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Bacterial influence on the maintenance of symbiotic yeast through *Drosophila* metamorphosis

Running title: Bacterial influence on yeast maintenance

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Summary statement

Bacterial symbionts of *Drosophila* influence yeast maintenance through fly metamorphosis, a novel observation that may have consequences for the evolution of insect-yeast-bacteria interactions.

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1 # Abstract

2 Interactions between microbial symbionts of metazoan hosts are emerging as key features of
3 symbiotic systems. Little is known about the role of such interactions on the maintenance of
4 symbiosis through host's life cycle. We studied the influence of symbiotic bacteria on the
5 maintenance of symbiotic yeast through metamorphosis of the fly *Drosophila melanogaster*.
6 To this end we mimicked the development of larvae in natural fruit. In absence of bacteria
7 yeast was never found in young adults. However, yeast could maintain through
8 metamorphosis when larvae were inoculated with symbiotic bacteria isolated from *D.*
9 *melanogaster* faeces. Furthermore, an Enterobacteriaceae favoured yeast transstadial
10 maintenance. Because yeast is a critical symbiont of *D. melanogaster* flies, bacterial influence
11 on host-yeast association may have consequences for the evolution of insect-yeast-bacteria
12 tripartite symbiosis and their cooperation.

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13 # Introduction

14 Metazoans often form associations with non-obligatory symbiotic microorganisms. Microbial
15 symbiont can influence host phenotype, and hosts determine symbiont multiplication and
16 dispersal (Ferrari and Vavre, 2011). The importance of interactions between microorganisms
17 is relatively better understood in the context of parasitism than in the context of beneficial
18 symbionts (Alizon et al., 2013; Tollenaere et al., 2016; Zélé et al., 2018). Beneficial microbial
19 symbionts can nonetheless interact in a wide variety of manners (Comolli, 2014; Seth and
20 Taga, 2014; Hassani et al., 2018) and affect both the dynamics of each microorganism and the
21 phenotype of their host (Wargo and Hogan, 2006; Newell and Douglas, 2014; Callens et al.,
22 2018; Gould et al., 2018; Sommer and Newell, 2019).

23 Fungi-bacteria interactions are well described microbial interactions due to their importance
24 in human health, industry and domestic life (Kobayashi and Crouch, 2009; Jouhten et al.,
25 2016; Carbonetto et al., 2018). In the wild, yeasts and other fungi associate with extracellular
26 bacteria in a wide variety of habitats, including decaying plant materials where they interact
27 with the larvae and adults of saprophagous insects such as *Drosophila* flies. Symbioses
28 between *Drosophila* and either yeast and bacteria have however been largely studied
29 separately. Each type of microorganism affects *Drosophila* physiology, nutrition,
30 reproduction and behavior (Ryu et al., 2008; Anagnostou et al., 2010; Shin et al., 2011;
31 Storelli et al., 2011; Becher et al., 2012; Broderick and Lemaitre, 2012; Wong et al., 2014)
32 and may maintain through - a variable part of - *Drosophila* life cycle (Bakula, 1969; Starmer
33 et al., 1988; Hoang et al., 2015; Pais et al., 2018). In natural fruit, *D. melanogaster* larval
34 development can be impossible, or largely compromised, in absence of yeast (Becher et al.,
35 2012), suggesting symbiosis with yeast may not be dispensable for larvae. A handful of
36 studies considering both fungi and bacteria showed that direct interaction between yeast and
37 bacteria can modulate fly behavior (Fischer et al., 2017) and that bacteria can affect fly
38 attraction to yeast (Leitão-Gonçalves et al., 2017). There is to date little data on how
39 interactions between microbial symbionts affect their transmission from the host or
40 maintenance among host life stages, which would have wide consequences for the ecological
41 dynamics of the microorganisms, and consequently, the evolution of their symbiosis with
42 flies.

43 It is established that *Drosophila* flies contribute to bacteria and yeast dynamics through
44 effects on local multiplication and dispersal (Gilbert, 1980; Ganter, 1988; Starmer et al.,

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45 1988; Chandler et al., 2012; Stamps et al., 2012; Buser et al., 2014). *Saccharomyces* yeast, for
46 example, attracts adult flies with volatiles, these adults then acquire the microorganism and
47 later inoculate it in fruit where larvae develop. An alternative mean of insect vectoring for
48 symbiotic bacteria and yeast of larvae would rely on the transstadial maintenance, from the
49 larval to the adult stage. It would enable the colonization of freshly emerged adults by larval
50 symbionts and, provided they maintain and are shed later in life, their possible dispersal to
51 new patches of resources. The maintenance of yeast or bacteria throughout the *Drosophila* life
52 cycle have been investigated (Rohlfs and Hoffmeister, 2005; Pais et al., 2018), including
53 maintenance from larvae to adults through metamorphosis (i.e. transstadial maintenance or
54 transstadial transmission) (Bakula, 1969; Starmer et al., 1988; Ridley et al., 2012; Duneau and
55 Lazzaro, 2018). To our knowledge, how interactions between microbial symbionts affect
56 symbiont maintenance through *Drosophila* life stages remains however unknown. Here, we
57 investigated the maintenance of a wild isolate of *Saccharomyces cerevisiae* yeast from larvae
58 to young adults *Drosophila melanogaster*. We found that yeast presence in adults only
59 occurred when larvae were associated with symbiotic bacteria and its frequency depended on
60 the identity of these bacteria.

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61 # Material and methods

62 ## Biological material

63 We used a *Drosophila melanogaster* (Meigen, 1830) Oregon-R strain usually maintained on
64 banana medium (233 g.L^{-1} banana, 62 g.L^{-1} sugar, 62 g.L^{-1} dead yeast, 25 g.L^{-1} ethanol, 10
65 g.L^{-1} agar and 5 g.L^{-1} nipagin) at 21°C with a 14 h/10 h day-night cycle. The four bacterial
66 strains used had been isolated from feces of adult Oregon-R flies and had been described in
67 Guilhot et al. (2019). Bacterial strains were identified as *Staphylococcus* sp. (accession
68 number MK461976 in the NCBI database), *Enterococcus* sp. (MK461977), an
69 Enterobacteriaceae (MK461978) and an Actinobacteria (MK461979). The taxonomic
70 resolution of our analyses is unfortunately modest and these bacteria do not usually dominate
71 the *Drosophila* microbiota. However similar strains had already been identified as associated
72 with Drosophilids (Chandler et al., 2011; Staubach et al., 2013). More importantly, microbial
73 species and strains can evolve rapidly and with large consequences on their effects on host
74 phenotype (Winans et al., 2017; Martino et al., 2018). For this reason, the proper description
75 of symbiont effects on host phenotype in relevant experimental conditions (Guilhot et al.
76 2019) may be more important than high taxonomical resolution to understand symbiosis.

77 The yeast *Saccharomyces cerevisiae* (Meyen ex Hansen, 1883) strain was isolated from a
78 wild Drosophilid in the *'Le Domaine de l'Hortus'* vineyard, near Montpellier in Southern
79 France.

80 ## Experimental design

81 The main experiment was conducted on sterile vials. Each experimental unit consisted of
82 twenty *D. melanogaster* eggs free of cultivable bacteria and yeast deposited on an artificial
83 wound of a surface-sterilized grape berry that was inoculated, or not, with specific
84 microorganisms. Eggs were laid by conventionally-reared *D. melanogaster* Oregon-R females
85 on solidified grape juice, which contained the antibiotic streptomycin (1 mg.L^{-1}, from a
86 standard streptomycin solution of 1 mg.ml^{-1} in 1 mM EDTA (Sigma-Aldrich ref. 85886)) in
87 order to remove parental bacteria. Grape berries were surface-sterilized; they were hence
88 dipped in a 2% bleach solution, rinsed with sterile water and dried before use. We ensure
89 these procedures were efficient: no cultivable bacteria or yeast were found in surface-
90 sterilized berries and eggs homogenates. Every grape berry received 10^4 *S. cerevisiae* yeast
91 cells suspended in 10 µl. Berries inoculated with *S. cerevisiae* were then allocated to six

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92 different bacterial treatments: no bacteria (control, n = 18); one of the four bacterial strains
93 described above (10^4 cells of the same type: n (*Staphylococcus*) = 13, n (*Enterococcus*) = 13,
94 n (Enterobacteriaceae) = 11, n (Actinobacteria) = 13); and a mixture of the four bacteria
95 (2.5×10^3 cells of each type, n = 9). Replicates were organized in eleven blocks launched over
96 four days.

97 Newly formed pupae were aseptically removed daily from their larval container and placed in
98 a sterile new vial until adult emergence. This procedure mimicked natural insect behavior as
99 *D. melanogaster* larvae usually crawl out of their substrate before pupation (Sokolowski et al.,
100 1986; Woltz and Lee, 2017), which incidentally prevent the exposure of young adults to the
101 microorganisms present in the larval substrate. It was not logistically feasible to process every
102 adult independently. We therefore elected to assess microbial content in groups of adults that
103 emerged on the same day. For each grape berry, we randomly selected a single pupa and
104 pooled all the adults, females as males, that emerged on the day as this pupa. This protocol
105 allowed the detection of yeast (and bacterial) cells associated with freshly emerged adults that
106 may have been present externally or internally. Sampled adults were homogenized in sterile
107 PBS using a Tissue Lyser II (Qiagen) and Ø3 mm glass balls. Serially diluted fly samples
108 were plated on Lysogeny Broth (LB) plates to count microbial cells after incubation at 24°C.
109 Colonies of the five microbial symbionts (yeast and bacteria) were distinguished according to
110 their morphology (see Guilhot et al. 2019).

111 Effects of bacteria on yeast development in the larval fruit may explain some of our results.
112 We hence collected the remaining juice from grape berries two days after the formation of the
113 last pupa. Although quantifying yeast in fruit when larvae were feeding would have been
114 informative too, we elected to not disturb the development of the larvae and waited the larvae
115 left their substrate. Serially diluted fruit samples were plated on LB plates to count yeast
116 colonies at 24°C. As above, microbial colonies were distinguished based on their
117 morphology.

118 In parallel to the main experiment, bacteria were also inoculated without yeast on cubes of
119 laboratory medium (which contains dead yeast) following the procedure above to assess
120 bacteria transstadial maintenance in their environment of origin (i.e. the banana medium used
121 to rear the fly colony and described above). The six different bacterial treatments were: no
122 bacteria (control, n = 12); one of the four bacterial strains described above (n
123 (*Staphylococcus*) = 12, n (*Enterococcus*) = 7, n (Enterobacteriaceae) = 11, n (Actinobacteria)

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124 = 11); and a mixture of the four bacteria (n = 14). Replicates were organized in fifteen blocks
125 launched over four days.

126 Statistical analyses

127 We hypothesized larval bacteria would affect yeast transstadial maintenance. We estimated
128 this phenomenon in groups of 1 to 11 freshly emerged adults (median = 5, IQR = 4). Yeast-
129 positive samples contained 1 to 150 cells per adult fly (Fig. S1). This variation was not
130 investigated statistically due to low statistical power. Whether live yeast cells were present or
131 not was analyzed using a generalized linear model with binomial distribution and logit link
132 function. Tested factors comprised bacterial treatment, number of adults in the groups, yeast
133 concentration in the fruit, age of the adults and experimental block. Statistical power did not
134 enable testing the interaction between bacterial treatment and number of adults in the groups
135 (but see Fig. S2). Because we used groups of adult flies it was mandatory to take into account
136 the number of individuals per pool. The biological material employed (i.e. wild and laboratory
137 strains and populations) informs on the factors that can influence transstadial symbiont
138 maintenance in a qualitative fashion, but could not indicate their quantitative occurrence in
139 the field. Backward model selection allowed to eliminate non-significant terms (i.e. yeast
140 concentration in the fruit and age of the pooled flies) from the initial complete model.
141 Contrasts were used to detect significant differences between bacterial treatment levels.
142 Numbers of replicates varied among bacterial treatments due to differential larval mortality.
143 However, the analysis of larval survival revealed no significant effect of the bacteria on this
144 trait (Guilhot et al., 2019).

145 To study the effect of the bacterial treatment on the yeast concentration (log-transformed) in
146 larval fruit substrate, we used a linear mixed model with Restricted Maximum Estimate
147 Likelihood. Experimental block was defined as a random factor.

148 Analyzes were performed with JMP (SAS, 14.1).

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149 # Results

150 Bacterial treatment significantly affected *S. cerevisiae* presence in freshly emerged adult flies
151 (χ^2 = 20.30, df = 5, p = 0.001). Yeast was never observed in adult flies that emerged from
152 control treatments, unlike treatments with bacteria at the larval stage (contrast 'All treatments
153 with bacteria' vs 'Control': χ^2 = 11.2, df = 1, p < 0.0001) (Fig. 1). Young adult flies that had
154 developed with the Enterobacteriaceae alone or in mixture with the other bacteria were more
155 likely to harbor live yeast cells than the other treatments with bacteria at the larval stage
156 (contrast 'With Enterobacteriaceae' vs 'All other treatments with bacteria': χ^2 = 4.52, df = 1, p
157 = 0.03) (Fig. 1). As expected, the number of individuals in the assayed pool significantly and
158 positively affected the likelihood of yeast observation (χ^2 = 7.54, df = 1, p = 0.01) (Fig. S2) –
159 supporting the need to include this factor in all the analyses. The age of freshly emerged adult
160 flies (χ^2 = 0.65, df = 1, p = 0.42) and the yeast concentration in the larval medium (χ^2 = 0, df =
161 1, p = 1) had not significant influence on yeast presence in adults.

162 The bacterial treatment had no significant effect on the yeast concentration in the medium two
163 days after the formation of the last pupa ($F_{5,49}$ = 1.18, p = 0.33) (Fig. 2). Yeast presence in
164 fruit flesh was detected in all replicates but one.

165 Bacteria could be observed in young adults that emerged from most combinations of larval
166 environment and bacterial treatment (Fig. S3). However, the proportion of bacteria-positive
167 groups never exceeded 25%. When bacteria were detected, load varied from 1 to 33 bacterial
168 cells per adult fly (data not analyzed statistically). The observation of the inoculated bacteria
169 in emerged adults shows these bacteria sampled in laboratory adults reared on artificial
170 medium could establish symbiosis with larvae, even in fruit substrate.

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171 # Discussion

172 Our most important result is that larval bacteria influenced yeast transstadial maintenance
173 (Fig. 1). In control treatments that were not inoculated by bacteria, yeast was never found in
174 freshly emerged adult flies. On the contrary, the presence of bacteria at the larval stage
175 favored yeast maintenance through host metamorphosis. In particular, inoculation by the
176 Enterobacteriaceae bacterium (alone or in mixture) led to greater *S. cerevisiae* transstadial
177 maintenance than the other bacterial treatments (Fig. 1). The propensity to favor yeast
178 maintenance hence seemed to vary among bacteria.

179 It is well known that coinfecting symbionts (mutualistic as parasitic) often affect each other's
180 horizontal transmission to new hosts in holometabolous insects (Azambuja et al., 2005;
181 Fellous and Koella, 2009; Gendrin and Christophides, 2013; Hegde et al., 2015) and other
182 multicellular organisms (Azambuja et al., 2005; Lass et al., 2013; Barret et al., 2016; Bonnet
183 et al., 2017; Zélé et al., 2018). However, we know a single other case of microbial interactions
184 affecting symbiont maintenance through complete metamorphosis: in *Galleria mellonella*
185 butterflies, the bacterium *Enterococcus mundtii* interacts with host immunity during the pupal
186 stage to shape adult bacterial microbiota (Johnston and Rolff, 2015). Although we used fresh
187 fruit and a wild yeast strain, flies and bacteria were laboratory sourced. Our experiment hence
188 shows bacteria affect yeast transstadial maintenance in *D. melanogaster*, but further work will
189 be necessary to unveil the pervasiveness of this phenomenon in the field.

190 What mechanisms may underlie symbiont transstadial maintenance, and how did bacteria
191 affect it? The maintenance of *S. cerevisiae* yeast and several bacterial strains through
192 *Drosophila* metamorphosis are congruent with previous reports of the transstadial
193 maintenance of extracellular microbial symbionts in Drosophilids (Bakula, 1969; Starmer et
194 al., 1988; Ridley et al., 2012; Duneau and Lazzaro, 2018; Téfit et al., 2018), other Dipterans
195 (Radvan, 1960; Capuzzo et al., 2005; Rochon et al., 2005; Damiani et al., 2008; Lauzon et al.,
196 2009; Gendrin and Christophides, 2013; Nayduch and Burrus, 2017; Majumder et al., 2020)
197 and other holometabolous insects (Hammer and Moran, 2019). Microbial symbionts could
198 maintain on inner or outer walls of the pupal chamber (Kaltenpoth et al., 2010; Wang and
199 Rozen, 2017). In *Drosophila melanogaster*, bacterial cells of *Escherichia coli* were found
200 associated with the inner pupal membrane (Bakula, 1969). Alternatively, adults might retrieve
201 symbionts by consuming their own meconium - the remaining of larval midgut that is
202 excreted right after adult emergence (Moll et al., 2001; Broderick and Lemaitre, 2012;

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203 Gendrin and Christophides, 2013). The mechanism of bacterial influence on yeast
204 maintenance through metamorphosis is not trivial either. The Enterobacteriaceae that favored
205 yeast maintenance, despite presenting a wide metabolic spectrum (Guilhot et al., 2019),
206 probably not improved fruit quality by concentrating or synthetizing nutrients (Ramiro et al.,
207 2016) as had no significant effect on fly phenotype in this context (Guilhot et al., 2019).
208 Besides, the concentration of yeast cells in fruit did not correlated with the presence of yeast
209 in the freshly emerged adults and was not affected by the bacterial treatment (Fig. 2). This
210 lack of quantitative relationships suggests yeast maintenance through metamorphosis may be
211 determined by qualitative processes rather than mere cell numbers. Several bacteria are
212 known to interact with *Drosophila* host signaling (e.g. Shin et al., 2011; Storelli et al., 2011).
213 Symbiotic bacteria could therefore elicit host or yeast physiological responses in a way that
214 would affect the likelihood of transstadial maintenance.

215 Yeast transstadial maintenance in *D. melanogaster* may have consequences for the spatial
216 spread of the yeast and the evolution of the symbiosis. Yeast needs active transport by insect
217 vectors to disperse among the ephemeral patch of resources formed by fruits (Starmer and
218 Lachance, 2011). *Drosophila* adults contribute to such yeast dispersal through two non-
219 excluding mechanisms. First, it is well established that yeasts produce chemical volatiles that
220 attract adult flies (Palanca et al., 2013; Buser et al., 2014; Scheidler et al., 2015; Anagnostou
221 et al., 2016; Bellutti et al., 2018; Günther et al., 2019; Lewis and Hamby, 2019), which favors
222 their acquisition and vectoring by insects to new resource patches (Buser et al., 2014).
223 Whether this phenomenon reflects yeast adaptation to insect vectoring is however debated
224 (Günther and Goddard, 2019). Second, yeast maintenance through *Drosophila* metamorphosis
225 - as demonstrated here - would enable the dispersal to new resource patches of larval
226 symbionts (e.g. fruit, possibly infested with insect larvae) by colonized emerging adults. Such
227 continuity in symbiosis over the life-cycle selects larval symbionts for beneficial effects on
228 host fitness (Ebert, 2013). The microbial strains the most beneficial to larval development (for
229 example in terms of larval survival) would be the ones best dispersed to new resources
230 patches by the vigorous or numerous adult hosts they favored the development of.
231 Furthermore, the maintenance of larval microbial symbionts until adult emergence may also
232 benefit the host as freshly emerged adults could be less susceptible to opportunistic pathogens
233 due to symbiont prior presence (Blum et al., 2013; Johnston and Rolff, 2015; Obadia et al.,
234 2017). To conclude, transstadial maintenance of larval symbionts has implication for the
235 dynamics and evolution of both hosts and microorganisms, the effects of bacteria on yeast we

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236 report may therefore affect important aspects of symbiosis. These are new and anticipated
237 consequences of insect association with bacteria.

238 Symbiont-symbiont interactions are emerging as key features of numerous taxa (Ferrari and
239 Vavre, 2011; Álvarez-Pérez et al., 2019, Mathé-Hubert et al., 2019), including *Drosophila*
240 flies (Fischer et al., 2017; Gould et al., 2018). Studying microbial symbionts one by one may
241 be more tractable, however our experiment illustrates understanding the nature and diversity
242 of host-symbiont relationships necessitates encompassing the complexity of natural
243 communities.

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248

249 ## Competing interests

250 No competing interests declared.

251

252 ## Author contributions

253 R.G., A.R. and S.F. designed the experiment. R.G., A.R., A.X. and S.F. ran the experiment.
254 K.H. collected the yeast isolate. R.G. and S.F. analyzed the data and wrote the manuscript.

255

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260

261 ## Data availability

262 The dataset is available in the open data repository Zenodo (doi: 10.5281/zenodo.3546129)
263 (Guilhot et al., 2020).

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Figures

Fig. 1. Transstadial maintenance of *Saccharomyces cerevisiae* in response to bacterial treatment. Symbols indicate the proportion of groups of freshly emerged adult flies containing yeast per bacterial treatment (n = number of adult groups per bacterial treatment). 95% binomial confidence intervals were calculated using normal approximation method. These results are qualitative as we used groups of adult flies to estimate yeast transstadial maintenance (Fig S2).

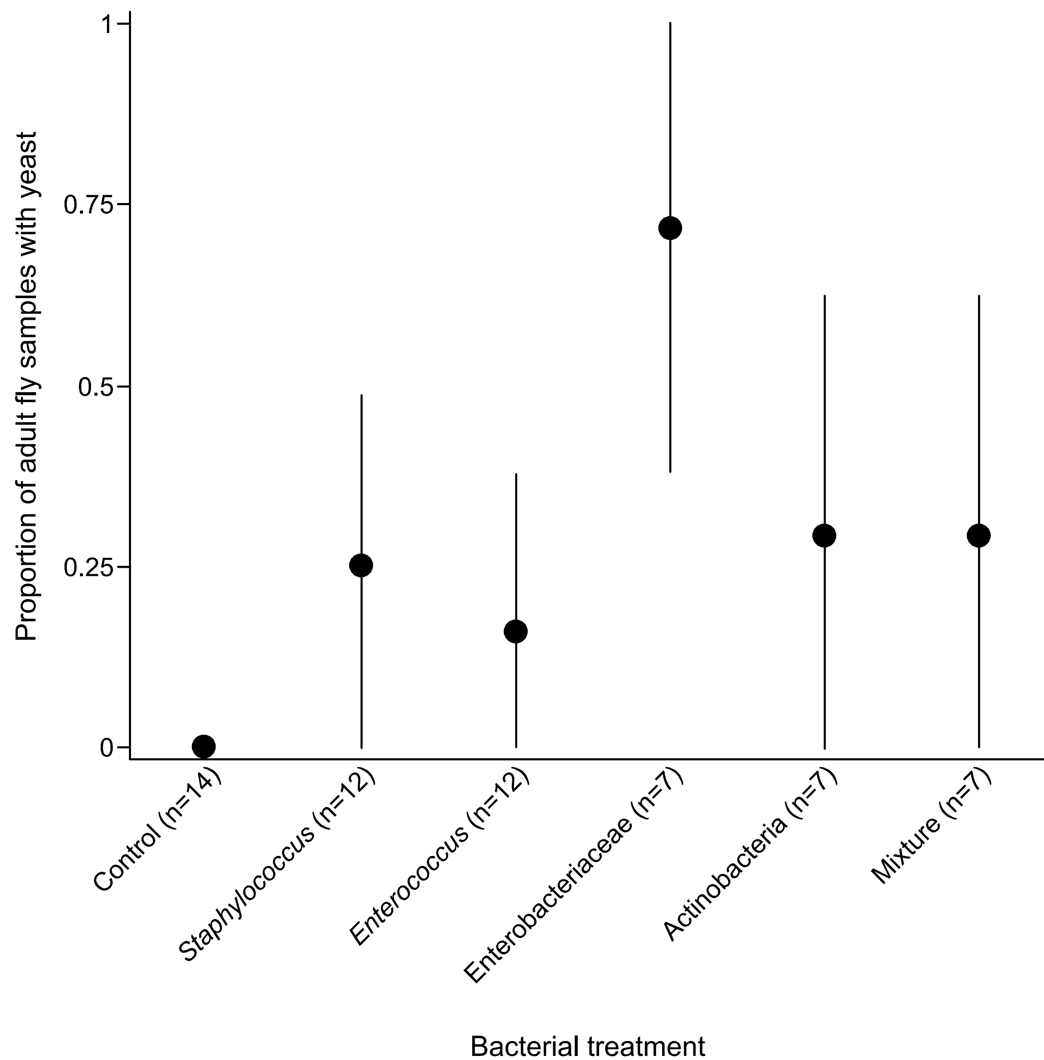

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Fig. 2. Yeast concentration in grape berry flesh after the formation of the last pupa. Concentration is expressed in number of yeast cells per 200 µl of fruit flesh. Symbols indicate mean ± s.e.m.