table S1, Fig. 2). The intergenic region between *ydhL* and *nemR* was
316 mutated in the remaining two Manuka honey-adapted isolates and the remaining two Medihoney™-
317 adapted isolates (Table S1, Fig. 2). An additional gene for which we found mutations in multiple isolates

318 serially passaged in Manuka honey is *yafS* (methyltransferase; Table S1, Fig. 2), located next to *gloB*,
319 the gene encoding glyoxalase II, an enzyme involved in detoxification of reactive aldehydes (Ozyamak
320 *et al.*, 2010). In control-evolved isolates, we found parallel mutations in *fimE*, a recombinase
321 responsible for on-to-off switching of type 1 fimbriae expression, and *flhD*, one of two transcriptional
322 activators of the *E. coli* flagellar regulon but also involved in cell division (Klemm, 1986; Iino *et al.*,
323 1988; Liu and Matsumura, 1994; Prüß and Matsumura, 1996), consistent with past work with LB-
324 adapted isolates (Knöppel *et al.*, 2018). In summary, we found parallelisms in both control-evolved and
325 honey-evolved isolates, and in particular every Manuka honey- and Medihoney™-evolved isolate had
326 a mutation in or affecting *nemR*. As we discuss below, this may reflect the role of *nemR* in
327 methylglyoxal degradation (Ozyamak *et al.*, 2013), an active component of *Leptospermum* honeys.
328

329 **Single-Gene Knockouts Support a Role for *nemR* in Honey Resistance**

330 We used single-gene knockout variants of several genes that were mutated in serially-passaged isolates
331 to test for further evidence that they play a role in honey resistance (Fig. 3 & S5). The knockout variant
332 Δ*nemR* had increased Manuka honey resistance (all replicates higher than all replicates of the wild-type
333 strain). This variant also showed increased average resistance to Medihoney™, although this was not
334 consistent across all replicate assays (Fig. 3 & S5). This is consistent with the above finding that all
335 isolates serially passaged in Manuka honey or Medihoney™ had mutations in or close to *nemR*, and all
336 were more resistant to Manuka honey and Medihoney™ than the ancestral strain. In Δ*clpP*, the
337 knockout of the other gene for which we observed independent mutations in multiple honey-evolved
338 isolates, we observed a similar pattern: increased resistance to Manuka honey (in all replicate assays)
339 and higher average Medihoney™ resistance (two out of three replicates higher than wild-type). This is
340 consistent with the increased resistance for these honeys we observed with the two Manuka honey-
341 selected isolates with mutations in *clpP* (Fig. 2 & S4). Two other knockout variants, Δ*nemA* and Δ*eda*,
342 had consistently altered resistance to Manuka honey, with Δ*eda* having a higher susceptibility than the
343 ancestral strain. In summary, independent deletion of two genes, *nemR* and *clpP,* which were also
344 directly or indirectly affected by mutations in several of our serially-passaged isolates, conferred
345 increased resistance to Manuka honey and Medihoney™.

346

347 **Impaired Growth in the Absence of Honey for Honey-Evolved Compared With Control-Evolved**
348 **Isolates**

349 Most serially passaged isolates (both honey-evolved and control-evolved) had increased population
350 growth rates relative to the ancestral strain in the absence of honey (Fig. 6). However, control-evolved
351 isolates had higher growth rates on average compared to all four types of honey-evolved isolates (linear
352 mixed-effects model: Manuka honey: $t_{20} = -4.355$, $p < 0.001$; Medihoney™: $t_{20} = -5.538$, $p < 0.001$;
353 SurgihoneyRO™: $t_{20} = -4.078$, $p < 0.001$; commercial honey: $t_{20} = -4.671$, $p < 0.001$). By contrast, we
354 found little effect of serial passaging on growth yield in evolved isolates compared to the ancestral
355 strain (Fig. S6). Comparing the growth yield in different evolution environments, we found no
356 significant difference between isolates serially passaged in LB and honey-evolved isolates (linear
357 mixed-effects model: all $p > 0.05$; Fig. S6). In summary, for most experimentally evolved isolates we
358 found a positive effect of serial passage on growth rate, but honey-adapted isolates had a lower growth
359 rate on average compared to isolates serially passaged in the absence of honey.

360

361 **No Evidence for Cross-Resistance Between Honey and Antibiotics**

362 We determined the phenotypic resistance of our serially-passaged isolates and the ancestral strain to
363 nine antibiotics of six different classes (Fig. 5; Fig. S7). We found only a single case out of 180
364 combinations (20 serially-passaged isolates × 9 antibiotics) where antibiotic resistance was consistently
365 altered compared to the ancestral strain (higher/lower IC_{90} for all mutant replicates compared to all
366 replicates of ancestor). This was for one Manuka honey-evolved isolate, Manuka_05, tested with
367 polymyxin B. For seven of the other eight antibiotics, the largest difference shown by individual
368 replicates relative to the median of the ancestral replicates was two-fold or less. For the remaining
369 antibiotic, kanamycin, some individual replicates had differences of four-fold compared to the ancestor.
370 The overall lack of major differences between evolved isolates and the ancestral strain was supported
371 by Analysis of Variance (testing each evolved-isolate-versus-wild-type combination separately, with
372 genotype (evolved vs wild type) and assay compound (antibiotic) as factors, including the interaction
373 term and with *p*-values adjusted using the Holm-Bonferroni method); this indicated a single significant

374 effect of genotype, for isolate Manuka_06). Thus, we found no evidence for appreciable changes in
375 sensitivity to antibiotics in serially-passaged honey-adapted isolates.
376
377 **Single-Step Screening Supports Similar Variation of Resistance Evolution Among Honey**
378 **Products**
379 We tested whether our finding that resistance evolution was more pervasive with Manuka honey than
380 the other honey compounds also holds true when bacteria are exposed to inhibitory honey
381 concentrations suddenly, rather than gradually. We did this by plating aliquots from multiple
382 independent, replicate overnight cultures directly onto honey-supplemented agar, and picking the
383 resulting colony isolates that showed a putative resistance phenotype. As for our serially passaged
384 isolates above, we observed the largest increases in resistance for colony isolates from the Manuka-
385 honey treatment, when assayed with Manuka honey (Fig. S8). For SurgihoneyRO™ and Medihoney™,
386 we observed colonies on some of our plates, but after picking six colonies for each compound and
387 testing their phenotypic resistance, none of them had consistently altered resistance to the honey they
388 were selected with. When we streaked out frozen stocks of these 12 putative honey-resistant colony
389 isolate on honey-supplemented agar at a concentration inhibitory to the parental strain, only 3 out of 12
390 isolates formed colonies (Medihoney_10, Surgihoney_09, Surgihoney_10). Thus, most putative
391 SurgihoneyRO™- and Medihoney™-resistant mutants showed unstable phenotypic resistance across
392 multiple rounds of isolation, culturing and restreaking (we discuss this further in the context of earlier,
393 similar observations below). For commercial honey, we obtained no viable colonies despite repeating
394 the assay an additional three times on different days (216 aliquots in total for commercial honey). In
395 summary, we isolated single-step resistant mutants with Manuka honey, but found no colony isolates
396 with robust resistance phenotypes to SurgihoneyRO™, Medihoney™ or commercial honey, consistent
397 with our finding in serial passage that resistance evolved most readily against Manuka honey.
398
399 **DISCUSSION**
400 We found *E. coli* does not easily develop large increases in honey resistance upon serial passage at
401 gradually increasing concentrations, or upon plating of many replicate populations at inhibitory

402 concentrations. However, we identified a set of genes important in adaptation to honey, which conferred
403 moderate increases in resistance to Manuka honey. The known physiological role of this genetic
404 pathway helps to explain why these genetic changes, and associated increases in phenotypic resistance,
405 were specific to *Leptospermum* honeys (detoxification of methylglyoxal, which contributes to
406 antibacterial activity of these honeys). This indicates the likelihood of honey resistance upon medical
407 application may depend critically on the type or combination of honey products used. Furthermore, we
408 found that honey adaptation *in vitro* had only minimal side-effects for antibiotic resistance.

409 The first important implication of our results is that *E. coli* does not readily become resistant to
410 honey, which is promising in the context of expanding medical use of honey. Previous studies also had
411 difficulty in isolating mutants with stable honey resistance, and/or found that honey resistance
412 phenotypes revert quickly in the absence of honey (Cooper *et al.*, 2010). This is consistent with our
413 observation that several putative resistant mutants from our single-step screen had unstable phenotypes,
414 and some serially passaged populations that attained the ability to grow at increased honey
415 concentrations subsequently lost this ability upon transfer to fresh medium (referred to by (Abdel-Azim
416 *et al.*, 2019) as *second transfer crash* (STC)). One possible explanation is that non-genetic changes such
417 as persister formation or other morphological changes enable temporary population survival or growth
418 in otherwise inhibitory concentrations. Consistent with honey inducing physiological responses that are
419 not necessarily heritable, Brudzynski and Sjaarda (2014) observed morphological changes in *E. coli*
420 cultures exposed to honey, including changes in cell shape / filamentation.

421 The second key implication of our results is that, to our knowledge, this is the first report of a specific
422 genetic mechanism linked to decreased honey susceptibility, namely changes affecting the *nemAR*
423 operon, and alternatively via changes affecting *clpP*. In support, *nemR* and its operon have previously
424 been described as being involved in physiological processes that we can expect to be beneficial during
425 honey exposure. Manuka honey and Medihoney™ are both *Leptospermum* honeys, whose high non-
426 peroxide antimicrobial activity is attributed to their relatively high content of methylglyoxal (Allen *et*
427 *al.*, 1991; Mavric *et al.*, 2008), a small amount of which bacteria produce intracellularly (Tötemeyer *et*
428 *al.*, 1998). Ozyamak *et al.* (2013) linked the *nemAR* operon to methylglyoxal detoxification. The *nemAR*
429 operon is located upstream from the glyoxalase system (Ozyamak *et al.*, 2013) which consists of GlxI

430 and GlxII (glyoxalase I and II, encoded by *gloA* and *gloB* respectively), the two most important enzymes
431 in the detoxification pathway of methylglyoxal (Clugston *et al.*, 1998; MacLean *et al.*, 1998;
432 Mannervik, 2008). Deletion of *nemR*, the repressor gene, results in increased transcription both of *nemA*
433 and *gloA* (Ozyamak *et al.*, 2013). Thus, the role we find for *nemAR* in manuka honey resistance is
434 consistent with its known role in methylglyoal detoxification. Similarly, *clpP*, another gene we found
435 independently mutated in multiple Manuka honey-adapted isolates, was shown by Jenkins *et al.* (2014)
436 to be overexpressed in *Leptospermum* honey-exposed methicillin-resistant *S. aureus*, supporting a role
437 for this gene in honey resistance in multiple species.

438 That methylglyoxal detoxification plays a key role in increased honey resistance is further supported
439 by the variation we observed among honey compounds. Robust resistant mutants emerged in our single-
440 step screen only with a *Leptospermum* honey (Manuka) with a high concentration of methylglyoxal,
441 but not with the other three compounds. During serial passage we saw a similar trend: the most
442 consistent increases in resistance were with the two *Leptospermum* honey products. By contrast,
443 resistance of putative single-step resistant mutants isolated from SurgihoneyRO™-supplemented agar
444 was not stable, and we could not isolate any spontaneous mutants for the commercial honey we used.
445 This commercial honey is a blend of honeys with unknown flower sources from different South-
446 American countries. Drawing a parallel to antibiotics, where combinations of antibiotics can make it
447 harder for bacteria to evolve resistance (Baym *et al.*, 2016), we speculate that resistance evolution
448 against this honey was rare in our experiments because it is a form of combination treatment. This raises
449 the possibility that effective application of honey in treatment may benefit from combining multiple
450 types of honey products with different mechanisms of action. We note that despite variation among
451 honey products, all isolates in our experiment were inhibited by honey concentrations comparable to
452 those found in medical-grade honey products (63–100% in products recommended/licensed for medical
453 wound care, http://www.medihoney.de/index.html). We hypothesize that the multi-faceted nature of
454 honey's antibacterial activity (Wang *et al.*, 2012; Nolan *et al.*, 2019) contributes to the difficulty of
455 bacteria evolving resistance to such high concentrations.

456 The third major implication of our results is that honey adaptation did not come with collateral
457 effects on antibiotic resistance. This is a second aspect that is promising in the context of wider medical

458 application of honey, and contrasts starkly with the frequent occurrence of cross-resistances between
459 antibiotics (Szybalski and Bryson, 1952; Gutmann *et al.*, 1985) and between antibiotics and some other
460 antimicrobial agents (Loughlin *et al.*, 2002; Braoudaki and Hilton, 2004; Baker-Austin *et al.*, 2006;
461 Allen *et al.*, 2017; Bischofberger *et al.*, 2020). We do not exclude that more significant changes in
462 antibiotic resistance might be observed in mutants or strains with larger changes in honey resistance
463 than the moderate increases we observed here, if such strains exist. A key avenue for future work is
464 therefore to determine whether any genetic variation in natural populations associated with variable
465 honey resistance is independent of variation in antibiotic resistance, as in our experiment. Similarly,
466 because honey has shown effective inhibition of a wide range of pathogens (Carter *et al.*, 2016; Hillitt
467 *et al.*, 2017; Yabes *et al.*, 2017), and the principal genes involved in honey resistance in our experiment
468 are conserved in multiple pathogenic species (e.g., *nemR* or analogues are present in *Klebsiella*
469 *pneumonia, Acinetobacter baumannii* and *Salmonella typhimurium*), our work identifies candidate loci
470 that may be involved in resistance in other species. This is another relevant area for future research in
471 the context of medical honey application.

472 Our results also provided indirect evidence of growth costs associated with honey adaptation.
473 Growth in the absence of selecting antibacterials is widely considered an important parameter in the
474 long-term spread of resistance (Andersson and Hughes, 2010). The larger increase in growth rate
475 observed in our control-evolved isolates compared to honey-adapted isolates suggests either (i) that
476 honey-adaptation constrained adaptation to other aspects of the environment, preventing acquisition of
477 other beneficial mutations, or (ii) that honey-resistance mutations combined with other types of
478 beneficial mutations conferred a smaller net increase in growth rate in honey-free growth medium
479 compared to other beneficial mutations alone. Our sequence data are more consistent with the former:
480 control-evolved populations had parallel mutations in flagella and fimbriae genes (*flhD* and *fimE*),
481 consistent with past work with LB-adapted *E. coli* (Knöppel *et al.*, 2018). These mutations were much
482 less common in honey-adapted colony isolates (Fig. 2). This could be due, for example, to epistatic
483 interactions between the different types of adaptive mutations (Scanlan *et al.*, 2015). A full
484 understanding of the growth costs and benefits associated with individual honey-resistance alleles and

485 their interactions with other types of beneficial mutations is beyond the scope of this paper, and was
486 not our aim here, but would make an interesting avenue for future work.

487 In conclusion, honey resistance in our experiment only evolved with a subset of the compounds we
488 tested, and only to a moderate degree. This is promising in the context of medical application of honey.
489 A further positive aspect is that we found no evidence of strong downstream effects on antibiotic
490 susceptibility in isolates adapted to honey *in vitro* via chromosomal mutation. Finally, we identified
491 putative genetic mechanisms involved in honey adaptation, via changes affecting genes involved in
492 detoxifying methylglyoxal, making this mechanism most relevant for *Leptospermum* honeys such as
493 Manuka honey.

494 **Tables**

495

496 **Table 1: Honey products used in this study.**

Product name	**Product description**	**Initial IC_{90} for *E. coli* K-12 MG1655**	**Manufacturer**
SurgihoneyRO™	medical honey; sterile, bio-engineered; undiluted; poly-origin (unspecified)	5%(w/v)	H&R Healthcare, Hull, UK
Medihoney™ Medical Honey	medical honey; sterile, undiluted; *Leptospermum* honey (methylglyoxal content unspecified)	16%(w/v)	sorbion austria, Zwölfaxing, Austria
MGO™ Manuka Honey MGO™550+	consumer product; natural, undiluted; *Leptospermum* honey (methylglyoxal content of 550 mg/kg)	10%(w/v)	Manuka Health New Zealand Ltd, Te Awamutu, New Zealand
Commercial honey (Fairtrade Liquid Blossom Honey)	consumer product; natural, undiluted; poly-origin (Chili, Guatemala, (Mexico))	10%(w/v)	Coop, Basel, Switzerland

497

498 **Supplementary Table 1. Mutations involved in adaptation to serial passaging in different honey**

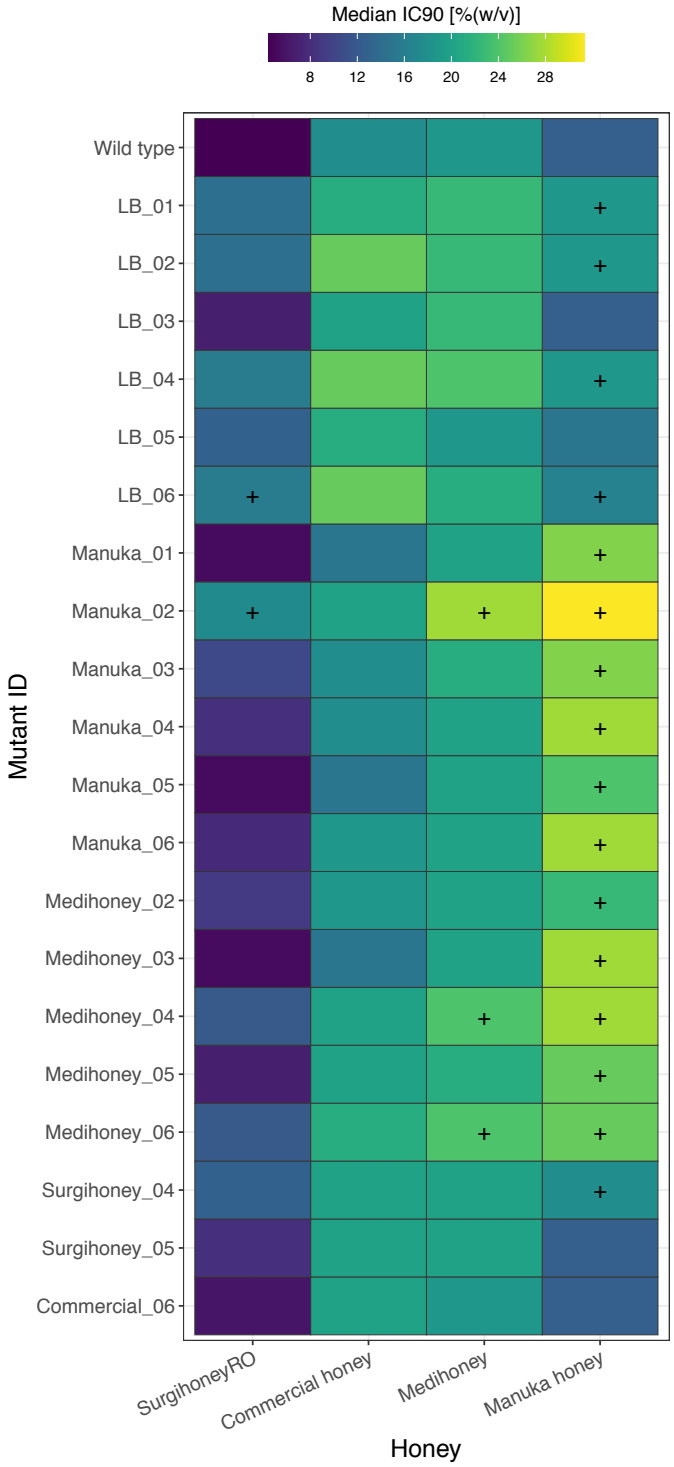
499 **products and in LB.** The table contains all mutations registered in serially-passaged isolates (14

500 isolates adapted to four different honeys, six control isolates) using the Illumina Hiseq 4000 platform.

501 Gene description according to breseq and the reference sequence of the ancestral isolate, E. coli K-12

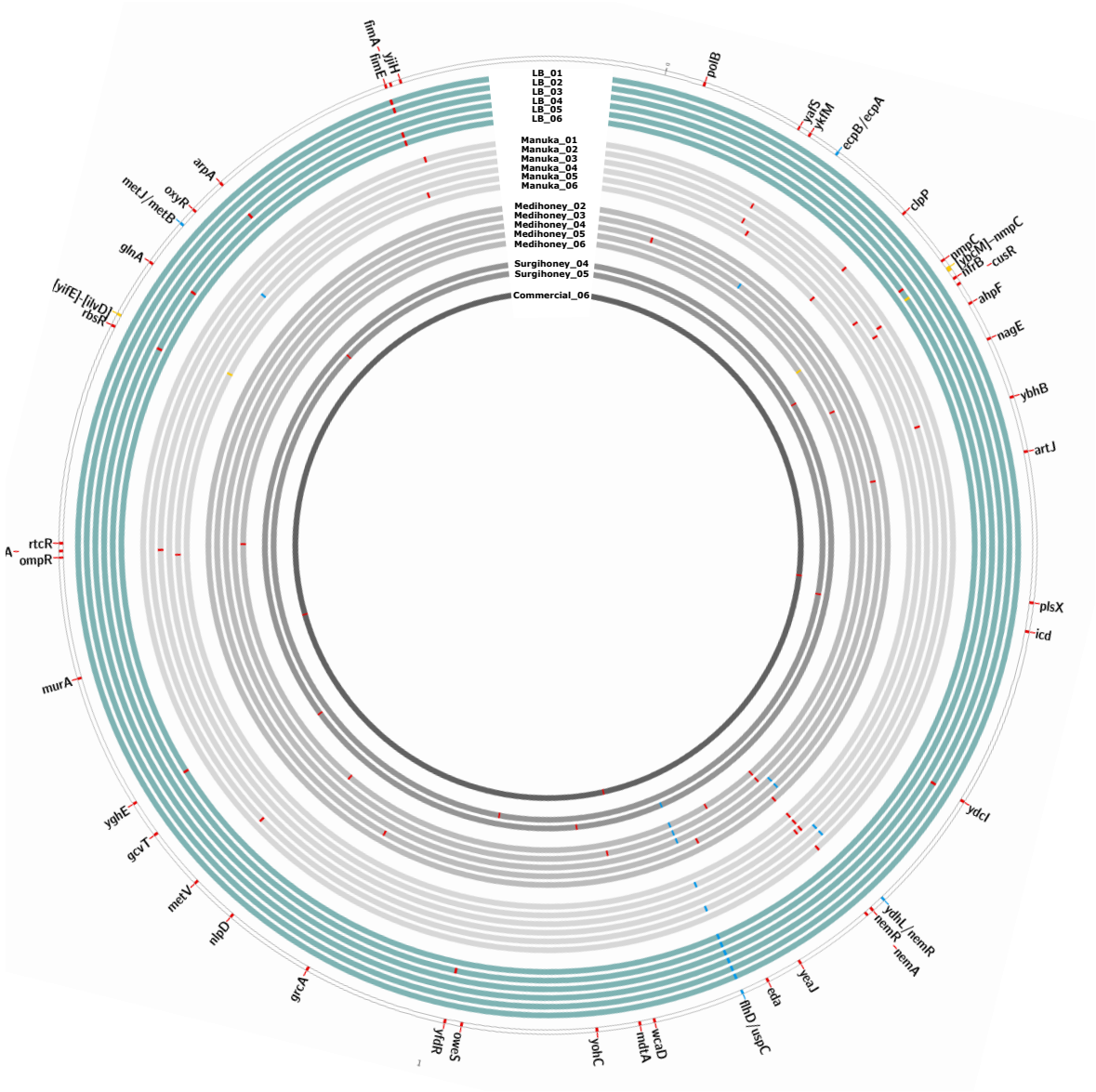
502 MG1655. (*see .xslx document "tableS1_Bischofbergeretal.xslx"*)

503 **Figures**



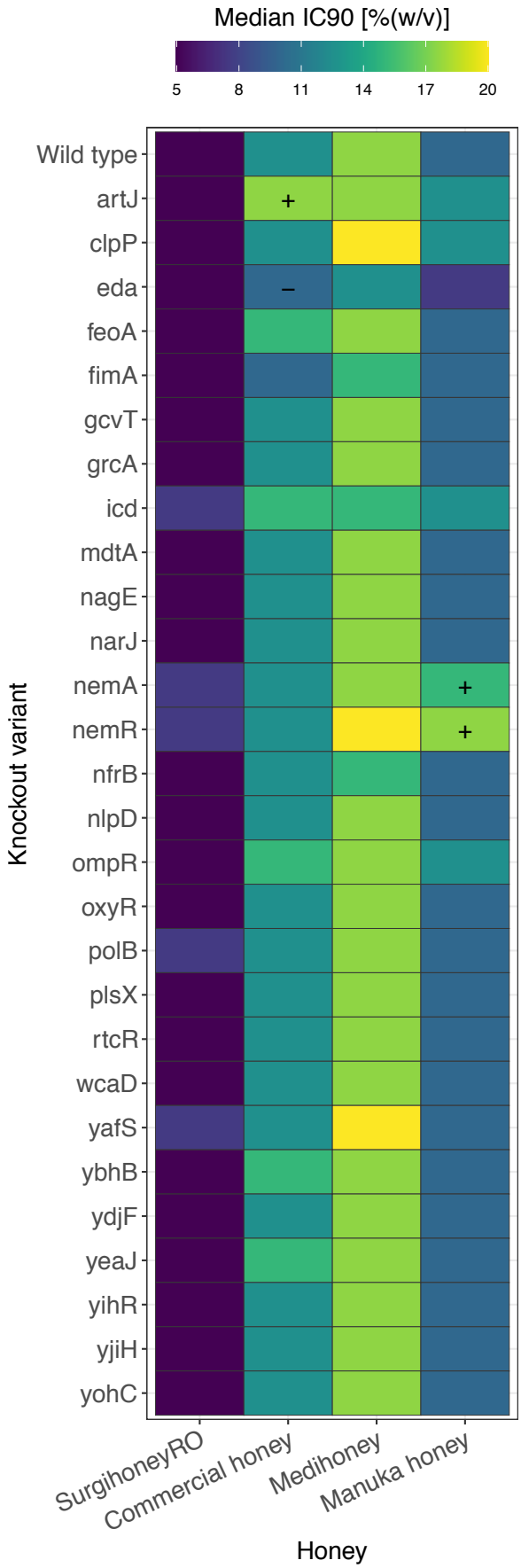
504 **Figure 1.**

505 **Figure 1. Susceptibility of serially-passaged, putative resistant mutants with four different honey**
506 **compounds.** Each cell gives the median IC_{90} for a given isolate (the ancestral strain K-12 MG1655, in
507 the top row, or a putative resistant mutant serially passaged with one of the four honey compounds,
508 labelled according to compound and replicate selection line) assayed with one of the four honey
509 compounds (columns). Each value is the median of four independent replicates; combinations where
510 all replicates of a putative resistant mutant were higher than all replicates of the ancestor in the same
511 treatment are indicated with a "+". Individual replicates for each strain are shown in Fig. S4.


LB_01
LB_02
LB_03
LB_04
LB_05
LB_06
Manuka_01
Manuka_02
Manuka_03
Manuka_04
Manuka_05
Manuka_06
Medihoney_02
Medihoney_03
Medihoney_04
Medihoney_05
Medihoney_06
Surgihoney_04
Surgihoney_05
Commercial_06
polB
yafS
ykfM
ecpB/ecpA
clpP
nmpC
[ybcM]-nmpC
nfrB
cusR
ahpF
nagE
ybhB
artJ
plsX
icd
ydcI
ydhL/nemR
nemR
nemA
yeaJ
eda
flhD/uspC
wcaD
mdtA
yohC
yfdR
oweS
grcA
nlpD
metV
gcvT
yghE
murA
ompR
rtcR
rbsR
[yifE]-[ilvD]
glnA
metJ/metB
oxyR
arpA
fimA
fimE
yjiH

512 **Figure 2.**

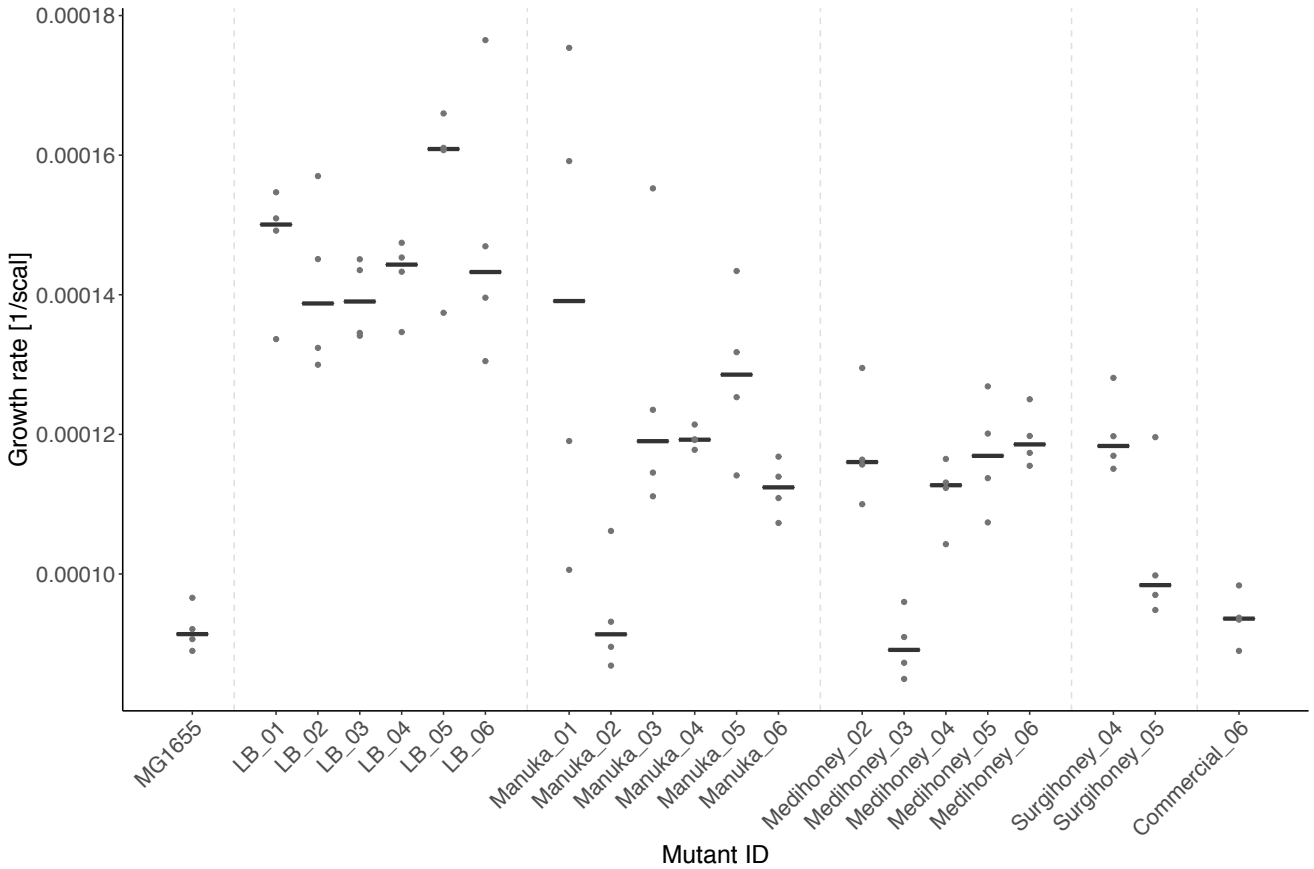
513 **Figure 2. Genetic changes in 20 serially-passaged isolates of *E. coli* K-12 MG1655.** The outermost
514 ring represents the ancestral isolate with coloured tiles for all genes in which we found mutations in
515 serially-passaged isolates. Each inner circle represents one serially-passaged isolate: in turquoise:
516 control treatment; in grey: four different honey treatments. Genetic mutations are indicated with
517 coloured tiles: red: mutations in one gene; yellow: intergenetic mutations; blue: deletions >1000bp.


Median IC90 [%(w/v)]
5
8
11
14
17
20
Wild type
artJ
clpP
eda
feoA
fimA
gcvT
grcA
icd
mdtA
nagE
narJ
nemA
nemR
nfrB
nlpD
ompR
oxyR
polB
plsX
rtcR
wcaD
yafS
ybhB
ydjF
yeaJ
yihR
yjiH
yohC
Knockout variant
+
–
+
+
SurgihoneyRO
Commercial honey
Medihoney
Manuka honey
Honey

518 **Figure 3.**

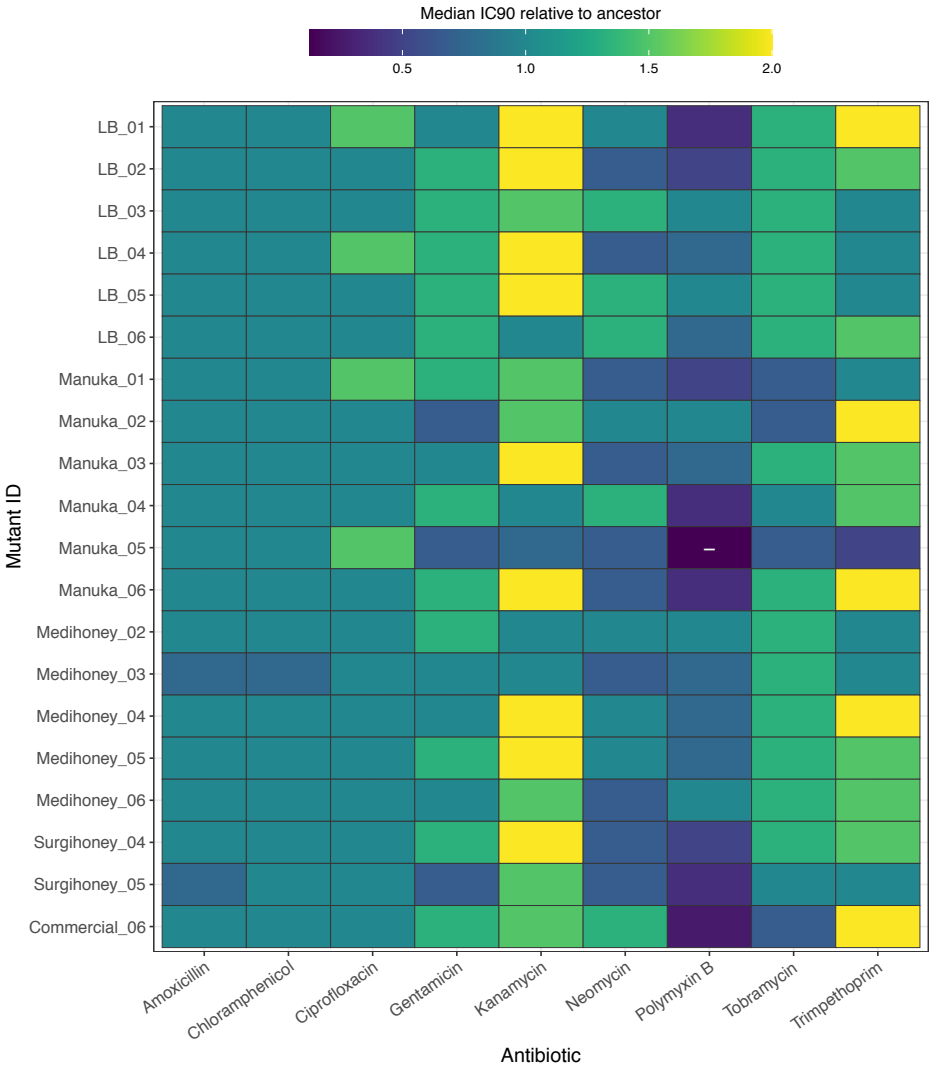
519 **Figure 3. Honey susceptibility of single-gene deletion variants.** Each cell shows the median IC_{90}
520 towards four different honeys (columns) for the ancestral strain *E. coli* K-12 BW25113 (top row) or
521 one of 28 single-gene knockout variants (other rows). Each cell is the median value of three independent
522 replicates; combinations where all replicates of a putative resistant mutant were higher/lower than all
523 replicates of the ancestor in the same treatment are indicated with a "+"/"-". Individual replicates for
524 each strain are shown in Fig. S5.

525



526 **Figure 4.**

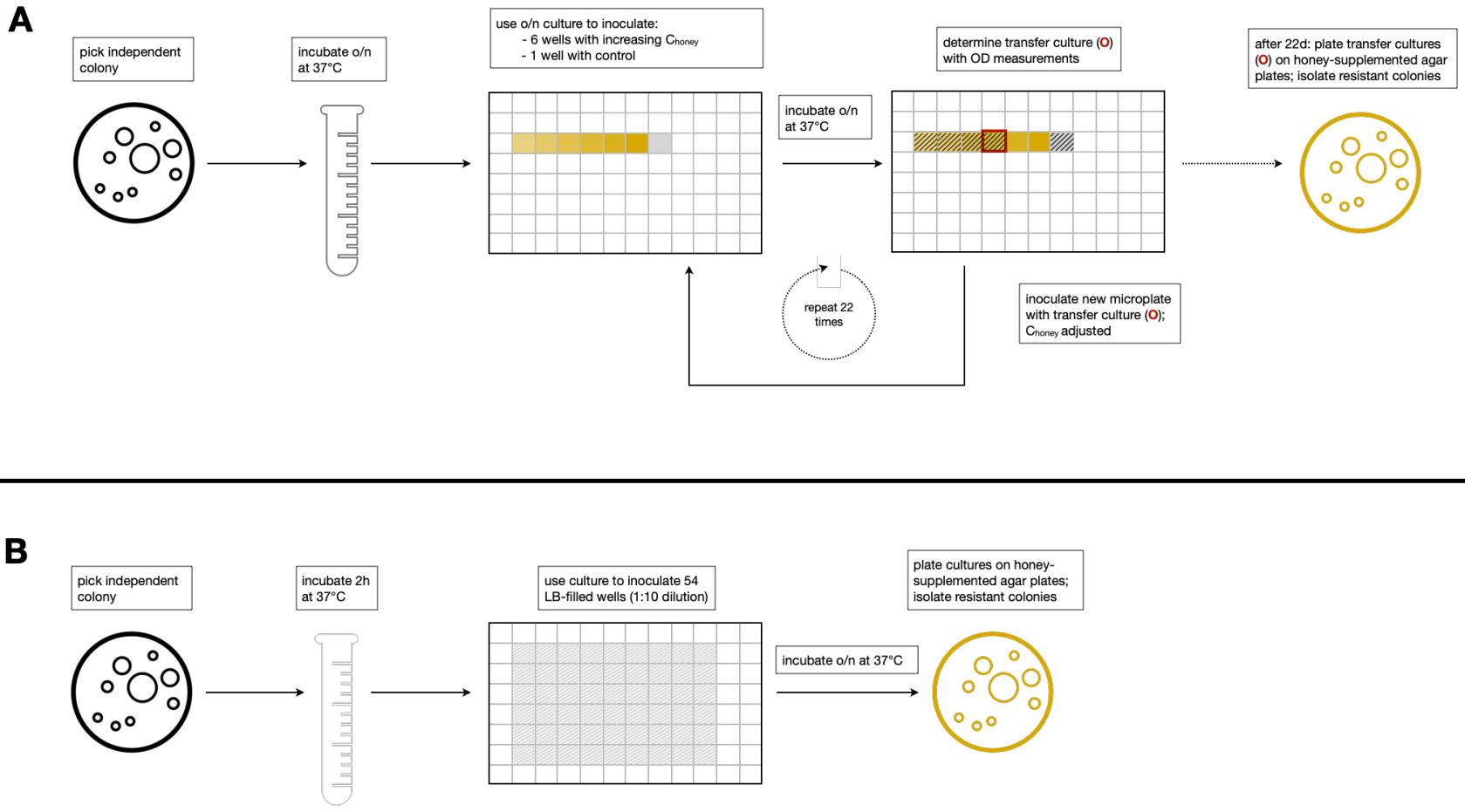
527 **Figure 4. Growth rate of experimentally evolved isolates in the absence of honey.** Population
528 growth rate (*y*-axis) is shown for the wild type (MG1655), six mutants serially passaged in honey-free
529 LB (LB_01-LB_06), and 14 mutants serially passaged in different honeys (labelled according to honey
530 and replicate; *x*-axis). Each black line gives the median of four replicates (replicates shown as dots).


Median IC90 relative to ancestor
0.5
1.0
1.5
2.0
Mutant ID
LB_01
LB_02
LB_03
LB_04
LB_05
LB_06
Manuka_01
Manuka_02
Manuka_03
Manuka_04
Manuka_05
Manuka_06
Medihoney_02
Medihoney_03
Medihoney_04
Medihoney_05
Medihoney_06
Surgihoney_04
Surgihoney_05
Commercial_06
Amoxicillin
Chloramphenicol
Ciprofloxacin
Gentamicin
Kanamycin
Neomycin
Polymyxin B
Tobramycin
Trimpethoprim
Antibiotic

531 **Figure 5.**

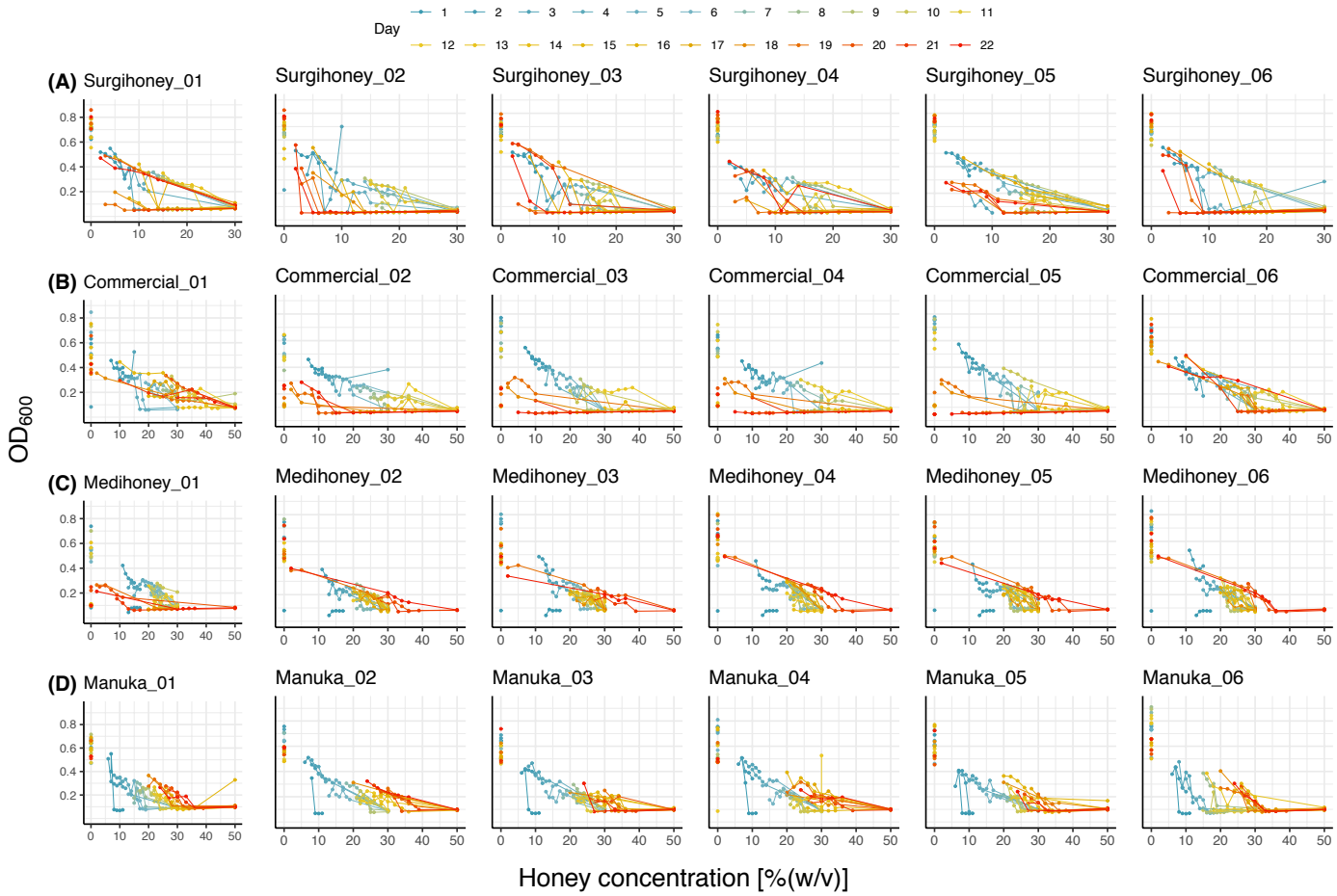
532 **Figure 5. Resistance of serially-passaged, putative honey-resistant mutants against nine different**
533 **antibiotics, relative to the wild type.** The matrix shows the median IC_{90} towards nine antibiotics of 20
534 serially-passaged isolates relative to the median IC_{90} of the parental strain, *E. coli* K-12 MG1655.
535 Blue/dark seagreen signify susceptibility, yellow/light green resistance relative to the parental strain;
536 combinations where all replicates of a given isolate were lower than all replicates of the ancestor in the
537 same treatment are indicated with a "-". Individual replicates for each strain are shown in Fig. S7.

538 **Supplementary Figures**



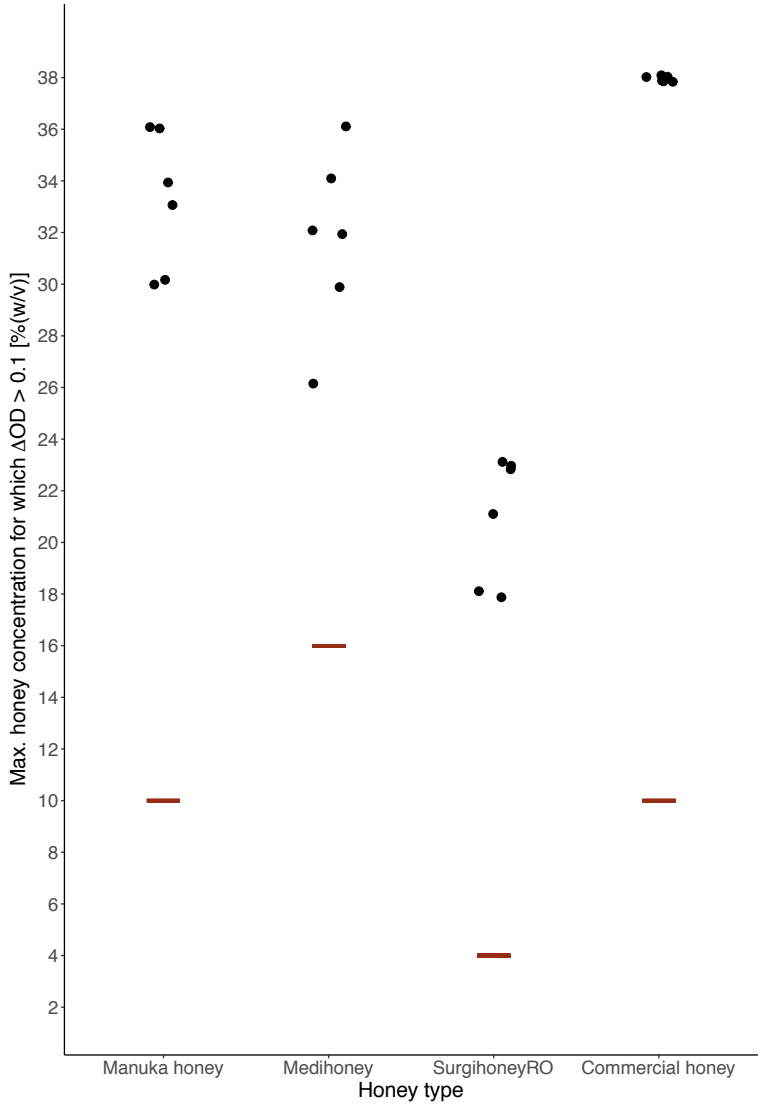
539 **Supplementary Figure 1.**

540 **Supplementary Figure 1. Schematic protocol of experimental resistance evolution.** (A) Serial passage: For each replicate selection line, an independent
541 overnight culture was used to inoculate a range of honey concentrations and honey-free medium (LB). The highest honey concentration that showed viable
542 growth ((OD_{600} 24h – OD_{600} 0h) > 0.1, transfer culture (outlined in red)) was used to inoculate wells filled with various concentrations in a new microplate.
543 Control treatment was performed by daily passaging in honey-free medium (LB). This was repeated daily for 22 days. (B) Single-step screening: for each
544 compound we made multiple overnight cultures before plating on honey-supplemented agar and screening for resistant mutants.



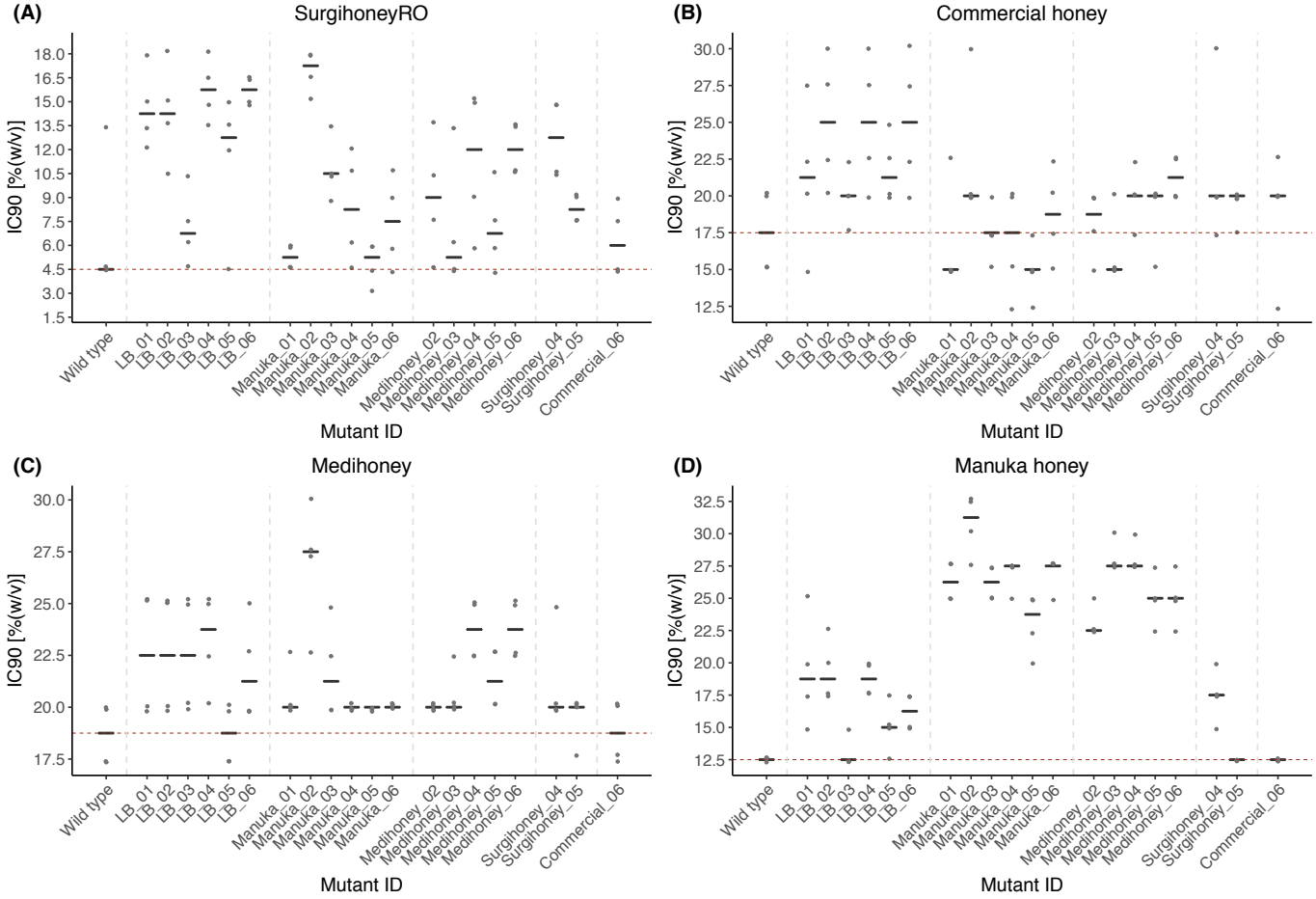
Supplementary Figure 2.

547 **Supplementary Figure 2. Population densities at different time points and honey concentrations**
548 **for 24 selection lines passaged with different honeys for 22 days.** Each row of panels (A-D) shows
549 population density, estimated by OD_{600} (*y*-axis) of six selection lines passaged with one of the four
550 honey compounds. The *x*-axis shows the concentration of the respective honey in % (w/v), and each
551 line gives the observed population densities across all tested concentrations on a given day (different
552 days/lines have different colours, going from blue to green to yellow to red over earlier/later time
553 points).



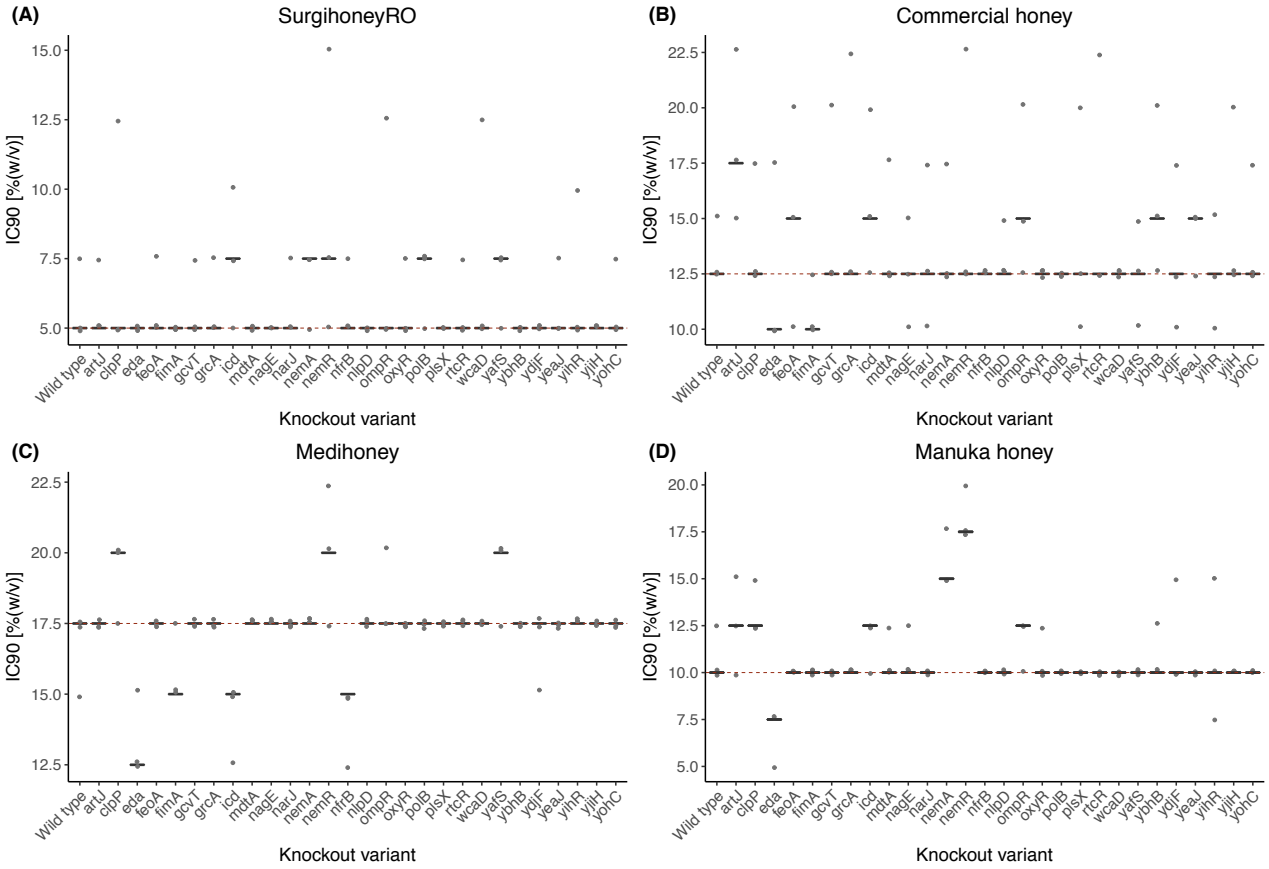
554 **Supplementary Figure 3.**

555 **Supplementary Figure 3. Highest honey concentration each selection line was able to grow at**
556 **during 22d of serial passage in honey-supplemented medium.** For each honey compound, we
557 passaged six selection lines by daily transfer into increasing concentrations of honey. Each point
558 represents the highest concentration for which an individual selection line showed $\Delta OD_{600} > 0.1$ after
559 24h incubation at any time during the experiment (points are jittered along the axes). The red line
560 represents the initial IC_{90} of the wild type strain, *E. coli* K-12 MG1655, for each honey.



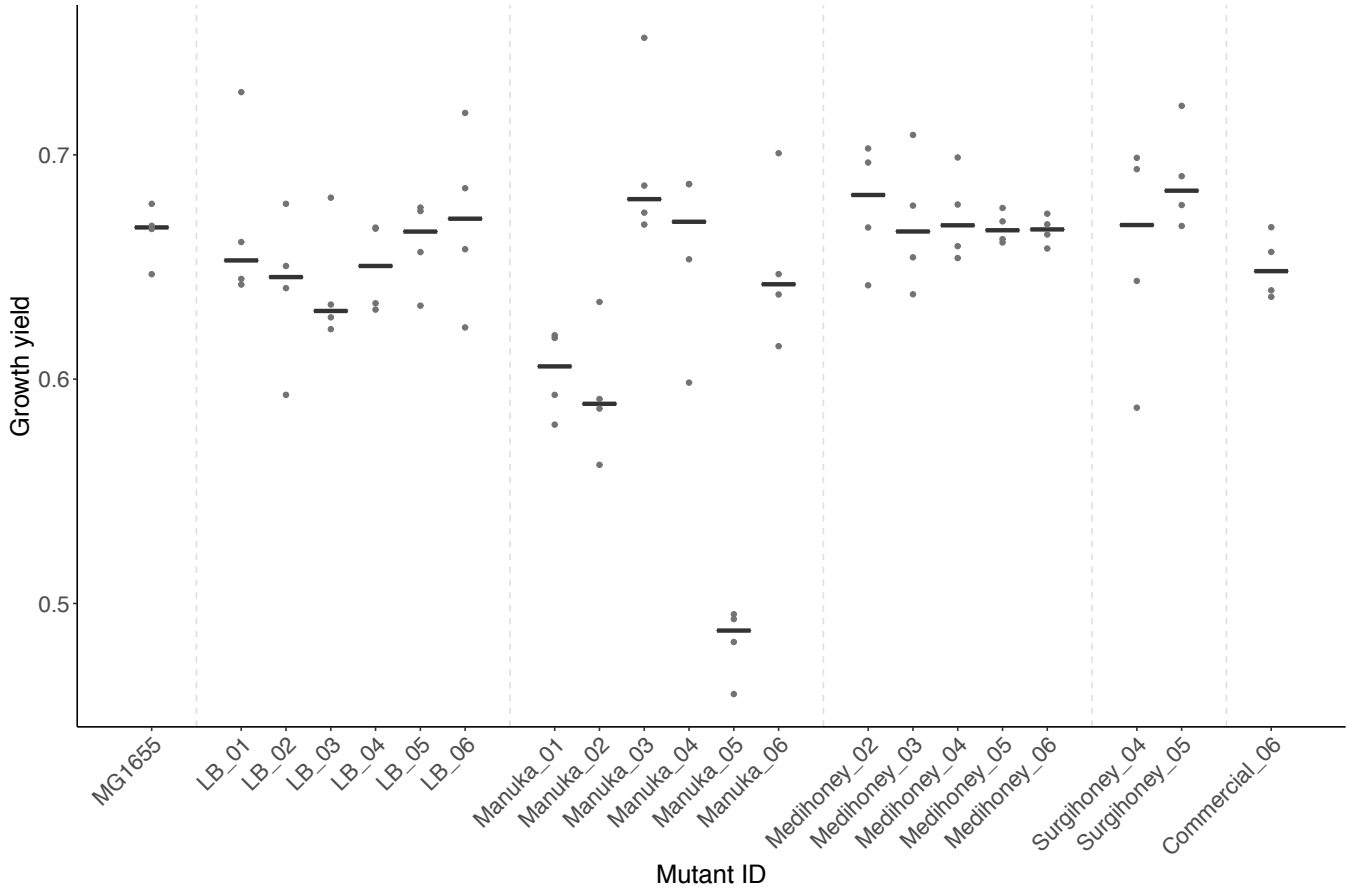
Supplementary Figure 4.

562 **Supplementary Figure 4. Resistance of serially-passaged putative resistant mutants to four**
563 **different honeys.** Each of the four panels shows the IC_{90} of 14 honey-adapted (and six control-adapted)
564 isolates and of the parental strain *E. coli* K-12 MG1655 to four honey products after 24h incubation.
565 The black lines represent the median of four independent replicates per serially-passaged isolate,
566 replicates are shown as dots (jittered along the *y*-axis). The red horizontal line represents the median
567 IC_{90} of four independent replicates of the parental strain.



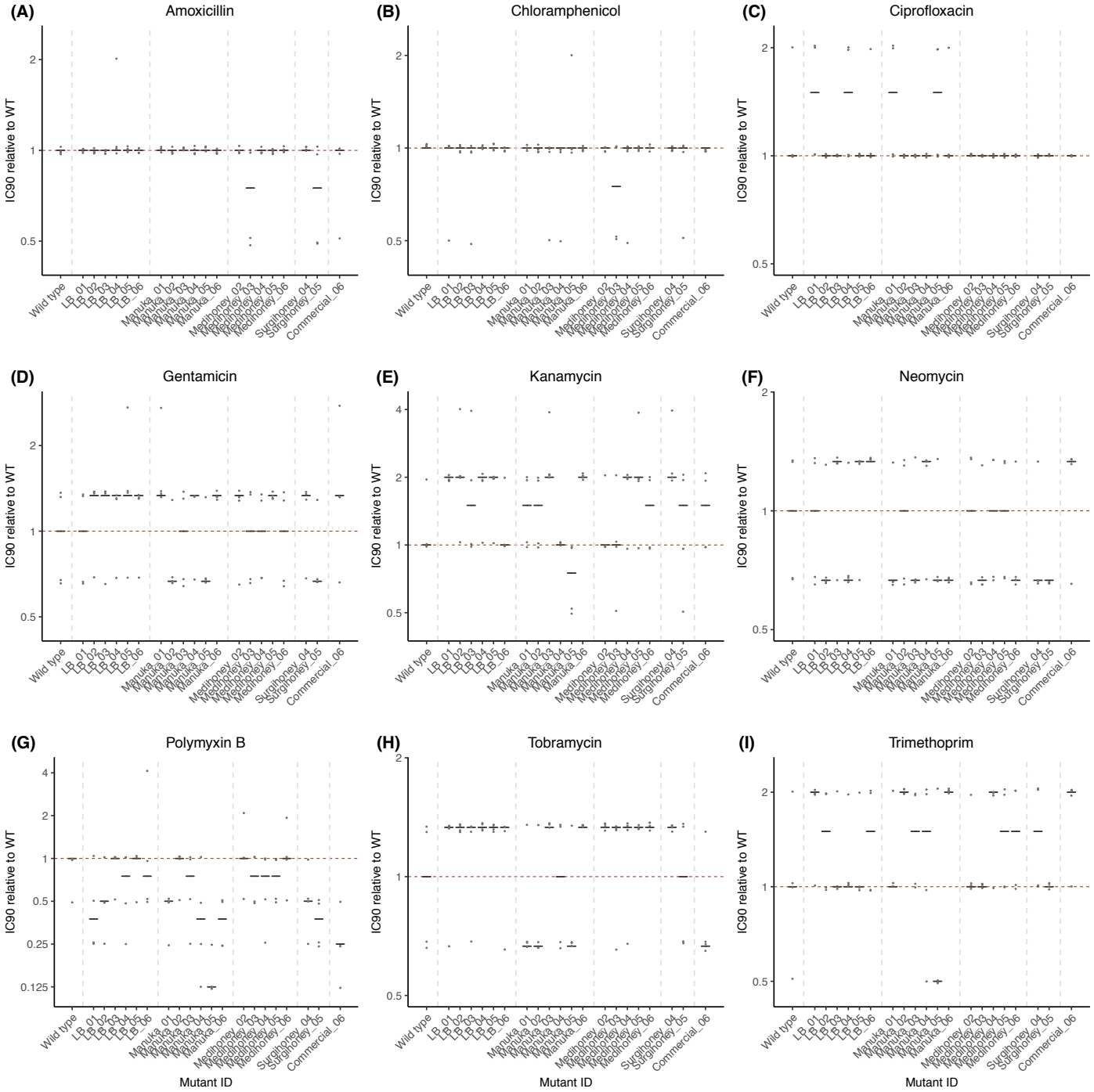
Supplementary Figure 5.

571 **Supplementary Figure 5. Honey resistance of single-gene deletion variants.** Each panel shows the
572 IC_{90} of 28 single-gene knockout variants from the Keio Knockout Collection and of the parental strain
573 *E. coli* K-12 BW25113 after 24h incubation. Each black line is the median of three replicates per
574 knockout variant; replicates are shown as dots (jittered along the *y*-axis). The dashed red line represents
575 the median IC_{90} of three replicates of the wild-type strain, *E. coli* K-12 BW25133.



576 **Supplementary Figure 6.**

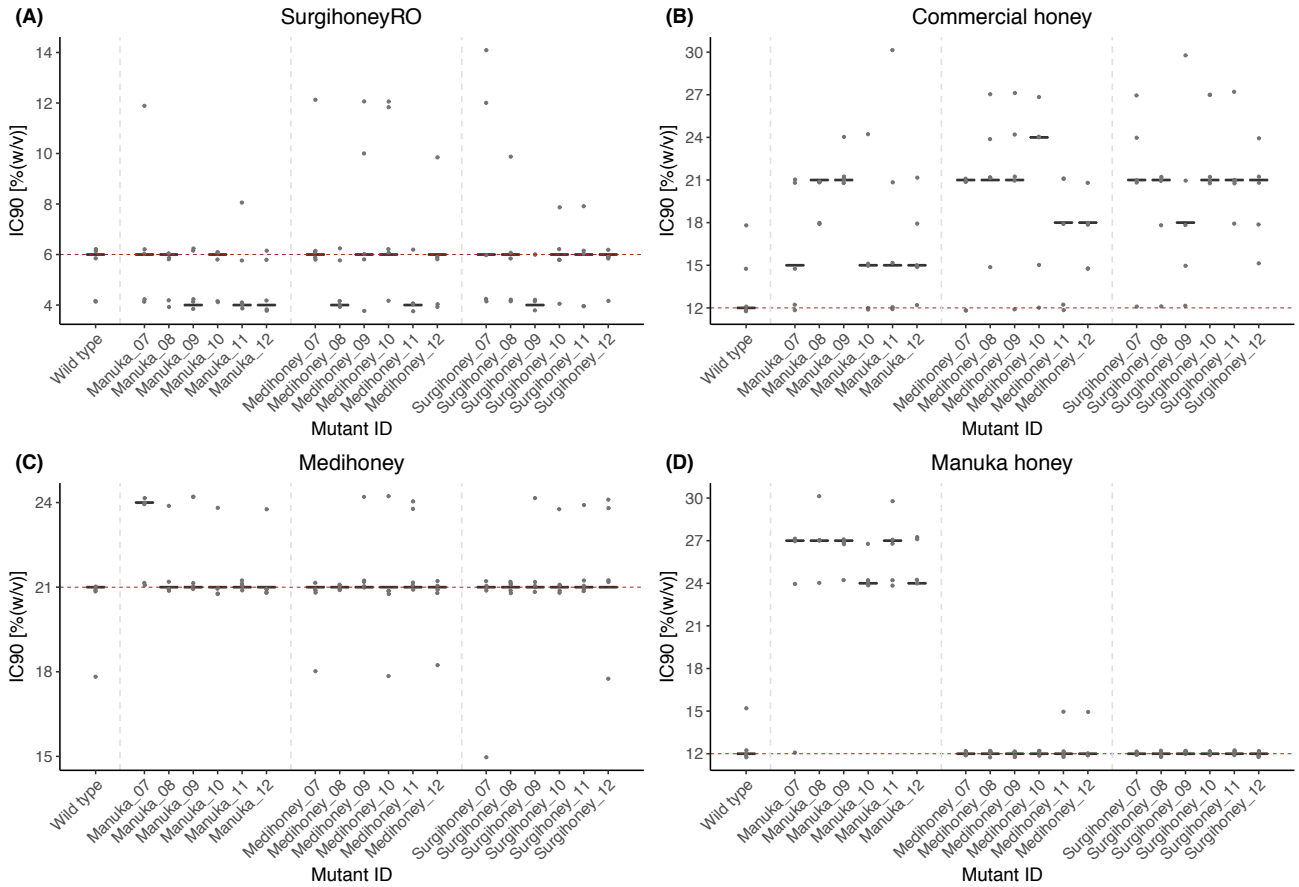
577 **Supplementary Figure 6. Growth yield of serially passaged, putative honey-resistant isolates in**
578 **honey-free growth medium (LB).** Growth yield (*y*-axis) is shown for the wild type (MG1655), six
579 isolates serially passaged in LB (LB_01-LB_06), and 14 isolates serially passaged in different honeys
580 (labelled according to honey and replicate) (*x*-axis). Each black line represents the median of four
581 replicates (points).



582 **Supplementary Figure 7.**

583 **Supplementary Figure 7. No evidence for cross-resistance between four honeys and nine**
584 **antibiotics.** Each panel shows the IC_{90} of 20 isolates that were serially-passaged in honey or LB relative
585 to the IC_{90} of the wild type (*E. coli* K-12 MG1655; individual replicates for the wild type are also shown
586 relative to the median for the wild type, which is the red horizontal line in each panel) towards one
587 antibiotic. Each black line is the median of four independent replicates per isolates; replicates are shown
588 as dots (jittered along the *y*-axis).

589



590 **Supplementary Figure 8.**

591 **Supplementary Figure 8. IC_{90} of 18 single-step putative resistant mutants to four different honeys.**
592 Each of the four panels shows the IC_{90} values of 18 single-step mutants and of the parental strain *E. coli*
593 K-12 MG1655 to four different honeys. The black lines represent the median of five independent
594 replicates per single-step putative resistant mutant, replicates are shown as dots (jittered along the *y*-
595 axis). The red horizontal line represents the median IC_{90} of five independent replicates of the wild-type
596 strain.

Acknowledgements

See title page.

Data Archiving Statement

The data that support the findings of this study will be made openly available at Dryad Digital Repository: to be completed after manuscript is accepted for publication.

603 **References**

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