

1 **Title page**

2 Insect pollination is an ecological process involved in the assembly of the seed  
3 microbiota

4 **Running title:** Insect pollination affects seed microbiota

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26 **ABSTRACT**

27         The assembly of the seed microbiota involves some early microbial seed  
28 colonizers that are transmitted from the maternal plant through the vascular system,  
29 while other microbes enter through the stigma. Thus, the seed microbiota consists of  
30 microbes not only recruited from the vascular tissues of the plant, but also from the  
31 flower. Flowers are known to be a hub for microbial transmission between plants and  
32 insects. This floral-insect exchange opens the possibility for insect-transmitted bacteria  
33 to colonize the ovule and subsequently the seed, and to pass then into the next plant  
34 generation. In this study, we evaluated the contribution of insect pollination to the seed  
35 microbiota through high-throughput sequencing. Oilseed rape (OSR) *Brassica napus*  
36 flowers were exposed to visits and pollination by honey bees (*Apis mellifera*) or red  
37 mason bees (*Osmia bicornis*), hand pollination, or autonomous self-pollination (ASP).  
38 Sequence analyses revealed that honey bee visitation reduced the bacterial richness  
39 and diversity, increased the variability in the seed microbial structure, and introduced  
40 bee-associated taxa. In contrast, mason bee pollination had minor effects on the seed  
41 microbiota. We highlight the need to consider insect pollination as an ecological  
42 process involved in the transmission of bacteria from flower to seeds.

43

44 **IMPORTANCE**

45 Insect pollinators and flowering plants have a very old mutualistic relationship in which  
46 animal mobility is used for the dispersal of pollen. The pollination services provided by  
47 insects are extremely important to many natural plant populations as well as  
48 agricultural crops. Here we show that while visiting flowers, insect pollinators can  
49 disperse bacteria that are able to colonize the developing seed via the flower. Hence,  
50 insect pollination participates in the assembly of the seed microbiota, the inoculum for

51 the next plant generation. This novel insight has important implications in terms of re-  
52 assessing pollinator services by including microbe transfer.

53

#### 54 **KEYWORDS**

55 Bee pollination, bacterial transmission, entomovectors, seed-associated microbes

56

#### 57 **INTRODUCTION**

58 In nature, plants live in close association with a wide diversity of micro- and  
59 macro-organisms, both inside and outside their tissues. Microbes play essential roles  
60 in plant growth and development, affecting plant biomass or disease resistance  
61 (Berendsen et al. 2012; Santhanam et al. 2015; Sugiyama et al. 2013). Although  
62 numerous studies have been focusing on the analysis of microbial assemblages  
63 associated to different plant organs (Bulgarelli et al. 2013, Junker et al. 2011), little is  
64 known about tripartite interactions between plants, their microbiomes and other  
65 multicellular organisms, like pollinators. Insect visitors acquire and deposit  
66 microorganisms onto flower surfaces during nectar and pollen collection (Adler et al.  
67 2018; Aizenberg-Gershtein et al. 2013; de Vega et al. 2013; Ushio et al. 2015), thus  
68 shaping the flower microbiota (Alekklett et al. 2014; Manirajan et al. 2016; McFrederick  
69 et al. 2017). Since the flower microbiota serves as one of several inocula for the plant  
70 ovule, and hence for the seed (Truyens et al. 2015), it is possible that by affecting the  
71 microbial community of the flower (including pollen), pollinators could modify the seed  
72 microbiota.

73 The role of insect vectors in the dispersal of bacteria and fungi to roots, stems,  
74 leaves, flowers, and fruits is well documented (Perilla-Henao et al. 2016; Shikano et  
75 al. 2017), while their role in the microbial assembly of the seed has yet to be described.  
76 During the seed-to-seed development cycle, some early microbial seed colonizers are

77 transmitted from the mother plant to the ovule through the vascular system (internal  
78 transmission) while others colonize the ovule through the stigma (floral transmission).  
79 Other microbes are subsequently incorporated to the seed via external transmission,  
80 due to the contact with microorganisms present on fruits, flowers or threshing residues  
81 (Maude, 1996). Thus, the assembly of the seed microbiota is a complex process,  
82 including microbes recruited not only from the vascular tissue of the plant, but also  
83 from the floral microbiota. By affecting floral traits, microorganisms inhabiting the flower  
84 can have beneficial or detrimental consequences for the reproductive success of the  
85 plant (Alekkett et al. 2014; Canto et al. 2008; Herrera et al. 2013). However, it is  
86 unknown whether microbes inhabiting the flower can be incorporated via the floral  
87 pathway and affect seed traits. It is therefore important to understand the drivers in the  
88 assembly of seed microbiota.

89 Previous community-profiling approaches performed on the seed microbiota of  
90 various plant species have identified a range of bacterial taxa that could be potentially  
91 insect-transmitted (Adam et al., 2018; Bergna et al., 2019; Barret et al. 2015, Klaedtke  
92 et al., 2016; Leff et al., 2017, Links et al., 2014, Rezki et al., 2016; Rybakova et al.,  
93 2017, Rodrigues et al., 2018; Truyens et al. 2015). For instance, bacterial taxa affiliated  
94 to the *Enterobacteriaceae*, such as the ubiquitous *Pantoea agglomerans*, are found  
95 both in flowers, seeds and insect visitors such as the honey bee *Apis mellifera*  
96 (Loncaric et al. 2009; Rezki et al. 2018). In addition, another *Enterobacteriaceae*  
97 species, *Rosenbergiella nectarea*, isolated from the nectar of different plant species  
98 (Halpern et al. 2013), has also been detected in seeds (Torres-Cortes et al. 2018),  
99 indicating a possible bacteria transmission to the seed through the floral pathway by  
100 insect pollinators. However, it is currently unknown if insect pollinators participate in  
101 shaping the structure of the seed microbiota.

102 In this study, we apply metabarcoding approaches to uncover the contribution  
103 of insect pollinators to the seed microbiota and to the transmission of seed-associated  
104 microorganisms. Since bees (*Apoidea: Anthophila*) are one of the most important  
105 pollinators and harbor bacteria that are shared with flowers (Mcfrederick et al. 2017),  
106 we have examined the effect of bee pollination on the seed microbiota of oilseed rape  
107 (OSR; *Brassica napus*) by performing pollination exclusion experiments. Our results  
108 show that bee pollination participates in the microbial assembly of the seed by reducing  
109 the bacterial richness and diversity, increasing the variability amongst plants (beta  
110 dispersion) and introducing bee-associated taxa. Collectively, these data suggest that  
111 insect pollination is an ecological process involved in the assembly of the seed  
112 microbiota.

113

## 114 **RESULTS**

115 The effect of bee pollination on OSR seed-associated microbial assemblages  
116 was assessed during two years (2017 and 2018) on two different plant lines: i) a male  
117 fertile F<sub>1</sub> hybrid (MF; cv 'Exocet') that was bagged to exclude insects and be  
118 autonomously self-pollinated or left open to bee visits with two pollinator species, the  
119 domestic honey bee (*Apis mellifera*) and the red mason bee (*Osmia bicornis*); and ii)  
120 its male sterile parent, thereafter referred to as MS line, that does not produce pollen  
121 and was either hand-pollinated with pollen from many different plants of the MF line or  
122 exposed to honey bee visits and pollination. In total, 198 samples corresponding to  
123 bees, nectar, pollen and seeds were analyzed by 16S rRNA gene amplicon Illumina  
124 sequencing (**Supp. Table 1**).

125

### 126 **Experimental design and sequence analysis**

127 To investigate if bee pollination services has an additional effect on the microbial  
128 assemblage of OSR seeds, bee and plant samples were collected and submitted to  
129 bacterial community profiling analyses. Pollinator exclusion experiments were carried  
130 out inside two insect proof tunnels. Prior to the experiment, all inflorescences were  
131 covered under gas permeable osmoflux bags to exclude any contact with insects.  
132 During the experiment, half of the inflorescences on each plant were uncovered and  
133 exposed to bee pollination (seeds issued from this treatment will be referred to as BP  
134 seeds). The other half of the inflorescences were left covered. These un-touched  
135 flowers produced seeds resulting from autonomous self-pollination, hereafter referred  
136 to as ASP seeds. Inflorescences of the MS line were pollinated by hand using a paint-  
137 brush with pollen of many different plants (HP seeds). In this way, ASP, BP and HP  
138 seeds, as well as nectar, pollen and bees were collected and their DNA was extracted.  
139 Amplicon libraries of the V4 region of 16S rRNA were constructed afterwards and  
140 sequenced to characterize the bacterial assemblages present in the samples. The raw  
141 sequencing data was first resolved into Amplicon Sequence Variants (ASVs) using the  
142 software DADA2 (Callahan et al. 2016). After quality filtering, chloroplast removing and  
143 taxonomic affiliation, a total of 6.3 million reads were classified in 2,764 ASVs.  
144 According to rarefaction curves, most samples reached an asymptote at 12,000  
145 sequences and were rarefied at this value (**Supp. Figure S2**). This analysis indicated  
146 that we were able to characterize most of the diversity found in the seed samples with  
147 an average of 95.7 ASVs per seed sample in the MF line and 67.4 ASVs per sample  
148 in the MS line.

149

150 **Taxonomic composition of bee, pollen, nectar and seed microbiota**

151 Analysis of the ASV taxonomic affiliation showed that *Proteobacteria* and  
152 *Firmicutes* were the dominant *phyla* in honey bee samples (**Supp. Figure S3**). These  
153 *phyla* contain the main taxa of the “core gut” microbiome in honey bee workers such  
154 as *Frischella*, *Gillamella*, *Snodgrassella*, *Lactobacillus* and *Buchnera* spp. (Engel et al.  
155 2012). Concerning OSR pollen, samples were also dominated by *Firmicutes* and  
156 *Proteobacteria* in both years. However, we observed very contrasting results for nectar  
157 samples between years (**Supp. Figure S3**). Nectar samples from 2017 were more  
158 diverse than the ones from 2018, which were dominated by the genus *Acinetobacter*.  
159 This taxa was also found in very high abundance in the seeds (see below).

160 In the case of microbial assemblages associated to seeds, the most common  
161 bacteria belonged to the *Actinobacteria*, *Bacteroidetes*, *Firmicutes* and *Proteobacteria*  
162 (**Figure 1a, 1c**), which is in agreement with other microbiome studies performed on  
163 OSR seeds (Rybakova et al. 2017). Seed samples from 2018 were dominated by the  
164 genera *Acinetobacter* and *Pantoea* (around 80% of the reads were affiliated to these  
165 two genera; **Figure 1c**). Honey bee pollination changed significantly (logarithmic LDA  
166 score higher than 2.5) the abundances of 3 ASVs in the seeds, while mason bee  
167 pollination changed the abundances of 15 ASVs (**Figure 1d; Supp. Table 3 and 4**).

168 In the seed samples collected during 2017, *Acinetobacter* and *Pantoea* were  
169 not the most abundant taxa; the genera *Sphingobium*, *Pseudomonas*, *Lactobacillus*  
170 and *Gillamella* were the most predominant instead. Interestingly, honey bee pollination  
171 affected the relative abundances of 7 ASVs (**Figure 1b; Supp. Table 2**), 4 of which  
172 are specifically associated with honey bees and a part of the bee core microbiota  
173 (**Figure 1a**). *Arsenophonus* (Yañez et al. 2016), *Frischella* (Engel et al. 2013),  
174 *Spiroplasma* and *Lactobacillus* are bee-associated taxa and were significantly more  
175 abundant in seeds issued from BP as opposed to those issued from ASP. Specifically,

176 the ASV0075 showed 99% identity with the honey bee pathogen *Spiroplasma apis*  
177 strain B31<sup>T</sup> (Ku et al. 2014) and the ASV0100 showed 100% identity with *Lactobacillus*  
178 *mellis* strain H1HS38N, a symbiotic bacterium inhabiting the bee stomach (Olofsson et  
179 al. 2014). The other two remaining taxa enriched in bee-pollinated samples belong to  
180 the genus *Moraxella*, which is highly abundant in nectar. The acetic acid bacteria of  
181 the genus *Bombella*, which are found in the gut of bumble bees and honey bees (Li et  
182 al. 2015), were only present in the bees themselves and in the seeds issued from bee  
183 pollination. However, due to its low relative abundance, the *Bombella* ASV did not  
184 achieve the 2.5 fold change criteria. These results suggest that insect pollinators can  
185 transfer bacteria (*Arsenophonus*, *Frischella*, *Spiroplasma*, *Lactobacillus* and  
186 *Bombella*) from the insect itself or from the nectar collected to the seed through the  
187 floral pathway.

188

### 189 **Seed microbial alpha diversity is modified by honey bee pollination**

190 To further investigate how bee pollination affects the structure of seed microbial  
191 assemblages, bacterial richness and diversity indexes were assessed for the seed-  
192 associated assemblages in all the different treatments (**Figure 2**). To compare the  
193 alpha diversity in seed samples, non-parametric Wilcoxon rank-signed tests were  
194 performed on the rarefied data set. No statistically differences were found in alpha  
195 diversity in the 2017 seed samples (N=11). In contrast, variations of ASV richness and  
196 diversity were observed between seed samples in relation to the pollination mode in  
197 2018 (N=53, **Figure 2a**). Bacterial richness significantly decreased in seeds issued  
198 from honey bee pollination ( $p < 0.0001$ ; **Figure 2a**). However, we do not observe the  
199 same trend in the seeds issued from mason bee pollination (**Figure 2a**). These  
200 discrepancies observed in the two types of pollinators used in this study are probably



201 due to differences in the foraging intensity of these insects. Indeed, honey bees visited  
202 the flowers more intensively (~ 1 visit per flower every 2 min) than mason bees (~ 1  
203 visit per flower every 10 min). Moreover, while the honey bee colony used in these  
204 experiments consisted of a population of ca. 5000 worker bees with a population of  
205 foragers of at least 1000 individuals, only 200 female and male cocoons of mason bees  
206 were introduced. It is also noteworthy that the visits to the experimental flowers during  
207 the mason bee experiment was done mostly by males that were searching for nectar  
208 between matings.

209         Seeds issued from hand pollination on the MS line (HP seeds), did not differ in  
210 bacterial richness from those issued from bee pollination, suggesting that the decrease  
211 in richness (**Figure 2a**) could be partly explained by the amount of pollen deposited on  
212 the stigma and not by the selection of insect-associated taxa. When checking the  
213 evenness in the samples, we observed a significant reduction due to mason bee  
214 pollination. Indeed, *Acinetobacter* is the dominant taxa in seeds issued from mason  
215 bee pollination.

216         To assess the impact of bee pollination on the diversity of the seed microbiota,  
217 different diversity indexes were calculated. As observed with richness, there was a  
218 significant reduction in bacterial diversity due to honey bee pollination (Shannon and  
219 Faith's PD phylogenetic diversity;  $p=0.003$  and  $p<0.0001$  respectively; **Figure 2c-d**).  
220 In this case, we observed a similar trend in the diversity of the microbial assemblages  
221 associated to the seeds issued from mason bee pollination (**Figure 2c**), where the  
222 diversity calculated with the Shannon index was also reduced ( $p=0.001$ ). Altogether,  
223 these results suggest that bee pollination decreases the diversity of the microbial  
224 assemblages associated with seeds.

225

## 226 **Effect of bee pollination on the structure of the seed microbiota**

227 Similarity in community composition between samples was then estimated  
228 through PCoA ordination of unweighted UniFrac distances (**Table 1** and **Figure 3** for  
229 2018 data). The relative contribution of pollination was then investigated through  
230 canonical analysis of principal coordinates (CAP) followed by PERMANOVA (**Table 1**).  
231 The bacterial composition differed between plant materials (seeds, nectar and pollen;  
232 **Figure 3a**), nectar and seeds clustering together in the ordination plot and separated  
233 from pollen. The type of plant material explained 35.54 and 9% of the variation in  
234 microbial composition in 2017 and 2018 respectively (**Table 1**;  $p=0.0002$  and  
235  $p=0.0001$ ). As expected, microbial assemblages associated to bees were distinct from  
236 the ones associated to plant tissues. Moreover, microbes associated with the surface  
237 of the highly social honey bees were very different to the microbes living inside the bee  
238 gut (Engel et al. 2012). However, this is not the case for the solitary mason bee, which  
239 harbored similar microbial assemblages on its surface and gut (**Figure 3b**).

240 Differences in microbial composition of ASP and BP seeds were not statistically  
241 different in 2017 (**Table 1**). However, in 2018, flowers pollinated by honey bees  
242 produced seeds with significant changes in microbial composition compared with those  
243 from ASP seeds, showing that honey bee pollination can change the composition of  
244 the seed microbiota (**Figure 3c**). Moreover, these BP seed microbial assemblages  
245 diverge from those of ASP seeds and are closer to those of pollen (**Figure 3a**).  
246 According to CAP analyses, honey bee pollination explained 12.3% ( $p = 0.001$ ) of the  
247 variation in bacterial community composition in the seed samples in 2018. In the  
248 experiments performed in 2017, we observed a similar trend, although the change was  
249 not statistically different due to the low size of the sample (**Table 1**). In contrast, we did  
250 not observe changes in the microbial composition of the seeds issued from flowers

251 pollinated by mason bees, probably due to the reduced visitation rates observed during  
252 the experiment.

253 During both years, the bacterial composition of the seeds produced by honey  
254 bee pollination did not differ from that of HP seeds on the MS line (**Table 1**). Ordination  
255 plots did not show a segregation between the seeds from the honey bee and the hand  
256 pollination treatments. In agreement with these results, the constrained analysis of  
257 principal coordinates revealed that the pollination mode of the sterile line had no  
258 significant effect on seed-associated microbial assemblages. This fact suggests that  
259 the differences observed between seeds that resulted from honey bee pollinated  
260 flowers and seeds that came from ASP flowers could be due to the amount and the  
261 varied origin of the pollen, and not necessarily to the insect associated taxa.

262

### 263 **Pollination by honey bees increases microbial beta dispersion among seeds**

264 The PCoA (**Figure 3c**) showed that following honey bee pollination, the  
265 bacterial assemblages associated to BP seeds were more variable than those of the  
266 ASP seeds. Our experimental design involved having two pollination modes  
267 represented within each plant, which allowed for the comparison of the variability in the  
268 bacterial composition of seeds issued from those pollination modes. We compared the  
269 distances of each plant in the principal coordinate space (created using the unweighted  
270 UniFrac distances) to the group (pollination mode) centroid (Anderson et al., 2006;  
271 Vannette et al. 2017). In this way, we assessed the beta dispersion as the variability  
272 of the ASV composition at the plant level (inter-plant variation, n=9, n=10 & n=10 for  
273 honey bee, mason bee and HP on the MS line respectively). We did not observe  
274 statistically significant changes in distance to the centroid (beta dispersion) between  
275 ASP and BP seed microbial assemblages in the mason bee treatment. No differences

276 in beta dispersion were detected between honey bee and hand pollinated seed  
277 microbial assemblages in the MS line. However, we did observe that the beta  
278 dispersion was significantly higher in seed communities that were exposed to honey  
279 bee pollination in contrast to those that came from flowers with ASP (Wilcoxon rank-  
280 signed test,  $p = 0.02$ ; **Figure 4**).

281

## 282 **DISCUSSION**

283         Currently, little is known about the neutral or niche-based ecological processes  
284 involved in the assembly of the seed microbiota. Niche processes, like selection by the  
285 environment, have been shown to shape the structure of seed-associated fungal  
286 assemblages (Klaedtke et al. 2015). Furthermore, host-filtering processes have been  
287 shown to be involved in the assembly of oilseed rape (Rybakova et al. 2017) and  
288 tomato (*Solanum lycopersicum*) seed microbiomes (Bergna et al. 2018). On the other  
289 hand, neutral processes related to ecological drift have been described as important  
290 drivers of the structure of the bacterial communities of radish (*Raphanus sativus*) seeds  
291 (Rezki et al. 2018). Taken together, these pioneer studies suggest that seed-  
292 associated microbial communities consist of a few dominant taxa that are probably  
293 niche-selected, and multiple scarce taxa whose distributions could be explained by  
294 neutral processes. The present study supports the hypothesis that insect pollination is  
295 a neutral ecological process (microbial dispersal) that participates in the dispersal of  
296 microbes between flowers and seeds and influences the assembly of the seed  
297 microbiota. Our results show that *Apis* pollination can change diversity of seed-  
298 associated bacterial assemblages and in some cases introduce bee-associated taxa.

299         The proximity between a floral structure and the developing seed suggests that  
300 microbes associated with the flower could eventually colonize the developing seeds

301 through the floral pathway. The floral pathway for the colonization of the seed tissue is  
302 less specialized than the vascular (internal) pathway. Indeed, plant-pathogenic  
303 bacteria can colonize both host and non-host seeds via the floral pathway (Darsonval  
304 et al., 2008). Thus, the mechanisms dispersing microbes into flowers (like insect  
305 pollination) could have important implications for the transmission of non-specialized  
306 microbes to the developing seeds. Previous studies have already shown that insect  
307 pollination modifies the composition of microbial assemblages associated to flowers  
308 (Ushio et al 2015), nectar (Aizenberg-Gershtein et al. 2013; de Vega et al. 2013;  
309 Vannette et al. 2017) and pollen (Manirajan et al. 2016); however, virtually nothing is  
310 known about the effect of insects pollinators on plant seed microbial communities.  
311 According to our data, the changes observed in the seed microbiota were mostly  
312 associated with the honey bee (*A. mellifera*) and very mild effects were observed with  
313 the red mason bee (*O. bicornis*). During our experiments, the bee density between the  
314 two bee species differed considerably (honey bees being an order of magnitude more  
315 abundant) and we observed a strong difference in visitation rates. These differences  
316 are reflected in our results on the seed microbiota, suggesting that the amount of  
317 contact between the insect and the flower might be a determining factor in the effect  
318 of the insect on the seed microbiota. Indeed, the time that a bee spends foraging on a  
319 flower is correlated with the transmission of bee pathogens (Adler et al. 2018). The  
320 potential role that foraging behaviour (especially the foraging intensity and the handling  
321 time, i.e. time spent per flower) has on the seed microbiota should be assessed in  
322 order to shed light on the role that different pollinators might have in the transmission  
323 of seed-borne diseases (Alekkett et al. 2014; Truyens et al. 2015).

324 The microbial assemblages associated with seeds examined in this study are  
325 dominated by members of the *Actinobacteria*, *Bacteroidetes*, *Firmicutes* and

326 *Proteobacteria* phyla (**Figure 1**). These phyla were also prevalent in other studies of  
327 the oilseed rape seeds (Rybakova et al. 2017). The taxonomic profiles were very  
328 different between the two years. This is not so surprising since biotic factors (such as  
329 the type of soil, the climatic conditions, and also the field management practices) have  
330 a strong influence on the seed microbiota (Klaedtke et al. 2016). *Acinetobacter* was  
331 the dominant genus of the seeds and the nectar in 2018. This is a widespread genus  
332 in animals, plants and the environment (Doughari et al. 2011). Specifically in plants,  
333 *Acinetobacter* spp. have been described as plant-growth-promoting bacteria (Suzuki  
334 et al. 2014). In our 2018 experiments, this taxon was already present in samples of  
335 pollen and nectar prior to the pollination exclusion experiments. It is thus possible that  
336 *Acinetobacter* acted as a pioneer taxon in seeds exerting a strong priority effect in the  
337 final microbial composition of the seed.

338 While mason bee carries pollen in its abdominal scopa, the honey bee  
339 aggregates pollen grains by adding regurgitated nectar or diluted honey to transport it  
340 to its nest in the corbiculae of its hind legs. The addition of nectar rises the humidity of  
341 the pollen grain, causing it to swell and expose the intine (Human & Nicolson 2008).  
342 The taxonomic profiles of the 2017 seed samples showed that honey bee pollination  
343 changed the seed microbiota by increasing the abundance of bee- or nectar-  
344 associated taxa. It is then tempting to speculate that the way the honey bee processes  
345 pollen allows for the transmission of bee-associated bacteria to the seed. Indeed,  
346 *Spiroplasma*, *Lactobacillus*, *Arsenophonus*, *Frischella* and *Bombella* are insect  
347 symbiotic bacteria living in the bee gut (Corby-Harris et al. 2014; Engel et al. 2013;  
348 Yanez et al. 2016; Yun et al. 2017) and were more abundant (and in some cases, only  
349 present) in seeds issued from BP as opposed to ASP. These results illustrate the  
350 possibility of insect-transmitted bacteria colonizing the seed. Interestingly, two of the

351 identified ASVs affiliated to *Spiroplasma* and *Arsenophonus* could act as bee  
352 pathogens, suggesting that plants could act as a reservoir for bee pathogens.

353 In our experimental design, the effect of bee pollination on the seed microbiota  
354 was assessed in two different plant genotypes: a male fertile line and a male sterile  
355 line that does not produce pollen. Our results on the male sterile line show that hand  
356 pollination did not differ from bee pollination. This would suggest that the diverse origin  
357 (many source plants) and copious amount of pollen delivered either by a bee or by a  
358 paintbrush have similar effects on the seed microbiota. On the contrary, the reduced  
359 amount and single origin of the pollen of ASP causes significant differences to the seed  
360 microbiota as compared with that of the seeds resulting from bee pollination. It is known  
361 that the amount of pollen delivered to the stigma has an impact on the germination  
362 rates of the pollen grains: small pollen populations germinate poorly. This population  
363 effect is partly explained by the availability of certain growth factors such as calcium  
364 ions (Brewbaker & Kwack 1963), flavonols (Taylor & Hepler 1997) and phytoalkaloids  
365 alpha (Chen et al. 2000). The greater availability of nutrients presented by large pollen  
366 populations might also provide nutrients for bacteria. The availability of nutrients  
367 fosters competition between the microbial taxa, which could explain the observed  
368 decrease in species richness observed in honey-bee-pollinated seeds.

369 Alternatively, the decrease in alpha diversity might be related to the period  
370 during which the stigma is receptive. Bacteria are generally unable to actively penetrate  
371 the plant tissue and rely on openings such as wounds, stomata, lenticels and  
372 nectarhodes (Gimenez-Ibanez et al. 2010). A possible route to enter the seed is  
373 provided by the stigma surface through which bacteria can penetrate (Compant et al.  
374 2011; Truyens et al. 2015), possibly during the penetration of the pollen tubes.  
375 Following flower opening, oilseed rape flowers require on average ~13h of exposure

376 to pollinators to complete their sexual function (Bell & Cresswell 1998). The removal of  
377 pollen triggers flower senescence (Bell & Cresswell 1998), so in our experiments the  
378 flowers visited by bees were likely to have senesced faster than the flowers left to self-  
379 pollinate autonomously. The increased duration of flower longevity in the ASP  
380 treatment may have facilitated the entry of bacteria, explaining then the higher diversity  
381 of the seed microbial communities. Future experiments should aim at disentangling  
382 how insect pollination and flower longevity affect the seed microbiome.

383 Honey bee pollination enhanced the variation in the structure of seed bacterial  
384 assemblages (**Figure 4a**). This result is in agreement with Vanette *et al.* (2017) who  
385 reported that pollinators increase dispersal of microorganisms and ultimately enhance  
386 dissimilarity between nectar microbial assemblages. These findings could be explained  
387 by a high stochasticity in the order in which bacterial species arrive (stochasticity of  
388 microbial dispersal), and due to strong priority effects, the composition of the  
389 assemblages can diverge (Vannette et al. 2017). Pollination by *Apis* would then favor  
390 the arrival of new species to the flower and seed, increasing the variability of the seed-  
391 associated microbial communities. Our data could serve as a foundation for additional  
392 experiments that target directly the effect of priority effects on beta dispersion and on  
393 the assembly of the seed microbial communities.

394

## 395 **Conclusions**

396 This study aimed to uncover the contribution of bee pollination to the seed  
397 microbiota. We have identified differences in richness, diversity and species  
398 composition in seeds issued from bee pollination, as compared to those from flowers  
399 with autonomous self-pollination. Our findings with two different bee species suggest  
400 that foraging behaviour (foraging rates/intensity) might mediate the insect's effect on  
401 the seed microbiota. Additionally, the amount and origin of the pollen might also have



402 an effect. These results provide novel insights about determinants involved in the  
403 transmission of bacteria from flower to seeds, and have important implications in terms  
404 of re-evaluating the services provided by pollinators, which could include microbe  
405 transfer.

406

## 407 **MATERIALS AND METHODS**

### 408 **Pollinator exclusion experiments**

409 Pollinator exclusion experiments were performed inside two 22 x 8 m insect  
410 proof tunnels at the INRA research station at Avignon during 2017 and 2018. Inside  
411 the tunnels, seeds of oilseed rape (*Brassica napus*) were sown in the soil in four 18 m  
412 long rows (20 plants per row). Two winter oilseed rape lines were sown side by side: a  
413 male fertile F<sub>1</sub> hybrid line 'Exocet' and its male sterile parent which does not produce  
414 pollen. Winter oilseed rape was chosen because it is a highly self-fertile plant that  
415 produces nectar attractive to bees, and because a male sterile line was available.  
416 Plants were watered two times a day with an automated water drip system.

417 In 2017, the pollinator exclusion experiment was only carried out using honey  
418 bees on 5 plants of the male fertile line in a single tunnel. In 2018, the pollinator  
419 exclusion experiments included 1) honey bees on the male fertile line in one tunnel 2)  
420 mason bees on the male fertile line in a second tunnel and 3) honey bees on the male  
421 sterile line in the first tunnel. For each experiment, ten plants were chosen as  
422 experimental plants based on their homogenous appearance. On each plant, six  
423 flowering panicles were marked using flower markers of two different colours (three  
424 panicles each) and bagged with hydrophilic plastic bags made of osmolux film (Pantek  
425 France, [www.pantek-france.fr/agriculture.html](http://www.pantek-france.fr/agriculture.html)). The osmolux bags are gas-  
426 permeable, but prevent all contact with insects, even small ones such as thrips

427 (Thysanoptera) (Perrot et al. 2018). On the day of the introduction of the bees into the  
428 tunnels, three color-coded panicles were un-bagged and exposed to bee visits (bee  
429 pollination treatment), while the three others remained covered (autonomous self-  
430 pollination treatment). Foraging bees were allowed to forage freely amongst the  
431 experimental plants and those that were not selected for the experiment. On the male  
432 sterile line, the panicles that remained bagged during the introduction of the bees were  
433 pollinated by hand using a fine paint brush with pollen coming from many flowers  
434 (>200) from several plants of the male fertile line (>10). After 48 hours, the bees were  
435 removed from the tunnels and the uncovered panicles were re-bagged. All panicles  
436 were kept bagged for an additional 48 hours to ensure that no bee was left in the  
437 tunnels, at which point all bags were removed.

438 One tunnel was used to perform experiments using honey bees (*Apis mellifera*)  
439 where we introduced a 5-frame hive (adult population ~5000). In the other tunnel, 100  
440 male and 100 female cocoons of the red mason bee (*Osmia bicornis*) were introduced.  
441 Male mason bees were highly active visiting flowers and mating with the females during  
442 the experiment. The female mason bees were mostly inactive after mating and due to  
443 our experimental design, they were removed from the tunnel before they started  
444 provisioning their nests.

445

#### 446 **Material collection**

447 Prior to the introduction of bees into the tunnels, pollen and nectar samples were  
448 collected from bagged flowers and kept at -20 °C until extraction. Pollen was collected  
449 by dissecting closed flower buds and separating the anthers. Anthers were dried for  
450 4 h at room temperature in glass Petri dishes. To recover the pollen, the dried anthers  
451 were placed in a steel tea ball and vibrated using a Vibri Vario tomato vibrator. Nectar

452 was collected with 2  $\mu$ l capillary tubes between the base of the anthers and then  
453 transferred to 2 ml Eppendorf containers using a pipette bulb. During the experiment,  
454 honey bee foragers were captured and their pollen loads removed (in the case of honey  
455 bees) and frozen for further analysis. Once mature (2 months after pollination), OSR  
456 fruits (siliques) were collected in large paper bags and taken into the lab. In aseptic  
457 conditions, seeds were removed from the siliques and placed in small paper bags.

458

### 459 **DNA extraction, amplicon library construction and sequencing**

460 For seed sample preparation, a total of 0.5 g of oilseed rape seeds of each  
461 sample were transferred to sterile tubes containing 2 ml of PBS supplemented with  
462 0.05% (vol/vol) of Tween 20. Samples were incubated for 2 h and 30 min at 4°C under  
463 constant agitation (150 rpm). In the case of bee samples preparation, to obtain bee  
464 surface microbial assemblages, bees were sonicated in 1 ml of PBS buffer with Tween  
465 20 0.05% (vol/vol). After removing the liquid, insect samples were re-suspended in  
466 1 ml of PBS and crushed to recover the microbes living inside the bees. All the  
467 suspensions were then centrifuged (12,000  $\times g$ , 20 min, 4°C) and pellets were stored  
468 at -20°C until DNA extraction. Total DNA extraction was performed with the PowerSoil  
469 DNA isolation kit (MoBio Laboratories) using the manufacturer's protocol.

470 Amplification, purification and pooling for amplicon library construction were  
471 conducted following the protocol described in Barret *et al.* 2015. Briefly, for amplicon  
472 construction two rounds of PCR were performed. The first round was designed to target  
473 the region V4 of the 16S rRNA with the PCR primers 515f/806s (Caporaso *et al.* 2011).  
474 All PCRs were performed with a high-fidelity polymerase (AccuPrime *Taq* DNA  
475 polymerase; Invitrogen) using the manufacturer's protocol and 10  $\mu$ l of environmental  
476 DNA suspension. After amplicon purification, a second round of amplification was

477 performed with 5 µl of purified amplicons and primers containing the Illumina adaptors  
478 and indexes. All amplicons were purified, quantified and pooled in equimolar  
479 concentrations. Finally, amplicons libraries were mixed with 10% PhiX control  
480 according to Illumina's protocols. Two sequencing runs were performed in this study  
481 with MiSeq reagent kit v2 (500 cycles) for the samples of 2017 and MiSeq reagent kit  
482 V3 (600 cycles) for the samples of 2018.

483

#### 484 **Data Analysis**

485 MiSeq runs were analysed separately. Primers sequences of fastq files were  
486 first cut off using Cutadapt 1.8 (Martin 2011). Files were then merged and processed  
487 with DADA2 v.1.8.0 (Callahan et al 2016) according to the recommendations of the  
488 workflow "DADA2 Pipeline Tutorial". The workflow was modified in the truncLen  
489 parameter to adjust it to the quality of the sequencing run. The 16S rRNA amplicon  
490 sequence variants (ASV) resulting from DADA2 were aligned with a naive Bayesian  
491 classifier against the Ribosomal Database Project training set 16 database. Statistical  
492 analyses were done with Rstudio v3.3 using the R package *phyloseq* v1.24.2  
493 (McMurdie & Holmes 2013). The *Metacoder* R package v 0.3.0.1 (Foster et al. 2017)  
494 was used to plot the distribution of ASV, associated with a taxonomic classification in  
495 heat trees. Observed taxa richness, evenness, and diversity were calculated on a  
496 rarefied dataset at 12,000 reads per sample and differences were assessed by  
497 Wilcoxon signed-rank tests. Variances in community composition between the different  
498 samples were assessed by unweighted UniFrac distance (Lozupone & Knight 2005).  
499 Principal coordinate analysis (PCoA) was used for ordination of UniFrac distances.  
500 Permutational multivariate analysis of variance (PERMANOVA, Anderson 2017) was  
501 calculated to investigate the effect of pollinators on microbial community profiles as

502 implemented by the package *vegan* v2.5-3 in R. Moreover, to quantify this contribution,  
503 a canonical analysis of principal coordinates was performed with the function *capscale*  
504 of the *vegan* package. Changes in relative abundance of ASV between the different  
505 seed samples were determined using linear discriminant analysis (LDA) effect size  
506 using the LefSe tool (Segata et al. 2011) available at  
507 <http://huttenhower.sph.harvard.edu/galaxy>.

508 To compare the beta dispersion amongst pollination modes, we quantified the  
509 variability in ASV composition within each pollination treatment using the *betadisper*  
510 function in the *vegan* package in R. Beta dispersion is measured by the distance to the  
511 centroid of each treatment group in the principal coordinate space (Anderson et al.  
512 2006).

513

#### 514 **Data availability**

515 The raw sequencing data is available at the European Nucleotide Archive (ENA)  
516 under the study accession PRJEB31847. Tables and scripts used in this work are  
517 publicly available in GitHub.

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686 analyses. We also want to apologize to any authors whose relevant work was not cited  
687 in this article due to space constraints.

688

689 **Authors' contributions**

690 AP, BV, MB and GTC designed the study and made substantial contributions to the  
691 analysis and interpretation of the results. AP and GTC carried out the data analysis.  
692 AP and BV performed the tunnel experiments. GTC and BM constructed the amplicon  
693 libraries. AP and GTC wrote the manuscript with input from the other authors. All  
694 authors read and approved the final manuscript.

695

696 **Conflict of interest statement**

697 The authors declare no conflict of interest.

698

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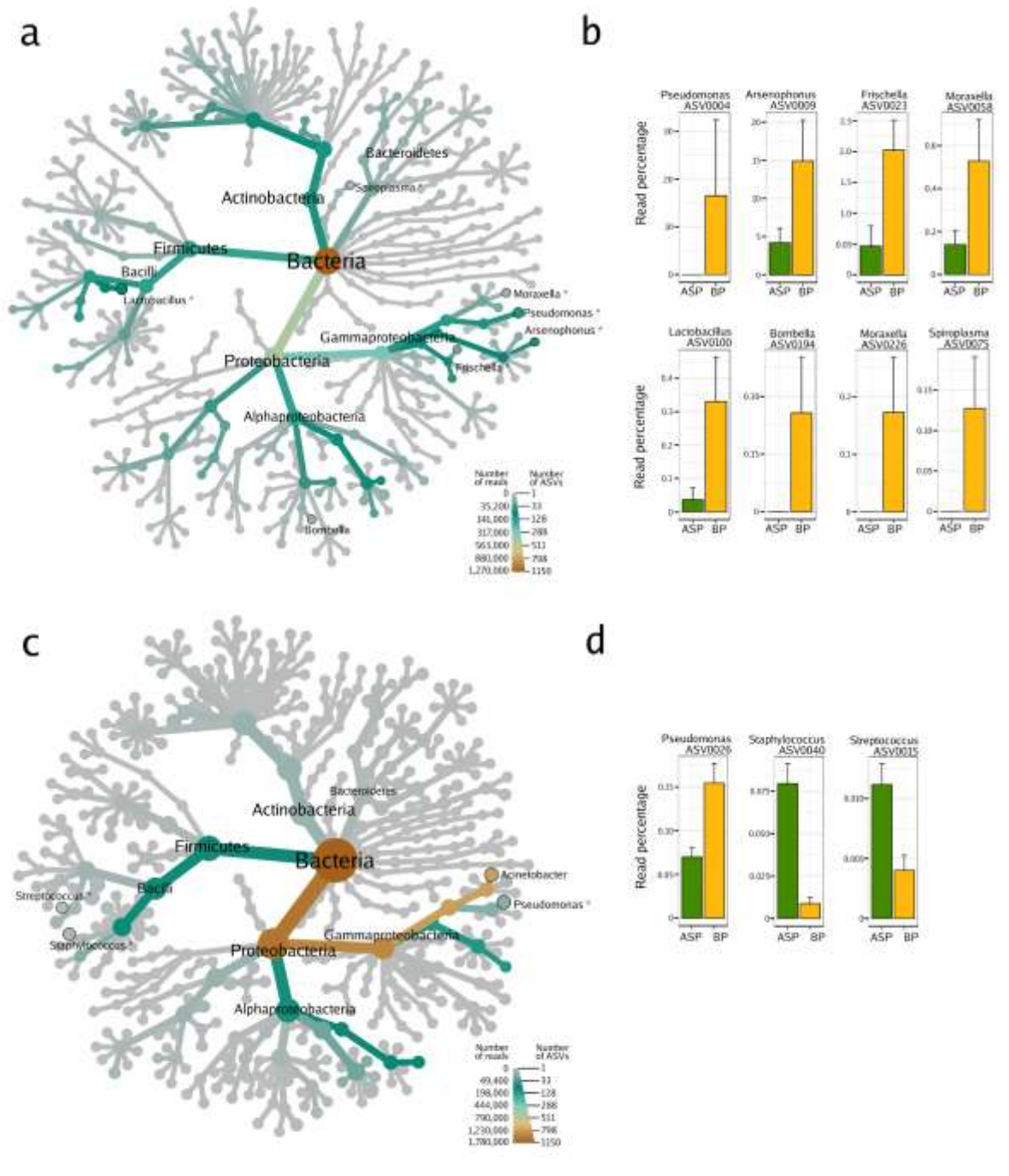
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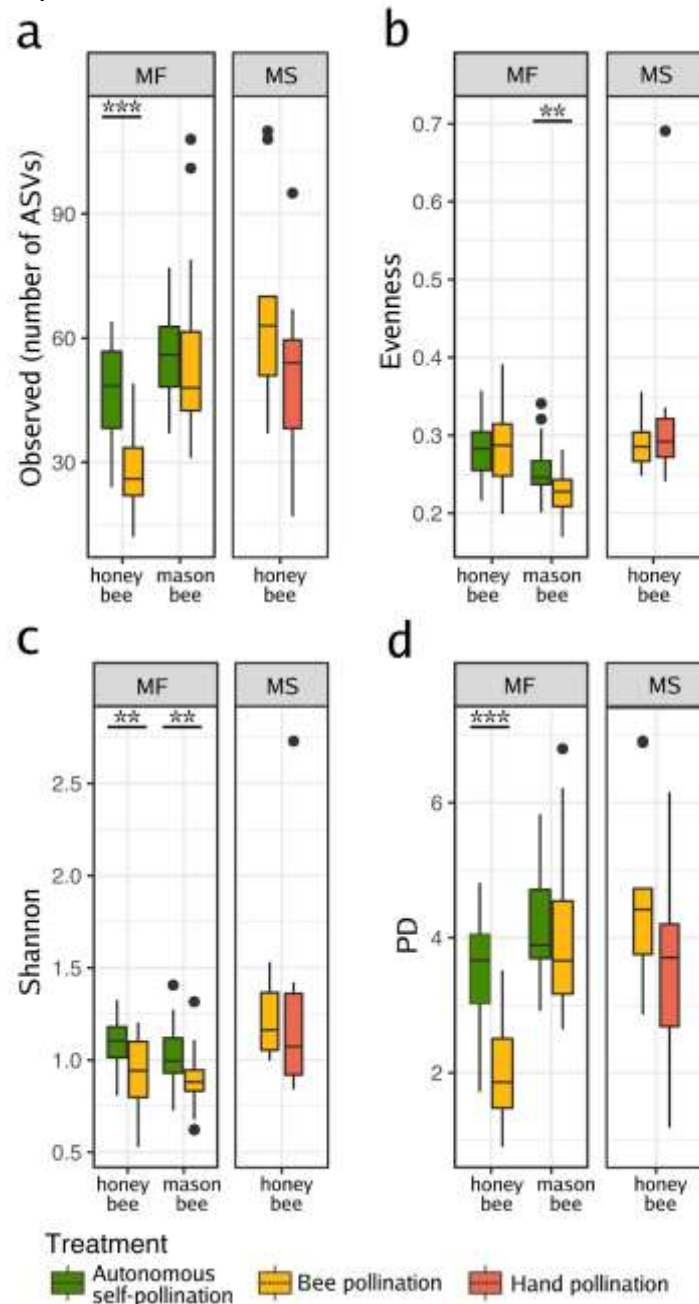
707 **Figures and figure legends**

708 **Figure 1. Microbial composition of oilseed rape seed samples issued from honey**  
 709 **bee pollination or autonomous self-pollination.** Heat trees show the microbial  
 710 composition of the seeds samples harvested in 2017 (**a**) and 2018 (**c**). The size of the  
 711 nodes refers to the number of ASVs of known identity and the color of the nodes and  
 712 edges represent the ASV read abundance. Asterisks (\*) indicate the taxonomic  
 713 affiliation of ASVs with significant changes in relative abundance (according to Linear  
 714 Discriminant Analysis Effect Size; LefSe) in relation to the pollination mode. ASVs with  
 715 significant changes in relative abundance are displayed on the right part of the figure  
 716 (**b, d**). BP: honey bee-pollination; ASP: autonomous self-pollination.



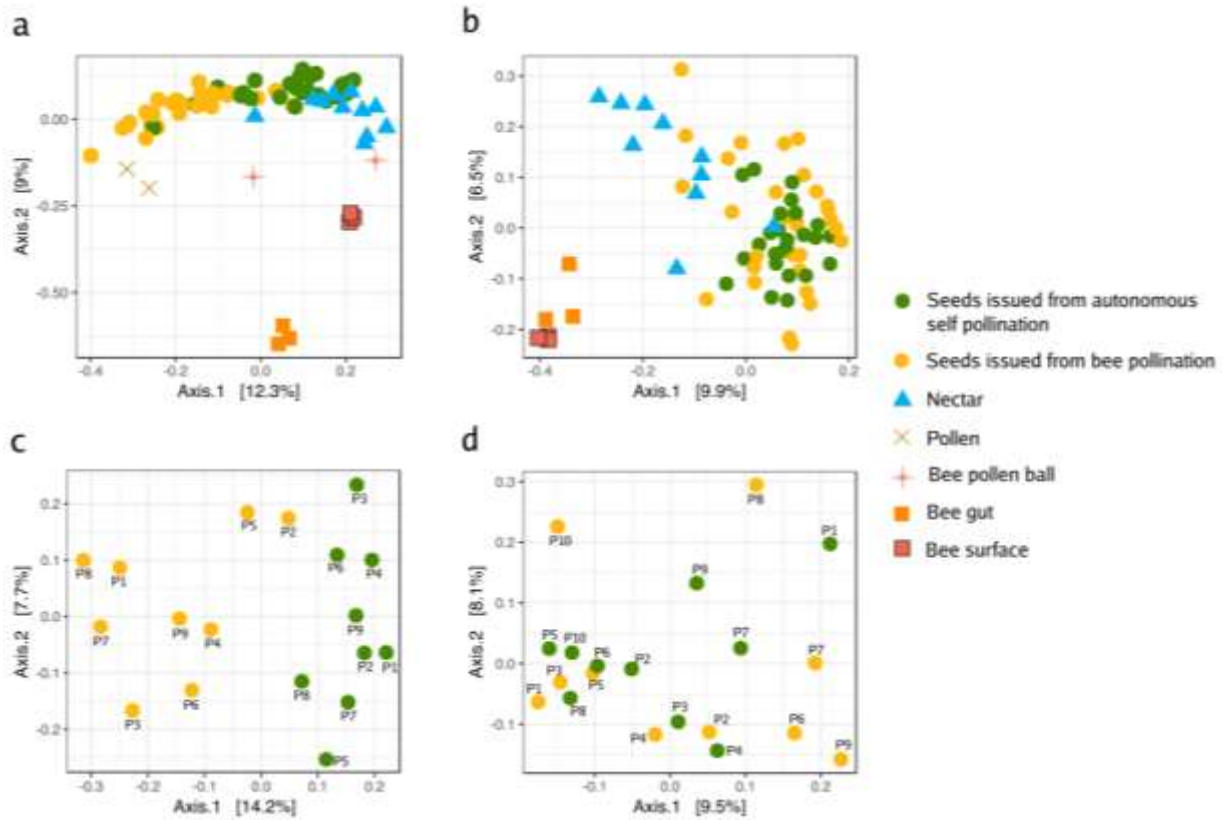
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719 **Figure 2. Changes in microbial richness and alpha diversity between seed**  
720 **samples.** Observed richness (a), evenness (b) and diversity (Shannon and Faith's PD  
721 phylogenetic diversity; c, d). The indexes were estimated in seed samples harvested  
722 from oilseed rape male fertile plants pollinated by bees or left for autonomous self-  
723 pollination. Additional indexes were calculated on seed samples harvested from a male  
724 sterile line that was hand- or insect-pollinated. Wilcoxon rank-signed tests were  
725 performed to assess the effect of pollination on richness and alpha diversity. Asterisks  
726 denote statistically significant differences between conditions considered as a *p*-  
727 *value*<0.05 (\*), a *p*-*value*<0.01 (\*\*), and a *p*-*value*<0.001 (\*\*\*). Richness, evenness and  
728 Shannon diversity were assessed with the number of ASVs rarefied at 12,000  
729 sequences per sample. MF: male fertile line; MS: male sterile line.



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732 **Figure 3. Ordination of unweighted UniFrac matrices with principal coordinate**  
733 **analysis (PCoA) showing variation in microbial composition.** PCoA plots show the  
734 ordination of all samples from the honey bee (a), and the mason bee (b) experiment.  
735 Seed microbial assemblages of each plant (pooled samples) are represented in panel  
736 (c) for HB pollination, and in panel (d) for MB.

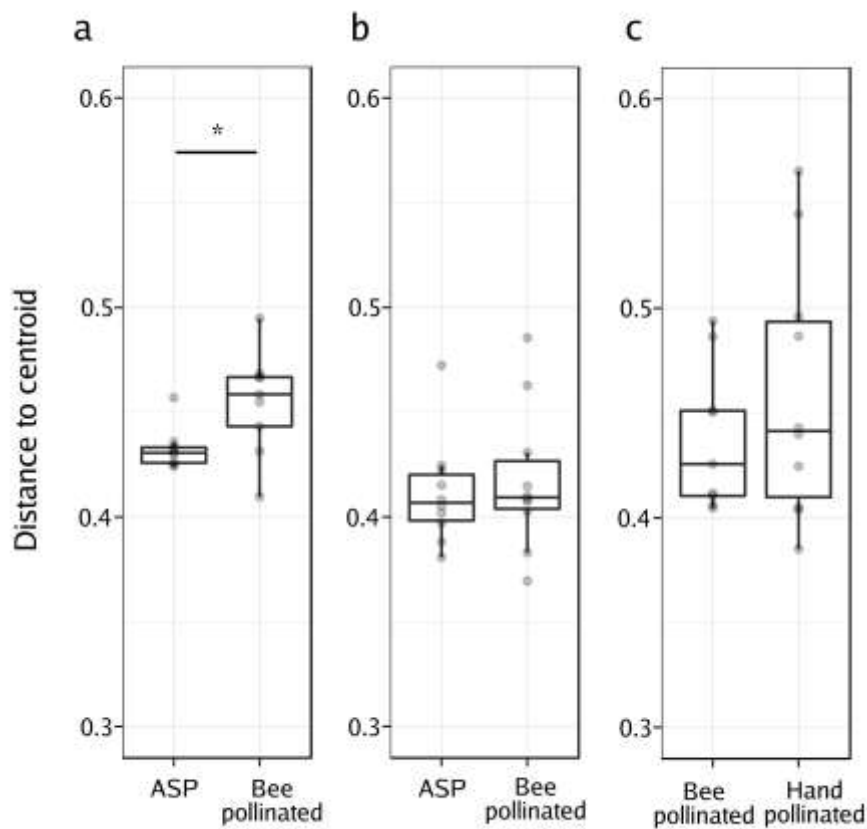


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741 **Figure 4. Effect of the pollination mode on the structure of seed microbial**  
742 **assemblages.**

743 Analysis of the multivariate homogeneity of group dispersions (variances). Boxplots  
744 represent the distance to the centroid of seed-associated microbial communities of  
745 male fertile (a, b) and male sterile plants (c) submitted to different modes of pollination.  
746 **a)** Distance to the centroid of seeds issued from male fertile plants subjected to  
747 autonomous self-pollination (ASP) or honey bee pollination, **b)** distance to the centroid  
748 of seeds issued from male fertile plants subjected to ASP or mason bee pollination, **c)**  
749 distance to the centroid of seeds issued from male sterile plants subjected to honey  
750 bee pollination or hand-pollinated with pollen of different plants. Wilcoxon signed-rank  
751 tests were performed to assess the effect of the pollination mode on the distance to  
752 the centroid. Asterisks denote statistically significant differences between conditions  
753 considered as a  $p$ -value < 0.05 (\*).



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758 **Table 1. Results of the constrained analysis of principal coordinates.** Proportion  
 759 of variance explained by the indicated variable on the different data sets. The  
 760 proportion of constrained inertia, F-values and *p*-values were calculated through a  
 761 canonical analysis of principal coordinates followed by PERMANOVA. Seeds issued  
 762 from autonomous self-pollination and bee pollination are referred to as ASP and BP  
 763 seeds, respectively.

Data set name	Year	Data set material	Explanatory variable	Samples	Proportion of constrained inertia (%)	F-value	<i>p</i> -value
All samples	2017 2018	ASP and BP seeds, nectar, pollen, bees	Year	198	<b>5.7</b>	<b>11.45</b>	<b>0.0001</b>
Experiment 2017	2017	ASP and BP seeds, nectar, pollen	Material	16	<b>35.54</b>	<b>3.584</b>	<b>0.0002</b>
Experiment 2017 male fertile line	2017	ASP and BP seeds	Pollination mode	10	NA	1.19	NS
Experiment 2017 male sterile line	2017	ASP and BP seeds	Pollination mode	12	NA	1.073	NS
Experiment 2018	2018	ASP and BP seeds (honey bee and mason bee), nectar, pollen	Tunnel	134	<b>4.5</b>	<b>6.22</b>	<b>0.0001</b>
Honey bee experiment 2018	2018	ASP and BP seeds, nectar, pollen	Material	69	<b>9.3</b>	<b>3.28</b>	<b>0.0001</b>
Honey bee experiment 2018 male fertile line	2018	ASP and BP seeds	Plant ID	18	NA	0.91	NS
Honey bee experiment 2018 male fertile line	2018	ASP and BP seeds	Pollination mode	18	<b>12.3</b>	<b>2.23</b>	<b>0.001</b>
Honey bee experiment 2018 male sterile line	2018	ASP and BP seeds	Pollination mode	21	NA	1.29	NS
Mason bee experiment 2018 male fertile line	2018	ASP and BP seeds	Plant ID	20	NA	1.33	NS
Mason bee experiment 2018 male fertile line	2018	ASP and BP seeds	Pollination mode	20	NA	1.14	NS

