# Long-term changes in soil properties and microbial communities are continuous cropping obstacles associated with American ginseng (*Panax quinquefolius* L.) cultivation

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**Abstract:** This study aims to verify the time-variant feature of American ginseng (AG) continuous cropping obstacles and to explore the factors impeding continuous cropping. We verified the feature with a plant-soil feedback pot experiment and then investigated the factors by comparing the properties of control soils that had not been previously used for growing ginseng (CS) with those of soils with a 10-year-crop-rotation cycle following the growth of AG (RS). It's found that the survival rate of AG in RS was lower than that in CS. The RS had lower pH, available potassium content, and urease activity. Additionally, *p*-coumaric, *p*-hydroxybenzoic, vanillic, caffeic, and cinnamic acid levels were lower in RS than in CS, but salicylic acid levels showed the opposite pattern. RS had higher *Rhodanobacter* and lower *Acidothermus*, *Sphingomona*s relative abundances in bacterial community. It's also found that many bacteria were substantially correlated with phenolic acids and soil physiochemical properties. Results indicate that even after 10-year crop rotation, the negative effects of prior continuous cropping of AG has not been eliminated. The growth of AG can be affected negatively with deterioration of soil physicochemical properties and with lower levels of phenolic acids which promote pathogen reproduction. Probiotics reduction also weighs. Moreover, biotic factors are interrelated with abiotic ones. Therefore, it can be inferred that the comprehensive change of soil properties is the main obstacle for continuous cropping.

**Keywords:** continuous cropping obstacles; *Panax quinquefolius* L.; phenolic acids; soil bacterial community composition; soil nutrients

## Introduction

Continuous cropping obstacles are a common phenomenon, not only in the cultivation of crops such as apples, melons, eggplants, and peanuts, but are also associated with the cultivation of many medicinal plants such as *Angelica sp.*, *Pinellia sp.*, *Atractylodes sp.* [1], and *Panax quinquefolius* L. (American ginseng, AG) [2]. AG is a perennial herb that belongs to the family Araliaceae. Clinical research has shown that AG, as a kind of medicinal plant with impressive medicinal properties, has the functions of boosting immune system, sobering up, anti-oxidation, anti-cancer, etc [3-5]. In addition, it's therapeutic in treatment of SARS, H5N1 and COVID-19, according to the research of traditional Chinese medicine [6,7]. It is often used as prescription drugs in medical treatment and disease prevention. However, serious continuous cropping obstacles restrict the replanting and the yield of AG, resulting in huge economic losses.

Crop rotation is one of the most effective practices to reduce or eliminate continuous cropping obstacles. While continuous cropping obstacles of most plants can be eliminated by employing a favorable rotation strategy [8-10], those of AG can last for more than 5 to 10 years or even decades [11,12]. The main detrimental factors to replanting AG in land restored by rotation are not clear.

Many studies have reported that crops under continuous cropping can be adversely affected by allelopathic or autotoxic effects [2,13]. Phenolic acids released during plant growth as the main allelochemicals, are the main continuous cropping obstacles to many terrestrial plants. They have been reported to exert inhibitory effects on many crops and medicinal plants [14,15]. Notably, in a study on the allelopathy of single and mixed phenolic acids on AG [16], nine phenolic acids including

44  cinnamic, vanillic, and ferulic acids were found to inhibit the growth of AG radicles at concentrations ranging from 0.1

45  mmol L$^{-1}$ to 10 mmol L$^{-1}$; this inhibition increased with increasing phenolic acid concentration which indicated that phenolic

46  acids had direct inhibitory effects on the growth of AG. However, there are also microorganisms that interact with phenolic

47  acids in soil, especially some AG probiotics (e.g., *Sphingomonas*, *Burkholderia*) and AG pathogens (e.g., *Fusarium*

48  *oxysporum*, *Phytophthora cactorum*). Kertesz et al. found that phenolic acids can be used as substrates by *Sphingomonas*

49  and *Burkholderia*, enhancing their population growth, and that phenolic acids also play a role in the formation of a stable

50  beneficial microbial community [17]. According to Hu et al. [18], at a concentration of 100 mg L$^{-1}$, ferulic acid promoted the

51  growth of cucumber fusarium wilt, whereas at 150 mg L$^{-1}$, it showed a significant inhibitory effect. Yuan et al. [19] found that

52  ferulic acid promoted the growth of *Fusarium oxysporum* at low concentrations, but inhibited its growth at high

53  concentrations. On one hand, phenolic acids can be used as substrates to support beneficial microorganisms, but on the other

54  hand, high phenolic acid concentrations can inhibit the growth of pathogenic bacteria, thereby promoting AG growth.

55  Therefore, higher concentrations of phenolic acids can not only inhibit the growth of AG as allelochemicals, but also

56  promote AG growth by stimulating beneficial microorganisms and reducing harmful microorganisms. The direct and

57  indirect effects of allelochemicals may have opposite effects on plant growth, which are similar to the allelopathic effects

58  of different plants on invasive *Phytolacca americana* [20,21].

59      In addition, some studies have also showed that planting AG leads to progressive imbalances in soil microbial

60  communities [22,23], and the depletion of available nutrients in the soil or soil acidification [24,25]. However, there is little research

61  examining whether these deleterious effects on the microbial community, soil nutrient availability, and soil acidification

62  can be reversed after a period of recovery (here, 10 years). Therefore, it is important to measure soil microbial diversity,

63  community composition, and soil physical and chemical properties after crop-rotation restoration in order to determine

64  which factors influencing AG replanting persist over the course of 10 years. Since previous studies have mostly focused on

65  fungi, equally important bacteria [26-28] have been often overlooked; therefore, here we focused on the bacterial communities.

66      In the present study, soil used for planting AG and then subjected to crop rotation for 10 years was collected and used

67  for replanting AG (second age AG seedlings). The growth of AG seedlings in rotation soil (RS) was observed to explore

68  the time-variant feature of continuous cropping obstacles and was compared to that in soil where no AG had been grown

69  (control soil (CS)) to verify if: 1) the change in soil phenolic acid content has an effect on soil microbial community

70  composition, and thereby an indirect effect on AG growth; 2) the change in bacterial community structure mainly reflects

71  an increase in pathogen abundance or a reduction in beneficial bacterial abundance; and 3) soil physicochemical properties

72  and enzyme activities remain unsuitable for AG growth.

## Results

**Pot experiment of AG planting** As shown in Fig. 1, the survival rate of the two groups decreased in the stable period

75  (20 days)—that of the RS group decreased to 65% and that of the CS group decreased to 72.5%. On the 21st day, the survival

76  rate of the two groups were recovered to 100% by replanting AG seedlings. Although there was no significant difference in

77  leaf area, aboveground biomass, and underground biomass between the two groups, the survival rate of RS was only 45%

78  on the 60th day, while that of CS was 67.5%. The survival rate of AG between the two groups was substantially different.

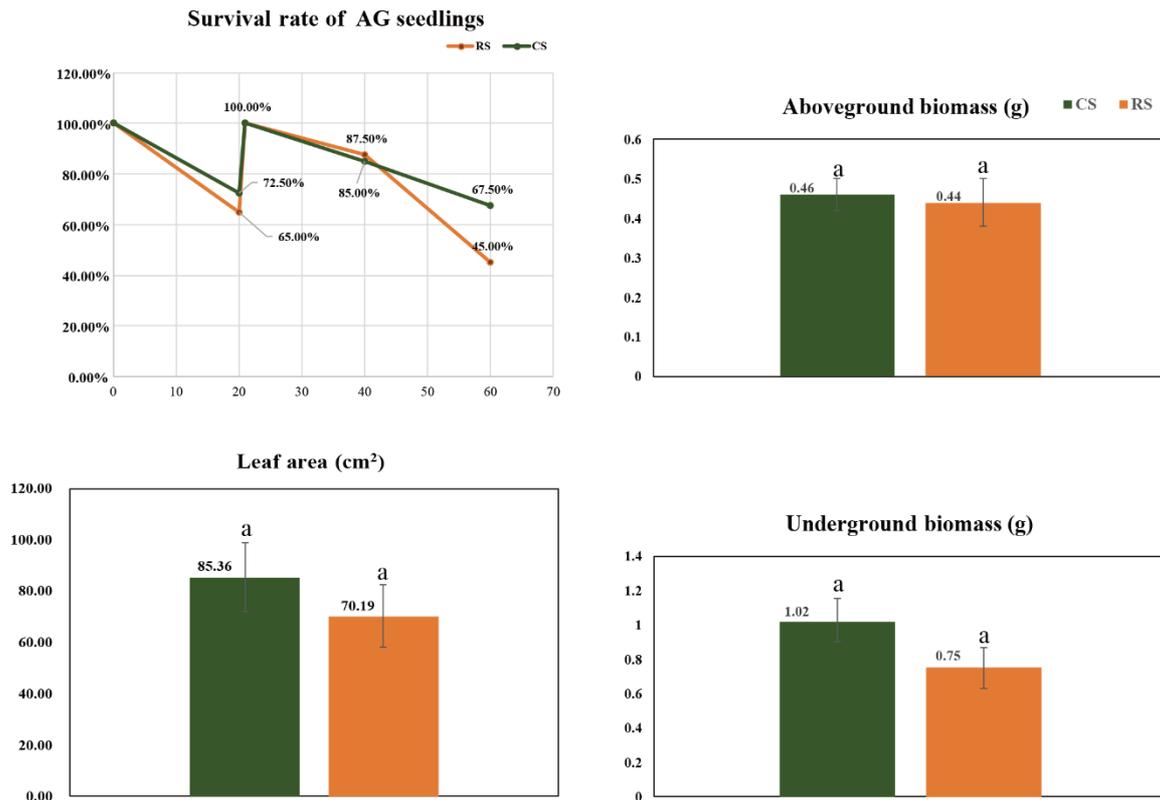



**Figure 1.** Survival rate and growth index of *Panax quinquefolium* in the pot experiment. The survival rate was calculated every 20 days. The aboveground and underground biomasses were calculated at the end of 60 days. Leaf area was calculated at the final harvest. CS: Plants in the CS group; RS: Plants in the RS group; a: means the difference is not significant ($P > 0.05$). RS: the 10-year post-ginseng rotation soil; CS: soil in which no ginseng was grown before ginseng planting.

**Soil physicochemical properties and enzyme activities** The water content of RS was significantly higher than that of CS. While, the pH of RS was significantly lower than that of CS. The content of available K in CS was also significantly (2.96-fold) greater than that in RS. There were no significant differences in the levels of total C, total N, ammonium N, nitrate N, and available P between CS and RS; however, in terms of available nutrients, the levels in CS tended to be higher than those in RS (Table 1).

**Table 1.** Comparison of physicochemical properties of 10-year post-ginseng rotation soil (RS) and soil in which no ginseng was grown (CS) before ginseng planting. * Statistically significant difference at $P < 0.05$; ** $P < 0.01$ (T test). Mean ± SE (n = 4) data shown.

| Physicochemical properties | RS (ginseng rotation) | CS (no ginseng) |
|---|---|---|
| Water content (%) | 15.92 ± 0.30* | 15.05 ± 0.00 |
| pH | 5.00 ± 0.04 | 5.19 ± 0.04* |
| Total N (%) | 0.21 ± 0.01 | 0.20 ± 0.02 |
| Total C (%) | 1.45 ± 0.45 | 1.14 ± 0.10 |
| Ammonium N (mg kg$^{-1}$) | 12.16 ± 0.56 | 13.72 ± 0.57 |
| Nitric N (mg kg$^{-1}$) | 60.09 ± 3.88 | 70.50 ± 4.27 |
| Available P (mg kg$^{-1}$) | 28.61 ± 6.65 | 37.83 ± 8.19 |
| Available K (mg kg$^{-1}$) | 0.45 ± 0.10 | 1.33 ± 0.12** |

97     There was a significant difference in urease activity between the CS and RS samples, with the activity in CS being

98     25.13% higher than that in RS; the activities of phosphatase, sucrase, and catalase were higher in CS than in RS, but these

99     differences were not significant (Table 2).

100

101     **Table 2.** Comparison of enzyme activities between 10-year post-ginseng rotation soil (RS) and soil in which

102     no ginseng was grown (CS) before ginseng planting. * Statistically significant difference at $P < 0.05$ (T test).

103     Mean $\pm$ SE (n = 4) data shown.

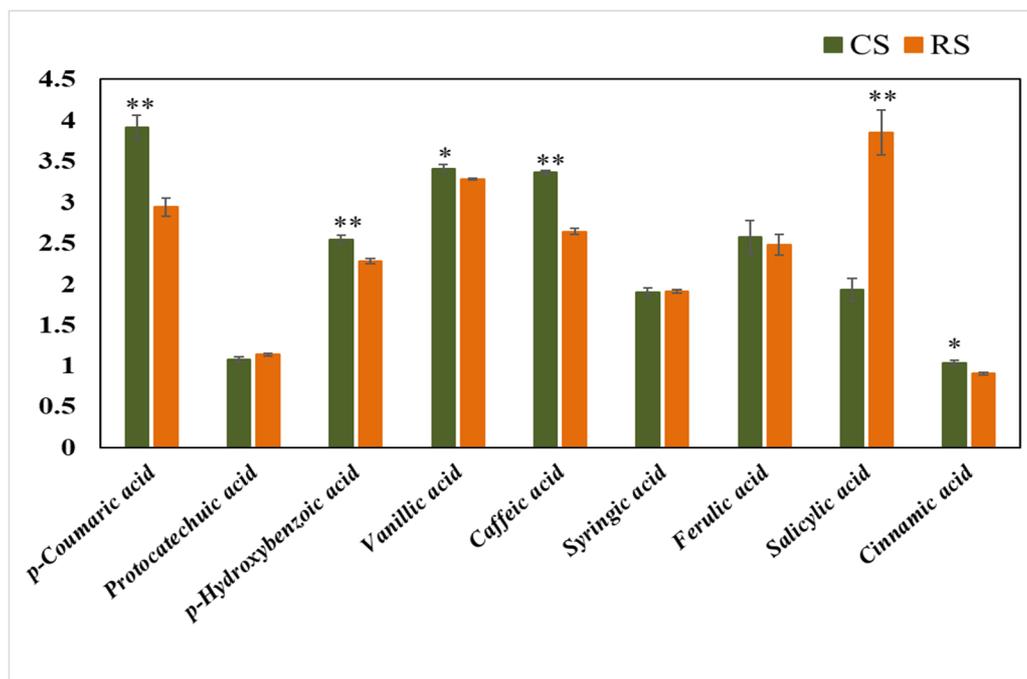
| Soil enzyme activity | RS (ginseng rotation) | CS (no ginseng) |
|---|---|---|
| Phosphatase (mg g$^{-1}$) | $79.26 \pm 34.42$ | $139.96 \pm 26.48$ |
| Sucrase (mg g$^{-1}$) | $0.07 \pm 0.01$ | $0.14 \pm 0.05$ |
| Catalase (mg g$^{-1}$) | $224.16 \pm 0.19$ | $225.02 \pm 0.33$ |
| Urease (mg g$^{-1}$) | $20.69 \pm 0.65$ | $25.89 \pm 0.88^*$ |

104

105     **Soil phenolic acids content** In terms of phenolic acids, the levels of vanillic and cinnamic acids were markedly

106     higher in CS than those in RS ($P < 0.05$); the levels of *p*-coumaric, caffeic, and *p*-hydroxybenzoic acids were

107     significantly higher in CS than those in RS ($P < 0.01$). However, salicylic acid showed the opposite trend ($P <$

108     0.01) (Fig. 2).

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110     **Figure 2.** Comparison of phenolic acid content between 10-year post-ginseng rotation soil (RS) and soil in

111     which no ginseng was grown (CS) before ginseng planting. *represent significant differences ($P < 0.05$)

112     according to Student's t-test (n=4). ** represent significant differences ($P < 0.01$).

113     **Soil bacterial diversity and community structure**

114     **Sequencing results, OTU cluster analysis, and α-diversity analysis** The V3–V4 region of the 16S rRNA gene

115     was sequenced. After removing the low quality, barcode, and primer sequences, 636,654 effective sequences were obtained

116     from the eight samples; 5421 and 5747 OTUs were contained in RS and CS samples, respectively, and the number of shared

117     OTUs was 3673 after clustering the effective sequences (see Supplementary Figure S4). The slopes of species

118     accumulation curves for bacterial species were flat at different similarity cutoff values, indicating that the identified bacterial

119    diversity was close to saturation and that an increase in the sequencing depth would not increase the number of detected
120    species (see Supplementary Figure S5). Species composition and abundance distributions for each sample, from phylum
121    to genus levels, were obtained using QIIME (see Supplementary Table S2). The diversity and richness of bacterial
122    communities in the soil samples were evaluated and compared using Chao1, Simpson's, Shannon's, and ACE indices. Each
123    index reflected higher microbial community diversity and richness in RS than in CS, although none of the differences were
124    significant (see Supplementary Figure S6).

125    **β -diversity** As shown in Table S3, ANOSIM for the unweighted UniFrac result (R = 0.7188, P = 0.030) supported the
126    NMDS analysis (Fig. 3a) for the unweighted UniFrac distance matrix, both indicating that the bacterial community structure
127    of RS and CS differed significantly without weighing. However, the weighted UniFrac result (R = 0.3646; P = 0.085)
128    indicated that the difference between the two groups of samples was not significant after considering evenness (Fig. 3b).
129    The overall β-diversity index analysis (Fig. 3a, b, c, d) showed that there were some differences between RS and CS.

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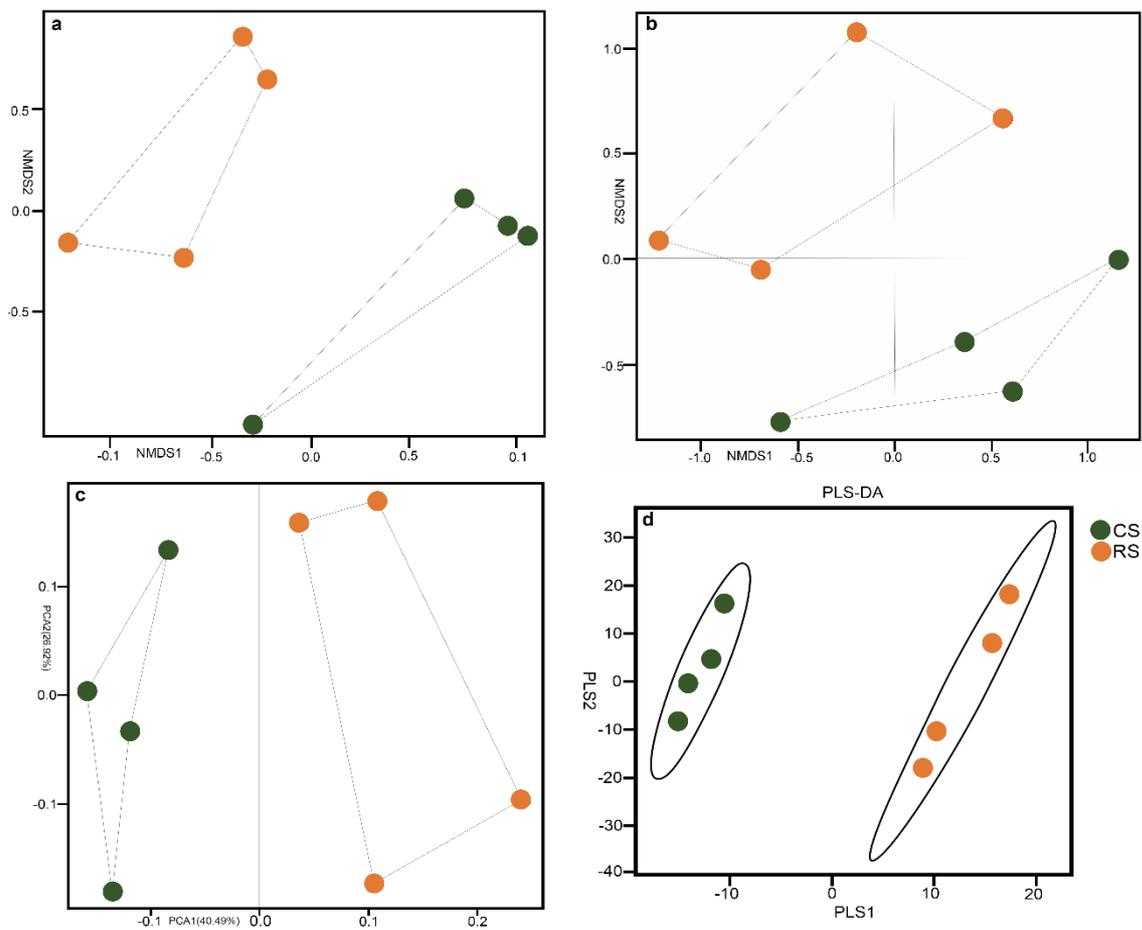
132    **Figure 3.** Nonmetric multidimensional scaling (NMDS) ordination plot of bacterial community structure
133    comparing 10-year post-ginseng rotation soil (RS) and soil in which no ginseng was grown (CS) before ginseng
134    planting. a, unweighted and b, weighted UniFrac distance matrix. c, PCA (principal component analysis) of
135    bacterial community structure; d, PLS-DA (partial least squares discriminant analysis) of bacterial community
136    structure. The shorter the distance between two points, the higher the similarity of the microbial community
137    structure between the two groups.

138

139    **Bacterial community structure and significantly different bacterial composition** The bacterial populations in
140    the different samples were analyzed by comparing the obtained 16S rRNA sequences with those of the Greengenes databases
141    using BLAST (Fig. 4). The dominant bacterial phyla were Proteobacteria, Actinobacteria, Acidobacteria, Chloroflexi,

142 Firmicutes, Gemmatimonadetes, and Bacteroidetes across the two groups (Fig. 4a). The most prominent difference between
143 the two groups was that the abundance of Chlamydiae in RS (0.28%) was significantly higher than that in CS (0.10%), and
144 further analysis at lower classification levels revealed that c__Chlamydiae, o__Chlamydiales, f__Simkaniaceae, and
145 g__uncultured were all significantly different (see Supplementary Table S2).

146     The most abundant bacterial genera were also the same across both groups, namely *Rhizomicrobium*, *Bacillus*,
147 *Bryobacter*, *Variibacter*, *Candidatus_solibacter*, *Gemmatimonas*, *Bradyrhizobium*, *Pseudolabrys*, *Crossiella*,
148 *Streptomyces*, *Burkholderia-Paraburkholderia*, *Granulicella*, and *Roseiarcus* (Fig. 4b). The proportions of
149 *Rhodanobacter* (3.45%), *Mizugakiibacter* (2.28%), *Clostridium_sensu_stricto_*1 (0.45%), *Arthrobacter* (0.43%),
150 *Holophaga* (0.20%), *Novosphingobium* (0.20%), and *Peptoclostridium* (0.20%) were higher in RS than in CS. The
151 proportions of *Sphingomonas* (2.83%), *Jatrophihabitans* (1.15%), *Nitrobacter* (1.13%), and *Thermosporothrix* (0.35%)
152 were higher in CS than in RS (Fig. 4).

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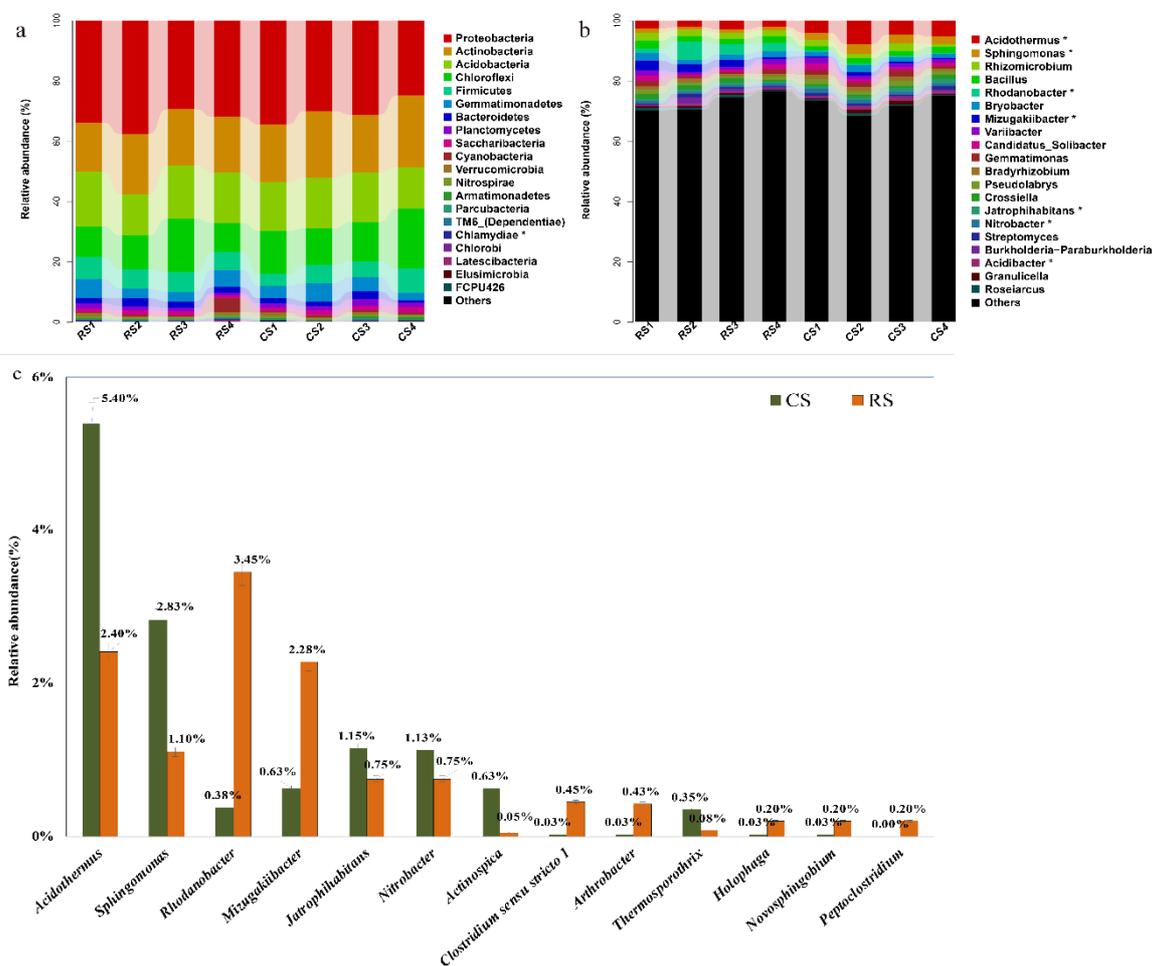
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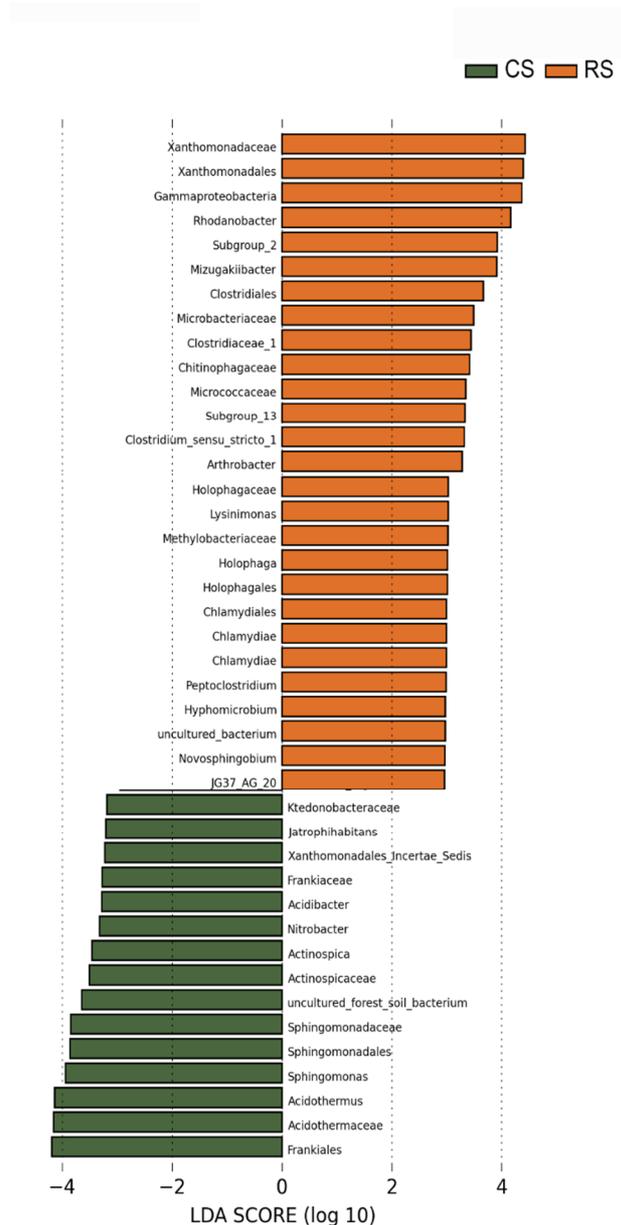
155     **Figure 4.** Composition and structure of the bacterial community from the 10-year post-ginseng rotation soil
156     (RS) and soil in which no ginseng was grown (CS) before ginseng planting. a: phylum, b: genus, c: significantly
157     different bacteria at the genus level, comparing RS and CS. The bacteria genera listed in c are all significantly
158     different (t-test, P < 0.05). Mean (n = 4) data are shown; error bars represent SE. *represents significant
159     differences (P < 0.05) according to the Metastats-test (n = 4).

160

161 **LEfSe analysis of the differentially abundant bacterial communities** LEfSe uses LDA scores to estimate the
162 effect size of each differentially abundant taxon, and to rank the relative differences among taxa that are discriminative

163   with biological consistency and statistical significance. The LEfSe analysis of RS and CS bacterial communities showed
164   42 differentially abundant taxonomic clades with an LDA score higher than 3.0 (Fig. 5). After combining these results
165   (Fig. 5) with the information in Supplementary Table S2, it was found that c (class)_Gammaproteobacteria_o
166   (order)_Xanthomonadales_f (family)_Xanthomonadaceae_g   (genus)_*Mizugakiibacter* and g_*Rhodanobacter*
167   (underscores represent relationships at the level of phylogeny); o_Holophagales_f_ Holophagaceae_g_*Holophaga* were all
168   significantly different (T-test, P<0.05) and had a higher abundance in RS. O_Sphingomonadales_f_
169   Sphingomonadaceae_g_*Sphingomonas* and _g_ *Novosphingobium* were significantly different (t-test, P<0.05); in
170   addition, they had a much higher abundance in CS, except for Novosphingobium. Furthermore,
171   f_Acidothermaceae_g_*Acidothermus* and f_Actinospicaceae_g_*Actinospica* were significantly different.

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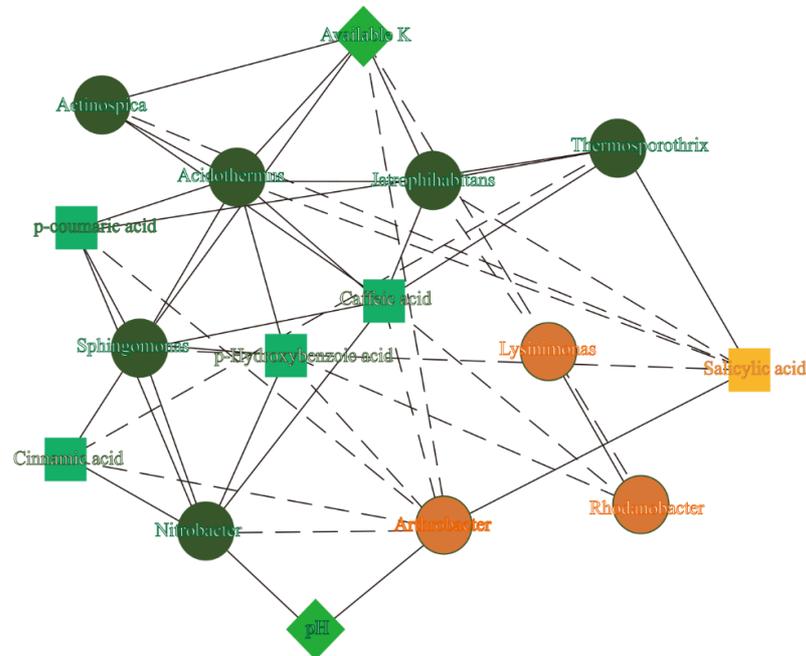
173       **Figure 5.** Differentially abundant bacterial taxa as assessed using linear discriminant analysis (LDA) with effect
174       size measurements (LEfSe) in the 10-year post-ginseng rotation soil (RS) and soil in which no ginseng was
175       grown (CS) before ginseng planting.

176

177   **Correlation analysis** To further examine the possible "collaborative" or "competing" relationships among the different
178   communities, Spearman's rank correlation coefficients between the most abundant genera were calculated using Mothur

179  software. The correlations among the 50 dominant bacterial genera were analyzed (see Supplementary Figure S7), and the
180  correlation analysis between the top ten bacterial genera (see Supplementary Table S4) and factors of interest—comprising
181  six different phenolic acids and three physicochemical properties—indicated a number of interesting relationships. As
182  shown in the Fig. 6 and Supplementary Table S4, Sphingomonas with low relative abundance in RS significantly positively
183  correlated with available K, concentration of phenolic acid, Acidothermus and Nitrobacter abundance and negatively
184  correlated with salicylic acid levels.

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186  **Figure 6.** The correlation network of phenolic acids with bacteria and soil physicochemical properties. When
187  the correlation factor is yellow, it means that the factor is enriched in the 10-year post-ginseng rotation soil (RS),
188  whereas when it is green, it represents the soil in which no ginseng was grown (CS) before ginseng planting.
189  The solid line represents the positive correlation among the correlation factors, while the dotted line is the
190  negative correlation. The circle represents bacteria, and the different arrangement of squares represents phenolic
191  acid and physicochemical properties.

192

## Discussion

193

194  **Pot experiment of AG planting** As shown in Fig. 1, compared to CS, the survival rate of 10-year rotation AG decreased,
195  indicating that 2-year-old AG survival rate in RS was lower than that of AG in CS. This confirmed the continued existence
196  of AG continuous cropping obstacles in RS.

197

198  **The decrease of physicochemical properties and enzyme activity** Plant growth requires water and nutrients.
199  Because soil physicochemical properties influence water and nutrient availability, changes in soil physicochemical
200  properties directly affect AG growth. In the present study, the water content of RS was significantly higher than that of CS
201  under the same management conditions (Table 1). Shu et al. [29] found that high soil water content induced root rot disease in
202  AG when sandy loam water content exceeded 30% or that of clay exceeded 50%. Similarly, according to Wang et al. [30], the
203  incidence of rust rot positively correlated with soil moisture and rainfall. Therefore, high soil water content, caused by
204  changes in soil physicochemical properties, may negatively affect AG replanting. Furthermore, the pH of RS was
205  significantly lower than that of CS (Table 1). According to Rahman and Punja [24], root rot severity at soil pH 5.05 was greater
206  than that at pH 7.0, indicating that acidic conditions can negatively affect AG health. In addition, the available K content in

207  RS was lower than that in CS (Table 1). Sun [31] found that AG should be fertilized from emergence to early flowering, when

208  its demand for potassium fertilizer is the highest, suggesting that AG has a high potassium requirement. The levels of

209  ammonium N, nitrate N, available P, and available K, but not of total N and total C, were generally lower in RS than in CS

210  (Table 1), indicating that the cultivation of AG may have long-term negative effects on these soil nutrients. The same trend

211  was observed for soil enzyme activity. Urease, a nickel-containing enzyme, catalyzes the hydrolysis of urea into carbonate

212  and ammonia. Here, urease activity was significantly higher in CS than in RS. Average phosphatase and sucrase activities

213  were also higher in CS than those in RS, although these differences were not significant (Table 2). Yang [32] also found that

214  the activities of sucrase, urease, and phosphatase decreased during AG cultivation. In summary, compared to that of CS, RS

215  had lower fertility, but higher soil water content and lower pH, two conditions which are conducive to AG disease, and that

216  may, therefore, present obstacles to AG replanting.

217  

218  **The dual effects of phenolic acids** The results showed that the content of salicylic acid in RS was significantly higher

219  than that in CS. Yang [16] found that among the various phenolic acids tested, salicylic acid had the strongest inhibitory effect

220  on AG radicle growth. In our study, higher salicylic acid content in RS may have posed direct autotoxicity to AG. As a

221  major defense hormone, salicylic acid has the function of enhancing immune signals and reprogramming defense

222  transcriptomes [33]. After planting AG, the soil salicylic acid content increased, which indicated that AG might release more

223  salicylic acid in the growth process to improve immune response to the surrounding environment. Therefore, the role of

224  salicylic acid in the continuous cropping obstacles to AG cultivation deserves further study.

225  In addition, we found that the content of most phenolic acids, such as *p*-coumaric, *p*-hydroxybenzoic, vanillic, caffeic,

226  and cinnamic acid, decreased after AG cultivation, and had not returned to the levels in CS even after 10 years of subsequent

227  crop rotation. AG requires a suitable environment for growth. Before germination in spring, the ginseng farmers' association

228  uses wheat straw to cover the soil, which not only reduces soil temperature and retains soil moisture, but also improves soil

229  quality and promotes the growth of AG seedlings. Jia et al. [34] detected the increase in ferulic, vanillic, cinnamic, and *p*-

230  hydroxybenzoic acid in a wheat-corn rotation area. In addition, Zheng et al. [35] found that straw return, a common method

231  for soil improvement, also increased the concentration of phenolic acids in soil. In our study, the increased phenolic acid

232  content in CS relative to RS may have been beneficial to the growth of AG. Similar to our research results, Jiao et al. [36] also

233  found that the content of phenolic acid substances such as syringic, vanillic, *p*-coumaric, and ferulic acid decreased by

234  49.1%–81% after adding AG root residues (simulating the seasonal AG leaf and fibrous root senescence). Therefore,

235  decreases in the soil contents of some phenolic acids after planting AG may underlie the decline of other soil properties,

236  which is not conducive to the subsequent growth of AG.

237  As described above, some phenolic acids may be beneficial to the growth of AG; if so, by what mechanism do these

238  beneficial phenolic acids exert their role? Li et al. [37] found that cinnamic acid inhibits *Cylindrocarpon destructans* (a

239  pathogen of ginseng) growth at high concentrations, while promoting it at low concentrations. Yang et al. [38] found that

240  vanillic acid promoted the growth of the pathogens *Rhizoctonia solani* and *Fusarium solani* at low concentrations, but

241  inhibited it at high concentrations; many phenolic acid compounds can inhibit the proliferation of *Phytophthora cactorum*

242  (a pathogenic bacterium that causes AG phytophthora disease) at high concentrations. In addition, Yuan et al. [39] found that

243  *p*-coumaric acid strongly suppressed the in vitro growth of fungi, significantly reducing the decay caused by *Alternaria*

244  *alternata*. Therefore, it can be seen that phenolic acids have inhibitory effects on pathogens at higher concentrations. With

245  a decrease in soil phenolic acid content, this inhibitory effect on pathogenic bacteria will be weakened, resulting in an

246  imbalance in the soil microbial composition that affects AG growth performance. Overall, soil phenolic acid content may

247  indirectly affect AG growth performance by affecting soil microorganisms.

248

249 **The change in the relative abundance of key bacteria** Our results showed that there was no significant difference
250 in bacterial α-diversity between 10-year post-ginseng RS and CS, but there were differences in β-diversity, which reflects
251 community composition and structure, between CS and RS. In other words, there were significant differences in the relative
252 abundance of key bacteria in the bacterial community, such as Chlamydiae (phylum level, RS: 0.28%, CS: 0.10%, P =
253 0.035), within this phylum, the c_Chlamydiae, o_Chlamydiales, f_Simkaniaceae, and g_uncultured; *Acidothermus* (genus
254 level, RS: 2.40%, CS: 5.40%, P = 0.030); Sphingomonadales (order level, CS: 2.98%, RS: 1.68%, P = 0.002),
255 Sphingomonadaceae (family level, CS: 2.88%, RS: 1.48%, P = 0.004), genera *Novosphingobium* (CS: 0.03%, RS: 0.20%,
256 P = 0.035) and *Sphingomonas* (CS: 2.83%, RS: 1.10%, P = 0.000); *Rhodanobacter* (CS: 0.38%, RS: 3.45%, P = 0.050);
257 *Arthrobacter* (CS: 0.03%, RS: 0.43%, P = 0.001); *Mizugakiibacter* (CS: 0.63%, RS: 2.28%, P = 0.048); *Jatrophihabitans*
258 (CS: 1.15%, RS: 0.75%, P = 0.048); *Pseudomonas* (RS: 0.15%, CS: 0.03%, P = 0.029) among others (Fig. 4, see
259 Supplementary Table S2).

260      There was no difference in soil bacterial α-diversity between RS and CS, which may be due to the recovery of soil
261 bacterial diversity after 10 years of rotation. However, the results of the pot experiment showed that RS still presented
262 continuous cropping obstacles, which indicated that restoring soil microbial α-diversity does not alleviate continuous
263 cropping obstacles for AG. Instead, differences in microbial community composition (i.e., β-diversity), particularly the
264 abundances of bacterial taxa that play key roles, may explain the persistence of AG continuous cropping obstacles in RS
265 after 10 years.

266      Among the differences in microbial community composition, CS had higher relative abundances of some bacterial
267 genera that may be beneficial bacteria. The genus *Acidothermus* had the highest abundance, and it contained a single species,
268 *A. cellulolyticus*, which is thermophilic, acidophilic, and has the ability to produce many thermostable cellulose-degrading
269 enzymes [40]. Therefore, higher cellulose-degrading capacity might exist in CS than that in RS. *Sphingomonas*, a bacterium
270 with the ability to decompose mono- and polycyclic aromatic compounds, as well as heterocyclic compounds, was more
271 abundant in CS than RS, suggesting that bacterial decay of recalcitrant plant compounds was also higher in CS than RS. In
272 addition, *Sphingomonas* not only decomposes monoaromatic phenolic acids but also improves plant stress resistance, and it
273 is considered a plant probiotic [41]. Similar to our results, Li and Jiang [23] found that *Jatrophihabitans* relative abundance in
274 soil used for AG for 4 years was significantly (P < 0.05) lower than that in soil used for other crops over the same period;
275 therefore, we speculate that AG planting has reduced the abundance of *Jatrophihabitans* as a potential beneficial bacterium
276 in soil. All in all, we found that previous AG planting reduced the relative abundance of some functionally-important
277 bacteria, i.e. those with the abilities to decay cellulose and monocyclic/heterocyclic aromatic compounds, as well as the
278 relative abundance of the plant-beneficial microbe *Sphingomonas* even after 10 years of crop rotation. These changes have
279 negative effects on the maintenance of soil microbial community stability and the promotion of AG growth.

280      Moreover, there are some genera with higher relative abundance in RS that may be harmful pathogens. Many reports
281 have shown that *Chlamydia* and its phylum Chlamydiae are pathogenic to humans and animals, and their host range may be
282 larger [42]; hence, whether *Chlamydia* contributes to the continuous cropping disorder of AG needs further study. Similar to
283 our research results, in a study conducted by Jiang et al. [43], the abundance rank for *Rhodanobacter* was healthy root group
284 > root rot group > control group; in addition, compared with CS, there was a higher abundance of *Rhodanobacter* in the soil
285 in which Korean ginseng (*Panax ginseng*) was grown [44]. We also found that this genus might be increased by the influence
286 of Panax plants, which warrants further study. Our results showed that *Arthrobacter* was higher in the RS group, and Jiang
287 et al. [43] also found that the relative abundance of *Arthrobacter* in the root rot group was higher than that in the healthy root
288 group; therefore, we speculate that *Arthrobacter* might be a factor causing root rot of *P. quinquefolius*, leading to a
289 continuous cropping obstacle to AG growth. Our results showed that the abundance of *Pseudomonas sp.* in RS was higher
290 than that in CS (RS: 0.15%, CS: 0.03%, P = 0.029, see Supplementary Table S2). Tan et al. [45] showed that *Pseudomonas*
291 *sp.* was the main pathogen causing root rot disease in *P. notoginseng*. In addition, Jiang et al. [43] also found that *Pseudomonas*
292 is abundant in the rhizosphere soils of diseased ginseng roots. Therefore, it is necessary to further study the effects of

293   *Pseudomonas* species on AG growth. To sum up, the relative abundances of a large number of bacteria that are either
294   confirmed or potentially harmful to other plants increased in RS, which may be an important factor leading to the occurrence
295   of continuous cropping obstacles in the 10-year post-ginseng rotation soil.

296        As shown in Fig. 6, there are many correlations among the three factors. The abundances of *Acidothermus*,
297   *Sphingomonas*, *Jatrophihabitans*, and *Actinospica* were each positively correlated with that of available K, caffeic acid, and
298   cinnamic acid, but negatively correlated with that of salicylic acid. Therefore, the interactions among phenolic acids,
299   microorganisms, and soil nutrients evidenced possible "synergistic" or "antagonistic" effects within the microecosystem.
300   Overall, these complex relationships are the main reason for AG continuous cropping obstacles, but it is still unknown which
301   of these factors plays the primary role. Finally, *Nitrobacter*, *Actinospica*, *Clostridium sensu stricto 1*, *Thermosporothrix*,
302   *Holophaga*, and *Peptoclostridium*, also showed significant differences in abundance between RS and CS (Fig. 4), which
303   also should receive more attention.

304

## Methods

305

306   **Study site** Two study sites were selected in March 2018; these were two adjacent farmlands in Houjia Town, Wendeng
307   District, Weihai, located 45 m above sea level at 122°13′17″ E, 37°4′34″ N, and has a continental monsoon climate. The
308   rotation plot had been used for 4-year AG cultivation, following which it was used for 10 years (rotation soil, RS); the
309   control plot was a cropland in which AG had never been planted (control soil, CS). Each plot was approximately 2 ha in
310   size. For the 10 years of crop rotation, crop types and plot management such as fertilization, watering, and weeding were
311   the same in both the plots. When sampled, both were planted with wheat.

312

313   **Soil sampling** Each plot was divided into four subplots. Soil samples were collected by the five-point sampling method
314   in each subplot. After removing surface stones, gravel, humus, and plant residues from each sampling point, approximately
315   100 g of surface soil (0–20 cm depth) was collected. The five soil samples from each subplot were evenly mixed to produce
316   one soil sample of approximately 500 g. Each soil sample was then divided as follows. Approximately 10 g of soil was
317   transferred into cryogenic vials and placed in a liquid nitrogen tank for low-temperature transportation and preservation;
318   after returning to the laboratory, these vials were refrigerated at −80 °C for use in high-throughput sequencing.
319   Approximately 200 g of soil was immediately stored in a refrigerator at 4 °C to determine soil water, nitrate nitrogen, and
320   ammonium nitrogen content. Approximately 300 g of soil was air-dried, filtered through a 2 mm sieve, and stored at room
321   temperature (18-25 °C) to determine physical and chemical properties, enzyme activity, and phenolic compound content.

322

323   **Pot experiment of plant-soil feedback** Sufficient soil was collected from the eight subplots of the two plots. The soil
324   collected from each plot was divided into 40 pots (pottery basin, 40 cm in diameter and 30 cm in height). Because 2-year-
325   old AG is prone to disease, two seedlings of this age were planted per pot (enough space for 2 years of AG growth).
326   Regardless of the scientific management of local planting technology, the growth of AG seedlings was regularly observed
327   and recorded in the first 20 days after planting. Because of this process, not only soil effects but also transplanting effects
328   were observed; however, transplanting may lead to fibrous root fracture of AG, thereby causing its death. Hence, we chose
329   to reseed pots on the 21st day to recalculate the survival rate, leaf area, biomass, and other indicators.

330

331   **Soil physicochemical properties and enzyme activities** Soil pH was measured using a Delta 320 electrode pH
332   meter (Mettler Toledo, USA) in a 1:2.5 (w/v) soil water suspension. Water content was measured by oven-drying the fresh
333   soil samples at 105 °C for 24 h. Total nitrogen (N) and carbon (C) contents were determined using a vario EL III elemental
334   analyzer (Elementar Analysensysteme, Germany). Ammonium N content was determined via the potassium chloride
335   extraction–indigo phenol blue colorimetric method; nitrate N content was determined using the phenol disulfonic acid

336  colorimetric method. Available phosphorus (P) content was determined using the sodium bicarbonate extraction–
337  molybdenum-antimony colorimetric method, and the content of available potassium (K) was determined via flame
338  spectrophotometry [46]. Soil urease and acid phosphatase activities were determined via colorimetry. Catalase activity was
339  determined via potassium permanganate titration. Sucrase activity was determined using sodium thiosulfate titration. The
340  determination of soil enzyme activity was based on the method proposed by Guan [47].

341

342  **Phenolic acids in soil** The method proposed by Hartley and Buchan [48] was used to extract phenolic acids from soil with
343  the following improvements. Air-dried soil (25 g) was added to 25 mL of 1 mol L$^{-1}$ NaOH solution, left overnight, and then
344  agitated for 30 min on a reciprocal shaker. After centrifugal separation of the soil suspension (5 min, 2860 g, 20 °C), the
345  supernatant was filtered through filter paper. The suspension was then acidified to pH 2.5 with 12 mol L$^{-1}$ hydrochloric acid.
346  After 2 h, humic acid was removed via centrifugation (same as above), and the supernatant was filtered through a 0.22-μm
347  filter to obtain the final extract. This final extract was analyzed using high performance liquid chromatography (HPLC; see
348  details below) on the e2695 HPLC system (Waters, USA) with a diode array detector using Uranus C18 (250 × 4.6 mm, 5
349  μm) and guard columns (20 × 4.6 mm, 5 μm). The results were converted to dry soil weight.

350  Standard phenolic acids (see Supplementary Figure S1) were purchased from Sigma (St. Louis, USA). Detection was
351  performed at 280 nm. Different phenolic acids were identified by their retention times compared to those of the purchased
352  standards (seeSupplementary Figure S2). The chromatographic data were recorded and processed using an Empower
353  workstation (Waters, USA). Standards of nine phenolic acids were prepared in different concentrations. The standard curve
354  equations of the nine phenolic acids were obtained by considering the peak area of the liquid chromatogram as Y and the
355  sample concentration as X. The concentration of each compound in each soil sample was obtained based on the peak areas
356  (see Supplementary Table S1).

357  The HPLC separations were conducted as follows: the mobile phase comprised aqueous formic acid solution (0.1%,
358  v/v) and acetonitrile; the column temperature was 30 °C; the injection volume was 20 μL and the flow rate was 1.0 mL
359  min−1. The gradients and timings were 6–10% acetonitrile (0–16 min), 10–22% (16–36 min), 22–50% (36–46 min), 50–
360  100% (46–48 min), and final hold for 5 min. The column was equilibrated for 10 min between injections.

361

362  **Genomic DNA extraction, PCR amplification, and high-throughput amplicon sequencing** Total DNA was
363  extracted from soil samples using the PowerSoil® DNA Isolation Kit (MoBio, USA), according to the manufacturer's
364  protocol. The concentration and purity of the obtained DNA samples were then determined using a UV-1200 UV
365  spectrophotometer (Shanghai Mapada, China) and agarose gel electrophoresis. The V3–V4 region of the 16S rRNA gene
366  was amplified using primers 338F (5′-ACTCCTACGGGAGGCAGCA-3′) and 806R (5′-
367  GGACTACHVGGGTWTCTAAT-3′) for bacterial community analysis[49]. PCR amplification was carried out in a total
368  volume of 25 μL containing 5 μL 5× reaction buffer, 5 μL 5× GC buffer, 2 μL dNTPs (2.5 mmol), 1 μL 338F primer (10
369  μmol), 1 μL 806R primer (10 μmol), 2 μL DNA template, 8.75 μL ddH2O, and 0.25 μL Q5 High-Fidelity DNA Polymerase
370  (NEB, USA)[50]. The PCR protocol consisted of an initial denaturation step at 98 °C for 2 min, followed by 28 cycles of
371  denaturation at 98 °C for 15 s, annealing at 55 °C for 30 s, and extension at 72 °C for 30 s; a final extension at 72 °C was
372  performed for 5 min and reaction mixtures were held at 10 °C until further analysis[51]. Three replicates were used per sample,
373  and these were pooled to minimize PCR bias. PCR products were detected via electrophoresis on 2.0% agarose gels, and
374  fragments of about 450 bp were purified with the AxyPrep DNA Gel Extraction Kit (Axygen, USA). PCR amplicons were
375  quantified using the PicoGreen dsDNA Assay Kit (Invitrogen, USA). Finally, paired-end 2×300 bp sequencing of bacterial
376  amplicons was carried out on the MiSeq platform (Illumina, USA) at Personal Biotechnology Co., Ltd (Shanghai, China).
377  The detailed experimental procedure is shown in Supplementary Figure S3.

378

379   **Sequence analyses** Using the unique barcodes obtained from the quantitative insights into microbial ecology (QIIME,

380   v1.8.0, qiime.org) pipeline [52], the raw sequences were assembled for each sample after removing the primer sequences and

381   adaptors. Low quality sequences were filtered out using the criteria proposed by [53,54]. Paired-end reads were assembled

382   using FLASH v1.2.7 [55]. After chimera detection, the remaining high-quality sequences were clustered into operational

383   taxonomic units (OTUs) at 97% sequence identity by UCLUST [56]. A representative sequence was selected from each OTU

384   using default parameters. The OTU taxonomic classification was conducted using the basic local alignment search tool

385   (BLAST) by comparing the representative sequences set against the Greengenes Database [57] and retrieving the best hit [58].

386   An OTU table was further generated to record the abundance of each OTU in each sample and its taxonomy. OTUs

387   containing less than 0.001% of total sequences across all samples were discarded.

388   To minimize the difference in sequencing depth across samples, an averaged, rounded, and rarefied OTU table was

389   generated by averaging 100 evenly resampled OTU subsets below the 90% minimum sequencing depth for further analyses.

390   Sequence data analyses were mainly performed using the QIIME v1.8.0 and R v3.2.0 (https://www.r-project.org/) packages.

391   Chao1, abundance-based coverage estimator (ACE), Shannon's, and Simpson's $\alpha$-diversity indices were calculated at the

392   OTU level using the OTU table in mothur (v1.25.1, https://mothur.org/). OTU-level ranked abundance curves were

393   generated to compare the richness and evenness of OTUs among samples [52]. To explore variation in bacterial community

394   structures across the analyzed soil samples, unweighted and weighted UniFrac distances were also calculated using R v3.2.0.

395   Nonmetric multidimensional scaling (NMDS) analysis, principal component analysis (PCA), and partial least squares

396   discriminant analysis (PLS-DA), were performed on the distance matrices, and coordinates were used to draw 2D graphical

397   outputs. The linear discriminant analysis (LDA) effect size (LEfSe) method was used to detect differentially abundant taxa

398   across groups using the default parameters through the Galaxy online analytics platform

399   (http://huttenhower.sph.harvard.edu/galaxy/) [59]. The difference in sequence size (i.e., absolute abundance) between samples

400   (groups) of each taxon at the phylum and genus levels was compared and tested, using mothur v1.25.1 with the statistical

401   algorithm of Metastats (http://metastats.cbcb.umd.edu/) [60]. mothur v1.25.1 was also used to calculate Spearman's rank

402   correlation coefficients among the dominant genera (i.e. those with abundance in the top 50) and to construct the correlation

403   network for the dominant genera whose Rho > 0.6 and P value < 0.01. This network was then imported into the Cytoscape

404   software (http://www.cytoscape.org/) [61]. We used analysis of similarities (ANOSIM) to determine the size of the intra- and

405   inter-group differences by ranking the distance between the samples and evaluated the statistical significance of the

406   differences between the original samples using the permutation test, calculated by QIIME v1.8.0 [62,63].

407

408   **Statistical analyses** The data concerning survival rate, leaf area, biomass of AG in each sample group, soil

409   physicochemical properties, enzyme activities, content of phenolic acids, $\alpha$-diversity indices, and the relative abundances

410   of bacterial taxa (phyla and genera) were compared using independent sample t-tests with significance accepted at $P < 0.05$,

411   as performed in SPSS v19.0 (IBM Corp., USA). Pearson's correlation analysis was used to identify correlations between

412   bacterial genera and each of the phenolic acids and physicochemical properties. R v3.2.0 and Photoshop CS6 (64 bit,

413   adobe.com) were used to draw analysis charts and output the results. The structural formula of phenolic acid was obtained

414   from indraw (5.1.0, http://indrawforweb.integle.com/).

415

416   **Data accession** Sequencing raw data can be found in the Sequence Read Archive at the National Center for Biotechnology

417   Information              under              Bioproject              ID              PRJNA612151

418   (https://dataview.ncbi.nlm.nih.gov/object/PRJNA612151?reviewer=lou3qt0rvghbih2cbthrqe76l8).

419   ## Conclusions

420   Compared to that in CS, the AG survival rate in RS was lower in the pot experiment, showing notable signs of

421   continuous cropping obstacles. We found higher water content and acidification, and lower nutrient levels in RS than in CS;

422   these factors were directly or indirectly unfavorable to AG growth. The lower levels of *p*-coumaric, *p*-hydroxybenzoic,
423   vanillic, caffeic, and cinnamic acids might be among the factors in RS that indirectly reduced AG growth by promoting the
424   growth and reproduction of its pathogens; in contrast, higher salicylic acid content might be one of the factors directly
425   impeding AG growth. Analysis of the bacterial community structure and composition showed that *Chlamydia* had higher
426   abundance in RS than in CS. In addition, at the genus level, *Acidothermus*, which degrades cellulose, and *Sphingomonas*,
427   which decomposes phenolic acids, had lower abundances in RS than in CS. Hence, according to the correlations we
428   observed, we conclude that complex relationships among water content, pH, phenolic acids, and bacteria play important
429   roles in the continuous cropping obstacles found in RS.
430

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563

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575

576  **Figure legends**

577  **Figure 1** Survival rate and growth index of *Panax quinquefolium* in the pot experiment. The survival rate was calculated
578  every 20 days. The aboveground and underground biomasses were calculated at the end of 60 days. Leaf area was calculated
579  at the final harvest. CS: Plants in the CS group; RS: Plants in the RS group; a: means the difference is not significant ($P >$
580  0.05). RS: the 10-year post-ginseng rotation soil; CS: soil in which no ginseng was grown before ginseng planting

581  **Figure 2.** Comparison of phenolic acid content between 10-year post-ginseng rotation soil (RS) and soil in which no
582  ginseng was grown (CS) before ginseng planting. *represent significant differences ($P < 0.05$) according to Student's
583  t-test (n=4). ** represent significant differences ($P < 0.01$)

584  **Figure 3.** Nonmetric multidimensional scaling (NMDS) ordination plot of bacterial community structure comparing 10-
585  year post-ginseng rotation soil (RS) and soil in which no ginseng was grown (CS) before ginseng planting. a, unweighted
586  and b, weighted UniFrac distance matrix. c, PCA (principal component analysis) of bacterial community structure; d, PLS-
587  DA (partial least squares discriminant analysis) of bacterial community structure. The shorter the distance between two
588  points, the higher the similarity of the microbial community structure between the two groups

589  **Figure 4.** Composition and structure of the bacterial community from the 10-year post-ginseng rotation soil (RS) and
590  soil in which no ginseng was grown (CS) before ginseng planting. a: phylum, b: genus, c: significantly different
591  bacteria at the genus level, comparing RS and CS. The bacteria genera listed in c are all significantly different (t-test,
592  $P < 0.05$). Mean (n = 4) data are shown; error bars represent SE. *represents significant differences ($P < 0.05$)
593  according to the Metastats-test (n = 4)

594     **Figure 5.** Differentially abundant bacterial taxa as assessed using linear discriminant analysis (LDA) with effect size
595     measurements (LEfSe) in the 10-year post-ginseng rotation soil (RS) and soil in which no ginseng was grown (CS)
596     before ginseng planting

597     **Figure 6.** The correlation network of phenolic acids with bacteria and soil physicochemical properties. When the
598     correlation factor is yellow, it means that the factor is enriched in the 10-year post-ginseng rotation soil (RS), whereas
599     when it is green, it represents the soil in which no ginseng was grown (CS) before ginseng planting. The solid line
600     represents the positive correlation among the correlation factors, while the dotted line is the negative correlation. The
601     circle represents bacteria, and the different arrangement of squares represents phenolic acid and physicochemical
602     properties

603     **Table**

604     **Table 1.** Comparison of physicochemical properties of 10-year post-ginseng rotation soil (RS) and soil in which no ginseng
605     was grown (CS) before ginseng planting. * Statistically significant difference at P < 0.05; ** P < 0.01 (T test). Mean ± SE
606     (n = 4) data shown

607     **Table 2.** Comparison of enzyme activities between 10-year post-ginseng rotation soil (RS) and soil in which no
608     ginseng was grown (CS) before ginseng planting. * Statistically significant difference at P < 0.05 (T test). Mean ± SE
609     (n = 4) data shown

610