1
2
3
4
5
6
7

Gut microbiota in the burying beetle, *Nicrophorus vespilloides*, provide colonization resistance against larval bacterial pathogens

11
12
13

14 Yin Wang and Daniel E. Rozen*
15
16
17
18 Institute of Biology, Leiden University, Sylviusweg 72, 2300 RA, Leiden, The
19 Netherlands
20
21
22
23 *- corresponding author: d.e.rozen@biology.leidenuniv.nl

24
25
26
27
28
29

Abstract

Carrion beetles, *Nicrophorus vespilloides,* are reared on decomposing vertebrate carrion where larvae are exposed to high-density populations of carcass-derived bacteria. We previously showed that larvae do not become colonized with these bacteria, but instead are colonized with the gut microbiome of their parents. These results suggested that bacteria in the beetle microbiome outcompete the carcass-derived species for colonization of the larval gut. Here we test this hypothesis directly and quantify the fitness consequences of colonization of the *Nicrophorus* larval gut with different bacterial symbionts, including the insect pathogen *Serratia marcescens*. First, we show that beetles colonized by their endogenous microbiome produce significantly heavier broods than those colonized with carcass-bacteria. Next, we show that bacteria from the endogenous microbiome, including *Providencia rettgeri* and *Morganella morganii*, are better colonizers of the beetle gut and can outcompete non-endogenous species, including *S. marcescens* and *Escherichia coli*, during *in vivo* competition. Finally, we find that *Providencia* and *Morganella* provide beetles with colonization resistance against *Serratia* and thereby reduce *Serratia*-induced larval mortality during co-inoculation. Importantly, this effect is eliminated in larvae first colonized by *Serratia*, suggesting that while competition within the larval gut is strongly determined by priority effects, these effects are less important for *Serratia*-induced mortality. Our work supports the idea that bacterial gut symbionts provide direct benefits to *Nicrophorus* larvae by outcompeting potential bacterial pathogens. They further suggest that one benefit of parental care in *Nicrophorus vespilloides* is the social transmission of the microbiome from caring parents to their offspring.

Key words: Microbiome, Bacterial symbionts, Colonization resistance, *Nicrophorus vespilloides*

Introduction

Animals are colonized by a diverse array of bacterial symbionts, the microbiome, that provide essential functions to their hosts (1–3). Animal microbiomes can alter nutrient uptake (4), development (5), parasite susceptibility (6), and even behaviors like mate choice (7). In addition, symbionts that reside within animal guts can provide their hosts with resistance to bacterial pathogens via a process called colonization resistance (8). For example, the gut bacterial community of locusts, *Schistocerca gregaria,* prevents invasion and disease from the insect pathogen *Serratia marcescens,* an outcome that depends in part on the diversity of the gut microbial community (9). Similarly, honeybees became more susceptible to *Serratia* infection following treatment with antibiotics that altered the structure of their endogenous microbiota (10). These results support the idea that gut bacteria can provide protection against pathogens while also highlighting the importance and timing of symbiont transmission in juvenile animals (11). However, it is often unclear if colonization resistance results from specific inhibition of invading pathogens or whether it results from the simple fact that symbionts get there first (9, 12, 13). In other words, is colonization resistance the result of specificity or priority?

To address this question we focus on the role of the endogenous microbiota of the burying beetle, *Nicrophorus vespilloides*. This system is especially well suited to this work given the high exposure of larval beetles to environmental bacteria (14–17), together with extensive data on the composition and transmission of the beetle microbiome from parents to offspring (18–20). *N. vespilloides* larvae are reared on small vertebrate carcasses where they feed directly from the carcass and are provided regurgitated food from parent beetles that care for developing broods (14, 21, 22). Parental beetles dramatically increase larval growth and fitness during brood rearing by investing in pre- and post-hatch care (15, 22, 23). During pre-hatch care, parents remove the fur and guts of the carcass and coat its surface in oral and anal secretions that have antimicrobial activity (14, 24–26). Post-hatch, parents defend their developing larvae from other insect species and also feed larvae with regurgitated food (21, 27). In a recent study we found that parents transmit their gut microbiome to their larvae by direct feeding. In addition, we found that the core members of this microbiota could even be transmitted to larvae indirectly, by bacteria deposited onto the carcass by parents (18). This unexpected result suggested that these core bacterial species were outcompeting the numerous microbes living on and inside the carcass within the larval gut, thus giving rise to the stable endogenous *Nicrophorus* microbiome (18, 19). However, the mechanisms of their increased competitiveness remained unclear as were the consequences of their colonization.

Here we carry out invasion experiments into sterile larvae to directly quantify competitive interactions taking place between endogenous and non-endogenous microbes from the *Nicrophorus* gut. We first quantify bacterial growth rates within the larval gut and then directly determine the competitive interactions between species during mixed inoculations and in different orders. Finally, we quantify whether members of the core microbiome provide colonization resistance against *Serratia marcescens*, and known insect pathogen. Briefly, we show that native gut species significantly outcompete foreign species within the host gut, irrespective of infection order. In addition, we find that the endogenous microbiota increases beetle fitness, both in terms of brood size and in terms of pathogen resistance in larvae. Our results

123   provide strong evidence that an important benefit of parental care in *N. vespilloides* is
124   the social transmission of the microbiome from caring parents to their offspring.
125
126   ## Methods and Materials
127
128   ### Beetle collection and rearing
129
130   Experimental beetles were taken from an outbred laboratory population derived from
131   wild-caught *N. vespilloides* individuals trapped near Leiden in The Netherlands,
132   between May and June 2015. Beetles were maintained in the laboratory at 20°C with
133   a 15:9 hour light:dark cycle and fed fresh chicken liver twice a week. Mating pairs
134   were established by placing a male and female in a small plastic container containing
135   ~ 1 cm soil overnight. Mated females were provided with a fresh carcass (20-23g) the
136   following morning to initiate egg laying.
137
138   To examine the impact of different microbial communities on *N. vespilloides* fitness,
139   we established independent treatment populations containing endogenous or carcass
140   derived gut bacteria, designated FC (full-care) and NC (no-care) beetles respectively.
141   Whereas parents and larvae in the FC treatment were reared in the presence of
142   parental care and thus acquired their microbiota primarily from their parents, larvae in
143   the NC group were reared in the absence of parental care (with an unprepared carcass
144   that we opened using a sterile scalpel), and acquired their microbiota from the carcass
145   and surrounding soil (18). Ten day old adults that had eclosed from FC and NC
146   broods were paired within treatments for mating (n = 15 / treatment) and subsequently
147   provided with a fresh mouse carcass (22-24g) for breeding. The fitness of both
148   parental treatment groups (NC and FC) was determined by quantifying total brood
149   size, total larval weight and mean larval mass.
150
151   ### Experimental bacterial inoculation of *N. vespilloides* larvae
152
153   To generate germ-free larvae, we collected eggs 15 hours after FC females were
154   provided with a fresh carcass. These were surface sterilized twice for 15 minutes in an
155   antimicrobial solution containing hen-egg white lysozyme (1 mg/ml), streptomycin
156   (500 µg/ml) and ampicillin (100 µg/ml), and followed by a sterile water wash. Next,
157   treated eggs were transferred onto 1% water agar plates to hatch. Previous
158   experiments have shown that eggs thus treated are free of bacteria (28). 0 - 24h old
159   first-instar larvae were transferred onto new sterile 1% water agar petri dishes
160   (100mm x 15mm) in groups of a maximum of 7 larvae. Larvae on each plate were
161   derived from independent breeding pairs. Larvae were fed a sterile diet developed
162   using Pasteurized chicken liver prepared via a "Sous vide" cooking approach. Fresh
163   chicken liver was sliced into 3g chunks using aseptic technique and transferred in
164   individual pieces to a 1.5ml eppendorf tube containing 100 µl sterile water. These
165   were then placed in a water bath at 65°C for 8 minutes, followed by immediate
166   cooling at -20°C. We determined the effectiveness of this method by plating liver
167   samples before and after pasteurization onto both 1/3 strength Tryptic Soy agar and
168   LB agar. The initial CFU of unpasteurized liver was ~ 1e^6/gram CFU while following
169   treatment the CFU was reduced to 0 (with a limit of detection of ~ 10 CFU/mL).
170   Larvae were offered this sterile diet, alone or coated with different bacterial inocula,
171   on new 1% water agar plates daily.
172

In vivo competition within larvae

To determine if "endogenous" bacteria can outcompete foreign strains during larval colonization we competed bacterial strains against one another within the larval gut, focusing on four different bacterial species. The bacterial species *Providencia rettgeri* and *Morganella morganii* are abundant *N. vespilloides* gut symbionts throughout development and are considered "endogenous" species (18, 19). By contrast, *Serratia marcescens* and *Escherichia coli*, which are found commonly in both soil and on decomposing carcasses, colonize larvae that are reared without parental care in NC broods (18). *S. marcescens* is also a known insect pathogen in several insect species (29–31), including *N. vespilloides*. *P. rettgeri* (P) and *M. morganii* (M) were isolated from *N. vespilloides* adults guts while *S. marcescens* (S) and *E. coli* (E) were isolated from decomposing mouse carcasses (18).

Bacteria for inoculations were cultured overnight at 30°C in 1/3 TSB medium. Overnight cultures of each species were pelleted and washed two times in sterile phosphate buffered saline (PBS, pH= 7.2), and diluted to an optical density at 600nm (OD_{600}) of 0.2 measured using a BIO-RAD SmartSpec^{TM} Plus spectrophotometer. Ten microliters of this solution, containing ~10^6 cells total, was used to coat sterile liver prepared as above. Inoculations with 2 species contained the same total bacterial density, with each species present at a 1:1 ratio. Larvae were provided with inoculated diet for six hours on a sterile water agar plate, after which they were transferred to a new agar plate containing new sterile diet. Subsequent transfers to plates containing fresh sterile food took place every 24 hours for 7 days, or until larvae were destructively sampled. In experiments where larvae were sequentially challenged with different bacterial species, we treated larvae the same as above, but larvae were inoculated with target strains in series: the first as above, and the second 6 hours later on a new plate containing diet coated with the second bacterial strain. As with the first exposure, larvae were exposed to bacteria in the second inoculum for 6 hours, after which they were returned to a sterile plate with sterile diet.

To examine competitive interactions within the *Nicrophorus* gut, larvae were inoculated either simultaneously or in series with two of the four species in the following pairings (Strain 1 vs Strain 2): P vs S; M vs S; P vs E; M vs E; P vs M; and S vs E. Within each treatment, larvae from independent families (n = 6) were inoculated as outlined above and then 6 larvae were destructively sampled for plating 6h or 24h later. These values were taken as estimates of input and final densities, respectively. Competition indices (CI) were calculated using the following equation: CI = (Strain 1 $_{output}$ /Strain 2 $_{output}$) / (Strain 1 $_{input}$ / Strain 2 $_{input}$), where input and output values refer to initial and final densities of each competitor, respectively. CI w log transformed, so that a CI of 0 indicates equal competitiveness, while CI > 0 indicates that the strain 1 is a stronger *in vivo* competitor. A two-tailed *t*-test was used to test if CI values for each strain differed significantly from 0.

 At each time point, larvae were sampled by sterilely dissecting individual larval guts with fine forceps and suspending these in 0.7 ml sterile PBS. Gut contents were serially diluted in PBS and plated to quantify CFU on a chromogenic medium (CHROMagar^{TM} Orientation), which can distinguish our experimental strains based on both color and morphology (Figure S1). Mortality rates for each treatment were:

222 PvsS (38.1%); MvsS (47.6%) and PvsM (35%), PvsE (54.5%); MvsE (64.7%) and
223 SvsE (71.4%).
224
225 **Larval fitness with different bacterial colonizers**
226
227 To determine the impact of different bacterial symbionts on larval survival, larvae
228 were inoculated as above and then monitored for survival through time. Larvae
229 exposed to sterile PBS (pH = 7.2) were used as a control in this experiment. A
230 minimum of 40 larvae from 9 - 15 families was collected for each bacterial treatment.
231 We monitored larval survival every 24 hours after inoculation. To reduce the high
232 rates of mortality in larvae reared on liver, all the experimental larvae were
233 transferred daily into a fresh petri dish containing fresh sterile diet.
234
235 **Statistical Analysis**
236
237 Parental fitness and bacterial colonization data were analyzed using ANOVA. Larval
238 survival data was analyzed by fitting a Cox proportional hazard model; this model
239 was constructed by fitting a saturated model using treatment, block and
240 treatment*brood interactions as covariates. The Wald's test was used to compare
241 mortality between treatments. All analyses were conducted using SPSS version 24
242 (IBM SPSS Inc., Chicago, IL, U.S.A.).
243
244 # Results
245
246 **The effects of gut microbiota on parental fitness**
247
248 To examine the role of the parental gut microbiome on beetle fitness, we reared larvae
249 either with (FC) or without parental care (NC) and then mated the dispersed adults
250 within treatments and allowed them to rear broods on fresh carcasses. Through this
251 treatment, all parents were given the opportunity to rear offspring under identical
252 conditions, and there were neither differences in carcass or maternal weight (both
253 NS). Previous results have shown that parents from these different rearing conditions
254 differ significantly in microbiome composition (18), the FC individuals containing an
255 endogenous symbiont population and the NC individuals a microbial population
256 derived from the soil and the decomposing carcass. Our results show that gut
257 microbiomes have a significant influence on parental fitness (Figure 1). FC parents
258 produced significantly heavier broods than NC parents, irrespective of brood size (2-
259 tailed ANCOVA: $F_{1,27}$ = 6.09, p = 0.021). In addition, the mean larval mass was
260 heavier in the FC group when controlling for brood size; however, this difference is
261 not significant (2-tailed ANCOVA: $F_{1,27}$ = 3.69, p = 0.067).
262
263 **Competitive interactions *in vivo***
264
265 To study competitive interactions between bacteria during larval colonization, we
266 selected four focal species to examine in detail. Two species, *Providencia rettgeri* and
267 *Morganella morganii* are common endogenous colonizers of the beetle gut (18, 19),
268 while the other two, *S. marcescens* and *E.coli*, are found more commonly in the guts
269 of beetles reared without parental care (18). We first inoculated beetles with each
270 species alone to measure growth and colonization. Figure 2 shows that while all four

271 species are able to colonize the larval gut, their ability to increase in density *in vivo*
272 varies significantly between strains (2-tailed ANOVA: $F_{3,12} = 43.13$, $p < 0.001$).
273
274 Competitive interactions between species were next determined using *in vivo* pairwise
275 assays where two species were simultaneously inoculated into 1 day old larvae.
276 Consistent with expectations based on mono-associated larvae, we observed clear
277 competitive differences between the strains. *Providencia* and *Morganella*
278 significantly outcompeted both *Serratia* (P vs S: $t_5 = 2.52$, $p = 0.053$; M vs S: $t_3 = 4.42$, $p = 0.022$) and *E. coli* (P vs E: $t_2 = 11.26$, $p < 0.001$; M vs E: $t_3 = 5.89$, $p = 0.01$), although to different degrees. By contrast, there were no significant competitive
281 differences between *Providencia* and *Morganella* (P vs M: $t_4 = 2.16$, $p = 0.097$)
282 (Figure 3A).
283
284 We next determined if competitive interactions between *Providencia* and *Serratia*
285 were influenced by the order of inoculation. Specifically, we were interested in
286 determining if the outcome of competition was reversed in larvae that were first
287 inoculated with *Serratia*. Our results in Figure 3B clarify that order is not an
288 important determinant of competitive fitness (2-tailed ANOVA: $F_{2,29} = 0.59$, $p = 0.56$). *Providencia* outcompetes *Serratia* in all cases to a similar degree regardless of
290 the order of inoculation.
291
Larval survival with different bacterial colonizers
293
294 Our results show that the endogenous microbiome provides likely benefits to
295 *Nicrophorus* by increasing total brood mass, and also that key members of this
296 microbiome can outcompete species that are predominantly found in larvae that do
297 not receive parental care. To test if these competitive interactions translate into
298 differences in larval fitness, we measured the survival of larvae inoculated with single
299 or multiple strains, as above. Results in Figure 4A show that larval mortality varies
300 significantly as a function of their bacterial colonists ($\chi^2 = 11.364$, df = 3, $P < 0.01$),
301 with increased mortality in larvae inoculated with *Serratia* compared to either
302 *Morganella*, *Providencia* or a PBS control (Wald statistic = 6.274; 4.794; 9.202,
303 respectively, all $P < 0.05$). This result is consistent with the known pathogenic effects
304 of *Serratia*. By contrast, there were no significant differences in mortality between
305 larvae inoculated with either *Providencia* or *Morganella* and the PBS control ($\chi^2 = 0.156$, df = 2, $P = 0.925$).
307
308 We also observed significant differences in survival when larvae were simultaneously
309 inoculated with *Serratia* and either of the endogenous species compared to survival
310 when *Serratia* is inoculated alone ($\chi^2 = 38.767$, df = 4, $P < 0.001$). Most importantly,
311 we found that co-inoculating *Serratia* with *Providencia* and/or *Morganella*
312 significantly increased larval survival, suggesting that these species provide
313 protection via colonization resistance for larvae (Wald statistic of PS; MS; PMS = 8.188; 3.697; 5.102, respectively, all $P < 0.05$, Figure 4B). However, this benefit of
315 colonization resistance disappeared when *Serratia* was able to become established
316 prior to inoculation with *Providencia*; there were no survival differences between
317 larvae inoculated with *Serratia* twice in series and larvae first inoculated with
318 *Serratia* and then followed by *Providencia* (Wald statistic of S/P = 0.077, P = 0.782,
319 Figure 4C). In light of results in Figure 3B showing that order of inoculation does not
320 affect *Providencia* competitiveness, these survival results indicate that *Serratia*

321 induced larval mortality is insensitive to bacterial competitiveness, thus supporting
322 the idea that initial establishment of the endogenous microbiota is crucial for
323 colonization resistance.
324
325

326 **Discussion**
327

328 *Nicrophorus* larvae are exposed to a highly diverse microbiota in their breeding
329 environment, first from the soil where they hatch and next from the microbes
330 proliferating on and within their carrion resource. In the absence of parental care,
331 larvae become colonized with these bacteria (18) which reduces their weight and
332 survival (15), and also leads to reduced brood mass when these larvae reproduce as
333 parents (Figure 1). However, when larvae are reared with parental care, their gut
334 microbiome resembles that of their parent, even if parental care is limited to carcass
335 preparation prior to larval hatch (18). These results suggested that the bacteria within
336 the parental gut are better competitors for the larval gut, but our earlier work neither
337 tested the colonization potential and competitiveness of the constituent species nor
338 determined the consequences of colonization with the *Nicrophorus* "endogenous"
339 microbiome. Our aims here were therefore to address these questions experimentally
340 by inoculating different endogenous or non-endogenous bacterial species into the guts
341 of developing larvae. We focus specifically on four species: *Providencia rettgeri* and
342 *Morganella morganii*, that are dominant members of the larval microbiome (18, 19),
343 and *Escherichia coli* and *Serratia marcescens*, which are non-endogenous species, but
344 which are either observed in the larval gut (*Serratia*) or have the potential to colonize
345 it through exposure on the mouse carcass (*E. coli*) (18, 32).
346

347 Using this approach, we first determined that there are clear differences in the
348 colonization potential of different bacterial species. While *Providencia*, *Morganella*
349 and *Serratia* increase in density more than 100-fold in 24 hours within the larval gut,
350 *E. coli* was a poor colonizer and only increased by ~ 10-fold over the same time
351 interval (Figure 2). In addition to clarifying these differences, these experiments also
352 established that it is feasible to experimentally colonize larval beetles via diet
353 manipulation. The growth differences between strains in monoculture were reflected
354 in their interactions *in vivo* during co-culture. Specifically, we saw competitive
355 dominance of *Providencia* and *Morganella* over *E. coli* and *Serratia* when pairs of
356 strains were simultaneously fed to larvae (Figure 3A). Moreover, in competition
357 experiments between *Providencia* and *Serratia*, we found that the order of inoculation
358 did not affect the competitive outcome between strains (Figure 3B). This latter result
359 suggested that priority effects are not realized in this system because *Serratia* could
360 be displaced even after a 24-hr head start in colonization. By contrast, another recent
361 study found that the colonization competitiveness of *Borrelia* strains within the mouse
362 gut are significantly determined by their order of presentation to the host mouse (33).
363

364 At present, we have limited understanding of the factors that mediate the competitive
365 differences between strains within the *Nicrophorus* larval gut. Differences in *in vivo*
366 growth rates are sufficient to explain the competition results during simultaneous
367 inoculation. However, the fact that *Providencia* can still invade an established
368 *Serratia*-colonized larva (Figure 3B), suggests the possibility that competitive
369 interactions are in part mediated by the host. For instance, host innate immunity could
370 be a direct factor in determining the competitive outcome and final population density

371 of bacterial species within hosts (34). Equally, commensal bacteria could prime the
372 host immune response to limit pathogen colonization by causing an up-regulation of
373 antimicrobial peptides, such as AMP molecules in *Aedes aegypti* mosquitoes and
374 islet-derived protein 3γ in mice (35, 36); however, these would need to be specifically
375 targeted to non-symbiont species. Host involvement in this system is further
376 suggested by other experimental results showing that *in vitro*, *Serratia* is able to
377 outcompete *Providencia* (YW unpublished data)*,* a result likely attributed to the faster
378 growth of this strain during *in vitro* culture. An important aim for future work will be
379 to clarify the factors that drive the competitive interactions between bacterial strains
380 during colonization.
381

382 To understand the consequences of the *Nicrophorus* microbiome for beetle fitness, we
383 quantified larval mortality following inoculation of monocultures or co-cultures of
384 different bacterial species. Consistent with results on colonization resistance in other
385 systems (9, 37), these experiments showed that *P. rettgeri* and *M. morganii* both
386 provide protection against *Serratia* infection, but with no added protection if both
387 endogenous species are present (Figure 4B). This is expected given the results from *in*
388 *vivo* competition assays. By contrast, when *Serratia* is inoculated first, the protection
389 provided by *Providencia* is abolished, in spite of the fact that *Providencia* can
390 outcompete *Serratia* in these conditions (Figure 4C). Interestingly, these results
391 indicate that the pathogenesis of *Serratia* is separate from its *in vivo* competitive
392 ability, perhaps owing to toxin production or invasion through the gut into the
393 haemocoel within the first 24 hours (38–40). Thus initial establishment of the
394 endogenous microbiota is apparently crucial for colonization resistance.
395

396 Although the mechanism of *Serratia*-induced mortality remain unknown in this
397 species, the fact that colonization resistance requires the prior or simultaneous
398 establishment of the *Nicrophorus* endogenous microbiota has important implications
399 for our understanding of the functions of parental care. Parents protect larvae and
400 provide nutrition in the form of regurgitated food (22). In addition, they transfer their
401 gut microbiome to larvae by direct feeding and via contamination of the carcass
402 surface (17, 18). The present results indicate that larvae benefit directly from the
403 acquisition of these bacteria (Figures 1 and 4B), and suggest that the microbiome, or
404 at least two of its key members, are mutualists with *Nicrophorus*. Thus while
405 *Nicrophorus* adults ensure transmission of these species from generation to
406 generation, the bacteria provide direct benefits to beetles within the highly
407 contaminated carcass environment (19). It remains possible that other advantages
408 exist, for example improved nutrient acquisition (41) or changes in the composition of
409 the decomposer microbial community on the carcass (17), but as yet these
410 possibilities have not been measured.
411

412 Our results provide strong evidence that members of the *Nicrophorus* microbiome
413 provide direct advantages to larvae and adults; however, it is important to note that
414 these advantages were not measured in the natural context of the carcass itself. While
415 this was necessary for the current work, it does mean that we may be underestimating
416 larval exposure to potential bacterial pathogens (42). In addition, our assay clearly
417 suffers from extremely high rates of larval mortality, irrespective of treatment.
418 Artificial diets that better mimic the larval environment and that improve larval
419 nutrition and survival are thus needed to more fully elucidate the functions of the
420 *Nicrophorus* microbiome. However, despite these limitations, our results point

421 towards yet another role of parental care in *Nicrophorus vespilloides*, and argue for
422 further comparative studies in other congeners that vary in their requirements for
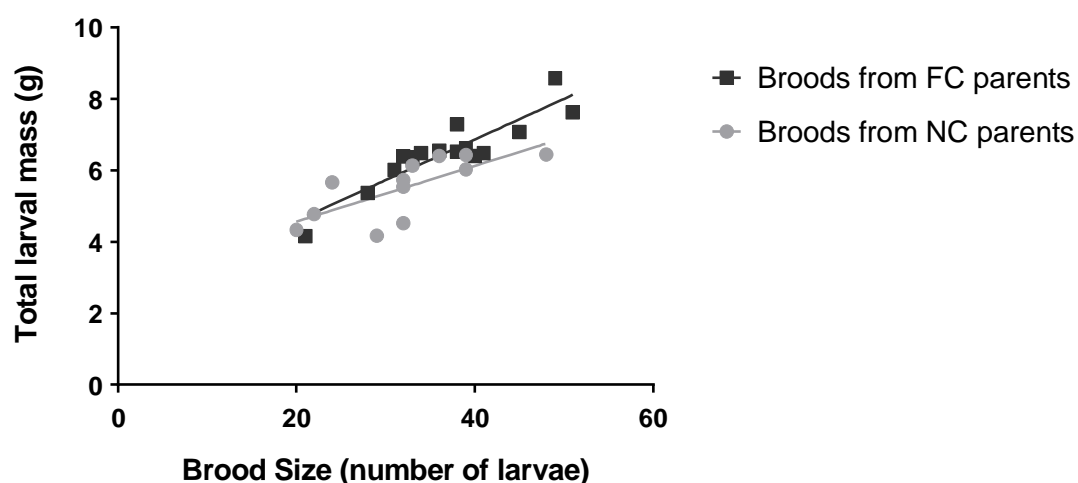423 parental care.
424

Acknowledgements

430

References

1. **McFall-Ngai M**, **Hadfield MG**, **Bosch TCG**, **Carey H V.**, **Domazet-Lošo T**, **Douglas AE**, **Dubilier N**, **Eberl G**, **Fukami T**, **Gilbert SF**, **Hentschel U**, **King N**, **Kjelleberg S**, **Knoll AH**, **Kremer N**, **Mazmanian SK**, **Metcalf JL**, **Nealson K**, **Pierce NE**, **Rawls JF**, **Reid A**, **Ruby EG**, **Rumpho M**, **Sanders JG**, **Tautz D**, **Wernegreen JJ**. 2013. Animals in a bacterial world, a new imperative for the life sciences. Proc Natl Acad Sci **110**:3229–3236.

2. **Rajagopal R**. 2009. Beneficial interactions between insects and gut bacteria. Indian J Microbiol **49**:114–119.

3. **Flórez L V.**, **Biedermann PHW**, **Engl T**, **Kaltenpoth M**. 2015. Defensive symbioses of animals with prokaryotic and eukaryotic microorganisms. Nat Prod Rep **32**:904–936.

4. **Hacquard S**, **Garrido-Oter R**, **Gonzalez A**, **Spaepen S**, **Ackermann G**, **Lebeis S**, **McHardy AC**, **Dangl JL**, **Knight R**, **Ley R**, **Schulze-Lefert P**. 2015. Microbiota and host nutrition across plant and animal kingdoms. Cell Host Microbe **17**:603–616.

5. **Sommer F**, **Bäckhed F**. 2013. The gut microbiota--masters of host development and physiology. Nat Rev Microbiol **11**:227–38.

6. **Schwarz RS**, **Moran NA**, **Evans JD**. 2016. Early gut colonizers shape parasite susceptibility and microbiota composition in honey bee workers. Proc Natl Acad Sci **113**:9345–9350.

7. **Sharon G**, **Segal D**, **Ringo JM**, **Hefetz A**, **Zilber-Rosenberg I**, **Rosenberg E**. 2010. Commensal bacteria play a role in mating preference of Drosophila melanogaster. Proc Natl Acad Sci **107**:20051–20056.

8. **Buffie CG**, **Pamer EG**. 2013. Microbiota-mediated colonization resistance against intestinal pathogens. Nat Rev Immunol **13**:790–801.

9. **Dillon RJ**, **Vennard CT**, **Buckling A**, **Charnley AK**. 2005. Diversity of locust gut bacteria protects against pathogen invasion. Ecol Lett **8**:1291–1298.

10. **Raymann K**, **Shaffer Z**, **Moran NA**. 2017. Antibiotic exposure perturbs the gut microbiota and elevates mortality in honeybees. PLoS Biol **15**:1–22.

11. **Macke E**, **Tasiemski A**, **Massol F**, **Callens M**, **Decaestecker E**. 2017. Life history and eco-evolutionary dynamics in light of the gut microbiota. Oikos **126**:508–531.

12. **Dillon RJ**, **Dillon VM**. 2004. The Gut Bacteria of Insects: Nonpathogenic Interactions. Annu Rev Entomol **49**:71–92.

13. **Jarosz J**. 1979. Gut flora of Galleria mellonella suppressing ingested bacteria. J Invertebr Pathol **34**:192–198.

14. **Scott MP**. 1998. The ecology and behaviour of burying beetles. Annu Rev Entomol **43**:595–618.

15. **Rozen DE**, **Engelmoer DJP**, **Smiseth PT**. 2008. Antimicrobial strategies in burying beetles breeding on carrion. Proc Natl Acad Sci U S A **105**:17890–17895.

16. **Hall CL**, **Wadsworth NK**, **Howard DR**, **Jennings EM**, **Farrell LD**, **Magnuson TS**, **Smith RJ**. 2011. Inhibition of microorganisms on a carrion breeding resource: the antimicrobial peptide activity of burying beetle (Coleoptera: Silphidae) oral and anal secretions. Environ Entomol **40**:669–78.

17. **Duarte A**, **Welch M**, **Wagner J**, **Kilner RM**. 2016. Privatization of a breeding resource by the burying beetle *Nicrophorus vespilloides* is associated with shifts in bacterial communities **44**.

18. **Wang Y**, **Rozen DE**. 2017. Gut Microbiota Colonization and Transmission in the Burying Beetle Nicrophorus vespilloides throughout Development. Appl Environ Microbiol **83**:e03250-16.

19. **Vogel H**, **Shukla SP**, **Engl T**, **Weiss B**, **Fischer R**, **Steiger S**, **Heckel DG**, **Kaltenpoth M**, **Vilcinskas A**. 2017. The digestive and defensive basis of carcass utilization by the burying beetle and its microbiota. Nat Commun **8**:15186.

20. **Kaltenpoth M**, **Steiger S**. 2014. Unearthing carrion beetles' microbiome: Characterization of bacterial and fungal hindgut communities across the Silphidae. Mol Ecol **23**:1251–1267.

21. **Smiseth PT**, **Darwell CT**, **Moore AJ**. 2003. Partial begging: an empirical model for the early evolution of offspring signalling. Proc Biol Sci **270**:1773–7.

22. **Eggert A-K**, **Reinking M**, **Mu JK**, **Ller }**. 1998. Parental care improves offspring survival and growth in burying beetles. Anim Behav **55**:97–107.

23. **Capodeanu-Nägler A**, **Keppner EM**, **Vogel H**, **Ayasse M**, **Eggert A-K**, **Sakaluk SK**, **Steiger S**. 2016. From facultative to obligatory parental care: Interspecific variation in offspring dependency on post-hatching care in burying beetles. Sci Rep **6**:29323.

24. **Arce AN**, **Johnston PR**, **Smiseth PT**, **Rozen DE**. 2012. Mechanisms and fitness effects of antibacterial defences in a carrion beetle. J Evol Biol **25**:930–937.

25. **Cotter SC**, **Kilner RM**. 2010. Sexual division of antibacterial resource defence in breeding burying beetles, Nicrophorus vespilloides. J Anim Ecol **79**:35–43.

26. **Hoback WW**, **Bishop AA**, **Kroemer J**, **Scalzitti J**, **Shaffer JJ**. 2004. Differences among antimicrobial properties of carrion beetle secretions reflect phylogeny and ecology. J Chem Ecol **30**:719–729.

27. **Milne LJ**, **Milne M**. 1976. The Social Behavior of Burying Beetles. Sci Am **235**:84–89.

28. **Jacobs CGC**, **Wang Y**, **Vogel H**, **Vilcinskas A**, **van der Zee M**, **Rozen DE**. 2014. Egg survival is reduced by grave-soil microbes in the carrion beetle, Nicrophorus vespilloides. BMC Evol Biol **14**:208.

29. **El Sanousi SM**, **El Sarag MSA**, **Mohamed SE**. 1987. Properties of Serratia marcescens Isolated from Diseased Honeybee (Apis mellifera) Larvae. Microbiology **133**:215–219.

30. **Nehme NT**, **Liégeois S**, **Kele B**, **Giammarinaro P**, **Pradel E**, **Hoffmann JA**, **Ewbank JJ**, **Ferrandon D**. 2007. A model of bacterial intestinal infections in Drosophila melanogaster. PLoS Pathog **3**:1694–1709.

31. **Renoz F**, **Noël C**, **Errachid A**, **Foray V**, **Hance T**. 2015. Infection dynamic of

521   symbiotic bacteria in the pea aphid Acyrthosiphon pisum gut and host immune
522   response at the early steps in the infection process. PLoS One **10**:1–18.

523 32. **Bäumler AJ**, **Sperandio V**. 2016. Interactions between the microbiota and
524   pathogenic bacteria in the gut. Nature **535**:85–93.

525 33. **Devevey G**, **Dang T**, **Graves CJ**, **Murray S**, **Brisson D**. 2015. First arrived
526   takes all: inhibitory priority effects dominate competition between co-infecting
527   Borrelia burgdorferi strains. BMC Microbiol **15**:381.

528 34. **Portal-Celhay C**, **Blaser MJ**. 2012. Competition and resilience between
529   founder and introduced bacteria in the Caenorhabditis elegans gut. Infect
530   Immun **80**:1288–1299.

531 35. **Kambris Z**, **Al E**. 2009. Immune Activation by Life-Shortening. Science (80-)
532   **134**:134–136.

533 36. **Kamada N**, **Seo S-U**, **Chen GY**, **Núñez G**. 2013. Role of the gut microbiota in
534   immunity and inflammatory disease. Nat Rev Immunol **13**:321–335.

535 37. **Sant'Anna MR**, **Diaz-Albiter H**, **Aguiar-Martins K**, **Al Salem WS**,
536   **Cavalcante RR**, **Dillon VM**, **Bates PA**, **Genta FA**, **Dillon RJ**. 2014.
537   Colonisation resistance in the sand fly gut: Leishmania protects Lutzomyia
538   longipalpis from bacterial infection. Parasit Vectors **7**:329.

539 38. **Andrejko M**. 1999. Exoproteinases of the type A in pathogenesis of insect
540   bacterial diseases. Folia Biol (Praha) **47**:135–141.

541 39. **Lauzon C**, **Bussert T**, **RE S**, **RJ P**. 2003. *Serratia marcescens* as a bacterial
542   pathogen of *Rhagoletis pomonella* flies (Diptera□: Tephritidae). Eur J Entomol
543   **100**:87–92.

544 40. **Tan B**, **Jackson TA**, **Hurst MRH**. 2006. Virulence of Serratia strains against
545   Costelytra zealandica. Appl Environ Microbiol **72**:6417–6418.

546 41. **Wilkinson TL**, **Koga R**, **Fukatsu T**. 2007. Role of host nutrition in symbiont
547   regulation: Impact of dietary nitrogen on proliferation of obligate and
548   facultative bacterial endosymbionts of the pea aphid Acyrthosiphon pisum.
549   Appl Environ Microbiol **73**:1362–1366.

550 42. **Reavey CE**, **Silva FWS**, **Cotter SC**. 2015. Bacterial infection increases
551   reproductive investment in burying beetles. Insects **6**:926–942.

552
553
554
555
556
557
558
559
560
561
562
563
564
565
566
567
568
569
570

571
572
573
574
575
576
577
578
579
580
581

582
583
584 **Figure 1.** Total larval mass as a function of brood size for maternal
585 beetles that were reared with either Full Care (FC) or No Care (NC).
586
587
588
589
590
591
592
593
594
595
596
597
598
599
600
601
602
603
604

605
606
607
608
609
610
611
612
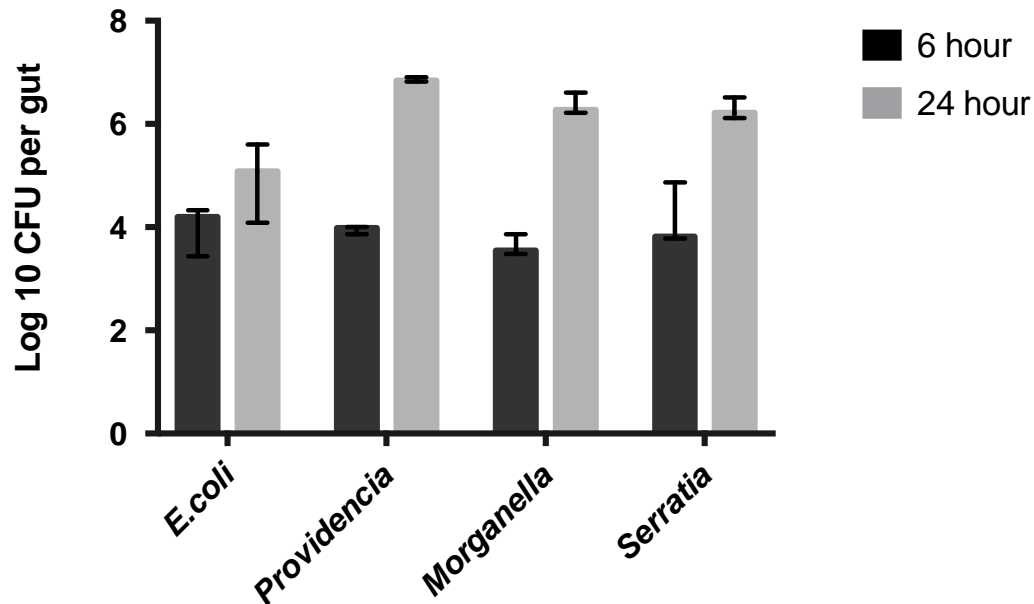
613
614
615
616 **Figure 2.** Growth and colonization of *N. vespilloides* symbionts within
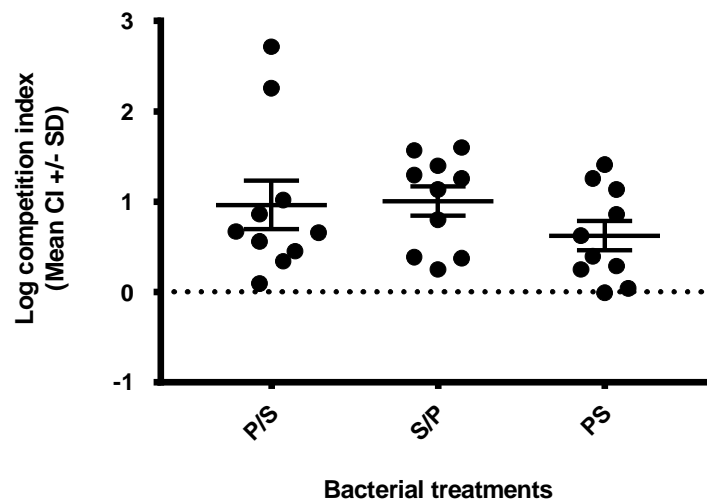617 the larval gut over 24 hrs. Values correspond to the mean +/- 95% CI.
618
619
620
621
622
623
624

625 **(A)**
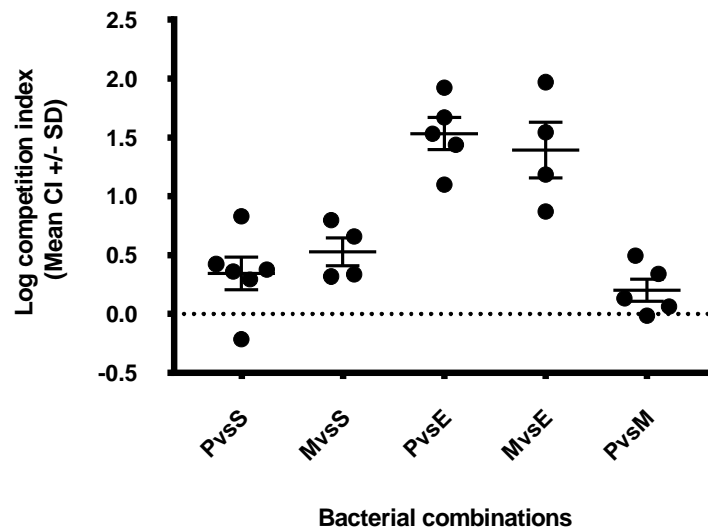
626

627 **(B)**

628
629
630 **Figure 3.** Competitive differences between different bacterial species *in*
631 *vivo* within the larval gut. Competition indices (CI) are given in reference
632 to the first species listed on the x-axis for (A) and with respect to
633 *Providencia* for (B). Strains were either inoculated simultaneously (A) or
634 in series (B) in cases where strains are separated with a / (e.g. P/S:
635 *Providencia* was inoculated first and then followed with *Serratia*,
636 whereas in PS both strains were co-inoculated). The dashed black line
637 illustrates a CI of 0, which indicates equal competitiveness of two strains.
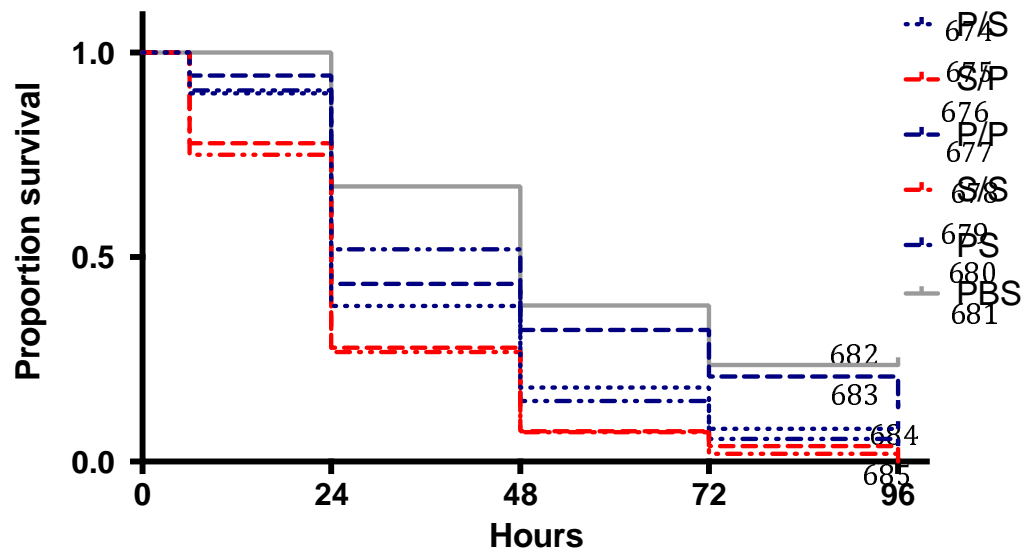638 Values > 0 indicates that strain 1 is a stronger *in vivo* competitor.
639

640   **(A)**

641
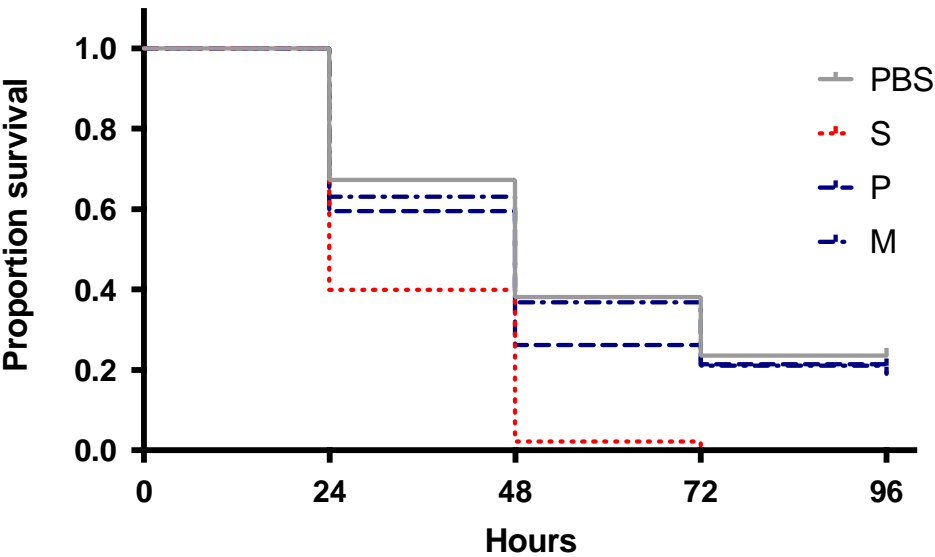
642

643

644   **(B)**

645

646
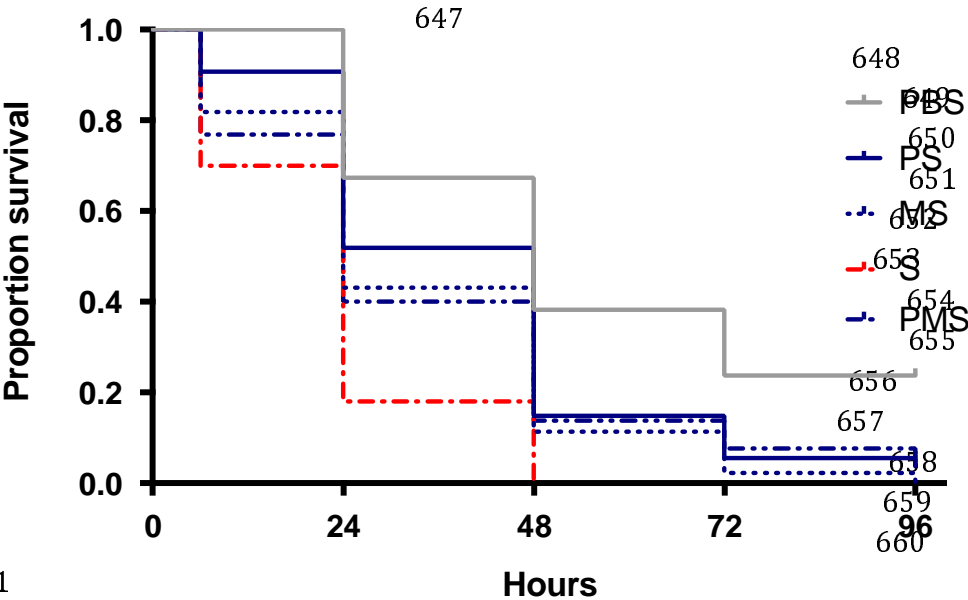
661

662

663

664

665

666

667

668
669
670 **(C)**
671
672
673

Figure 4. Larval survival when inoculated with different bacterial species. Larvae were inoculated with (A) single bacterial species in monoculture, (B) > 1 species in coculture simultaneously, or (C) bacteria either simultaneously or in series. Bacteria inoculated simultaneously are designated with the first letter of the species name (e.g. PS = *Providencia* with *Serratia*), while species inoculated in series are given in the same way with a slash (e.g. P/S = *Providencia* followed by *Serratia*). A PBS (phosphate buffered saline) control was set up for all the experiments.

711
712
713
714
715
716
717
718
719
720
721
722
723
724
725
726
727
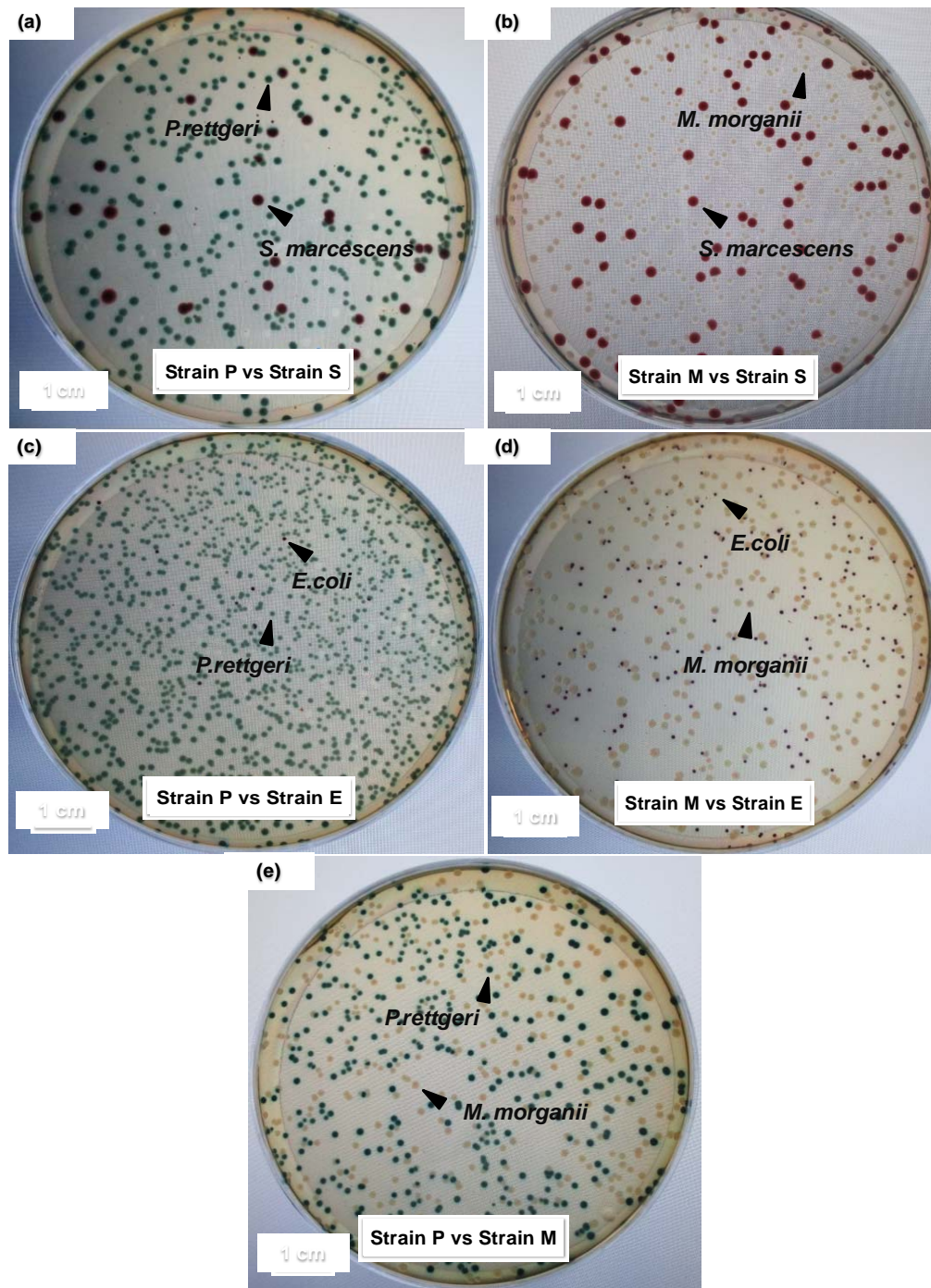
Figure S1. Color and morphology of experimental strains on chromogenic agar plates (CHROMagar^{TM} Orientation) used for bacterial competition assays. Bacterial combinations shown are: P vs S (a); M vs S (b); P vs E (c); M vs E (d); P and M (e).