

1 **Running title:** Biocontrol bacteria alleviate replanting problem

2

3 **Inoculation of biocontrol bacteria alleviated *Panax ginseng* replanting problem**

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11

12 **Highlight:** Changes in rhizospheric microbial communities driven by ginseng plants
13 of different ages and developmental stages could cause microecological degradation.
14 Biocontrol using microbial antagonists effectively alleviated the replanting problem.

15

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27 **Linlin Dong and Jiang Xu have equal contributions to this work.**

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30

31 **Abstract**

32 Replanting problem is a common and serious issue hindering the continuous
33 cultivation of *Panax* plants. Changes in soil microbial community driven by plant
34 species of different ages and developmental stages are speculated to cause this
35 problem. Inoculation of microbial antagonists is proposed to alleviate replanting
36 issues efficiently.

37 High-throughput sequencing revealed that bacterial diversity evidently decreased,
38 and fungal diversity markedly increased in soils of adult ginseng plants in the root
39 growth stage. Relatively few beneficial microbe agents, such as *Luteolibacter*,
40 Cytophagaceae, *Luteibacter*, *Sphingomonas*, Sphingomonadaceae, and Zygomycota,
41 were observed. On the contrary, the relative abundance of harmful microorganism
42 agents, namely, Brevundimonas, Enterobacteriaceae, *Pandoraea*, Cantharellales,
43 *Dendryphion*, *Fusarium*, and Chytridiomycota, increased with plant age. Furthermore,
44 *Bacillus subtilis* 50-1 was isolated and served as microbial antagonists against
45 pathogenic *Fusarium oxysporum* of ginseng root-rot, and its biocontrol efficacy was
46 67.8% using a dual culture assay. The ginseng death rate and relative abundance of
47 *Fusarium* decreased by 63.3% and 46.1%, respectively, after inoculation with 50-1
48 in replanting soils. Data revealed that changes in the diversity and composition of
49 rhizospheric microbial communities driven by ginseng of different ages and
50 developmental stages could cause microecological degradation. Biocontrol using
51 microbial antagonists was an effective method for alleviating the replanting problem.

52

53 **Keywords**

54 *Panax ginseng*; microbial communities; plant age; developmental stage; replanting
55 problem; biocontrol.

56

57 **Introduction**

58 *Panax ginseng* C.A. Meyer demonstrates anti-inflammatory and antitumor effects,
59 and it is commonly used in traditional Chinese medicine (Choo *et al.*, 2008; Ernst,
60 2010). The current annual global market value of this species is approximately 3.5
61 billion dollars (Hong *et al.*, 2006). Wild ginseng resources are becoming scarce
62 because of excessive and predatory exploitation, and wild ginseng is gradually
63 substituted with cultivated ginseng in mainstream market (Li *et al.*, 2012; Wu *et al.*,
64 2013). Ginseng is continuously cultivated in fixed plots for 4–5 years, and
65 subsequent replanting usually fails because of continuous cropping obstacles (Ying
66 *et al.*, 2012a). Several years of crop rotation is needed in successful replanting.
67 Large-scale deforestation increases to satisfy the market demand for ginseng.
68 Moreover, conflict between the operation of ginseng industry and environmental
69 conservation has worsened. Replanting issue is a severe drawback hindering the
70 development of ginseng industry. Hence, this problem must be addressed urgently.

71 Factors such as deterioration of soil physicochemical properties,
72 allelopathy/autotoxicity, outburst of soil-borne disease, and soil microbial
73 community changes cause replanting problem (Ogweno & Yu, 2006; Wu *et al.*, 2008;
74 Huang *et al.*, 2013). Changes in soil microbial community is one of the main factors
75 hindering crop replantation (Nayyar *et al.*, 2009; Manici *et al.*, 2013). Xiao *et al.*
76 (2016) reported that the lack of balance in rhizospheric microbial communities is one
77 of the key factors causing discontinuous ginseng cultivation. Microbial communities
78 exhibit evident changes during ginseng cultivation, thereby causing an imbalance in
79 microbial community (Ying *et al.*, 2012b). Increase in the relative abundance of
80 pathogenic microorganism could lead to the occurrence of soil-borne disease. Taken
81 together, changes in rhizospheric microbial community are suggested to cause the
82 replanting issues.

83 Many biotic or abiotic factors lead to changes in rhizospheric microbial
84 communities. Plants of different ages can alter microbial community (Wu *et al.*,
85 2015). Differences in diversity and composition of soil microbial community were
86 observed under continuous cropping of *P. notoginseng*; moreover, fungal diversity

87 and microbial taxa in soil microbial community are related to seedling mortality
88 (Dong *et al.*, 2016). Li *et al.* (2012) found that differences between rhizospheric and
89 non-rhizospheric soil microbial community in a particular site tend to become
90 increasingly significant with ginseng growth. *Panax* plants with different ages in a
91 site drive changes in microbial community. Plant developmental stage influences
92 microbial community structure and activity, and marked shifts in diversity and
93 relative activity are observed in soil microbial communities of different
94 developmental stages (Houlden *et al.*, 2008). However, the information whether
95 *Panax* plants of different ages and developmental stages mediate microbial
96 community is unclear. We hypothesized that changes in the rhizospheric microbial
97 community driven by ginseng plants of different ages and developmental stages
98 could cause microecological degradation.

99 Root rot is a severe disease that hinders the replantation of *Panax* plants (Guo *et*
100 *al.*, 2009). This soil-borne disease is caused by the pathogenic fungus *Fusarium*
101 *oxysporum*, which is the main agent in *Panax* plants (Miao *et al.*, 2006; Dong *et al.*,
102 2016). The relative abundance of *F. oxysporum* increased with the notoginseng
103 cultivation and death rate of seedlings was significantly related to its abundance
104 (Dong *et al.*, 2016). Thus decrease in the abundance of pathogenic *F. oxysporum*
105 could alleviate the occurrence of root-rot. Biological control using microbial
106 antagonists has attracted interest as an effective method to control plant pathogens
107 due to its non-toxic nature (Zheng *et al.*, 2011). Many studies reported that
108 biocontrol bacteria, such as *Pseudomonas fluorescens* and *Bacillus*
109 *amyloliquefaciens* RWL-1, show evident growth inhibition activity against *F.*
110 *oxysporum* in tomato (Kamilova *et al.*, 2006; Shahzad *et al.*, 2017). Nevertheless,
111 microbial antagonists against ginseng root rot is scarce, and application of biocontrol
112 bacteria could be effective to alleviate the replanting problem.

113 Recently, herbgenomics has been raised for medicinal plants research using
114 genomic tools including investigating rhizospheric environment using metagenomic
115 sequencing (Chen *et al.*, 2015). In the present study, high-throughput sequencing
116 analysis of 16S and 18S rRNA genes was used to demonstrate changes in the

117 diversity and composition of soil microbial communities in the rhizosphere of
118 ginseng seedlings of different ages and developmental stages. Furthermore,
119 biocontrol bacteria against *F. oxysporum* were isolated using a dual culture technique,
120 and their inhibition activity in a replanting soil was confirmed. These results
121 increased our understanding of the reasons behind replanting issues caused by
122 rhizospheric microbial community. Data provided an effective method for soil
123 bioremediation to alleviate issues in replanting of Chinese herbal medicine.

124

125 **Materials and Methods**

126 **Field experiment and soil extraction**

127 This field experiment was performed in a ginseng plantation in Jingyu, Jilin
128 Province (42°20'N, 126°50'E, 775 m a.s.l.), which is the main ginseng-producing
129 region in China. This region receives a northern temperate continental climate and an
130 annual precipitation of approximately 767 mm. The plough layer in the plantation
131 consists of gray brown soil.

132 Ginseng seedlings are consecutively grown for 4–5 years in a fixed site before
133 their root can be harvested. The disease and mortality of ginseng seedlings generally
134 occur after being consecutively grown for 2 years. Thus, we analyzed the influence
135 of two-, three-, and four-year-old transplanted seedlings on rhizospheric microbial
136 communities. In this experiment, two-, three-, and four-year-old ginseng seedlings
137 were transplanted in each plot in our plantation and denoted as 2-y, 3-y, and 4-y,
138 respectively. In our plantation, field plots were arranged in a completely randomized
139 block design, and three replicate plots (1.7 m × 8.0 m) were used for each plant age.
140 Ginseng was cultivated strictly in accordance with the standard operating procedures
141 of good agricultural practice (Heuberger *et al.*, 2010; Zhang *et al.*, 2010).

142 The distinct stages of ginseng development are as follows: vegetative,
143 flowering, fruiting, root growth, and annual dormancy (Table S1). During dormancy,
144 the aboveground plant parts of ginseng wither, and the root becomes dormant. In this
145 stage, the underground root activities weaken, and soil samples obtained during this
146 stage are not included in analyses. This experiment included 36 soil samples

147 obtained from two-, three-, and four-year-old ginseng seedlings under four
148 developmental stages, namely, vegetative (2-Ve, 3-Ve, and 4-Ve), flowering (2-Fl,
149 3-Fl, and 4-Fl), fruiting (2-Fr, 3-Fr, and 4-Fr), and root growth (2-Ro, 3-Ro, and
150 4-Ro). Six ginseng seedlings were randomly obtained from each plot (1.7 m × 8.0 m);
151 the soils were removed, and rhizosphere fractions were brushed and pooled into one
152 sample. Soil samples were obtained from three replicates per treatment. Thirty-six
153 soil samples were homogenized by passing through a 2 mm sieve for further
154 processing. The characteristics of the soil samples are described in Table S2.

155

156 **Calculation of emergence and survival rates**

157 The ginseng emergence rate was analyzed in May. The emergence rate in each plot
158 was calculated as follows: number of emerging ginseng seedlings divided by the
159 total number of transplanted seedlings. The mortality of ginseng seedlings was
160 observed from June to August, and the survival rate was determined at the end of
161 August. The survival rate of ginseng in each plot was calculated as follows: number
162 of living seedlings divided by the total number of emerging seedlings in the area.
163 Three plots per treatment served as three replicates.

164

165 **DNA extraction and PCR amplification**

166 Total soil DNA was extracted from 0.1 g of freeze-dried soil using a MoBio
167 Powersoil Kit (MoBio Laboratories Inc., Carlsbad, CA) according to the
168 manufacturer's instructions; the DNA was stored at -20 °C for further processing.
169 For each sample, fragments of 16S and 18S rRNA genes were amplified using the
170 conserved primers 27F/338R (Fierer *et al.*, 2008) and 817F/1196R (Rousk *et al.*,
171 2010). The forward and reverse primers contained an eight-base pair barcode (Table
172 S3). Amplification reactions and purification were performed as previously
173 described (Rodrigues *et al.*, 2013). Purified PCR products were quantified by
174 Qubit®3.0 (Life Invitrogen, Germany), and these amplicons were pooled in
175 equimolar ratios for sequencing.

176

177 **High-throughput sequencing**

178 The pooled DNA product was used to construct Illumina pair-end library according
179 to the Illumina's genomic DNA library preparation procedure. The amplicon library
180 was subsequently paired-end sequenced (2×250) on an Illumina HiSeq platform
181 (Shanghai Biozeron Co., Ltd.) according to standard protocols.

182 Raw FASTQ files were demultiplexed and quality-filtered using QIIME with
183 the following pipeline (Caporaso *et al.*, 2010). Operational taxonomic units were
184 clustered using 97% similarity cutoff by using UPARSE (version 7.1
185 <http://drive5.com/uparse/>), and chimeric sequences were identified and removed
186 using UCHIME. The phylogenetic affiliation of each 16S and 18S rRNA gene
187 sequence was analyzed using Ribosomal Database Project (Wang *et al.*, 2007) and
188 Silva schemes (Quast *et al.*, 2013). Rarefaction analysis based on Mothur v.1.21.1
189 (Schloss *et al.*, 2009) was performed to reveal diversity indexes, including Chao 1
190 and Shannon diversity (H') indexes. We clustered the taxa obtained from the RDP
191 Classifier through complete linkage hierarchical clustering by using R package
192 HCLUST (<http://sekhon.berkeley.edu/stats/html/hclust.html>). PCoA was used to
193 compare groups of samples based on unweighted UniFrac distance metrics in QIIME
194 (Caporaso *et al.*, 2010). Linear discriminant analysis effect size (LEfSe)
195 (<http://huttenhower.sph.harvard.edu/lefse/>) was used to characterize the features
196 differentiating the bacterial and fungal communities in soils according to the
197 methods described by Segata *et al.* (2011). All metagenomic data were submitted to
198 the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov>);
199 the accession numbers of 16S rRNA and 18S rRNA genes are SRP103168 and
200 SRP103176, respectively.

201

202 **Quantitative PCR of *Fusarium***

203 To compare the relative abundance of *Fusarium* in soils of ginseng seedlings, the
204 copy numbers of *Fusarium* were calculated using the ITS-Fu-F/ITS-Fu-R
205 (Abd-Elsalam *et al.*, 2003). Quantitative PCR was performed according to the
206 description of Rousk *et al.* (2010) with minor modification. Standard curves were

207 generated using 10-fold serial dilution of a plasmid containing a full-length copy of
208 *F. oxysporum* 18S rRNA gene to estimate the copy numbers of *Fusarium*. qPCR
209 reactions (25 µL) were performed using a SYBR Green PCR Master mix (Takara,
210 Toyobo, Japan). *Fusarium* copy numbers were generated using a regression equation
211 for each assay relating the cycle threshold value to the known number of copies in
212 the standards.

213

214 **Isolation and selection of antagonistic bacteria**

215 Biological control is one of the most remarkable potential approaches for plant
216 disease control. Due to the increase in the abundance of pathogenic agents of
217 ginseng root rot,
218 a dual culture assay was used to screen microbial antagonists against *F. oxysporum*.
219 Rhizosphere soils of three-year ginseng seedlings under root growth stage were used
220 to isolate and select the antagonistic bacteria against *F. oxysporum*. The pathogenic *F.*
221 *oxysporum* was isolated and confirmed in our previous study (Wang *et al.*, 2016).
222 Soil (10 g) was homogenized in 100 mL of sterile distilled water, and the bacteria
223 were isolated through serial dilution technique. The isolated single strain was
224 screened on the basis of its antagonistic activity against *F. oxysporum* in a dual
225 culture according to the description of Shahzad *et al.* (2017). The experiment was
226 replicated three times. The zone of inhibition was measured following the
227 description of Kaiser *et al.* (2005) to screen the antagonistic bacterium and examine
228 the antagonistic activity of the candidates.

229

230 **Identification of antagonistic bacterial strain**

231 Antagonistic bacterium (named strain 50-1) was identified by morphological and
232 molecular methods. The morphology of strain 50-1 was recorded after it was
233 incubated on Luria–Bertani (yeast extract (5 g), peptone (10 g), NaCl (10 g), and
234 agar (10 g) in 1 L of water) medium for 24 h later. Strain 50-1 was molecularly
235 identified by amplifying 16S rRNA according to the description by Cai *et al.* (2012).
236 The amplified PCR product was analyzed on a 3730 XL sequencer (Applied

237 Biosystems, Foster City, CA, USA), and the generated sequence (accession number
238 KY962803) was submitted to GenBank. Neighbor-joining trees were constructed in
239 MEGA v6.0 to generate Kimura 2-parameter distance matrixes for each sequence
240 following standard parameters. The numbers at the branch knots were bootstrap
241 values based on 1000 resamplings for the maximum likelihood.

242 To further identify the strain 50-1, total genomic DNA from strain 50-1 was
243 extracted (Tiangen, Beijing, China) and purified by RNase-free DNase I (Takara,
244 Kyoto, Japan) to analyze its genome. The complete genome sequence was assembled
245 using a hybrid sequencing strategy combining PacBio RS II and Illumina HiSeq
246 sequencing platforms (Koren *et al.*, 2012). Genome sequencing was performed with
247 Illumina HiSeq2500 using the PE250 strategy following the manufacturer's protocol.
248 The reads obtained with Illumina PCR adapter and the filtered low-quality reads
249 were assembled by SOAPdenovo (Li *et al.*, 2008; Li *et al.*, 2010) to generate
250 scaffolds. Gene prediction genome assembly was performed using Glimmer and
251 gene functions were annotated by BLASTP against NR, COG and KEGG databases.
252 GeneMarkS (Besemer *et al.*, 2001) with an integrated model combining the
253 GeneMarkS-generated parameters and Heuristic model parameters. A genome
254 overview with annotation information was created using Circos (Krzywinski *et al.*,
255 2009). The whole genome was deposited in NCBI (BioProject ID PRJNA383782).

256

257 **Evaluation of the biocontrol efficacy of antagonists in replanting soils**

258 A pot experiment was performed to assess the biocontrol efficacy of bacterium 50-1
259 in replanting soils in our phytotron. Each pot contained 1 kg of soils cultivated with
260 ginseng seedlings for three years and happened root rot. Two-year-old ginseng
261 seedlings (three plants) were transplanted in each pot. The pots were placed in the
262 phytotron under the following conditions: 26 ± 2 °C, 60% humidity, and 14 h of light
263 alternating with 10 h of darkness. The rhizospheres of uniform ginseng seedlings
264 were inoculated with 1 mL (10^6 cfu mL⁻¹) of cultures containing biocontrol strains
265 when the seedlings were cultivated for one week. Pots were inoculated with cultures
266 containing inactivated strains as controls. Five pots served as one replicate, and three

267 replicates were prepared. After a two months following inoculation, the death rate of
268 ginseng seedlings was calculated as follows: number of dead seedlings divided by
269 the total number of transplanted seedlings in each treatment. The rhizosphere soils of
270 three seedlings were randomly selected from each treatment, and they served as one
271 sample for the analysis of the relative abundance of *Fusarium*. The experiment was
272 replicated three times.

273

274 **Statistical analyses**

275 SPSS version 16.0 software (SPSS Inc., Chicago, IL, USA) was used for statistical
276 analyses. Variables were considered for all treatment replicates and subjected to
277 ANOVA. Mean values were compared by calculating the least significant difference
278 at 5% level.

279

280 **Results**

281 **Emergence and survival rates of ginseng seedlings**

282 The ginseng plants grown on field showed a healthy appearance with no sign of
283 disease during the growing season (Figs. 1A–1C). The emergence and survival rates
284 exhibited no significant difference among two-, three-, or four-year-old ginseng
285 seedlings (Figs. 1D, 1E). The emergence rate of two-, three-, or four-year-old
286 ginseng seedlings ranged from 82.3% to 86.9% (Fig. 1D), and their survival rate was
287 94.1%–97.1% (Fig. 1E). The growth of ginseng seedlings of different ages was
288 favorable, which guaranteed the success of the field experiment.

289

290 **Taxonomic diversity of bacterial and fungal communities**

291 A total of 2,296,684 classifiable 16S rRNA sequence reads were obtained from 36
292 soil samples for analysis (Table S3). The mean number of classifiable sequences per
293 sample was 63,796 (dominant length: 283–293 bp). The bacterial diversity indexes
294 (H' and Chao 1) markedly changed in the rhizosphere of ginseng seedlings of
295 different ages in late developmental stages (Fig. 2). H' and Chao 1 values were
296 significantly higher in the rhizosphere of 4-y seedlings than those in the rhizosphere

297 of 2-y seedlings in fruiting stage (Figs. 2A, 2B). During root growth, H' and Chao 1
298 values evidently decreased in the rhizospheres of 3-y and 4-y seedlings than those in
299 the rhizosphere of 2-y seedlings.

300 A total of 1,321,521 classifiable fungi sequences with a mean of 36,709
301 sequences were obtained per soil sample (dominant length: 411–414 bp; Table S3).
302 H' and Chao 1 values markedly decreased in the rhizosphere with increasing ginseng
303 age under vegetative stage (Figs. 2C, 2D). During root growth, H' and Chao 1 values
304 were evidently higher in the soils of 3-y and 4-y seedlings than those in the soil of
305 2-y seedlings.

306

307 **Variation in bacterial and fungal community compositions**

308 PCoA ordination and Bray–Curtis distance matrix revealed evident difference in
309 bacterial communities in soils of ginseng plant of different ages and developmental
310 stages (Figs. 3, S1). In vegetative and flowering stages, the second principal
311 components (14.41% and 14.22% contributions, respectively) demonstrated that the
312 bacterial communities in soils of 2-y seedlings differed from those in soils of 4-y
313 seedlings (Figs. 3A, 3B). In fruiting stage, the first principal component axis (22.00%
314 contributions) indicated that the bacterial communities in soils of 2-y seedlings
315 significantly differed from those in soils of 3-y seedlings (Fig. 3C). Additionally, the
316 first principal component axis (22.29% contributions) indicated that the bacterial
317 communities in soils of 3-year-old seedlings significantly differed from those in soils
318 of 2-y and 4-y seedlings; furthermore, the second principal component (13.98%
319 contributions) demonstrated that the bacterial communities in soils of 2-y seedlings
320 differ from those in soils of 4-y seedlings (Fig. 3D). LEfSe revealed differences in
321 the rhizospheric bacterial communities of ginseng seedlings of different ages and
322 developmental stages (Fig. S2).

323 PCoA ordination and Bray–Curtis distance matrix revealed that fungal
324 communities obviously differed in the soils of ginseng seedlings of different ages
325 and developmental stages (Figs. 3, S1). In vegetative stage, the first principal
326 component axis (33.94% contribution) of the fungal communities in soils of 4-y

327 seedlings markedly differed from those of the fungal communities in soils of 2-y
328 seedlings. Moreover, the second principal component axis (17.55% contribution) of
329 the fungal communities in soils of 2-year-old seedlings considerably differed from
330 those of the fungal communities in the soils of 3-y seedlings (Fig.3E). At the
331 flowering and fruiting stages, the first principal components (25.71% and 38.96%
332 contributions, respectively) in the fungal communities in soils of 2-y ginseng
333 seedlings significantly differed from those of the fungal communities in the soils of
334 3-y and 4-y seedlings (Figs. 3F, 3G). During root growth, the first principal
335 component (26.97% contribution) of fungal communities in soils of 3-y seedlings
336 considerably differed from those of fungal communities in soils of 2-y and 4-y
337 seedlings, and the second principal component (14.93% contribution) in fungal
338 communities in soils of 2-y seedlings differed from those of fungal communities in
339 soils of 4-y seedlings (Fig. 3H). According to LEfSe analysis, fungal composition
340 differed in the soils of ginseng seedlings of different ages and developmental stages
341 (Fig. S3).

342

343 **Changes in the relative abundance of bacterial taxa**

344 The relative abundance of bacterial groups changed in soils of ginseng plants of
345 different ages and developmental stages (Fig. 4). The relative abundance of
346 Chthoniobacteraceae, Chthonomonadales, Chthonomonadetes, *Chthoniobacter*,
347 *Granulicella*, and *Blastocatella* declined with plant age in vegetative stage (Fig. 4A).
348 The relative abundance of *Arthrobacter*, *Brevundimonas*, Micrococcaceae,
349 Rhodobiaceae, Intrasporangiaceae, and Micrococcales was significantly higher in
350 soils of 3-y and 4-y seedlings than that in soils of 2-y seedlings. The relative
351 abundance of *Luteolibacter*, Phyllobacteriaceae, *Acidovorax*, *Moheibacter*, and
352 Cytophagaceae in soils of 3-y and 4-y seedlings evidently decreased, and the
353 abundance of Elusimicrobia and Armatimonadetes was markedly increased in the
354 flowering stage (Fig. 4B). The relative abundance of *Bacillus*, Enterobacteriales,
355 Enterobacteriaceae, *Brevundimonas*, and Anaerolineae was significantly higher in
356 soils of 3-y and 4-y seedlings than that in soils of 2-y seedlings; additionally, the

357 abundance of *Luteibacter*, Clostridia, and Clostridiales declined in soils of 3-y and
358 4-y seedlings in fruiting stage (Fig. 4C). The relative abundance of *Paralcaligenes*,
359 Sphingomonadaceae, Saccharibacteria, *Sphingomonas*, and Alcaligenaceae
360 significantly decreased with plant age, whereas the relative abundance of *Pandoraea*,
361 Chlamydiales, and Chlamydiae was obviously higher in soils of 3-y and 4-y
362 seedlings than that in soils of 2-y seedlings in root growth stage (Fig. 4D).

363

364 **Changes in the relative abundance of fungal taxa**

365 The relative abundance of fungal taxa changed in soils of ginseng plants of different
366 ages and developmental stages (Fig. 5). The relative abundance of
367 Cystofilobasidiales, Ophiostomataceae, *Ophiostoma*, Ophiostomatales, and
368 Cystofilobasidiaceae significantly declined with seedling age, and the abundance of
369 Pezizales, Cantharellales, *Dendryphion*, Pezizomycetes, and Tubeufiaceae was
370 evidently higher in soils of 4-y seedlings than those in soils of 2-y and 3-y seedlings
371 in the vegetative stage (Fig. 5A). The relative abundance of Microascales, *Helicoma*,
372 and Tubeufiaceae was obviously higher in soils of 4-y seedling than those in soils of
373 2-y and 3-y seedlings in the flowering stage (Fig. 5B). The relative abundance of
374 Tremellales, Acrospermales, *Occultifur*, *Acrospermum*, Cystobasidiales,
375 Cystobasidiaceae, and Cystobasidiomycetes was markedly higher in the soils of 4-y
376 seedlings than that in the soils of 2-y and 3-y seedlings in fruiting stage (Fig. 5C).
377 The relative abundance of Zygomycota was obviously lower in soils of 3-y and 4-y
378 seedlings than that in soils of 2-y seedlings, and the abundance of Tremellomycetes,
379 Chytridiomycota, and Sordariales significantly increased in soils of 4-y seedlings
380 than those in soils of 2-y and 3-y seedlings in root growth stage (Fig. 5D).

381 The relative abundance of *Fusarium* showed increasing trends in the soils of the
382 adult ginseng plants under later developmental stages (Figs. 5E, S4). According to
383 the high-throughput sequencing analysis, the relative abundance of *Fusarium*
384 increased by 22.5%–25.0%, 35.7%–50.0%, and 18.2%–36.4% in soils of 3-y and 4-y
385 seedlings in the flowering, fruiting, and root growth stages, respectively (Fig. 5E).
386 The copy numbers of *Fusarium* also showed similar trends based on the analysis of

387 quantitative PCR (Fig. S4). These results revealed that the relative abundance of
388 pathogenic agent increased in soils during ginseng growth.

389

390 ***Bacillus subtilis* 50-1 were responsible for the biocontrol of *F. oxysporum***

391 Due to the relative abundance of pathogenic agent (*Fusarium*) increase in the
392 ginseng soils, dual culture techniques were used to isolate microbial antagonists
393 against *F. oxysporum* to control root rot. Antagonistic bacterium, namely, *B. subtilis*
394 50-1, was isolated and confirmed using a dual culture technique (Fig. 6). Strain 50-1
395 exhibited a broad spectrum of growth inhibition activity against *F. oxysporum*,
396 thereby resulting in 67.8% inhibition percentage (Fig.6A). Strain 50-1 is a
397 Gram-positive, oxidase- and catalase-positive, rod-shaped bacterial species (Fig.6B,
398 Table S4). Analysis of the 16S rRNA sequences revealed that strain 50-1 belonged to
399 *B. subtilis* with the bootstrap value of 100% (Fig. 6C). Strain 50-1 was further
400 confirmed according to the genome sequencing. The complete genome was
401 composed of a circular chromosome of 4,040,837 bp with 43.86% GC content (Fig.
402 6D, Table S5). The total numbers of genes were 4,193, which covered 88.6% of the
403 genome encoding 3,176 proteins. The function of these annotated genes was mainly
404 energy production and conversion, amino acid transport and metabolism,
405 transcription, carbohydrate transport and metabolism, inorganic ion transport and
406 metabolism, and signal transduction mechanisms based on the COG function
407 classification (Fig. S5).

408

409 **Inoculation of biocontrol bacteria reduced ginseng death rate in a replanting** 410 **soil**

411 The pot experiment analysis revealed that ginseng death rate and the relative
412 abundance of *Fusarium* significantly decreased by 63.3% and 46.1% in the
413 replanting soils inoculated with strain 50-1, respectively (Fig. 7). Furthermore, the
414 height and leaf area increased by 62.7% and 22.5%, respectively, after inoculation
415 with strain 50-1 (Fig. S6). These results revealed that inoculation of biocontrol
416 bacteria alleviated the ginseng replanting problem.

417

418 **Discussion**

419

420 Replanting problem is a common and serious issue in cultivation of medicinal plants.
421 Following replantation, *Rehmannia glutinosa* suffers from disease, and its biomass
422 and tuber quality decline (Qi *et al.*, 2009; Wu *et al.*, 2011; Yang *et al.*, 2011). The
423 survival rate of ginseng seedlings is lower than 25% after replantation for three years
424 (Zhao *et al.*, 2001). Multiple factors have caused this replanting problem, and
425 researchers suggested that changes in microbial communities influence soil health
426 and crop yield (Bisseling *et al.*, 2009; Bulgarelli *et al.*, 2013). In addition,
427 rhizospheric microbial community is governed by plant species and growth (Incroglu
428 *et al.*, 2011). Thus, we hypothesized that changes in the rhizospheric microbial
429 community were driven by ginseng seedlings of different ages and development
430 stages, which caused the replanting problem of *Panax* plants. Reducing the
431 occurrence of the disease is a useful strategy to alleviate the replanting issues.
432 Biological control is one of the most remarkable potential approaches for plant
433 disease control because of its safety and environmental friendliness.

434 Diversity in bacterial and fungal communities changed in the rhizosphere of
435 ginseng seedlings of different ages and developmental stages. H' and Chao1 values
436 revealed that bacterial diversity was obviously low, whereas fungal diversity was
437 significantly higher in the rhizosphere of adult ginseng seedlings in the root growth
438 stage. A similar study reported that increase in ginseng cultivation ages reduced
439 bacterial diversity and increased fungal diversity (Xiao *et al.*, 2016). Moreover,
440 diversity of microbial community in the rhizosphere of *Pseudostellaria heterophylla*
441 decreases with increased number of cropping years (Zhao *et al.*, 2016). Additionally,
442 the developmental stage of crops is an important driver of microbial community
443 structure (Houlden *et al.*, 2008). Plant ages and developmental stages alter the
444 microbial diversity during growth. The diversity of soil microorganisms is critical in
445 the maintenance of soil health and quality, and it serves as a sensitive bioindicator
446 (He *et al.*, 2008). The death rate of notoginseng and fungal diversity are significantly

447 negatively correlated, which suggest that fungal diversity is a potential bioindicator
448 of soil health (Dong *et al.*, 2016). A relationship exists between microbial diversity
449 and root disease suppression (Nitta, 1991; Workneh & van Bruggen, 1994). Mazzola
450 (2004) reported that reduced soil microbial diversity is responsible for the
451 development of soil-borne diseases. Reduced bacterial diversity in response to adult
452 plants is possibly the key indicator of ecological variations and functional
453 impairment in a root growth stage.

454 The bacterial and fungal compositions evidently differed in the rhizospheres of
455 ginseng plants of different ages and developmental stages. Variation in bacterial and
456 fungal community composition is observed during continuous cropping of
457 notoginseng (Dong *et al.*, 2016). Dynamics of microbial species is occurred in the
458 rhizosphere during ginseng growth (Li *et al.*, 2012). Sugiyama *et al.* (2014) reported
459 that soybeans affected the bacterial communities under vegetative stage, with further
460 alterations possibly occurring during later growth stages. The most divergent
461 community structures occur in the young plant stage of tomato, and the flowering
462 and senescence stages show increasingly similar community structure (Inceoğlu *et al.*
463 *et al.*, 2011). The composition of microbial community was affected by plant age and
464 development stage. In our study, the agents of beneficial microbes were relatively
465 few, whereas those of harmful microorganisms increased with plant age. The agents
466 of beneficial microbes include the microorganisms that degrade compounds
467 (*Luteolibacter*, Cytophagaceae, *Luteibacter*, and *Sphingomonas*), and resist disease
468 and improve growth (Sphingomonadaceae and Zygomycota). The agents of harmful
469 microorganisms mainly infect plants (*Brevundimonas*, Enterobacteriaceae,
470 *Pandoraea*, Cantharellales, *Dendryphion*, and Chytridiomycota). Another study has
471 showed similar results, i.e., the population of beneficial microbe decreases, whereas
472 that of harmful microorganisms increases with increasing number of cropping years
473 (Zhao *et al.*, 2016). Soil microbial community is an important bioindicator of soil
474 function (Zuppinger-Dingley *et al.*, 2014). Changes in functional groups revealed
475 that micro-ecological environment was degraded in the rhizosphere with increasing
476 age of ginseng.

477 Rhizospheric microbial community can be influenced by soil characteristics and
478 plant species (Lauber *et al.*, 2008; Berg and Smalla, 2009). In our study, the pH and
479 available K and organic matter contents exhibited no significant difference in
480 ginseng plants of different ages and developmental stages (Table S2). Plant species
481 could be one of the most important factors that influence rhizobacterial communities
482 (Micallef *et al.*, 2009). Root exudates offer substrates for microbial metabolism and
483 act as intermediates for biogeochemical reactions in rhizosphere (Uren, 2000; Berg
484 and Smalla, 2009). In the early developmental stages of *Arabidopsis*, cumulative
485 secretion levels of sugars and sugar alcohols are high, which enhanced the richness
486 of rhizospheric microbial community (Chaparro *et al.*, 2014). By contrast, root
487 exudates contain allelochemical that disturbs the balance in a microbial community
488 (Wu *et al.*, 2015). These results imply that root exudates are one of the main drivers
489 of changes in rhizospheric microbial communities during ginseng growth. Moreover,
490 our results showed that the diversity of microbial communities markedly changed in
491 the rhizosphere of ginseng plants with different ages in root growth stage. This
492 phenomenon is possibly a result of different root types and root exudates. The
493 composition of *Arabidopsis* root exudates changes throughout developmental
494 gradient in plants, and tomato root exudates in reproductive stage are more
495 phytotoxic than those in vegetative stage (Yu and Matsui, 1994; Chaparro *et al.*,
496 2013). Ginseng root rapidly grows after cropping for three years before harvest,
497 especially in root growth stage. Ginseng root growth stage is possibly characterized
498 by a specific but different root exudation pattern that drives different bacterial
499 communities.

500 Biological control using microbes is an environmentally friendly approach of
501 controlling disease (Bargabus *et al.*, 2003; Tjamos *et al.*, 2005). In our study, we
502 isolated *B. subtilis* 50-1 as an effective antagonist against *F. oxysporum*; the results
503 of antagonist inoculation revealed that biocontrol bacterium reduced the ginseng
504 morbidity and alleviated the replanting problem. *Bucillus* strains, as potent biological
505 control agents of plant diseases, have been reported in many studies (Zouari *et al.*,
506 2016; Shahzad *et al.*, 2017). The biocontrol efficacy of *B. amyloliquefaciens* 54

507 against bacterial fruit blotch is evident in a greenhouse (Jiang *et al.*, 2015). *B.*
508 *megaterium* (B5), *B. cereus* sensu lato (B25), and *Bacillus* sp. (B35) display the
509 highest antagonistic activity against *Fusarium verticillioides*, and antagonistic
510 activity reveals that these strains produce glucanases, proteases, chitinases,
511 siderophores, and auxins (Figueroa-López *et al.*, 2016). Furthermore, data suggest
512 that microbial strains producing bioactive constituents can help the inoculated plant
513 to reduce the negative effects of pathogenesis and abiotic stresses (Shahzad *et al.*,
514 2017). The microbial antagonists could be used for efficient control of plant
515 pathogens and comprise an environmentally friendly approach. Additionally, genome
516 sequence analysis provides insights into the pathways of functional bacteria and
517 facilitates their exploration. The genome sequence analysis of *Ganoderma lucidum*
518 revealed key genes encoding cytochrome P450s in secondary metabolism (Chen *et*
519 *al.*, 2012). In our study, the genome sequence of strain 50-1 helps accelerate the
520 development and application of biological inoculant.

521

522 **Supplementary data**

523 The details were online.

524 **Table S1** Description of developmental stages of ginseng and the sampling times.

525 **Table S2** Soil chemical characteristics in soils of ginseng cropping.

526 **Table S3** Barcodes, numbers of bacterial and fungal sequences and average length in
527 each sample.

528 **Table S4** Physiological and biological characteristics of *Bacillus subtilis* 50-1.

529 **Table S5** Genome features of *Bacillus subtilis* strain 50-1.

530 **Figure S1** Changes in bacterial and fungal community in rhizosphere of ginseng
531 seedlings of different ages and developmental stages.

532 **Figure S2** LEfSe identified the taxa in the soil bacterial communities of plants of
533 different ages in vegetative (A), flowering (B), fruiting (C), and root growth stages
534 (D).

535 **Figure S3** LEfSe identified the taxa in the fungal communities of plants of different
536 ages in vegetative (A), flowering (B), fruiting (C), and root growth stages (D).

537 **Figure S4** Relative abundance of *Fusarium* in the soils based on the analysis of
538 real-time PCR.

539 **Figure S5** COG function classification in the *Bacillus subtilis* strain 50-1.

540 **Figure S6** The height (A) and leaf area (B) of ginseng seedlings after inoculation of
541 strain 50-1 in a replanting soil.

542

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546

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548 **Figure captions**

549

550 **Figure 1** Growth status of ginseng seedlings. (A–C) Two-, three-, and four-year-old
551 ginseng seedlings, respectively. (D) Emergence rate of ginseng seedlings. (E)
552 Survival rate of ginseng seedlings. 2-y, 3-y, and 4-y represents the transplanted two-,
553 three-, and four-year-old seedlings, respectively. Data are presented as mean \pm SD (n
554 = 3). Identical letters denote nonsignificant difference at 0.05 significance level.

555

556 **Figure 2** Bacterial and fungal diversities in the rhizosphere of ginseng seedlings of
557 different ages and developmental stages. (A, B) show the Chao 1 and Shannon
558 diversity (H') of bacterial community. (C, D) show the Chao 1 and H' of fungal
559 community. Ve, Fl, Fr, and Ro represent vegetative, flowering, fruiting, and root
560 growth stages, respectively. Data are presented as mean \pm SD ($n = 3$). Non-identical
561 letters denote significant difference in the same developmental stage of ginseng plant
562 with different ages at 0.05 significance level.

563

564 **Figure 3** Changes in bacterial and fungal communities in the rhizosphere of ginseng
565 seedlings of different ages and developmental stages. (A–D) PCoA ordination plots
566 display the relatedness of samples separated using Unweighted UniFrac distance of
567 classified 16S rRNA gene sequences at vegetative, flowering, fruiting, and root
568 growth stages, respectively. (E–H) PCoA ordination plots show the relatedness of
569 samples separated using Unweighted UniFrac distance of classified 18S rRNA gene
570 sequences at the Ve, Fl, Fr, and Ro stages, respectively. Red, green, and blue
571 represent the samples in the rhizosphere of 2-y, 3-y, and 4-y transplanted ginseng
572 seedlings, respectively.

573

574 **Figure 4** Relative abundance of the bacterial taxa detected by the linear discriminant
575 analysis effect size (LEfSe) as biomarker. (A–D) represent the vegetative, flowering,
576 fruiting, and root growth stages, respectively. 2-, 3- and 4- represent the samples in
577 the rhizosphere of 2-y, 3-y, and 4-y transplanted ginseng seedlings. Data represent

578 the mean values of $n = 3$.

579

580 **Figure 5** Relative abundance of the fungal taxa detected by LEfSe as biomarker and
581 *Fusarium*. (A–D) represent the vegetative, flowering, fruiting, and root growth
582 stages, respectively. (E) Relative abundance of *Fusarium*. Data are presented as
583 mean \pm SD ($n = 3$).

584

585 **Figure 6** *Bacillus subtilis* 50-1 antagonized *Fusarium oxysporum*. (A) Colony
586 diameter measured in a dual culture. (B) Morphological features of bacterium 50-1.
587 (C) Relationships of 16S rRNA sequences between *B. subtilis* strain 50-1 (black
588 body) and published 16S rDNA sequences. (D) Genome map of strain 50-1. The six
589 circles (outer to inner) represent the scale line, forward strand CDSs (color by COG
590 categories), reverse strand CDSs (color by COG categories), RNA genes, GC content,
591 and GC skew. From outside to center: genome size, genes on the forward strand
592 (color by COG categories), genes on the reverse strand (color by COG categories),
593 RNA genes (tRNAs, orange; rRNAs, red), GC content (red and blue), and GC skew.
594 Only bootstrap values higher than 70% are shown. Bars represent the mean \pm SE (n
595 = 3). Asterisks denote significant differences between the colony diameters of *F.*
596 *oxysporum* and *F. oxysporum* + strain 50-1 at $P < 0.05$.

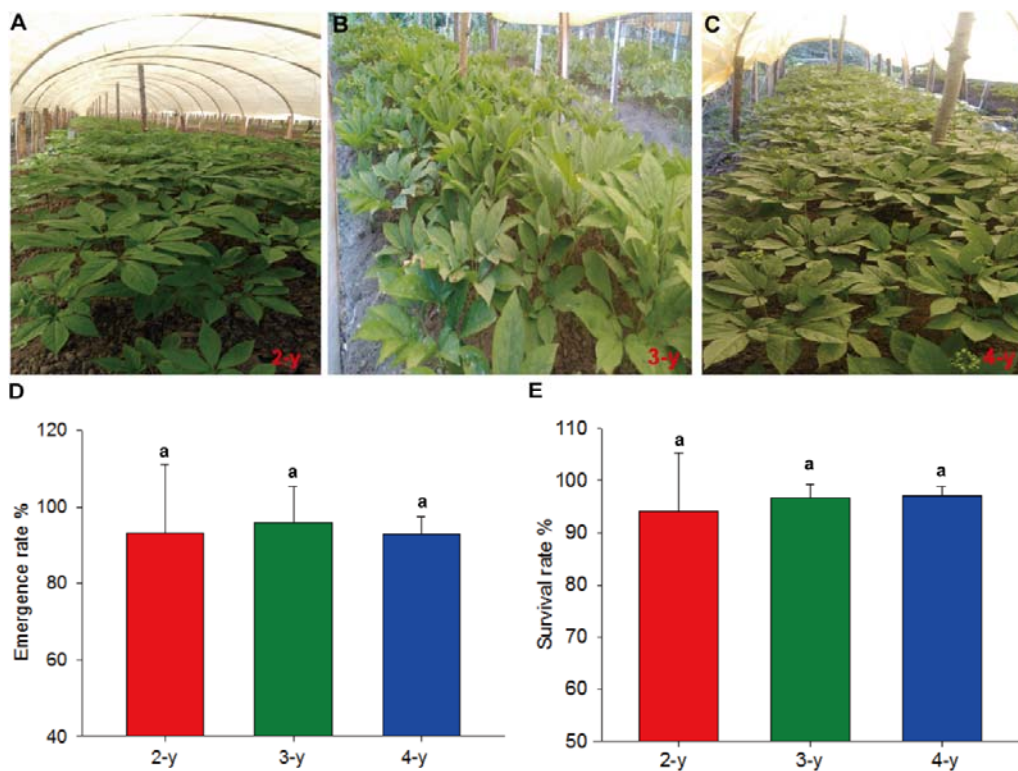
597

598 **Figure 7** Inoculation of strain 50-1 in ginseng replanting soils. (A) Death rate of
599 ginseng after inoculation with strain 50-1. (B) Copy numbers of *Fusarium* in soils.
600 No-inoculation and inoculation represent the samples inoculated with cultures
601 containing inactivated and activated strain 50-1, respectively. Data are presented as
602 mean \pm SD ($n = 3$). Asterisks denote significant differences between the
603 No-inoculation and inoculation at $P < 0.05$.

604

605

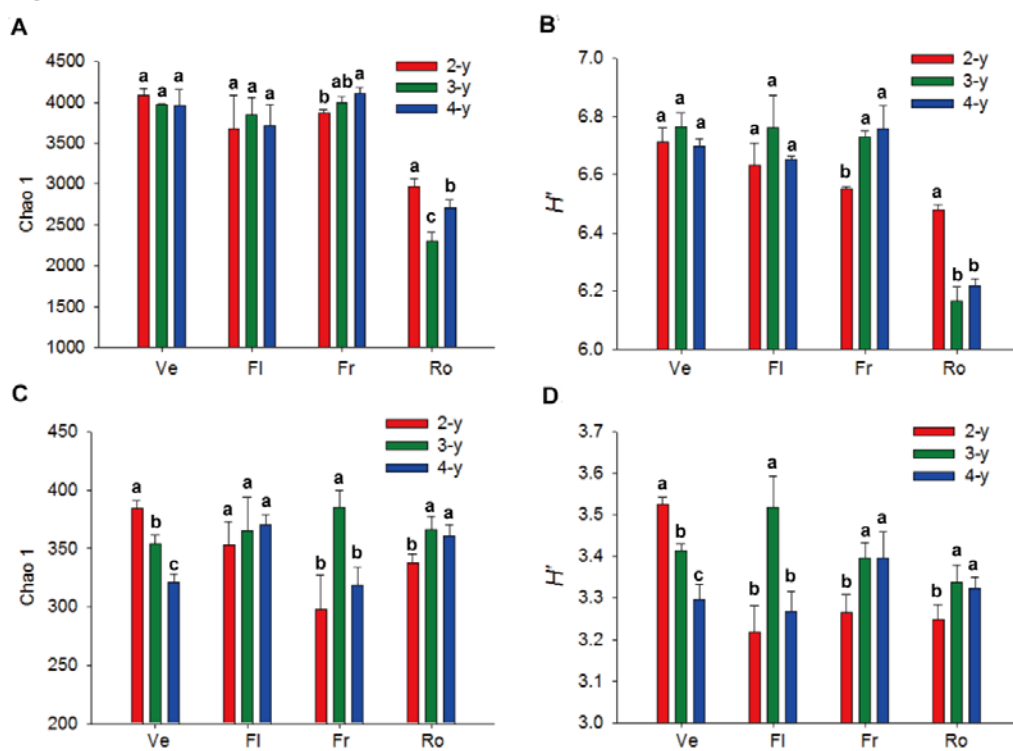
606 **Figure 1**



607

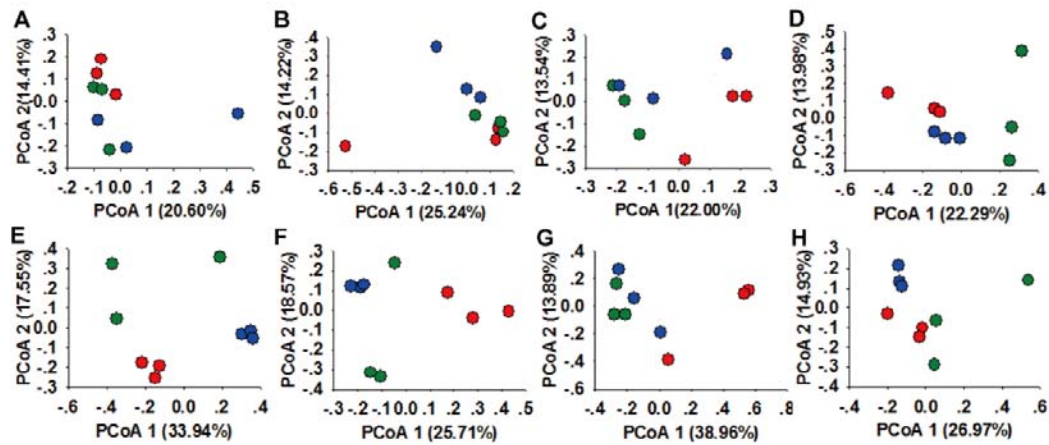
608

609 **Figure 2**



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611

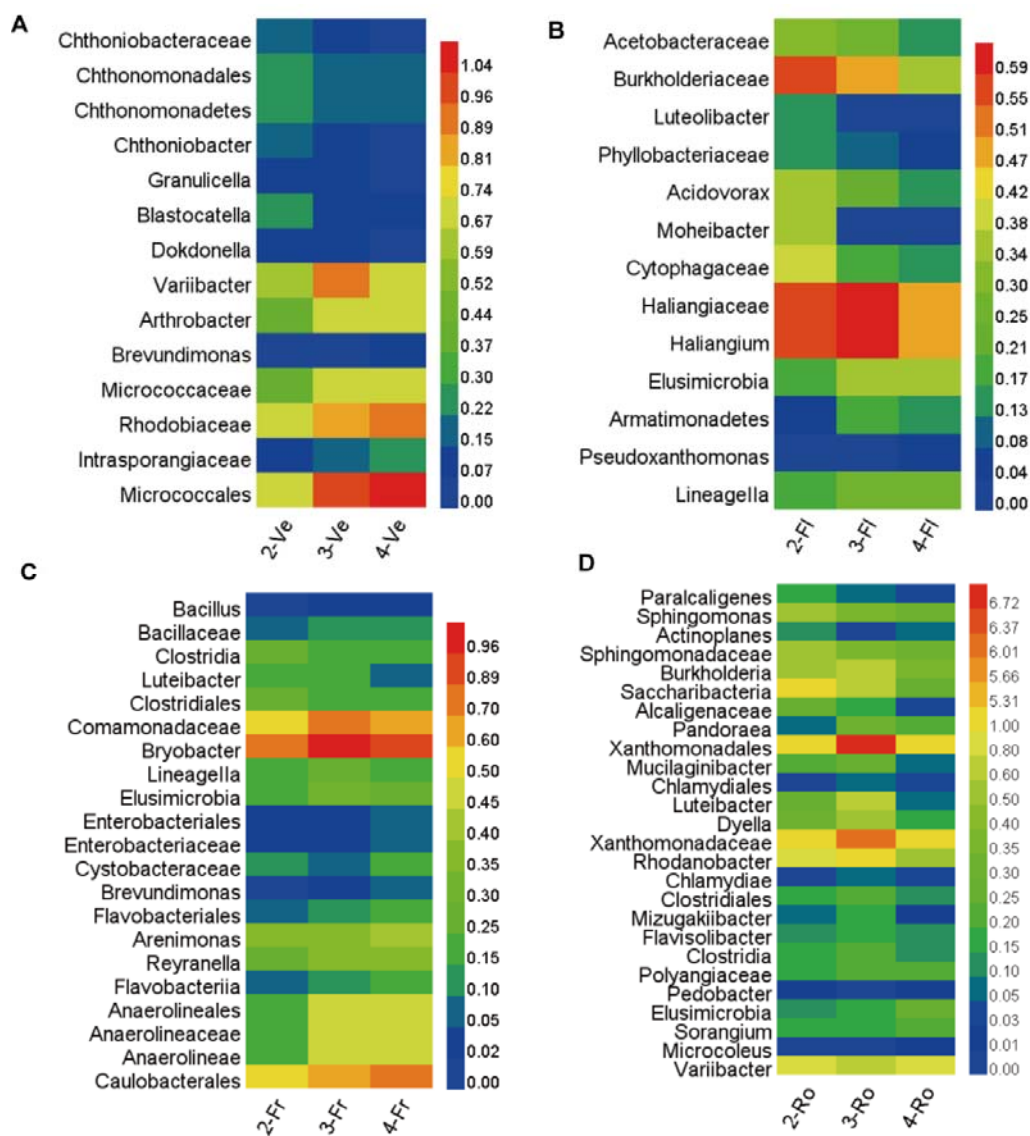
612 **Figure 3**



613

614

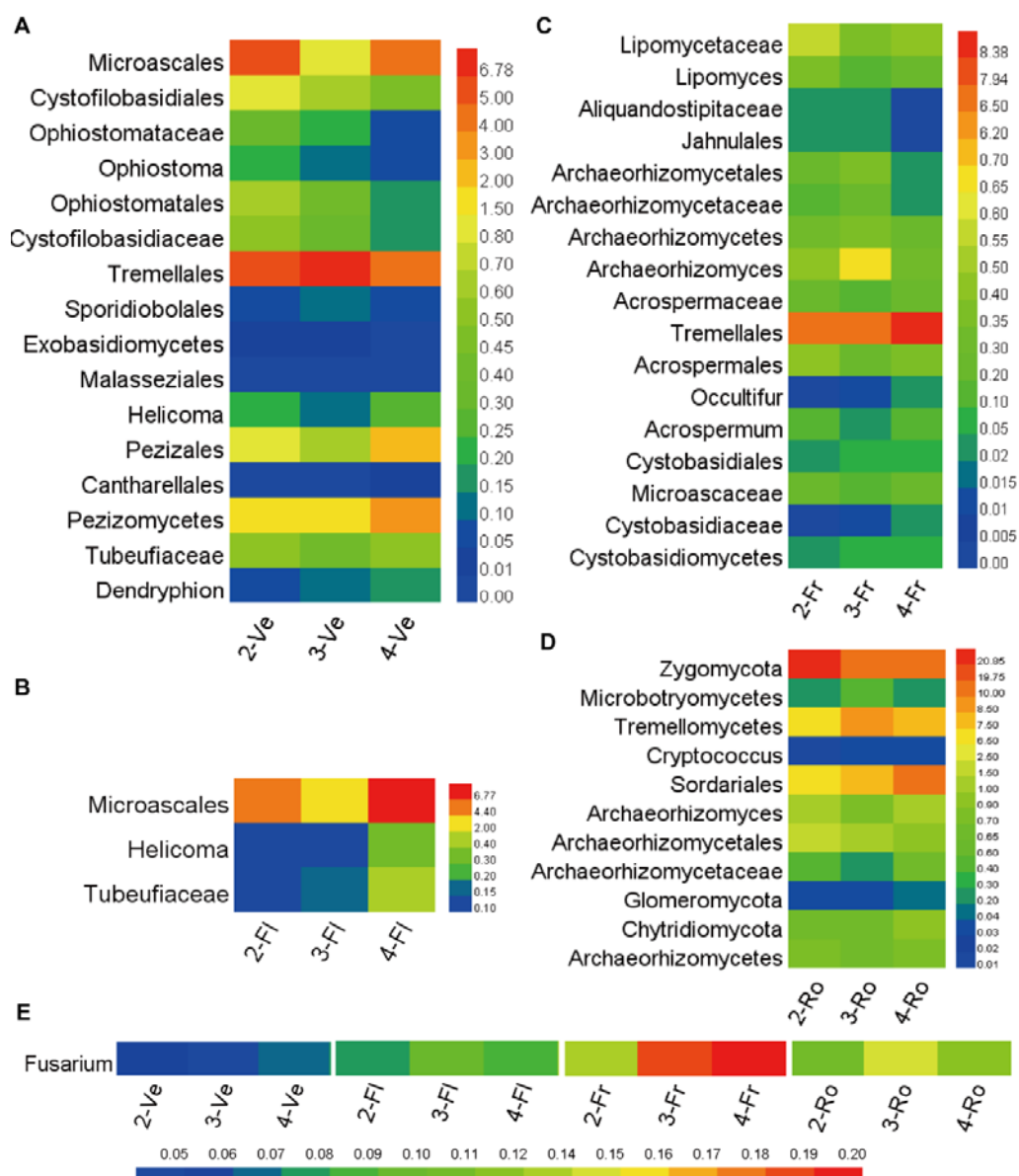
615 **Figure 4**



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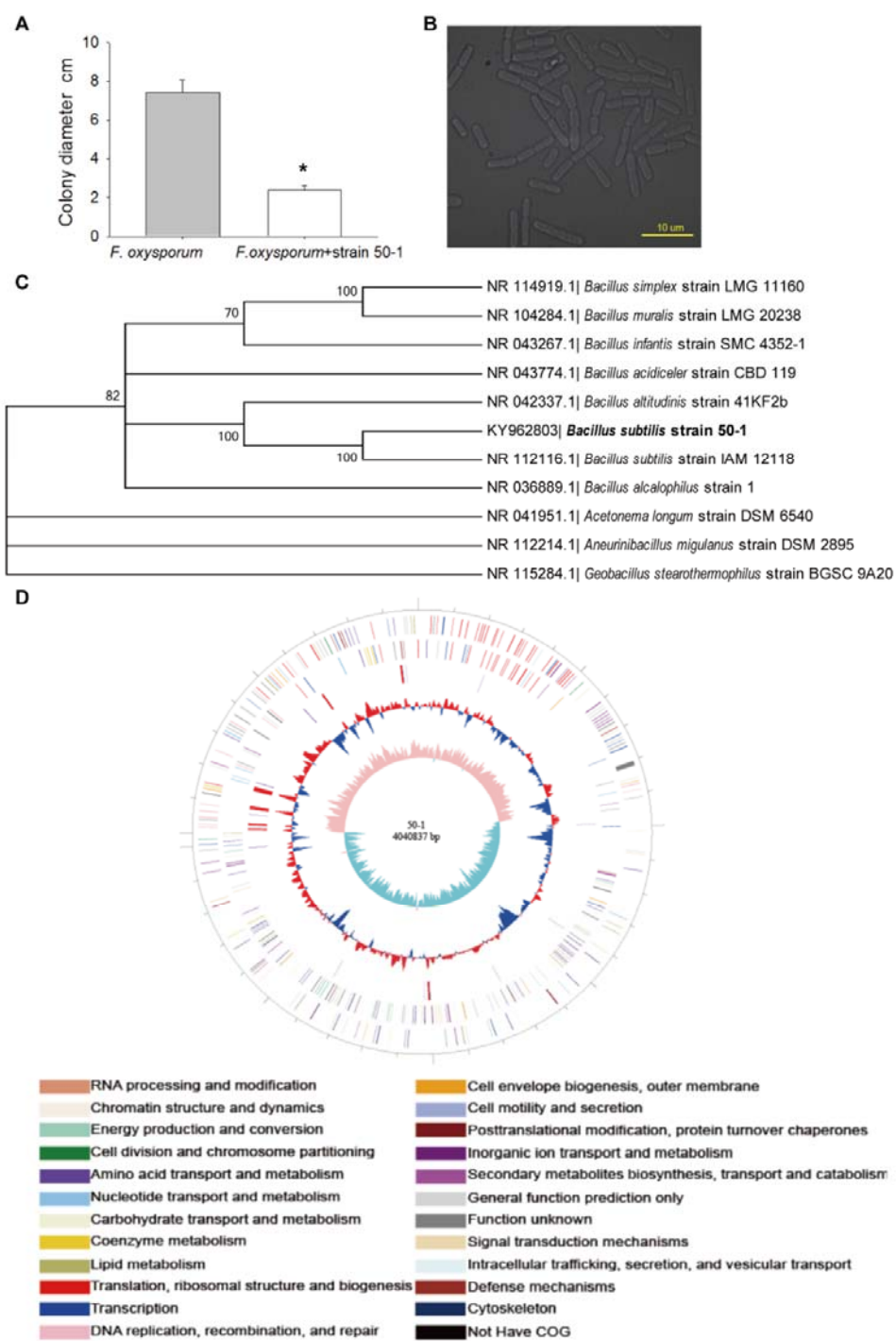
618 **Figure 5**



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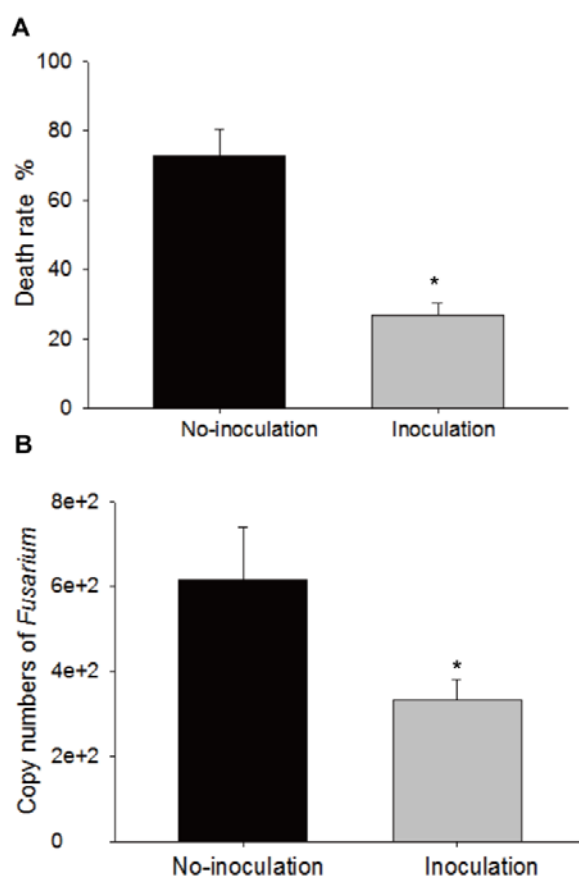
621 **Figure 6**



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624 **Figure 7**



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