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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

1. 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 the high medicinal value of AG, and it is widely used as both food and medicine [3-5]. 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 [6-8], those of AG can last for more than 5 to 10 years or even decades [9, 10]. The main detrimental factors to replanting AG in land restored by rotation are not clear.

42   Many studies have reported that crops under continuous cropping can be adversely affected by
43   allelopathic or autotoxic effects [2, 11]. Phenolic acids released during plant growth as the main
44   allelochemicals, are the main continuous cropping obstacles to many terrestrial plants. They have
45   been reported to exert inhibitory effects on many crops and medicinal plants [12, 13]. Notably, in a
46   study on the allelopathy of single and mixed phenolic acids on AG [14], nine phenolic acids including
47   cinnamic, vanillic, and ferulic acids were found to inhibit the growth of AG radicles at concentrations
48   ranging from 0.1 mmol L^{-1} to 10 mmol L^{-1}; this inhibition increased with increasing phenolic acid
49   concentration which indicated that phenolic acids had direct inhibitory effects on the growth of AG.
50   However, there are also microorganisms that interact with phenolic acids in soil, especially some AG
51   probiotics (e.g., *Sphingomonas*, *Burkholderia*) and AG pathogens (e.g., *Fusarium oxysporum*,
52   *Phytophthora cactorum*). Kertesz et al. [15] found that phenolic acids can be used as substrates by
53   *Sphingomonas* and *Burkholderia*, enhancing their population growth, and that phenolic acids also play
54   a role in the formation of a stable beneficial microbial community [15]. According to Hu et al. [16], at
55   a concentration of 100 mg L^{-1}, ferulic acid promoted the growth of cucumber fusarium wilt, whereas
56   at 150 mg L^{-1}, it showed a significant inhibitory effect. Yuan et al. [17] found that ferulic acid promoted
57   the growth of *Fusarium oxysporum* at low concentrations, but inhibited its growth at high
58   concentrations. On one hand, phenolic acids can be used as substrates to support beneficial
59   microorganisms, but on the other hand, high phenolic acid concentrations can inhibit the growth of
60   pathogenic bacteria, thereby promoting AG growth. Therefore, higher concentrations of phenolic
61   acids can not only inhibit the growth of AG as allelochemicals, but also promote AG growth by
62   stimulating beneficial microorganisms and reducing harmful microorganisms. The direct and
63   indirect effects of allelochemicals may have opposite effects on plant growth, which are similar to the
64   allelopathic effects of different plants on invasive *Phytolacca americana* [18, 19].

65   In addition, some studies have also showed that planting AG leads to progressive imbalances in
66   soil microbial communities [20, 21], and the depletion of available nutrients in the soil or soil
67   acidification [22, 23]. However, there is little research examining whether these deleterious effects on
68   the microbial community, soil nutrient availability, and soil acidification can be reversed after a
69   period of recovery (here, 10 years). Therefore, it is important to measure soil microbial diversity,
70   community composition, and soil physical and chemical properties after crop-rotation restoration in
71   order to determine which factors influencing AG replanting persist over the course of 10 years. Since
72   previous studies have mostly focused on fungi, equally important bacteria [24-26] have been often
73   overlooked; therefore, here we focused on the bacterial communities.

74   In the present study, soil used for planting AG and then subjected to crop rotation for 10 years
75   was collected and used for replanting AG (second age AG seedlings). The growth of AG seedlings in
76   rotation soil (RS) was observed to explore the time-variant feature of continuous cropping obstacles
77   and was compared to that in soil where no AG had been grown (control soil (CS)) to verify if: 1) the
78   change in soil phenolic acid content has an effect on soil microbial community composition, and
79   thereby an indirect effect on AG growth; 2) the change in bacterial community structure mainly
80   reflects an increase in pathogen abundance or a reduction in beneficial bacterial abundance; and 3)
81   soil physicochemical properties and enzyme activities remain unsuitable for AG growth.

82   **2. Results**

83   *2.1. Pot experiment of AG planting*

84   As shown in Figure 1, the survival rate of the two groups decreased in the stable period (20
85   days)—that of the RS group decreased to 65% and that of the CS group decreased to 72.5%. On the
86   21st day, the survival rate of the two groups were recovered to 100% by replanting AG seedlings.
87   Although there was no significant difference in leaf area, aboveground biomass, and underground
88   biomass between the two groups, the survival rate of RS was only 45% on the 60th day, while that of
89   CS was 67.5%. The survival rate of AG between the two groups was substantially different.

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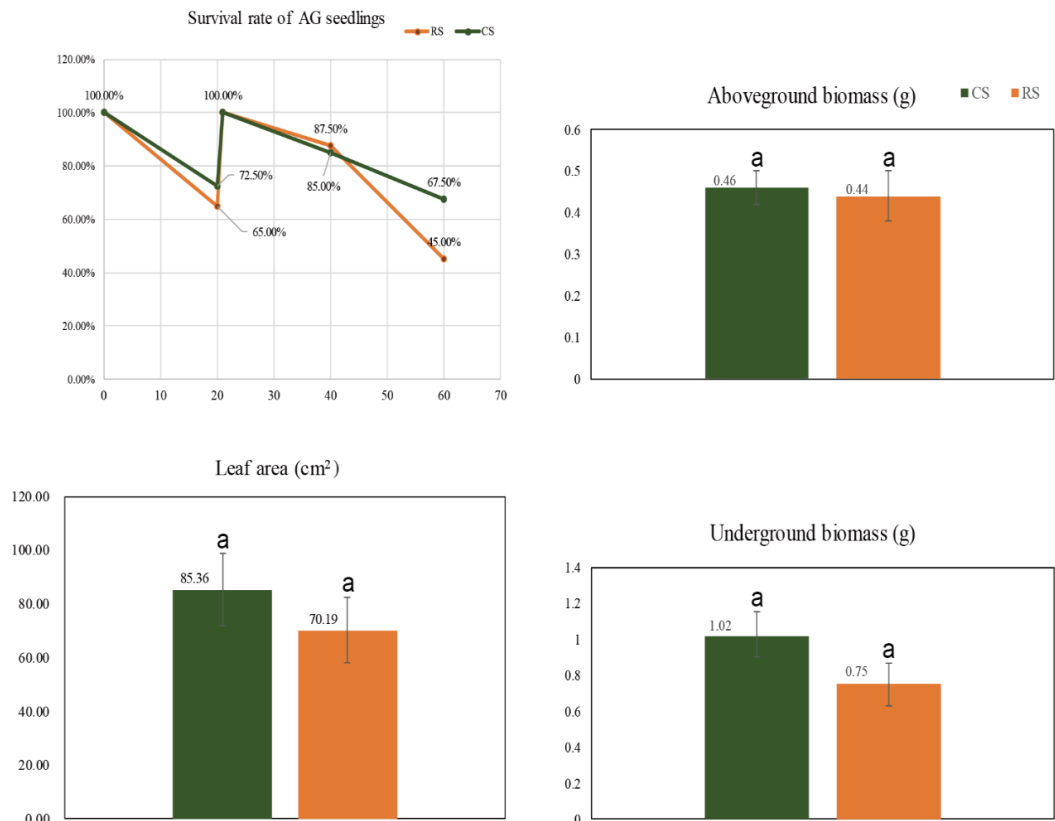
92   **Figure 1** Survival rate and growth index of *Panax quinquefolium* in the pot experiment. The survival
93   rate was calculated every 20 days. The aboveground and underground biomasses were calculated at
94   the end of 60 days. Leaf area was calculated at the final harvest. CS: Plants in the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

97

98   *2.2. Soil physicochemical properties and enzyme activities*

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

104

105   **Table 1** Comparison of physicochemical properties of 10-year post-ginseng rotation soil (RS) and
106   soil in which no ginseng was grown (CS) before ginseng planting

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**

107   * Statistically significant difference at $P < 0.05$; ** $P < 0.01$ (T test). Mean ± SE (n = 4) data shown

108

109   There was a significant difference in urease activity between the CS and RS samples, with the
110   activity in CS being 25.13% higher than that in RS; the activities of phosphatase, sucrase, and catalase
111   were higher in CS than in RS, but these differences were not significant (Table 2).

112

113   **Table 2** Comparison of enzyme activities between 10-year post-ginseng rotation soil (RS) and soil in which no
114   ginseng was grown (CS) before ginseng planting

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

115   * Statistically significant difference at $P < 0.05$ (T test). Mean ± SE (n = 4) data shown

116   2.3. Soil phenolic acids content

117   In terms of phenolic acids, the levels of vanillic and cinnamic acids were markedly higher in CS
118   than those in RS ($P < 0.05$); the levels of *p*-coumaric, caffeic, and *p*-hydroxybenzoic acids were
119   significantly higher in CS than those in RS ($P < 0.01$). However, salicylic acid showed the opposite
120   trend ($P < 0.01$) (Figure 2).
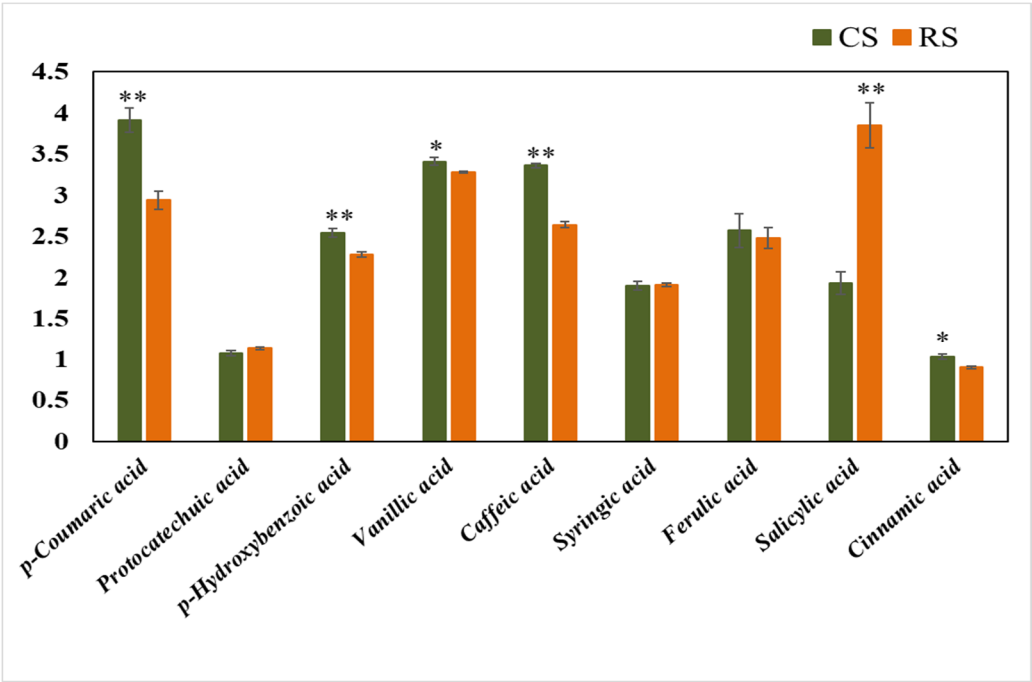
Figure 2. Comparison of phenolic acid content between 10-year post-ginseng rotation soil (RS) and soil in which no ginseng was grown (CS) before ginseng planting. *represent significant differences (P < 0.05) according to Student's t-test (n=4). ** represent significant differences (P < 0.01)

2.4. Soil bacterial diversity and community structure

2.4.1. Sequencing results, OTU cluster analysis, and α-diversity analysis.

The V3–V4 region of the 16S rRNA gene was sequenced. After removing the low quality, barcode, and primer sequences, 636,654 effective sequences were obtained from the eight samples; 5421 and 5747 OTUs were contained in RS and CS samples, respectively, and the number of shared OTUs was 3673 after clustering the effective sequences (Figure S4). The slopes of species accumulation curves for bacterial species were flat at different similarity cutoff values, indicating that the identified bacterial diversity was close to saturation and that an increase in the sequencing depth would not increase the number of detected species (Figure S5). Species composition and abundance distributions for each sample, from phylum to genus levels, were obtained using QIIME (Table S2). The diversity and richness of bacterial communities in the soil samples were evaluated and compared using Chao1, Simpson's, Shannon's, and ACE indices. Each index reflected higher microbial community diversity and richness in RS than in CS, although none of the differences were significant (Figure S6).

2.4.2. β-diversity

As shown in Table 3, ANOSIM for the unweighted UniFrac result (R = 0.7188, P = 0.030) supported the NMDS analysis (Figure 3a) for the unweighted UniFrac distance matrix, both indicating that the bacterial community structure of RS and CS differed significantly without weighing. However, the weighted UniFrac result (R = 0.3646; P = 0.085) indicated that the difference between the two groups of samples was not significant after considering evenness (Figure 3b). The

147   overall β-diversity index analysis (Figure 3a, b, c, d) showed that there were some differences
148   between RS and CS.

149

150   **Table 3.** UniFrac analysis of similarities between the bacterial communities present in 10-year post-ginseng
151   rotation soil (RS) and soil in which no ginseng was grown (CS) before ginseng planting

Method name	R statistic	P value	Number of permutations
Unweighted	0.7188	0.030	999
Weighted	0.3646	0.085	999

152   Note: the R value is the statistics of Anosim, which is between - 1 and 1, indicating the difference
153   between the differences between groups and within groups. The closer r value is to 1, the greater the
154   difference between groups, and the smaller the difference within groups, the better the grouping
155   effect; if r = 0, it means that the grouping effect of samples is equal to random allocation, and there is
156   no observable statistical difference between sample groups; if R is negative, it means that the
157   difference within groups exceeds the difference between groups, indicating that the grouping effect
158   is poor. P value reflects the statistical significance of anosim analysis results. The smaller the p value
159   is, the higher the significance of the difference between the sample groups is

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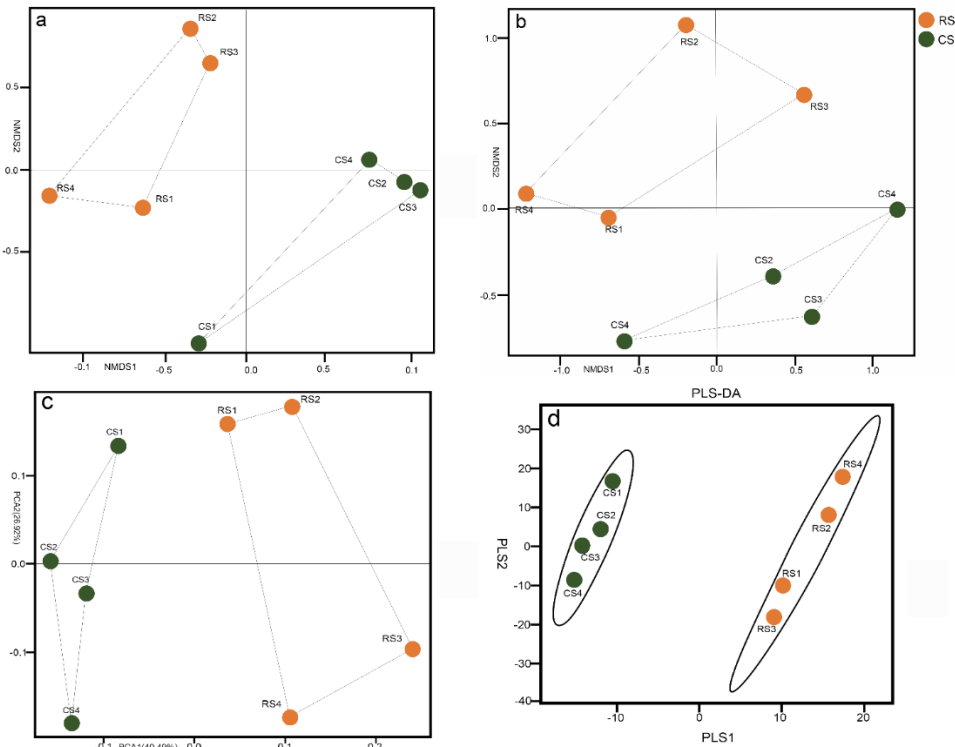
162   **Figure 3.** Nonmetric multidimensional scaling (NMDS) ordination plot of bacterial community
163   structure comparing 10-year post-ginseng rotation soil (RS) and soil in which no ginseng was grown
164   (CS) before ginseng planting. a, unweighted and b, weighted UniFrac distance matrix. c, PCA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

168

169   2.4.3. Bacterial community structure and significantly different bacterial composition

170   The bacterial populations in the different samples were analyzed by comparing the obtained 16S
171   rRNA sequences with those of the Greengenes databases using BLAST (Figure 4). The dominant
172   bacterial phyla were Proteobacteria, Actinobacteria, Acidobacteria, Chloroflexi, Firmicutes,
173   Gemmatimonadetes, and Bacteroidetes across the two groups (Figure 4a). The most prominent
174   difference between the two groups was that the abundance of Chlamydiae in RS (0.28%) was
175   significantly higher than that in CS (0.10%), and further analysis at lower classification levels revealed
176   that c__Chlamydiae, o__Chlamydiales, f__Simkaniaceae, and g__uncultured were all significantly
177   different (Table S2).

178   The most abundant bacterial genera were also the same across both groups, namely
179   *Rhizomicrobium*, *Bacillus*, *Bryobacter*, *Variibacter*, *Candidatus_solibacter*, *Gemmatimonas*, *Bradyrhizobium*,
180   *Pseudolabrys*, *Crossiella*, *Streptomyces*, *Burkholderia-Paraburkholderia*, *Granulicella*, and *Roseiarcus* (Figure
181   4b). The proportions of *Rhodanobacter* (3.45%), *Mizugakiibacter* (2.28%), *Clostridium_sensu_stricto*_1
182   (0.45%), *Arthrobacter* (0.43%), *Holophaga* (0.20%), *Novosphingobium* (0.20%), and *Peptoclostridium*
183   (0.20%) were higher in RS than in CS. The proportions of *Sphingomonas* (2.83%), *Jatrophihabitans*
184   (1.15%), *Nitrobacter* (1.13%), and *Thermosporothrix* (0.35%) were higher in CS than in RS (Figure 4).

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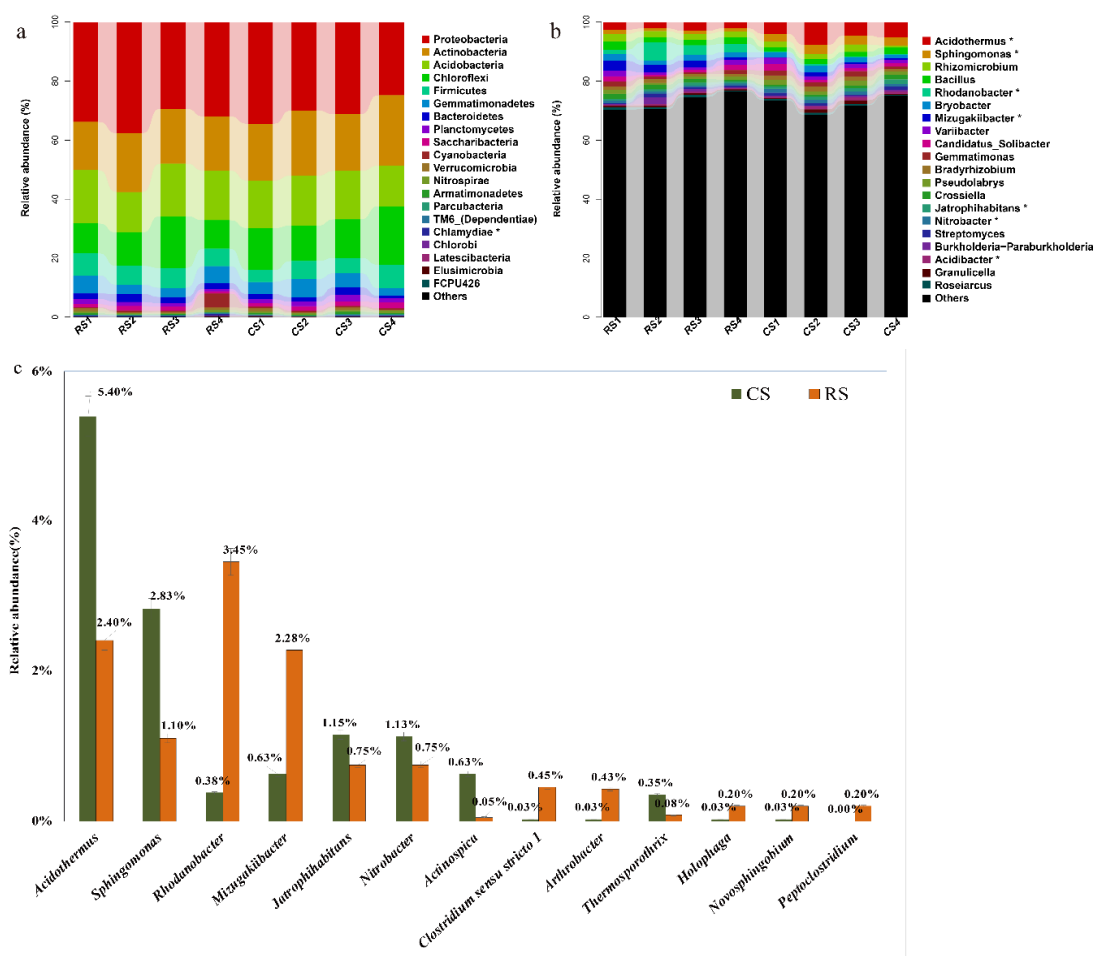
187   **Figure 4.** Composition and structure of the bacterial community from the 10-year post-ginseng
188   rotation soil (RS) and soil in which no ginseng was grown (CS) before ginseng planting. a: phylum, b:

189   genus, c: significantly different bacteria at the genus level, comparing RS and CS. The bacteria genera
190   listed in c are all significantly different (t-test, P < 0.05). Mean (n = 4) data are shown; error bars
191   represent SE. *represents significant differences (P < 0.05) according to the Metastats-test (n = 4)

192

193   2.4.4. LEfSe analysis of the differentially abundant bacterial communities

194   LEfSe uses LDA scores to estimate the effect size of each differentially abundant taxon, and to
195   rank the relative differences among taxa that are discriminative with biological consistency and
196   statistical significance. The LEfSe analysis of RS and CS bacterial communities showed 42
197   differentially abundant taxonomic clades with an LDA score higher than 3.0 (Figure 5). After
198   combining these results (Figure 5) with the information in Table S2, it was found that c
199   (class)_Gammaproteobacteria_o   (order)_Xanthomonadales_f   (family)_Xanthomonadaceae_g
200   (genus)_*Mizugakiibacter* and g_*Rhodanobacter* (underscores represent relationships at the level of
201   phylogeny); o_Holophagales_f_ Holophagaceae_g_*Holophaga* were all significantly different (T-test,
202   P<0.05)   and   had   a   higher   abundance   in   RS.   O_Sphingomonadales_f_
203   Sphingomonadaceae_g_*Sphingomonas* and _g_ *Novosphingobium* were significantly different (t-test,
204   P<0.05); in addition, they had a much higher abundance in CS, except for Novosphingobium.
205   Furthermore,   f_Acidothermaceae_g_*Acidothermus*   and   f_Actinospicaceae_g_*Actinospica*   were
206   significantly different.

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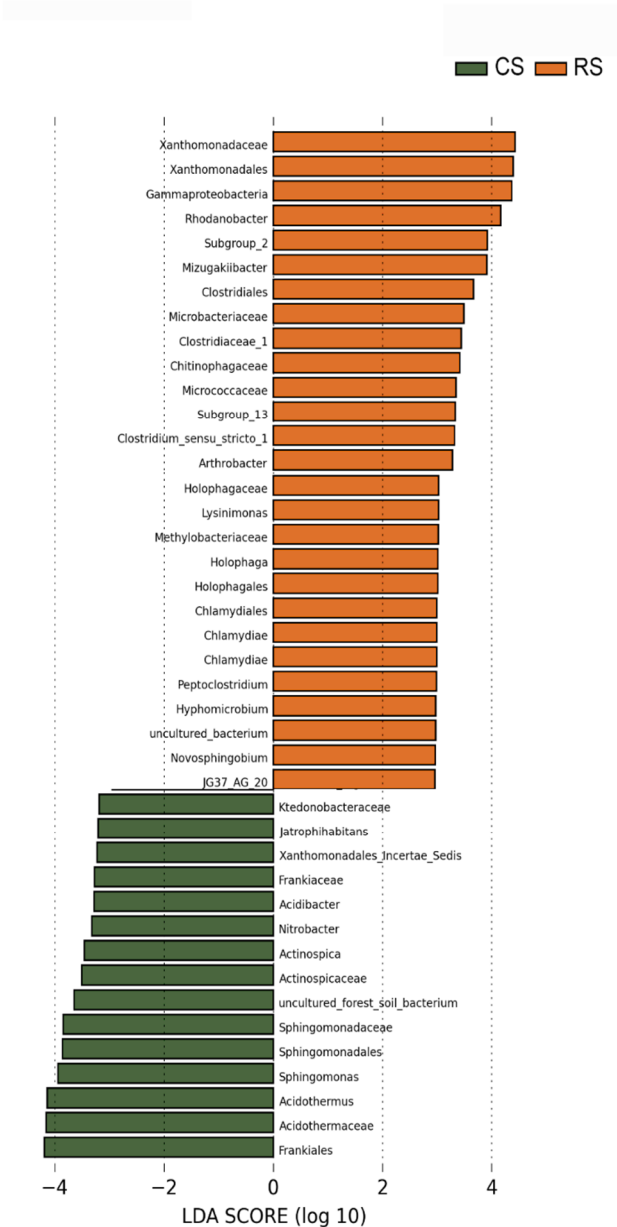
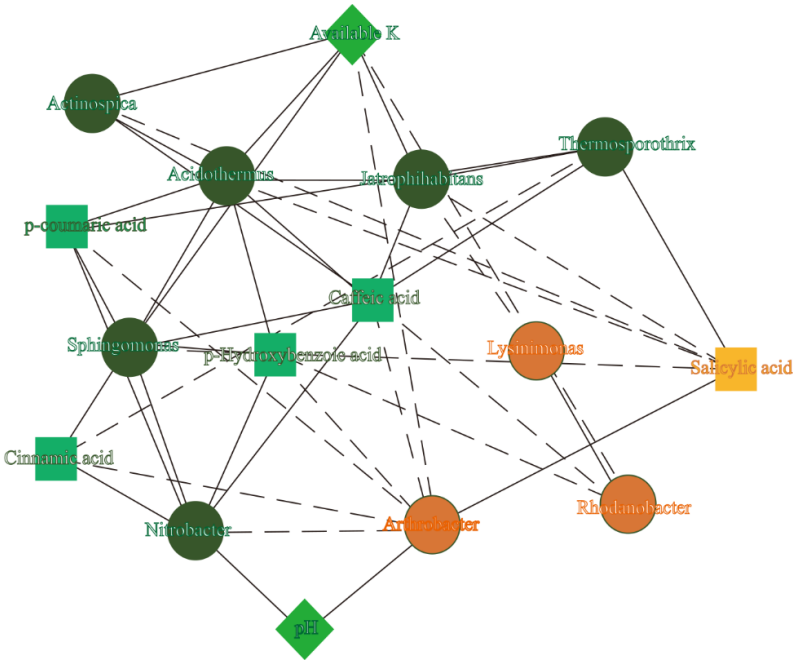


Figure 5. Differentially abundant bacterial taxa as assessed using linear discriminant analysis (LDA)
with effect size measurements (LEfSe) in the 10-year post-ginseng rotation soil (RS) and soil in which
no ginseng was grown (CS) before ginseng planting

211

2.5. Correlation analysis

To further examine the possible "collaborative" or "competing" relationships among the different
communities, Spearman's rank correlation coefficients between the most abundant genera were
calculated using Mothur software. The correlations among the 50 dominant bacterial genera were
analyzed (Figure S7), and the correlation analysis between the top ten bacterial genera (Table 4) and
factors of interest—comprising six different phenolic acids and three physicochemical properties—
indicated a number of interesting relationships. As shown in the Figure 6 and Table 4, Sphingomonas
with low relative abundance in RS significantly positively correlated with available K, concentration

220 of phenolic acid, Acidothermus and Nitrobacter abundance and negatively correlated with salicylic
221 acid levels.

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

229

230 **Table 4.** R values of Pearson correlation analysis between the top 10 most abundant bacterial genera
231 rank order and the phenolic compounds and physicochemical properties of 10-year post-ginseng
232 rotation soil (RS) and soil in which no ginseng was grown (CS) before ginseng planting

Bacterial genus	Phenolic Compounds					Physicochemical Properties		
	p-hydroxy-benzoic acid	Caffeic acid	Cinna-mic acid	Salicylic acid	*p*-coumaric acid	Available K	pH	Water Content
Acidothermus	0.751*	0.796*	-	-0.737*	0.864**	0.888**	-	-
Sphingomonas	0.847**	0.946**	0.792*	-0.877**	0.919**	0.953**	-	-
Rhodanobacter	-0.771*	-0.752*	-	-	-	-0.863*	-	-
Mizugakiibacter	-	-0.749*	-	-	-	-	-	-
Jatrophihabitans	-	0.759*	-	-0.800*	-	0.747*	-	-0.773*
Nitrobacter	0.946**	0.758*	0.865**	-	0.868**	-	0.790*	-

Actinospica	-	0.736*	-	-0.792*	-	0.707*	-	-
Clostridium sensu stricto 1	-0.736*	-0.772*	-0.853**	-	-	-0.817*	-	0.792*
Thermosporothrix	-	0.762*	-0.775*	0.804*	0.757*	-	-	-
Arthrobacter	-0.803*	-0.895**	-0.907**	0.825*	-0.810*	-0.858**	0.825*	-

233   * Statistically significant correlation at P < 0.05; ** P < 0.01. Mean ± SE (n = 4) data shown. Missing values

234   represent no significant correlation

3. Discussion

3.1. Pot experiment of AG planting

237   As shown in Figure 1, compared to CS, the survival rate of 10-year rotation AG decreased,

238   indicating that 2-year-old AG survival rate in RS was lower than that of AG in CS. This confirmed

239   the continued existence of AG continuous cropping obstacles in RS.

3.2. The decrease of physicochemical properties and enzyme activity

241   Plant growth requires water and nutrients. Because soil physicochemical properties influence

242   water and nutrient availability, changes in soil physicochemical properties directly affect AG growth.

243   In the present study, the water content of RS was significantly higher than that of CS under the same

244   management conditions (Table 1). Shu et al. [27] found that high soil water content induced root rot

245   disease in AG when sandy loam water content exceeded 30% or that of clay exceeded 50%. Similarly,

246   according to Wang et al. [28], the incidence of rust rot positively correlated with soil moisture and

247   rainfall. Therefore, high soil water content, caused by changes in soil physicochemical properties,

248   may negatively affect AG replanting. Furthermore, the pH of RS was significantly lower than that of

249   CS (Table 1). According to Rahman and Punja [22], root rot severity at soil pH 5.05 was greater than

250   that at pH 7.0, indicating that acidic conditions can negatively affect AG health. In addition, the

251   available K content in RS was lower than that in CS (Table 1). Sun [29] found that AG should be

252   fertilized from emergence to early flowering, when its demand for potassium fertilizer is the highest,

253   suggesting that AG has a high potassium requirement. The levels of ammonium N, nitrate N,

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

255   (Table 1), indicating that the cultivation of AG may have long-term negative effects on these soil

256   nutrients. The same trend was observed for soil enzyme activity. Urease, a nickel-containing enzyme,

257   catalyzes the hydrolysis of urea into carbonate and ammonia. Here, urease activity was significantly

258   higher in CS than in RS. Average phosphatase and sucrase activities were also higher in CS than those

259   in RS, although these differences were not significant (Table 2). Yang [30] also found that the activities

260   of sucrase, urease, and phosphatase decreased during AG cultivation. In summary, compared to that

261   of CS, RS had lower fertility, but higher soil water content and lower pH, two conditions which are

262   conducive to AG disease, and that may, therefore, present obstacles to AG replanting.

3.3. The dual effects of phenolic acids

264   The results showed that the content of salicylic acid in RS was significantly higher than that in

265   CS. Yang [14] found that among the various phenolic acids tested, salicylic acid had the strongest

266 inhibitory effect on AG radicle growth. In our study, higher salicylic acid content in RS may have
267 posed direct autotoxicity to AG. As a major defense hormone, salicylic acid has the function of
268 enhancing immune signals and reprogramming defense transcriptomes [31]. After planting AG, the
269 soil salicylic acid content increased, which indicated that AG might release more salicylic acid in the
270 growth process to improve immune response to the surrounding environment. Therefore, the role of
271 salicylic acid in the continuous cropping obstacles to AG cultivation deserves further study.

272   In addition, we found that the content of most phenolic acids, such as *p*-coumaric, *p*-
273 hydroxybenzoic, vanillic, caffeic, and cinnamic acid, decreased after AG cultivation, and had not
274 returned to the levels in CS even after 10 years of subsequent crop rotation. AG requires a suitable
275 environment for growth. Before germination in spring, the ginseng farmers' association uses wheat
276 straw to cover the soil, which not only reduces soil temperature and retains soil moisture, but also
277 improves soil quality and promotes the growth of AG seedlings. Jia et al. [32] detected the increase
278 in ferulic, vanillic, cinnamic, and *p*-hydroxybenzoic acid in a wheat-corn rotation area. In addition,
279 Zheng et al. [33] found that straw return, a common method for soil improvement, also increased the
280 concentration of phenolic acids in soil. In our study, the increased phenolic acid content in CS relative
281 to RS may have been beneficial to the growth of AG. Similar to our research results, Jiao et al. [34]
282 also found that the content of phenolic acid substances such as syringic, vanillic, *p*-coumaric, and
283 ferulic acid decreased by 49.1%–81% after adding AG root residues (simulating the seasonal AG leaf
284 and fibrous root senescence). Therefore, decreases in the soil contents of some phenolic acids after
285 planting AG may underlie the decline of other soil properties, which is not conducive to the
286 subsequent growth of AG.

287   As described above, some phenolic acids may be beneficial to the growth of AG; if so, by what
288 mechanism do these beneficial phenolic acids exert their role? Li et al. [35] found that cinnamic acid
289 inhibits *Cylindrocarpon destructans* (a pathogen of ginseng) growth at high concentrations, while
290 promoting it at low concentrations. Yang et al. [36] found that vanillic acid promoted the growth of
291 the pathogens *Rhizoctonia solani* and *Fusarium solani* at low concentrations, but inhibited it at high
292 concentrations; many phenolic acid compounds can inhibit the proliferation of *Phytophthora cactorum*
293 (a pathogenic bacterium that causes AG phytophthora disease) at high concentrations. In addition,
294 Yuan et al. [37] found that *p*-coumaric acid strongly suppressed the in vitro growth of fungi,
295 significantly reducing the decay caused by *Alternaria alternata*. Therefore, it can be seen that phenolic
296 acids have inhibitory effects on pathogens at higher concentrations. With a decrease in soil phenolic
297 acid content, this inhibitory effect on pathogenic bacteria will be weakened, resulting in an imbalance
298 in the soil microbial composition that affects AG growth performance. Overall, soil phenolic acid
299 content may indirectly affect AG growth performance by affecting soil microorganisms.

300 *3.3. The change in the relative abundance of key bacteria*

301   Our results showed that there was no significant difference in bacterial α-diversity between 10-
302 year post-ginseng RS and CS, but there were differences in β-diversity, which reflects community
303 composition and structure, between CS and RS. In other words, there were significant differences in
304 the relative abundance of key bacteria in the bacterial community, such as Chlamydiae (phylum level,
305 RS: 0.28%, CS: 0.10%, P = 0.035), within this phylum, the c_Chlamydiae, o_Chlamydiales,
306 f_Simkaniaceae, and g_uncultured; *Acidothermus* (genus level, RS: 2.40%, CS: 5.40%, P = 0.030);
307 Sphingomonadales (order level, CS: 2.98%, RS: 1.68%, P = 0.002), Sphingomonadaceae (family level,
308 CS: 2.88%, RS: 1.48%, P = 0.004), genera *Novosphingobium* (CS: 0.03%, RS: 0.20%, P = 0.035) and

309 *Sphingomonas* (CS: 2.83%, RS: 1.10%, P = 0.000); *Rhodanobacter* (CS: 0.38%, RS: 3.45%, P = 0.050);
310 *Arthrobacter* (CS: 0.03%, RS: 0.43%, P = 0.001); *Mizugakiibacter* (CS: 0.63%, RS: 2.28%, P = 0.048);
311 *Jatrophihabitans* (CS: 1.15%, RS: 0.75%, P = 0.048); *Pseudomonas* (RS: 0.15%, CS: 0.03%, P = 0.029) among
312 others (Figure 4, Table S2).
313   There was no difference in soil bacterial α-diversity between RS and CS, which may be due to
314 the recovery of soil bacterial diversity after 10 years of rotation. However, the results of the pot
315 experiment showed that RS still presented continuous cropping obstacles, which indicated that
316 restoring soil microbial α-diversity does not alleviate continuous cropping obstacles for AG. Instead,
317 differences in microbial community composition (i.e., β-diversity), particularly the abundances of
318 bacterial taxa that play key roles, may explain the persistence of AG continuous cropping obstacles
319 in RS after 10 years.
320   Among the differences in microbial community composition, CS had higher relative abundances
321 of some bacterial genera that may be beneficial bacteria. The genus *Acidothermus* had the highest
322 abundance, and it contained a single species, *A. cellulolyticus*, which is thermophilic, acidophilic, and
323 has the ability to produce many thermostable cellulose-degrading enzymes [38]. Therefore, higher
324 cellulose-degrading capacity might exist in CS than that in RS. *Sphingomonas*, a bacterium with the
325 ability to decompose mono- and polycyclic aromatic compounds, as well as heterocyclic compounds,
326 was more abundant in CS than RS, suggesting that bacterial decay of recalcitrant plant compounds
327 was also higher in CS than RS. In addition, *Sphingomonas* not only decomposes monoaromatic
328 phenolic acids but also improves plant stress resistance, and it is considered a plant probiotic [39].
329 Similar to our results, Li and Jiang [21] found that *Jatrophihabitans* relative abundance in soil used for
330 AG for 4 years was significantly (P < 0.05) lower than that in soil used for other crops over the same
331 period; therefore, we speculate that AG planting has reduced the abundance of *Jatrophihabitans* as a
332 potential beneficial bacterium in soil. All in all, we found that previous AG planting reduced the
333 relative abundance of some functionally-important bacteria, i.e. those with the abilities to decay
334 cellulose and monocyclic/heterocyclic aromatic compounds, as well as the relative abundance of the
335 plant-beneficial microbe *Sphingomonas* even after 10 years of crop rotation. These changes have
336 negative effects on the maintenance of soil microbial community stability and the promotion of AG
337 growth.
338   Moreover, there are some genera with higher relative abundance in RS that may be harmful
339 pathogens. Many reports have shown that *Chlamydia* and its phylum Chlamydiae are pathogenic to
340 humans and animals, and their host range may be larger [40]; hence, whether *Chlamydia* contributes
341 to the continuous cropping disorder of AG needs further study. Similar to our research results, in a
342 study conducted by Jiang et al. [41], the abundance rank for *Rhodanobacter* was healthy root group >
343 root rot group > control group; in addition, compared with CS, there was a higher abundance of
344 *Rhodanobacter* in the soil in which Korean ginseng (*Panax ginseng*) was grown [42]. We also found that
345 this genus might be increased by the influence of Panax plants, which warrants further study. Our
346 results showed that *Arthrobacter* was higher in the RS group, and Jiang et al. [41]also found that the
347 relative abundance of *Arthrobacter* in the root rot group was higher than that in the healthy root group;
348 therefore, we speculate that *Arthrobacter* might be a factor causing root rot of *P. quinquefolius*, leading
349 to a continuous cropping obstacle to AG growth. Our results showed that the abundance of
350 *Pseudomonas sp.* in RS was higher than that in CS (RS: 0.15%, CS: 0.03%, P = 0.029, Table S2). Tan et al.
351 [43] showed that *Pseudomonas sp.* was the main pathogen causing root rot disease in *P. notoginseng*. In
352 addition, Jiang et al. [41]also found that *Pseudomonas* is abundant in the rhizosphere soils of diseased

353   ginseng roots. Therefore, it is necessary to further study the effects of *Pseudomonas* species on AG
354   growth. To sum up, the relative abundances of a large number of bacteria that are either confirmed
355   or potentially harmful to other plants increased in RS, which may be an important factor leading to
356   the occurrence of continuous cropping obstacles in the 10-year post-ginseng rotation soil.

357   As shown in Figure 6, there are many correlations among the three factors. The abundances of
358   *Acidothermus*, *Sphingomonas*, *Jatrophihabitans*, and *Actinospica* were each positively correlated with that
359   of available K, caffeic acid, and cinnamic acid, but negatively correlated with that of salicylic acid.
360   Therefore, the interactions among phenolic acids, microorganisms, and soil nutrients evidenced
361   possible "synergistic" or "antagonistic" effects within the microecosystem. Overall, these complex
362   relationships are the main reason for AG continuous cropping obstacles, but it is still unknown which
363   of these factors plays the primary role. Finally, *Nitrobacter*, *Actinospica*, *Clostridium sensu stricto 1*,
364   *Thermosporothrix*, *Holophaga*, and *Peptoclostridium*, also showed significant differences in abundance
365   between RS and CS (Figure 4), which also should receive more attention.
366

367   **4. Materials and Methods**

368   *4.1. Study site*

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

376   *4.2. Soil sampling*

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

388   *4.3. Pot experiment of plant-soil feedback*

389   Sufficient soil was collected from the eight subplots of the two plots. The soil collected from each
390   plot was divided into 40 pots (pottery basin, 40 cm in diameter and 30 cm in height). Because 2-year-
391   old AG is prone to disease, two seedlings of this age were planted per pot (enough space for 2 years
392   of AG growth). Regardless of the scientific management of local planting technology, the growth of

393 AG seedlings was regularly observed and recorded in the first 20 days after planting. Because of this
394 process, not only soil effects but also transplanting effects were observed; however, transplanting
395 may lead to fibrous root fracture of AG, thereby causing its death. Hence, we chose to reseed pots on
396 the 21st day to recalculate the survival rate, leaf area, biomass, and other indicators.

397 *4.4. Soil physicochemical properties and enzyme activities*

398   Soil pH was measured using a Delta 320 electrode pH meter (Mettler Toledo, USA) in a 1:2.5
399 (w/v) soil water suspension. Water content was measured by oven-drying the fresh soil samples at
400 105 °C for 24 h. Total nitrogen (N) and carbon (C) contents were determined using a vario EL III
401 elemental analyzer (Elementar Analysensysteme, Germany). Ammonium N content was determined
402 via the potassium chloride extraction–indigo phenol blue colorimetric method; nitrate N content was
403 determined using the phenol disulfonic acid colorimetric method. Available phosphorus (P) content
404 was determined using the sodium bicarbonate extraction–molybdenum-antimony colorimetric
405 method, and the content of available potassium (K) was determined via flame spectrophotometry
406 [44]. Soil urease and acid phosphatase activities were determined via colorimetry. Catalase activity
407 was determined via potassium permanganate titration. Sucrase activity was determined using
408 sodium thiosulfate titration. The determination of soil enzyme activity was based on the method
409 proposed by Guan [45].

410 *4.5. Phenolic acids in soil*

411   The method proposed by Hartley and Buchan [46] was used to extract phenolic acids from soil
412 with the following improvements. Air-dried soil (25 g) was added to 25 mL of 1 mol L^{-1} NaOH
413 solution, left overnight, and then agitated for 30 min on a reciprocal shaker. After centrifugal
414 separation of the soil suspension (5 min, 2860 g, 20 ℃), the supernatant was filtered through filter
415 paper. The suspension was then acidified to pH 2.5 with 12 mol L^{-1} hydrochloric acid. After 2 h, humic
416 acid was removed via centrifugation (same as above), and the supernatant was filtered through a
417 0.22-μm filter to obtain the final extract. This final extract was analyzed using high performance
418 liquid chromatography (HPLC; see details below) on the e2695 HPLC system (Waters, USA) with a
419 diode array detector using Uranus C18 (250 × 4.6 mm, 5 μm) and guard columns (20 × 4.6 mm, 5 μm).
420 The results were converted to dry soil weight.
421   Standard phenolic acids (Figure S1) were purchased from Sigma (St. Louis, USA). Detection was
422 performed at 280 nm. Different phenolic acids were identified by their retention times compared to
423 those of the purchased standards (Figure S2). The chromatographic data were recorded and
424 processed using an Empower workstation (Waters, USA). Standards of nine phenolic acids were
425 prepared in different concentrations. The standard curve equations of the nine phenolic acids were
426 obtained by considering the peak area of the liquid chromatogram as Y and the sample concentration
427 as X. The concentration of each compound in each soil sample was obtained based on the peak areas
428 (Table S1).
429   The HPLC separations were conducted as follows: the mobile phase comprised aqueous formic
430 acid solution (0.1%, v/v) and acetonitrile; the column temperature was 30 °C; the injection volume
431 was 20 μL and the flow rate was 1.0 mL min^{-1}. The gradients and timings were 6–10% acetonitrile
432 (0–16 min), 10–22% (16–36 min), 22–50% (36–46 min), 50–100% (46–48 min), and final hold for 5 min.
433 The column was equilibrated for 10 min between injections.

434 *4.6. Genomic DNA extraction, PCR amplification, and high-throughput amplicon sequencing*

435   Total DNA was extracted from soil samples using the PowerSoil® DNA Isolation Kit (MoBio,

436 USA). The concentration and purity of the obtained DNA samples were then determined using a UV-

437 1200 UV spectrophotometer (Shanghai Mapada, China) and agarose gel electrophoresis. The V3–V4

438 region of the 16S rRNA gene was amplified using primers 338F (5′-ACTCCTACGGGAGGCAGCA-

439 3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) for bacterial community analysis. PCR

440 amplification was carried out in a total volume of 25 μL containing 5 μL 5× reaction buffer, 5 μL 5×

441 GC buffer, 2 μL dNTPs (2.5 mmol), 1 μL 338F primer (10 μmol), 1 μL 806R primer (10 μmol), 2 μL

442 DNA template, 8.75 μL ddH2O, and 0.25 μL Q5 High-Fidelity DNA Polymerase (NEB, USA). The

443 PCR protocol consisted of an initial denaturation step at 98 °C for 2 min, followed by 28 cycles of

444 denaturation at 98 °C for 15 s, annealing at 55 °C for 30 s, and extension at 72 °C for 30 s; a final

445 extension at 72 °C was performed for 5 min and reaction mixtures were held at 10 °C until further

446 analysis. Three replicates were used per sample, and these were pooled to minimize PCR bias. PCR

447 products were detected via electrophoresis on 2.0% agarose gels, and fragments of about 450 bp were

448 purified with the AxyPrep DNA Gel Extraction Kit (Axygen, USA). PCR amplicons were quantified

449 using the PicoGreen dsDNA Assay Kit (Invitrogen, USA). Finally, paired-end 2×300 bp sequencing

450 of bacterial amplicons was carried out on the MiSeq platform (Illumina, USA) at Personal

451 Biotechnology Co., Ltd (Shanghai, China). The detailed experimental procedure is shown in Figure

452 S3.

453 *4.7. Sequence analyses*

454   Using the unique barcodes obtained from the quantitative insights into microbial ecology

455 (QIIME, v1.8.0, qiime.org) pipeline [47], the raw sequences were assembled for each sample after

456 removing the primer sequences and adaptors. Low quality sequences were filtered out using the

457 criteria proposed by [48, 49]. Paired-end reads were assembled using FLASH v1.2.7 [50]. After

458 chimera detection, the remaining high-quality sequences were clustered into operational taxonomic

459 units (OTUs) at 97% sequence identity by UCLUST [51]. A representative sequence was selected from

460 each OTU using default parameters. The OTU taxonomic classification was conducted using the basic

461 local alignment search tool (BLAST) by comparing the representative sequences set against the

462 Greengenes Database [52] and retrieving the best hit [53]. An OTU table was further generated to

463 record the abundance of each OTU in each sample and its taxonomy. OTUs containing less than

464 0.001% of total sequences across all samples were discarded.

465   To minimize the difference in sequencing depth across samples, an averaged, rounded, and

466 rarefied OTU table was generated by averaging 100 evenly resampled OTU subsets below the 90%

467 minimum sequencing depth for further analyses. Sequence data analyses were mainly performed

468 using the QIIME v1.8.0 and R v3.2.0 (https://www.r-project.org/) packages. Chao1, abundance-based

469 coverage estimator (ACE), Shannon's, and Simpson's α-diversity indices were calculated at the OTU

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

471 curves were generated to compare the richness and evenness of OTUs among samples [47]. To

472 explore variation in bacterial community structures across the analyzed soil samples, unweighted

473 and weighted UniFrac distances were also calculated using R v3.2.0. Nonmetric multidimensional

474 scaling (NMDS) analysis, principal component analysis (PCA), and partial least squares discriminant

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

graphical outputs. The linear discriminant analysis (LDA) effect size (LEfSe) method was used to detect differentially abundant taxa across groups using the default parameters through the Galaxy online analytics platform (http://huttenhower.sph.harvard.edu/galaxy/) [54]. The difference in sequence size (i.e., absolute abundance) between samples (groups) of each taxon at the phylum and genus levels was compared and tested, using mothur v1.25.1 with the statistical algorithm of Metastats (http://metastats.cbcb.umd.edu/) [55]. mothur v1.25.1 was also used to calculate Spearman's rank correlation coefficients among the dominant genera (i.e. those with abundance in the top 50) and to construct the correlation network for the dominant genera whose Rho > 0.6 and P value < 0.01. This network was then imported into the Cytoscape software (http://www.cytoscape.org/) [56]. We used analysis of similarities (ANOSIM) to determine the size of the intra- and inter-group differences by ranking the distance between the samples and evaluated the statistical significance of the differences between the original samples using the permutation test, calculated by QIIME v1.8.0 [57, 58].

4.8. Statistical analyses

The data concerning survival rate, leaf area, biomass of AG in each sample group, soil physicochemical properties, enzyme activities, content of phenolic acids, α-diversity indices, and the relative abundances of bacterial taxa (phyla and genera) were compared using independent sample t-tests with significance accepted at $P < 0.05$, as performed in SPSS v19.0 (IBM Corp., USA). Pearson's correlation analysis was used to identify correlations between bacterial genera and each of the phenolic acids and physicochemical properties. R v3.2.0 and Photoshop CS6 (64 bit, adobe.com) were used to draw analysis charts and output the results. The structural formula of phenolic acid was obtained from indraw (5.1.0, http://indrawforweb.integle.com/).

4.9. Data accession

Sequencing raw data can be found in the Sequence Read Archive at the National Center for Biotechnology Information under Bioproject ID PRJNA612151 (https://dataview.ncbi.nlm.nih.gov/object/PRJNA612151?reviewer=lou3qt0rvghbih2cbthrqe76l8).

5. Conclusions

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

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