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Title page

Manure application did not enrich antibiotic resistance genes in root endophytic bacterial microbiota of cherry radish

Running title: Resistomes in root endophytes of cherry radish

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Abstract

Growing evidence suggests that livestock manure used as organic fertilizer in agriculture may lead to potential propagation of antibiotic resistance genes (ARGs) from “farm to fork”. However, little is known about the impacts of manure fertilization on the incidence of ARGs in the plant-associated microbiomes (including rhizosphere, endosphere and phyllosphere), which hampers our ability to assess the dissemination of antibiotic resistance in the soil-plant system. Here, we constructed a pot experiment to explore the effects of poultry and cattle manure applications on the shifts of resistome in the plant microbiome of harvested cherry radish. A total of 144 ARGs conferring resistance to eight major classes of antibiotics were detected among all the samples. Rhizosphere and phyllosphere microbiomes harbored significantly higher diversity and abundance of ARGs than root endophytic microbiomes of cherry radish. Manure application significantly increased the abundance of ARGs in the rhizosphere and phyllosphere, but not in the endophytes of root, which is the edible part of cherry radish. Soil and plant microbiomes changed dramatically after manure applications and clustered separately according to different sample types and treatments. Structural equation modelling revealed that bacterial abundance was the most important factor modulating the distribution patterns of soil and plant resistomes after accounting for multiple drivers. Taken together, we provide evidence that the enrichment of resistome in the rhizosphere and phyllosphere of cherry radish is more obvious compared with the endosphere after manure application, suggesting that manure amendment might not enhance the ARGs dissemination into the root of vegetables in the pot experiment.

Importance

Our study provides important evidence that manure application increased the occurrence of ARGs in the rhizosphere and phyllosphere of cherry radish, compared with the endophytic

bacterial microbiota of root, which is the edible part of cherry radish. Our findings suggest that although manure amendment is a significant route of ARGs entering agricultural soils, these manure-derived ARGs may be at low risk of migrating into the endophytes of root vegetables.

Keywords

Antibiotic resistance genes; resistome; plant microbiome; endophyte; phyllosphere

Introduction

The extensive use of antibiotics in livestock production and land application of animal manure have been regarded as a significant pathway for drug residues and antibiotic resistance genes (ARGs) flowing into agricultural soils (1-5). Animal manure is a large reservoir of mobile genetic elements (MGEs) such as plasmids, transposons and integrons, which potentially promote the horizontal gene transfer of environmental ARGs (1, 6). Antibiotic residues in animal manures exert strong selective pressure on soil indigenous bacteria and accelerate the development and evolution of resistance (7). Meanwhile, manure application influences the soil microbiomes and resistomes directly by spreading faecal microbes, including antibiotic resistant bacteria, to arable land or indirectly by altering soil physicochemical properties such as pH, organic matter, nitrogen and phosphorus (8-10). Numerous studies have examined the effect of manure application on soil resistome from various perspectives such as: different manure types including poultry, cattle and swine manures (5, 11, 12); different pre-application treatments including anaerobically digested, composted and mechanically dewatered manures (13-15); as well as different manure application rates (16). These studies concluded that manure amendments enhanced the level of ARGs conferring resistance to multiple classes of antibiotics in the soil environment and increased the risk of environmental ARGs being migrated into the human food chain.

The consumption of raw green vegetables and fruits has been assumed as a potential avenue for dissemination of environmental resistome to humans (17, 18). ARGs in the plant microbiome overlapped considerably with soil resistome, indicating that soil might be a major origin of plant-associated resistome (19). Soil and manure associated antibiotic resistant bacteria may find their way to the plant microbiome through colonizing on vegetable roots, which are in direct contact with soil, or on aboveground parts of vegetables potentially through air particulates or motility of root endophytes (20, 21). Moreover, plants can take up

antibiotic residues from manure-amended soil, which may impose long-term selective pressure on the emergence and spread of the plant resistome (22). Therefore, both root and leafy vegetables are at a risk of being contaminated by manure or soil ARGs. Majority of previous studies focused on merely the impacts of manure application on soil resistome (11-16), the phyllosphere ARGs of leafy vegetables (8, 23-25), or a limited number of ARGs (26-28). MGEs such as class I integron-integrase gene (*intI1*) and genes encoding transposases have been detected in leaf endophyte and phyllosphere of lettuce (20, 29) and *Brassica chinensis* L (8), phyllosphere of maize (24) and *Coriandrum sativum* L (25). However, the potential transfer of environmental resistome to the endophytic bacterial microbiota of root, which is the edible part of root vegetables and in direct contact with rhizosphere (a hotspot of ARGs), remains largely understudied.

In this study, we established a pot experiment to investigate the effects of poultry or cattle manure application on the transmission of resistome from manured soils to different compartments (rhizosphere, root endophyte and phyllosphere) of a root vegetable, cherry radish. The high-throughput quantitative PCR was employed to analyze the abundance and diversity of 285 ARG subtypes encoding resistance to all the major categories of antibiotics. We hypothesized that (i) application of poultry and cattle manures can lead to elevated levels of antibiotic resistance in soils and considerably change the soil bacterial community compositions; (ii) the changes in soil ARG contents induced by manure application will ultimately have consequences for the plant-associated resistome; and (iii) the changes in ARG patterns have close associations with bacterial communities, which have been recognized an important determinant of ARGs in natural settings.

Results

Resistomes in manure, soil and cherry radish

The number and absolute abundance of detected ARGs in the poultry manure were slightly higher (but not statistically significant) than the cattle manure samples (Fig. 1a and b). ARGs conferring resistance to aminoglycoside, Macrolide-Lincosamide-Streptogramin B resistance (MLSB), multidrug and tetracycline were the most abundant ARG subtypes in manure samples (Fig. 1b). Among the rhizosphere soil, root endophyte and phyllosphere samples of cherry radish, the number of detected ARGs ranged from 7 to 72 in individual samples (n = 27), with the rhizosphere soil sample in the cattle manure treatment (CM) and root endophyte sample in the control treatment harboring the highest and lowest diversity of ARGs, respectively (Fig. 1c). Manure application showed significant impacts on the number of ARGs ($P < 0.01$) in rhizosphere soil and root endophyte samples (Fig. 1c).

The absolute abundance of ARGs (copies g^{-1} solid) in rhizosphere soil (ranging from 5.3×10^9 to 1.3×10^{10}) was 20~50-fold higher than that in root endophyte samples (ranging from 2.0×10^8 to 6.4×10^8) (Fig. 1d). Multidrug resistance genes, a dominant ARG type in manure samples (Fig. 1b), were the most common ARG type in both soil and phyllosphere samples as well. Beta-lactam resistance genes were dominant in root endophyte samples (Fig. 1d). The relative abundance of ARGs showed a similar pattern to the absolute abundance of ARGs (Fig. S1). The application of manure significantly increased the absolute and relative abundances of ARGs in rhizosphere soil and phyllosphere ($P < 0.01$) (Fig. 1d and S1). Non-metric multidimensional scaling ordinations based on the Bray-Curtis dissimilarity matrices revealed that the ARGs in different treatments (i.e. poultry manure, cattle manure and control) and different sample types (i.e. rhizosphere soil, root endophyte and phyllosphere) were clustered separately from each other (Fig. 2a). The hierarchical clustering analysis revealed that the root endophyte cluster was separated from the cluster including manure, rhizosphere soil and phyllosphere samples (Fig. 2b), which was statistically supported by the Adonis test ($P < 0.001$, $R^2 = 0.60$).

Ten MGEs, including eight transposase genes, one class 1 integron-integrase gene (*int*1) and one clinical class 1 intergron-integrase gene (*intI*1) were detected in this study. No significant difference was observed for the number of MGEs across different treatments (Fig. 1c). As for the relative abundance of MGEs, the rhizosphere soil and phyllosphere samples harbored a significantly higher abundance of MGEs than root endophyte samples ($P < 0.01$) (Fig. 1d). The impact of manure application on MGEs was significant in the poultry manure amended soil samples (Fig. 1d). Mantel test revealed that the absolute abundance of MGEs was significantly and positively correlated with individual ARG classes including aminoglycoside, fluoroquinolone, quinolone, florfenicol, chloramphenicol, and amphenicol resistance genes (FCA), MLSB, multidrug, sulfonamide and tetracycline (Table 1) ($P < 0.01$).

Effects of manure application on the resistomes of rhizosphere soils

The absolute abundances of ARGs and MGEs in the manure-amended rhizosphere soils were compared with those in the control treatment, and the significantly enriched ARGs and MGEs were identified by Student t-test ($P < 0.01$). In the poultry manure treatment, the absolute abundances of 12 ARGs and 2 MGEs were significantly elevated in the manure amended soil compared with control. The enriched genes occupied a small portion of the total number of ARGs (18.4%), but accounted for 74.7% of the absolute abundance of total ARGs (Fig. 3a). The enriched genes were associated with multidrug, sulfonamide, aminoglycoside, MLSB, beta-lactam and tetracycline resistance (Fig. 3b). The ARG subtypes *oprJ*, *sul2*, *strB* *oleC* and the MGE subtype *intI1* were the most abundant enriched genes (Fig. 3b), and most of the enriched genes showed consistently high abundances in poultry manure samples (i.e. *ermT-02*, *aadA2-03*, *aadA2-02* and *tetM-02*) (Fig. 3c). In the cattle manure treatment, 16 ARGs conferring resistance to multidrug, beta-lactam, aminoglycoside, MLSB, and tetracycline were enriched, accounting for 64.1% of the absolute abundance of total ARGs in the cattle manure amended soils (Fig. 3d and 3e). The abundances of six ARGs (including

mexF, *aadA-02*, *aadA-03*, *aadA1*, *mefA* and *tetW-01*) were higher than 2.0×10^9 copies g^{-1} solid in the cattle manure samples (Fig. 3f).

Effects of manure application on microbiomes in soil and cherry radish

The microbial communities were assembled into 11 phyla dominated by Proteobacteria, Firmicutes, Actinobacteria and Bacteroidetes (Fig. S2). Poultry and cattle manure applications caused significant impacts on the alpha-diversity of the microbial communities in root endophyte and phyllosphere samples, respectively (Fig. S3). Moreover, different sample types showed significantly different alpha-diversity, with rhizosphere soil samples harbouring the most diverse phylotypes (Fig. S3). The Non-metric multidimensional scaling and hierarchical clustering analyses revealed that all the samples were clustered according to the sample types (Adonis test, $P = 0.001$, $R^2 = 0.69$) (Fig. S4). Procrustes analysis on the basis of Bray−Curtis dissimilarity metrics demonstrated that the bacterial community compositions were significantly correlated with ARG contents (sum of squares $M^2 = 0.19.7$, $P < 0.001$, 9999 permutations) (Fig. S5).

We further characterized shifts of individual bacterial taxa (at the class level) following manure application (Fig. 4). For the phyllosphere microbiomes, there was a significant enrichment of Betaproteobacteria and a decline of Gammaproterbacteria and Alphaproteobacteria after poultry manure application (Fig. 4a). Application of cattle manure significantly increased the abundance of Alphaproteobacteria, Betaproteobacteria and Flavobacteria, while decreased Bacilli and Gammaproteobacteria (Fig. 4b).

Multiple drivers accounting for the patterns of resistome

The direct and indirect effects of multiple drivers on the profiles of resistomes were evaluated via structural equation model (Fig. 5). Our model explained 84% and 90% of the variation in detected ARGs and MGEs, respectively (Fig. 5a). Sample types affected ARGs indirectly via its direct effect on MGEs ($\lambda = -1.29$, $P < 0.001$), bacterial diversity ($\lambda = 0.65$, P

< 0.001) and bacterial abundance (λ = 0.86, $P < 0.001$). Manure application affected the abundance of ARGs directly (λ = -0.12, $P < 0.05$) and indirectly by its significant impact on MGEs, which had a direct positive effect on the abundance of ARGs (λ = 0.65, $P < 0.01$). Bacterial abundance imposed an indirect effect on ARGs via its direct effect on MGEs (λ = 1.76, $P < 0.001$) (Fig. 5a). The standardized total effect of each driver was decomposed into direct and indirect effects to expatiate the strength of these multiple drivers (Fig. 5b). For the total effect, bacterial abundance showed the highest positive effect on ARG profiles, followed by MGEs and manure application (Fig. 5b). On the contrary, bacterial diversity showed a negative effect on the abundance of ARGs. Sample type had the weakest impact on ARGs because of the neutralization of negative direct effect and positive indirect effect (Fig. 5b).

Discussion

Application of organic waste as fertilizer represents a major inflow route of ARGs into agricultural lands (20, 23, 29-31). However, the impacts of organic farming on ARGs dissemination in the human food chain remain less understood. By using high-throughput quantitative PCR, this study provides important insights into the transmission of manure-derived ARGs in the plant-soil system and the potential impacts of manure application on the resistome in rhizosphere soil, phyllosphere and endosphere of cherry radish. A total of 12 and 16 enriched genes accounted for 74.7% and 64.1% of the total resistomes in poultry manure and cattle manure amended soils, respectively (Figs. 3a and 3d), and most of enriched genes were also present in high abundances in poultry manure and cattle manure samples (Figs. 3c and 3f). These results indicated that the enriched ARGs likely originated from manure and became dominant in soil resistomes after manure application. Moreover, the abundances of two MGEs (*intI1* and *tnpA-05*) significantly increased after poultry manure application (Fig. 3b), indicating that manure application not only directly introduced ARGs to soil, but also may increase the horizontal gene transfer potential of ARGs. This finding was further

supported by structural equation model, with manure application significantly impacting ARG profiles both directly and indirectly via its effect on MGEs. Although high-throughput qPCR is one of the most advanced technologies for ARG profiling in environmental resistome monitoring, this PCR-based method has inevitable drawbacks such as limited availability of ARG primers and PCR amplification bias (32). With the development of metagenomic sequencing and "omics" analysis, metagenomic approach is becoming popular in characterizing the environmental resistome in freshwater reservoir (32), sludge (33), swine and murine gut microbiome (34, 35). Compared with the high-efficiency and relatively lower cost of high-throughput qPCR, metagenomic approach is more suitable for characterizing ARGs in samples with low microbial diversity such as phyllosphere and endosphere, while is costly if it is applied to complex environmental soil samples.

A key finding of this study is that manure application significantly increased the absolute abundance of ARGs in the rhizosphere and phyllosphere of cherry radish, while had no significant effect on the ARG abundance in endophytic bacterial microbiota of root, which is the edible part of cherry radish. Consistent with previous studies (8, 23-25), land application of animal manure could enhance the incidence of ARGs in the phyllosphere of vegetables, possibly through the deposition of ARGs-bearing bacteria originated from manured soils and air particulates (36, 37), on the leaf surface of vegetables. Previous studies have recognized rhizosphere and phyllosphere as hotspots for horizontal gene transfer due to the enhanced nutrient input and water fluxes in the rhizosphere and high possibility of cell cluster and microcolonies/biofilms forming in the phyllosphere (22, 38). However, in this short-term pot experiment, the enrichment of ARGs in the rhizosphere and phyllosphere did not result in an elevated abundance of ARGs in root endophytic microbiota, suggesting that the transmission of manure/soil ARGs to the surface of vegetables is the predominant dissemination route, while these ARGs-bearing microbes can barely transfer into the plant tissues and colonize as root endophytes. This is supported by the hierarchical clustering

analysis which revealed that the ARG compositions in the phyllosphere, rhizosphere and manure were clustered into the same cluster, which were separated from the root endophyte cluster (Fig. 2b), suggesting the low risk for the transmission of external ARGs into the plant interior tissues. Taken together, we argue that root vegetables like cherry radish might be in lower risk of being contaminated by ARGs compared with leafy vegetables like lettuce (39). Despite of this finding, it should be noted that the actual agricultural field is far more complex than the pot experiment under laboratory conditions. In the future, field-based experiment needs to be done with various root and leafy vegetables to provide comprehensive evidence for the transfer of ARGs from soil to the plant-associated microbiomes.

The microbial community compositions of root endophyte and phyllosphere were dominated by the phyla Proteobacteria, Firmicutes, Actinobacteria and Bacteroidetes (Fig. S2), which have been reported previously as the dominant members in plant microbiomes among various host species such as *Brassica chinensis* (8), maize (24), *Arabidopsis thaliana* (40, 41), soybean (42) and grapevine (43). The phyllosphere microbiome harboured more diverse OTUs compared with root endophyte samples (Fig. S3), which may be explained by previous findings that phyllosphere could recruit bacteria from broader origins including air environments (36, 37). This finding again suggests that the root endophytic bacterial microbiota of cherry radish might be less influenced by the exterior rhizosphere bacterial communities, compared with the phyllosphere microbiota. Compared with the control treatment, a total of five bacterial taxa in phyllosphere microbiomes significantly changed following poultry manure or cattle manure application (Fig. 4). Among the enriched bacterial taxa, most of them were affiliated into the phyla including Proteobacteria, Actinobacteria and Bacteroidetes, which were all identified as the dominant bacterial phyla in rhizosphere soil samples (Figs. 4 and S2). This result suggests that soil and manure might be important sources that contribute to the profiles of plant phyllosphere microbiomes (20, 44). Manure amendment could directly introduce unique fecal microbes, including antibiotic resistant

bacteria, to arable land and subsequently change the structure of microbiomes and profiles of resistome in plant phyllosphere (45).

Conclusions

Our study provides comprehensive evidence that poultry and cattle manure applications increased the incidence of ARGs in the phyllosphere of cherry radish, but not in the endophytic bacterial microbiota of root, which is the edible part of cherry radish. Some ARG subtypes originated from manure samples may colonize on the surface of vegetable leaf through the deposition of air particulates originated from the manured soil, but not be able to transfer into the interior tissues of vegetables during the short-term pot experiment. Our findings have implications that, compared with leafy vegetables such as lettuce, the edible part of root vegetables such as cherry radish might have a relatively lower risk of being contaminated by manure-derived ARGs.

Materials and methods

Soil sampling and glasshouse experiment

The top 15 cm surface soil samples were collected from a long-term research vegetable farm (38°07′S, 145°19′E) in Victoria, Australia, without known history of organic fertilizer application during the preceding five years. Soil properties were as follows: pH 6.3, total carbon 2.4%, total nitrogen 0.27%, NH_4^+-N 19 mg kg^{-1} and NO_3^--N 240 mg kg^{-1}. The soil was passed through a 4.0 mm sieve and mixed with commercial composted cattle or poultry manure (purchased from Bunnings warehouse, Australia) to achieve a final concentration of 80 mg manure g^{-1} dry soil, corresponding to a typical agricultural rate of 60 m^3 manure per hectare.

Glasshouse experiment was established with three sets of treatments with three replicates: untreated soils (Control), poultry manure-amended soils (PM) and cattle manure-

amended soils (CM). Each plastic pot (15 cm in diameter and 30 cm in height) contained 2.0 kg of untreated soils or manured soils. Cherry radish (*Raphanus sativus L. var.radculus pers*) was germinated from commercial seeds (Bunnings warehouse, Australia) in moist vermiculite for 7 days and then transplanted into the designated pots. Three cherry radish plants were grown in each pot. The pots were incubated in a climate-controlled glasshouse maintained at 25 °C in daylight (14 h) and 20 °C in dark (10 h) (70% humidity). Plants were watered every two days with deionized water and harvested at day 90 when the vegetables reached maturity. Soils attached to the taproot of cherry radish were collected as rhizosphere soil samples. Aboveground and belowground parts of cherry radish were separated using ethanol-sterilized scissor and put into individual sampling bags for root endophyte and phyllosphere DNA extraction.

Genomic DNA extraction

Soil genomic DNA was extracted from 0.25 g of soil using the MoBio PowerSoil DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA, USA) following the supplier's manual. Root endophyte DNA was extracted according to Zhu *et al*. (20). Briefly, root samples were immersed in 30% hydrogen peroxide for 30 min to remove the bacterial community residing on the surface of roots and washed with sterilized water. The samples were then immersed in 70% ethanol for 1 min and washed with sterilized water. Around 0.5 g of root samples were used for endophytic DNA extraction with the MoBio PowerSoil DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA, USA). For the phyllosphere DNA extraction, around 5 g of cherry radish leaves were transferred into a 50 ml centrifuge tube containing 45 ml of 0.01 M phosphate buffered saline, and shaken at 200 rpm and 30 °C for 2 h. The washing solution was filtered with a sterilized nylon net and centrifuged at 7500 rpm for 30 min. The pellets were subjected to DNA extraction using MoBio PowerSoil DNA Isolation Kit. The concentration and purity of the extracted DNA were assessed using the NanoDrop ND2000c

spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

High-throughput quantitative PCR

High-throughput qPCR was performed to investigate the diversity and abundance of ARGs and MGEs. We used 296 primer sets, including 285 primer sets targeting eight major classes of ARGs, 8 transposases, class 1 integron, clinical class 1 integron and 16S rRNA gene (46). The SmartChip with 5184 nanowells was loaded into the Wafergen SmartChip Real-Time PCR Cycler for PCR reaction using the protocol described previously (47). Only well data with single effective melting peak and the amplification efficiencies within 1.7-2.3 were remained. A threshold cycle (C_T) value of 31 was used as the detection limit (46). Three technical replicates were included for each sample, and reactions with both a C_T value < 31 and three positive replicate samples were retained as positive quantification. The absolute abundance of 16S rRNA gene was quantified separately on a Bio-Rad CFX384 Real-Time PCR Detection System (Bio-Rad, Herculers, USA) using the same primer set and thermal-cycling conditions to the high-throughput qPCR analysis above. All samples were performed in technical triplicates with DNA template-free negative controls.

Bacterial 16S rRNA gene sequencing and data processing

To characterize bacterial communities in soil and plant samples, the V5-V7 region of 16S rRNA gene was amplified with chloroplast-excluding primers 799F/1193R (48). The 25 μl reaction mixtures contained 12.5 μl of Redmix (Bioline, London, UK), 0.5 μl of each primer (10 μM), 2 μl of DNA template and 9.5 μl of nuclease-free water. The thermal-cycling program was: 95 °C for 10 min, followed by 25 cycles of 95 °C for 20 s, 55 °C for 20 s, 72 °C for 20 s and a final extension at 72 °C for 10 min. The amplicons were purified, quantified and pooled as the final library and sequenced on an Illumina MiSeq sequencer using 2 × 301 bp sequencing kits at the Western Sydney University Central Genomic Facility.

Raw pair-end reads were sifted to remove adaptor sequences, low quality sequences containing three or more ambiguous nucleotides, reads with a low (< 20) average quality score and short reads (<100 nt) (10). After the above data filtering process, all the high-quality sequences were processed and analysed using QIIME (49). Operational taxonomic units (OTUs) were identified at 97% sequence similarity using the UCLUST algorithm (50). Chimeric sequences, mitochondrial, cytoplasmic OTUs and singletons were discarded from the final OTU data set. The taxonomic classification of each OTU was determined using the database from the Ribosomal Database Project at an 80% confidence threshold (51). Raw sequences were deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) under the accession number SRP201741.

Statistical analyses and data visualization

All statistical analyses were performed using SPSS 19 (IBM, Armonk, NY, USA) with significance level at $P < 0.01$. Mantel test was conducted to identify the relationship between MGEs and ARGs. Non-metric multidimensional scaling analyses based on the absolute abundance of ARGs and relative abundance of bacterial taxa were performed to visualize the Bray-Curtis dissimilarity matrices among different treatments (i.e. PM, CM and Control) and different sample types (i.e. soil, root endophyte and phyllosphere). Adonis test was performed in R with the "vegan" package to test the significance of difference in resistomes and microbiomes among different sample types (52). Procrustes test for correlation analysis between ARGs and bacterial communities based on Bray–Curtis dissimilarity matrices was performed using "vegan" in R (52).

The structural equation model was constructed to evaluate the effects of manure application, sample type, bacterial abundance and diversity, as well as MGEs, on the ARG patterns (53). An *a prior* model was established based on the known effects of these drivers on ARG profiles (Fig. S6). The pairwise correlations among these variables were determined

via Mantel test using the "Ecodist" package in R (54), and the resultant covariance matrix was imported into AMOS Graphics software (AMOS IBM, USA) for the structural equation model construction. The goodness-of-fit index (GFI > 0.90), χ^2 test ($P > 0.05$), and the root mean square errors of approximation (RMSEA < 0.05) were used to assess the overall goodness of the model fit. We calculated standardized direct effects, indirect direct and total effects of each factor on the ARG abundance.

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524 **Table 1** Mantel test between the absolute abundance of ARGs and MGEs in all the samples.

	Transposase		Integrase	
	R-value	*P*-value	*R*-value	*P*-value
Total ARGs	**0.57**	**< 0.01**	**0.19**	**0.01**
Aminoglycoside	**0.65**	**< 0.01**	**0.29**	**< 0.01**
β-lactam	0.03	0.30	0.16	0.02
FCA	0.52	0.02	**0.29**	**< 0.01**
MLSB	**0.77**	**< 0.01**	**0.37**	**< 0.01**
Multidrug	**0.52**	**< 0.01**	0.14	0.04
Sulfonamide	**0.69**	**< 0.01**	**0.31**	**< 0.01**
Tetracycline	**0.51**	**< 0.01**	**0.21**	**< 0.01**
Vancomycin	0.05	0.25	0.18	0.02
Others	0.10	0.13	0.07	0.55

525 Bold text indicates statistically significant correlations ($P < 0.01$).

526 **Figure captions**

527 **Figure 1** The average number (a and c) and absolute abundance (copies g^{-1} solid) (b and d) of
528 ARGs and MGEs detected in manure samples (a and b) and rhizosphere soil, root endophyte
529 and phyllosphere samples of cherry radish (c and d). Different letters above the bars indicate
530 a significant difference ($P < 0.01$) among different treatments within the same sample type.
531 (Abbreviations: FCA, fluoroquinolone, quinolone, florfenicol, chloramphenicol, and
532 amphenicol resistance genes; MLSB, Macrolide-Lincosamide-Streptogramin B resistance;
533 PM, poultry manure treatment; CM: cattle manure treatment).

534 **Figure 2** (a) Non-metric multidimensional scaling ordination plot depicts the Bray-Curtis
535 dissimilarity matrices between different samples based on the absolute abundance of ARGs
536 and (b) hierarchical clustering of the same samples as (a).

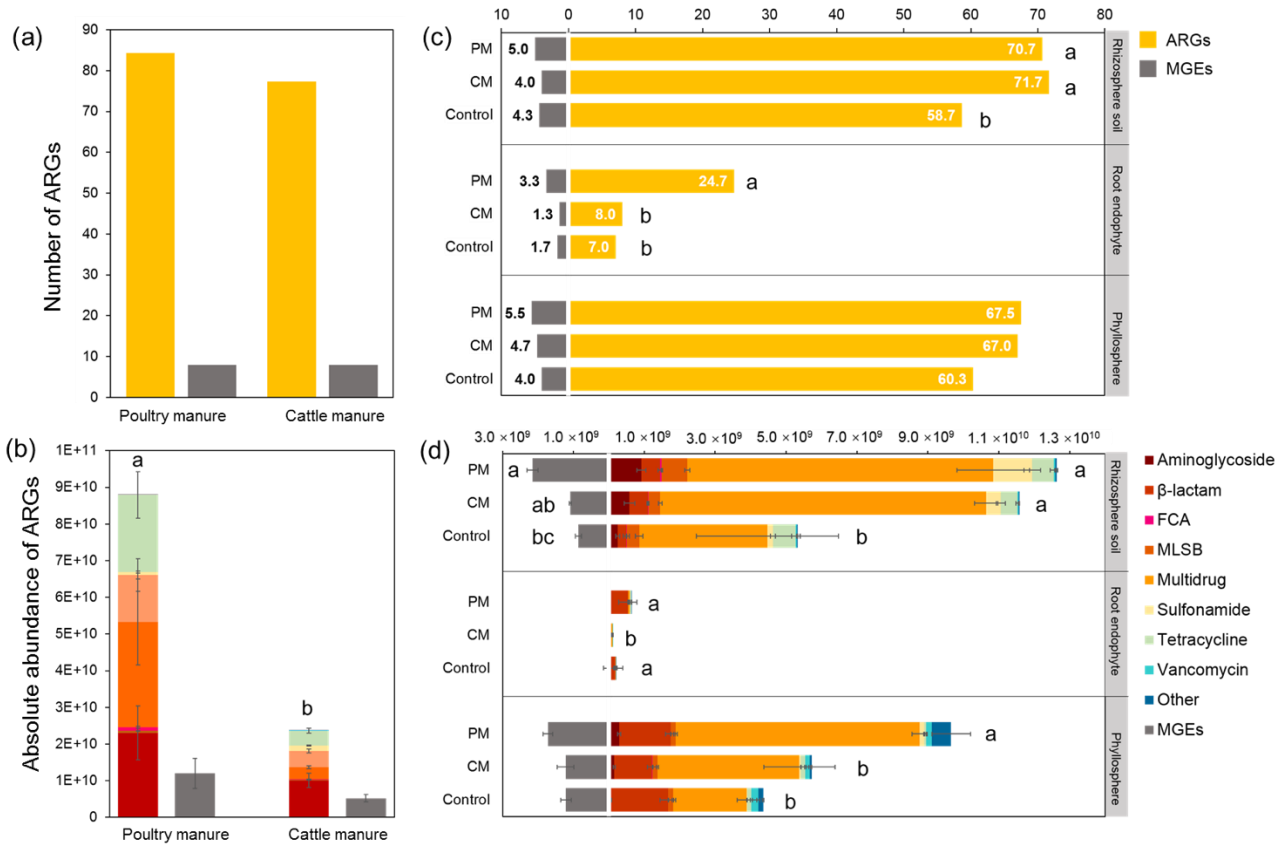
537 **Figure 3** The percentage of the number and absolute abundance of enriched ARGs and
538 MGEs compared to the total ARGs in the poultry manure (a) and cattle manure (d) amended
539 soil samples; the taxonomic composition of the enriched ARGs and MGEs in the poultry
540 manure (b) and cattle manure treatment (e); the absolute abundance of the enriched ARGs
541 and MGEs in the poultry manure (c) and cattle manure samples (f).

542 **Figure 4** Changes in relative abundance of bacterial taxa at the class level in phyllosphere
543 samples with poultry manure (a) and cattle manure applications (b). Changes in relative
544 abundance were determined by $R_{PM/CM} - R_{Control}$, where $R_{PM/CM}$ is the relative abundance of
545 bacteria community in poultry /cattle manure treatments and $R_{Control}$ is the relative abundance
546 of bacteria community in the control treatment. * represents the difference reaching the
547 significant level by Student t-test. (* $P < 0.05$; ** $P < 0.01$).

548 **Figure 5** Structural equation modelling of the effects of manure application, sample type,
549 bacterial abundance and diversity, as well as MGEs on the ARG patterns (a). The goodness-
550 of-fit statistics are: $\chi^2 = 14.8$, P = 0.11, RMSEA = 0.049, df = 2, GFI = 0.94. Solid and dash

lines represent positive and negative effects, respectively. The linewidth is proportional to the strength of the standardized path coefficients (as indicated by the numbers adjacent to lines). The R^2 value indicates the proportion of variance explained by the variable. *$P < 0.05$, **$0.05 > P > 0.01$, ***$P < 0.001$. (b) Standardized total effects, direct effects and indirect effects of all the factors on the ARG profiles based on the structural equation model results.

556 **Fig. 1**



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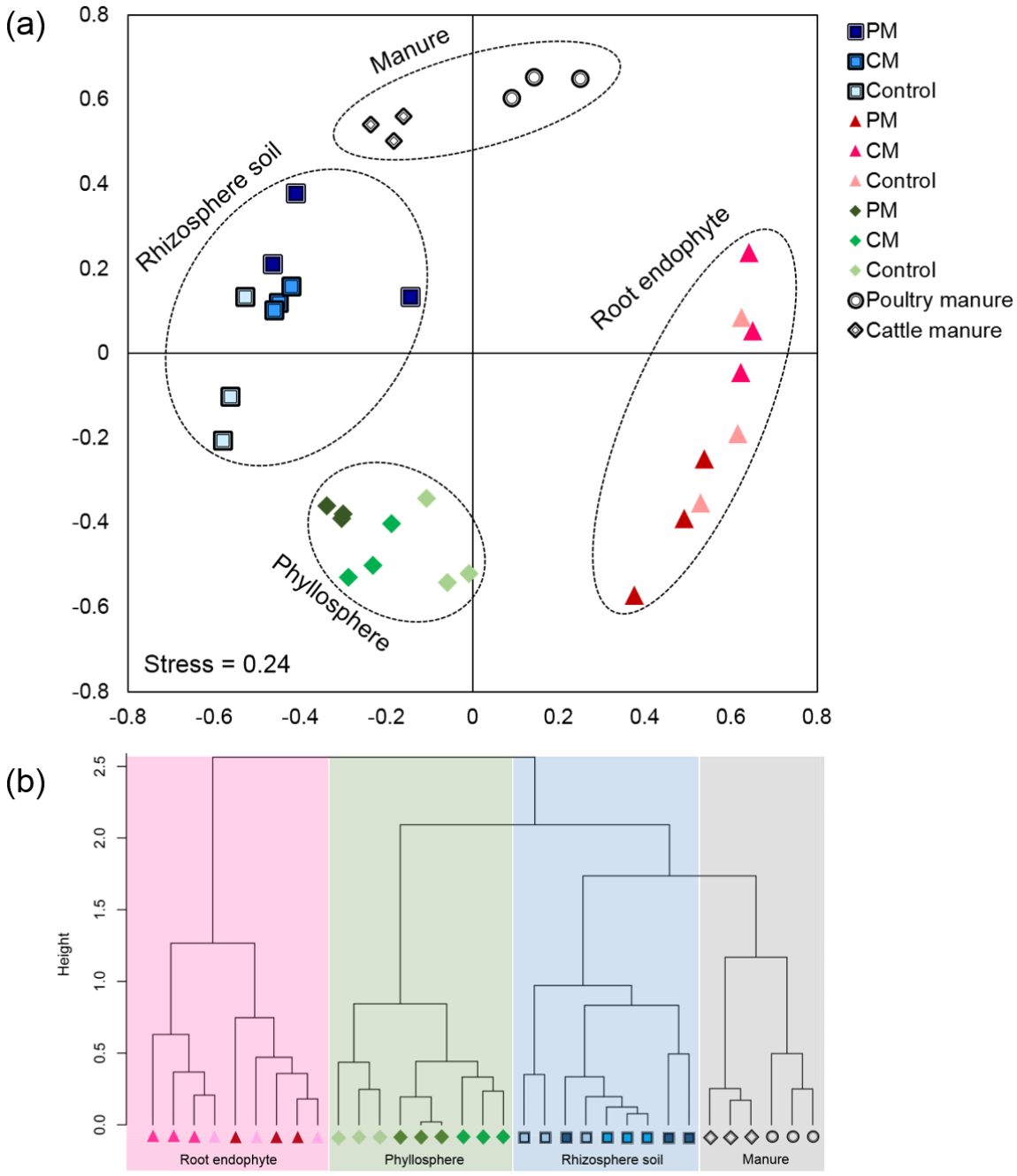
560 **Fig. 2**



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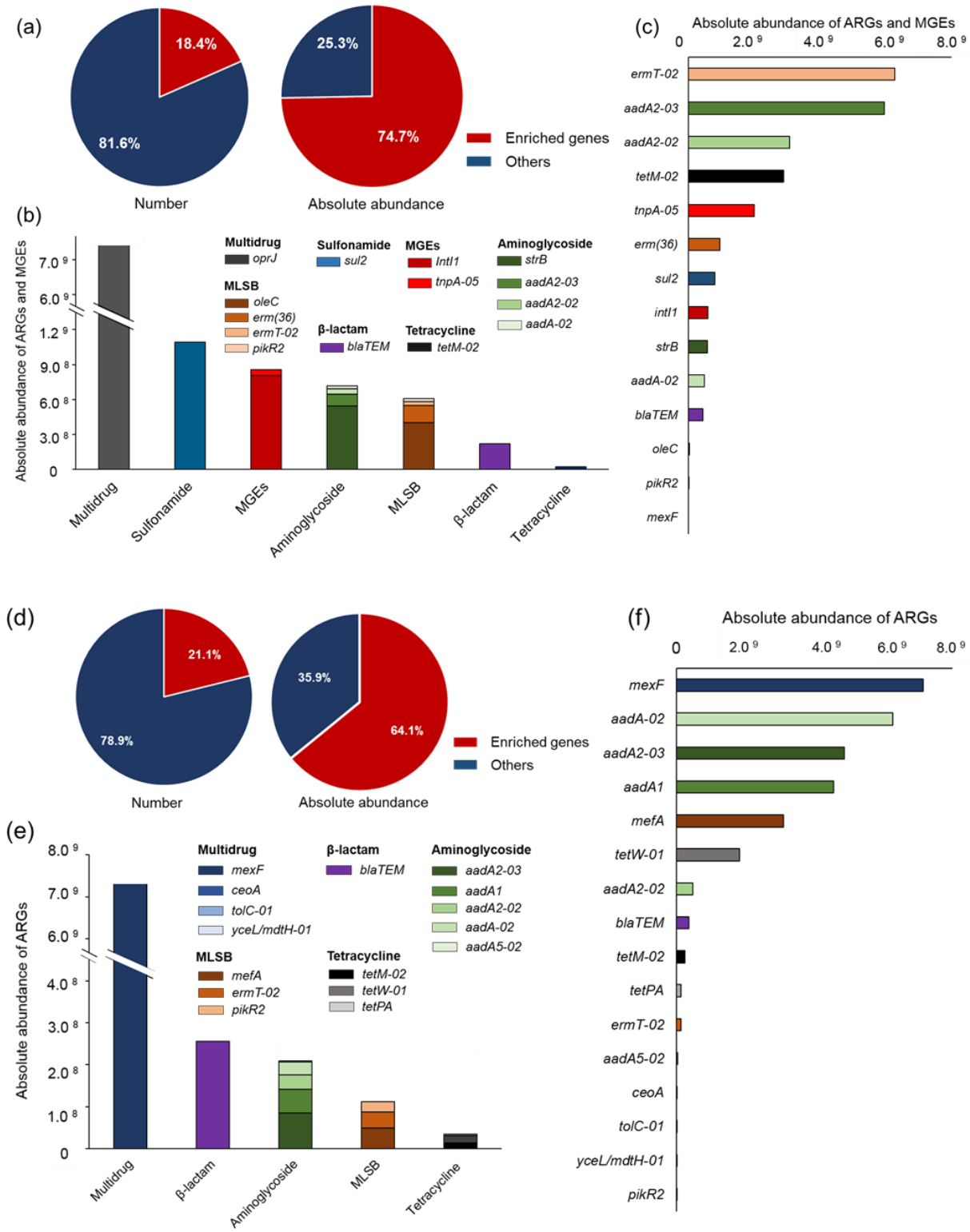
562 **Fig. 3**



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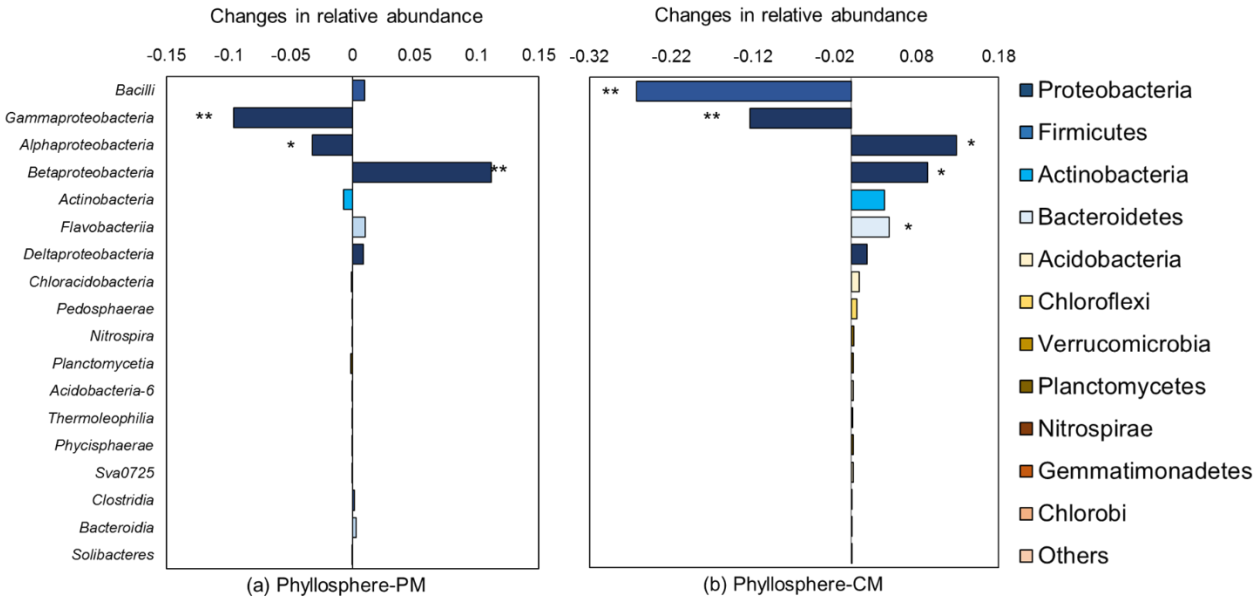
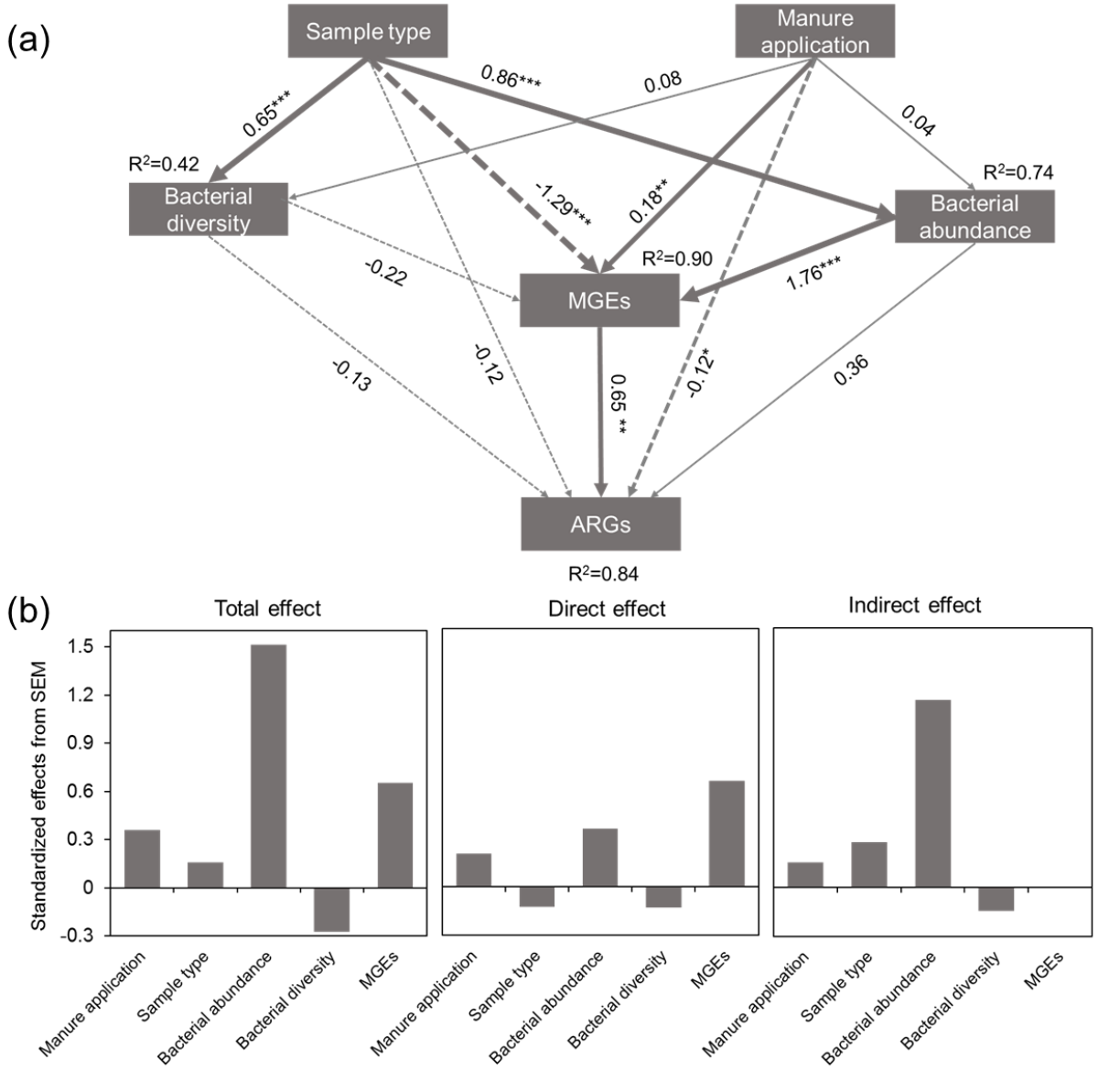
565 **Fig. 4**


Changes in relative abundance
-0.15 -0.1 -0.05 0 0.05 0.1 0.15
Changes in relative abundance
-0.32 -0.22 -0.12 -0.02 0.08 0.18
Bacilli
Gammaproteobacteria
Alphaproteobacteria
Betaproteobacteria
Actinobacteria
Flavobacteriia
Deltaproteobacteria
Chloracidobacteria
Pedosphaerae
Nitrospira
Planctomycetia
Acidobacteria-6
Thermoleophilia
Phycisphaerae
Sva0725
Clostridia
Bacteroidia
Solibacteres
Proteobacteria
Firmicutes
Actinobacteria
Bacteroidetes
Acidobacteria
Chloroflexi
Verrucomicrobia
Planctomycetes
Nitrospirae
Gemmatimonadetes
Chlorobi
Others
(a) Phyllosphere-PM
(b) Phyllosphere-CM

566

567 **Fig. 5**


(a)
Sample type
Manure application
Bacterial diversity
Bacterial abundance
MGEs
ARGs
R²=0.42
R²=0.74
R²=0.90
R²=0.84
0.65***
0.86***
0.08
0.04
-1.29***
0.18**
-0.22
1.76***
-0.13
-0.12
0.65**
-0.12*
0.36
(b)
Total effect
Direct effect
Indirect effect
Standardized effects from SEM
1.5
1.2
0.9
0.6
0.3
0
-0.3
Manure application
Sample type
Bacterial abundance
Bacterial diversity
MGEs

568