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Dung beetle improves soil bacterial diversity and enzyme activity and enhances growth and antioxidant content of Chinese cabbage (*Brassica rapa* ssp. *pekinensis*)

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ABSTRACT

Purpose

Dung beetles have been used to enhance soil health and crop growth and yields but their effects on soil enzymology and microbial diversity are seldom reported. The present work reports on beneficial effects of dung beetles on soil enzyme activity, bacterial diversity, and growth and antioxidant content of Chinese cabbage (*Brassica rapa* ssp. *pekinensis*).

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Methods

The soil amended with cow dung only (CD), or with dung broken down by dung beetles (DB), was compared to untreated control (CO). Soil bacterial diversity was determined through 16S rRNA gene sequencing, and enzymological tests were used for soil enzyme activity.

Results

Dung beetles significantly increased acid phosphatase, urease, cellulase, catalase, and β-glucosidase activities in the soil compared to CD or control. Bacterial alpha-diversity significantly increased in DB when compared to CD or control. Microbial carbon (C) and nitrogen (N) were significantly higher in DB than in CD, and in CD than control. The DB and CD treatments also significantly enhanced plant growth and the content of antioxidants (total flavonoids, polyphenols, and vitamin C) and phytohormones indole-3-acetic acid (IAA), *trans*-zeatin riboside (ZR), and gibberellic acid (GA_3) compared to control, while a significant reduction was noted in abscisic acid (ABA) in DB compared to CD or control plants. Correlation analysis confirmed that the enzyme activities are pH dependent, as pH was significantly lowered by DB compared to CD or control.

Conclusion

Our results suggest that use of dung beetles in conventional practices could be an effective strategy to improve soil nutrient availability and microbial activity and antioxidant content of Chinese cabbage.

Keywords: Chinese cabbage; dung beetle; microbial biomass; soil bacterial diversity; soil enzymes.

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1 Introduction

Chinese cabbage (*Brassica rapa* ssp. *pekinensis*) is one of the essential food crops in Asia. It is commonly used for the production of lightly fermented vegetables and is widely cultivated in many countries, especially in Korea and China (Cao et al. 2006). It is believed to have anticarcinogenic properties due to the high content of antioxidant substances such as phenolics, flavonoids, phenylpropanoids, and glucosinolates that are beneficial for human health and nutrition (Managa et al. 2020). Due to beneficial health effects, its consumption has increased in daily diets (Shawon et al. 2020).

On the other hand, increased pressure on crop production requires more fertiliser inputs, either inorganic or organic. Large-scale use of chemical fertilisers has negative impacts on the environment, such as freshwater eutrophication, or impoverishment of soil organic matter content which also reflects on the nutritional quality of crops (Liu et al. 2009). The diversity and biochemical activity of the soil microbiome is important for maintaining soil health (Chisanga et al. 2020). Organic farming, relying on the use of organic amendments for enhancing the organic content of the soil, represents an acceptable, sustainable alternative to chemical fertilisation (Padel et al. 2009). With the growing popularity of organic farming, the cultivation acreage under this practice is steadily increasing (Brenes-Muñoz et al. 2016; Squalli and Adamkiewicz 2018; Willer 2021). The nutrients released after the biodegradation of soil organic matter are essential for plant growth in organic farming (Fernandez et al. 2006). Soil enzyme activities are a suitable indicator of soil fertility as they reflect the effects of soil properties, pedological amendments and tillage (Chang et al. 2007). The release of nutrients upon decomposition of organic matter is a crucial step in organic farming, in which an important role is played by soil macrofauna such as annelids and arthropods (Bagyaraj et

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al. 2016). Among the arthropods, particular attention is being paid to dung beetles, as they have been proven to contribute to the recycling of nutrients from dung in an array of various ecosystems worldwide (Nichols et al. 2008). Dung beetles have been shown to positively contribute to soil aeration (Bang et al. 2005) and to its hydrological properties (Brown et al. 2010). Changes in the chemical composition of dung result from environmental and microbial changes caused by dung beetles (Kazuhira et al. 1991). Recently it has been shown that their activity is beneficial to the intensity of soil microbial respiration (Menéndez et al. 2016) and to the bacterial diversity of soil (Slade et al. 2016). However, despite the evident involvement of the soil microbiome in the dung beetle-driven nutrient cycling, the connection between these factors is not being given sufficient attention (Slade et al. 2016).

In our previous study, *Catharsius molossus* dung beetles showed beneficial effects on plant growth, physiology, and metabolite content, and improved soil nutrient status (Kaleri et al. 2020) but the effects of dung beetles on soil microbial diversity and resulting enzyme activities have not been studied. In this work, therefore, we evaluate whether the addition of cow dung and dung beetles positively affects soil enzyme activity, bacterial diversity, and growth and antioxidant content of Chinese cabbage (*Brassica rapa* ssp. *pekinensis*) in order to assess the potential benefits of the inclusion of this type of organic fertilisation in farming practices.

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2 Material and Methods

2.1 Experimental Enclosures

The experiment was conducted during the growing season of 2019-2020 in the net house of Southwest University of Science and Technology (SWUST) in Mianyang, Sichuan, China. The experimental approach to soil treatments was developed and designed according to previously published studies on the effects of dung beetles on soil health (Bang et al., 2005; Badenhorst et al., 2018; Kaleri et al. 2020) as described below. The soil used in the experiment was collected from uncultivated land near farmlands of Mianyang city adjoining areas (31.550074º N–104.640345º E). The soil is silty clay and covered with scattered wild vegetation of herbs and shrubs. The area has a subtropical climate with mean annual temperature ranging from 14.7 to 17.3 °C, mean annual rainfall of 826 to 1417 mm, and mean frost-free period of 252 to 300 days. The soil was collected in bulk, homogenised, and processed for physico-chemical analysis. The adult dung beetles were collected from farmlands near Xichang City, Sichuan Province, China. The beetles were brought to the laboratory and identified as *Catharsius molossus* L. using available relevant literature. The beetles were measured for body size using an ordinary ruler scale, and individuals with an equal body size of 30-31 mm length were selected. The cow dung used in the experiment was collected from a cattle dairy farm in Mianyang city, Sichuan. The bulk of fresh cow dung was obtained and brought to the experimental site on the day when the incubation phase (see below) started. A sub-sample was dried and processed for initial analysis.

2.2 Experimental Setup

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The experiment was conducted in a net house in two phases, the first phase comprised the incubation of cow dung with dung beetles at 22 ± 4 °C, 50-60% relative humidity for 30 days until more than 90% cow dung was broken down and mixed with the soil by dung beetles. In the second phase, treated soil was used to grow Chinese cabbage.

For investigation of the effects of cow dung and dung beetles on soil microbial diversity and soil enzyme activity, the cow dung and dung beetles were incubated in plastic buckets (33 cm diameter and 35 cm deep) filled halfway with sampled soil. The buckets were manipulated to restrict the possible escape of dung beetles from the setup. A total of nine buckets, divided into three groups, were used in this phase of the experiment. The first group comprising three buckets was amended with dung + beetles (DB), the second with cow dung (CD), and the third was kept as control (CO), i.e., only soil. This generated a simple completely randomised design (CRD) with three treatments each replicated three times. Each of the DB buckets was incubated with 300 g fresh cow dung pats and 20 dung beetles. The dung pats were placed so that the soil surface was covered with dung. The first application of dung was placed alternatingly and the second application was used to fill the gaps that had not been covered by the first application of cow dung. The large size of buckets allowed free roaming of dung beetles for dung processing and subsequent nutrient release.

The similar setup of cow dung placement was repeated in CD treatment but without beetles, while control treatment contained unamended soil. Dung beetles were removed from the buckets when more than 90% of the dung pats were broken down and homogenised in soil by the activity of dung beetles. The remaining dung pats, in DB and CD treatments, were manually homogenised in the soil so that each bucket was amended with around 5 kg of

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homogenised soil. Treated and untreated soil was transferred to pots of 21 × 27 cm size, and Chinese cabbage was sown at 10 seeds per pot. The pots were rinsed twice a week with equal quantities of tap water. Plants were harvested 42 days after sowing.

2.3 Soil Sampling and Analysis

From each treatment, soil samples were taken at the termination of incubation and mixing of remaining cow dung in soil. Electrical conductivity (EC) and pH were measured using a Hanna multi parameter meter (Corwin and Scudiero 2020). Total nitrogen (N) content was measured by the Kjeldahl method as described by Luitel et al. (2020). Soil phosphorus (P) was measured according to a modification of Olsen's colorimetric method as described by (Wolf and Baker 1990), whereas potassium (K), calcium (Ca) and magnesium (Mg) were determined by atomic-absorption spectrophotometry according to David (1960). Soil microbial biomass carbon (MBC) and nitrogen (MBN) were determined by the fumigation extraction method as described by Lu et al. (2015).

2.4 Soil Enzyme Activities

A 200 g soil sample was collected randomly from each experimental pot and was analysed through enzyme kits manufactured by Ningbo Zhongding Biotechnology Co Ltd China according to manufacturer's recommendations. The enzyme assays are summarised as follows. Urease activity was determined by sodium phenoxide-sodium hypochlorite colorimetry (Guan 1986). Catalase activity in the soils was determined using the $KMnO_4$ titrimetric method according to (Guan 1986). Acid phosphatase and β-glucosidase activities were determined by a colorimetric measurement of *p*-nitrophenol following the hydrolysis of *p*-nitrophenyl-phosphate (Tabatabai and Bremner 1969) or *p*-nitrophenyl-β-D-glucoside (Eivazi

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and Tabatabai 1988), respectively. Cellulase activity in soils was determined by a method based on the quantification of reducing sugars released upon hydrolysis of the substrate carboxymethylcellulose, as described by (Criquet 2002).

2.5 Determination of Bacterial Diversity

Bacterial diversity was assessed in control, CD, and DB-treated soils at the termination of incubation and mixing of remaining cow dung in soil. The DNA was extracted from 0.25 g fresh soil that had previously been frozen at -80°C in a sterile freezer bag. Isolation of DNA, the subsequent qPCR amplification, Illumina MiSeq sequencing of 16S rRNA and data analysis were performed as described by (Lévesque et al. 2020).

2.6 Plant Growth Measurements

Growth parameters, such as plant height, leaf number, mean leaf width and length, were documented 10 days after germination. At harvest time, plant roots and leaves were separated carefully for each treatment. The length of the root was measured with a ruler, according to (Song et al. 2017). The fresh weight of the root was measured, and for dry weight determination, roots were kept for incubation at 80 °C for 2–3 days. The fresh leaf samples were cut with a knife, then immediately placed into liquid nitrogen and then stored at -80°C for further use according to (Siddiqui 2004).

2.7 Determination of Total Flavonoid, Polyphenols, Vitamin C, and Chlorophyll Content

Flavonoid quantification was carried out by a colorimetric method following aluminium nitrate-ethanol extraction as described by Sen et al. (2013). Total polyphenols were checked according to the Folin–Ciocalteu method as described by Dini et al. (2020). Vitamin C was

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determined by the titration method with iodine (Suntornsuk et al. 2002). Chlorophyll was quantified using the colorimetric method following acetone extraction as described by Ahmed et al. (2019).

2.8 Plant Hormone Quantification

Chinese cabbage leaves were sampled from each treatment and placed immediately in liquid nitrogen. Furthermore, the protocols for the extraction and purification of indole-3-acetic acid (IAA), gibberellic acid (GA_3), zeatin riboside (ZR), and abscisic acid (ABA) were carried out as described by (Shixi et al. 2020). Moreover, the quantification of IAA, ZR, and ABA was performed by an enzyme-linked immunosorbent assay (ELISA). The concentration (w/w) of phytohormones (IAA, ZR, GA_3, ABA) in leaf tissue (ng g^{-1}) was calculated as previously described (Kaleri et al. 2020; Shixi et al. 2020).

2.9 Statistical Analysis

Alpha diversity metrics, including observed operational taxonomic units, Shannon, and Simpson index were calculated using the “diversity” and “richness” functions of the bacterial community. Repeated ANOVAs were performed to test the effects of dung beetle, and cow dung and their interactions on the bacterial diversity indices. One-way ANOVA was performed to test the effects of treatments on bacterial community structure, soil enzymes, plant growth, antioxidant and plant hormone contents. The differences between the mean values were determined using Fischer LSD test at $p < 0.05$ significance level. Effects of dung beetle and cow dung on bacterial community structure were further tested by Principal Coordinate Analysis (PCoA) using OTUs. Pearson correlation analysis was used to assess the relationships between plant growth, soil enzyme, microbial C and N and soil physico-chemical properties. Both PCoA and Pearson analyses were performed using the VEGAN

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package (Koutecký 2015) in R (R Core Team 2018). Other statistical analyses were performed using SPSS 20.0 and Microsoft Excel 2016. The statistical differences were considered significant at the $p < 0.05$ level.

3 Results

3.1 Soil Physico-Chemical Properties and Microbial C and N

Results showed a significant difference in soil physico-chemical properties between treatments. Soil EC and pH decreased significantly in DB treatment, while increases were noted in N, P, K, Ca, and Mg content when compared with CD or control soil (Table 1). MBC (Fig. 1) significantly increased by 85.4% and 28.0% in DB and CD treatments, respectively, as compared to control. MBC was also significantly higher in DB than CD treated soil. Furthermore, MBN significantly increased by 150.7% and 51.4% respectively, in DB and CD when compared with control. The C:N ratio showed a significant difference between treatments, wherein the values for DB and CD had a significantly lower C:N ratio than control.

3.2 Soil Enzyme Activities

In general, DB increased enzyme activities in treated soils, in comparison with soils in CD or control. However, the responses were dependent on the type of enzyme (Fig. 2). No significant difference was observed between CD and the control group for any of the tested enzymes except for cellulase and catalase (Fig. 2). The acid phosphatase activity was significantly higher in DB, marking an increase by 191.7%, as compared to control. Urease activity was significantly increased in DB treatment by 243.1%, as compared to control. Cellulase activity was significantly increased in DB and CD treatment, by 318.8% and 22.5%

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respectively, compared to control. Furthermore, catalase activity showed significant increase in DB and CD treatment by 22.0% and 4.0% respectively, as compared with control. β-glucosidase activity was significantly increased in DB treatment by 299.0% as compared to control.

3.3 Correlation Analysis

Fig. 3 shows a heat map diagram for Pearson correlation coefficients among various soil properties, and plant height as the most prominent morphological parameter dependent on soil fertility and health. Fig. 3a shows the overall correlation among soil parameters and plant height and includes all treatments (n = 12), while Fig. 3b shows the correlation of plant height, urease, acid phosphatase, catalase, cellulase, and β-glucosidase, within each individual treatment group, with other soil quality parameters. The overall correlation between plant height, K, Ca, Mg, N, P, MBC, MBN, and different enzymes was significantly positive except soil pH and EC. Within individual treatment groups, most positive correlations were found in DB for plant height, urease, catalase, acid phosphatase, cellulase, and β-glucosidase with other soil parameters except soil EC (Fig. 3b). Interestingly, for many parameters in Fig. 3b, the order of correlation is: DB > CD > control, for instance, the correlation between total Mg and plant height is highest in the DB treatment, lower in CD, and the lowest in control.

3.4 Bacterial Diversity

Fig. 4 shows α-diversity index values of bacterial communities. The highest value for all three indices was observed in dung beetle-amended soil compared to CD or control treatments. PCoA, used to identify the soil bacterial community structure in all samples (Fig.

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5), revealed significant differences in bacterial communities between the treatments. DB showed higher variation with respect to other treatments. At the class and family level, the classified sequences were affiliated to 20 groups. Bacterial community composition varied among treatments at the family level (Fig. 6). For instance, the relative abundance of Xanthomonadaceae, Burkholderiaceae, Rhizobiaceae, Sphingomonadaceae and Caulobacteraceae was significantly increased in the DB treatment when compared with CD and control group, whereas the opposite was found for Dongiaceae. However, no significant differences were found on the bacterial family level between CD and control.

At the class level, 20 bacterial classes were observed in different treatments (Fig. 6). For example, in the DB treatments, Gammaproteobacteria, Alphaproteobacteria, Bacilli and Bacteroidia had higher relative abundance when compared with CD or control. However, Holophagae and Acidobacteria showed opposite trends. No significant differences were observed between CD and control plots at the class level. Microbial community structure in DB showed an increase in abundance on the family and class level when compared with CD or control.

3.5 Plant Growth Parameters

Significant increases in the growth of Chinese cabbage were noted when plants were treated with DB or CD (Table 2). DB treatment significantly increased the germination efficiency, plant height, leaf number, leaf length, leaf width, root length, and root fresh and dry weight when compared with control. These parameters also increased in CD as compared with control, but the effect of CD was significantly lower than that of the DB treatment.

3.6 Total Flavonoid, Polyphenol, Vitamin C, and Chlorophyll Content

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A significant increase in flavonoid content by 13.9% was noted in Chinese cabbage in DB when compared with control plants. In CD plants, flavonoids were also higher than control (Fig. 7). DB showed a significant increase in polyphenol content as compared with CD or control. Furthermore, CD showed significant differences from the control. DB and CD plants showed a significant increase, 110.1% and 76.7% respectively, in vitamin C content when compared with control. However, there was no significant difference between CD and DB for vitamin C. DB plants had significantly increased chlorophyll content as compared to CD or control. However, the CD treatment did not contribute to significant increase in chlorophyll when compared with control.

3.7 Plant Hormones

DB and CD treatments resulted in alterations of phytohormone content in Chinese cabbage. The content of ZR, GA_3, and IAA was significantly higher in DB plants when compared with CD or control, while a reduction was noted in ABA content when compared with the CD or control plants (Fig. 8). Furthermore, the CD treatment significantly increased the ZR, GA_3, and IAA contents compared to the control group, while ABA was lower than in control.

4 Discussion

In our study, addition of cow dung to the soil significantly changed the soil properties, while the addition of the *Catharsius molossus* beetles to the dung-amended soil further increased these changes. Thus, soil EC and pH value decreased significantly in DB treatment, while increases were noted in N, P, K, Ca, and Mg content when compared with CD or control soil. Cow dung is a rich source of minerals, bacteria, and microbial enzymes (Gupta et al. 2016) and its addition enriches the mineral content of the soil; however, when the dung remains

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aboveground, these resources are largely unutilised. Tunnelling dung beetles burrow under the dung pat and transfer dung fragments underground for feeding and nesting, enriching the soil with the nutrients, enzymes and microorganisms from the dung (Nichols et al. 2008). The dung removal and bioturbation activity of the species *C. molossus*, used in our study, has been recently documented and quantified in detail, providing valuable insight into the dynamics of this process (Singh et al. 2021). Thus, by facilitating the transfer of the dung into the soil, the burrowing activity of *C. molossus* contributed to the changes in soil EC, pH and mineral composition in our study.

Furthermore, our results show that CD increased the soil MBC and MBN content compared with control, but further increase in these organic nutrients occurred when dung beetles were added. Losses of MBC and MBN from dung pats through microbial respiration and ammonium volatilisation, respectively, are reduced in the presence of tunneller-type dung beetles, which introduce the organic matter from the dung pat into the soil, thereby transferring microbial respiration underground (Menéndez et al. 2016) and facilitating the mineralisation of MBN within the soil, further positively affecting plant biomass outputs (Nervo et al. 2017; Maldonado et al. 2019) as N is rate-limiting for plant growth (Kant et al. 2012). A low MBC:MBN ratio is indicative of high N availability in the soil, as soil microorganisms are able to adjust their metabolism, including the production of extracellular enzymes, to the availability of nutrients (Zhu et al. 2021). Hence, our results corroborate the thesis that dung beetles enhance both the content and the availability of organic nutrients in the soil.

Soil enzyme activity was accepted as a key indicator of microbial function for nutrient conservation and nutrient transformations related to soil fertility and health (Lu et al. 2015).

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In our experiment, only soil cellulase and catalase activities were positively affected by the addition of CD to the soil, whereas urease, acid phosphatase, and β-glucosidase were not significantly altered. However, the addition of dung beetles to cow dung significantly increased the activity of all measured enzymes, with increases in cellulase, urease, acid phosphatase, and β-glucosidase activity to values 3-fold higher than control, indicating that the enrichment of soil with the enzymes from the dung is importantly enhanced by the tunnelling activity of dung beetles.

Moreover, our results show significant positive correlation between soil enzyme activities, MBC, MBN, soil physico-chemical properties and plant growth, indicating that all these parameters are affected by the applied treatments in a directional way. Additionally, high correlation between the soil parameters further suggests mutual interdependence between these parameters. For instance, the increase in the activity of soil enzymes may be caused not only by direct transfer of enzymes present in the dung to the soil, but also by the increase in the biomass of the microorganisms that produce them. These microbial enzymes are responsible for subsequent mineralisation of the organic matter present in the soil and its output on plant growth, as confirmed by the results of the correlation analysis. Cellulase and β-glucosidase activity are important determinants of the composting process as they catalyse the breakdown of polysaccharides in detritus and turn them into available C (Liu et al. 2020). Urease activity is a highly reliable soil enzymatic indicator for mineralisation of N of organic origin (Nourbakhsh and Monreal 2004). Microbial phosphatases play an important role in the solubilisation of phosphates, making them available to the plants for uptake (Rawat et al. 2021). Microbial antioxidant enzymes such as catalases, contribute to plant tolerance to a variety of stresses (Kaushal 2020). Importantly, plant height, as well as the activities of

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individual groups of enzymes, showed particularly pronounced positive correlation with most other parameters in the DB treatment but less so in CD and control, indicating that the presence of dung beetles acted in a deterministic manner, i.e., affecting the measured soil parameters and the relationships between them, as well as their output on plant growth, in a highly predictable fashion.

In addition, our results showed that the presence of dung beetles significantly enhances bacterial diversity in cow dung-amended soil. By breaking the interface between the dung and the soil, dung beetles enrich the soil with the bacteria originating from the dung (Slade et al. 2016). Additionally, the complex changes in soil properties, caused by dung beetles as shown in our study, might contribute to the changes in the composition of bacterial taxa through the alteration of their environment. It has been suggested that bacterial diversity is relevant to soil health and fertility (Hatfield and Walthall 2015; Tautges et al. 2016). A growing amount of evidence on the relationships between microbial diversity and soil organic C content, points to the importance of microbial diversity for the efficacy of C turnover in the soil and enhancement of soil fertility (Prasanna et al. 2012). The diversity of soil bacteria is assumed to contribute to soil health and fertility through the provision of a balanced complement of diverse microbial ecological functions, most of which are still poorly understood (Brandt et al. 2015).

Moreover, dung beetles, but not cow dung alone, importantly altered the bacterial community composition at both the class and family level in our study. At the family level, the relative abundance of Xanthomonadaceae, Burkholderiaceae, Rhizobiaceae, Sphingomonadaceae and Caulobacteraceae was significantly enhanced in DB when compared with CD or control, whereas the opposite was found for Dongiaceae. It is possible that Dongiaceae have lesser

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relative environmental benefit from enriching the soil with dung (and/or beetles) compared to most other soil bacteria, causing them to be less competitive and reproduce relatively slower in comparison to other bacterial taxa, which results in the decrease of their relative abundance in the soil microbiome. The relative abundance of *Dongia sp.* was reported to decrease in the presence of the larvae of the Japanese rhinoceros beetle (*Trypoxylus dichotomus septentrionalis*), a scarabeid relative of dung beetles, that feeds on rotting wood (Eo et al. 2017). The relative abundance of phylogenetic groups within Rhizobiaceae and Burkholderiaceae was previously reported to correlate with the availability of nutrients in the soil (Ai et al. 2018). Furthermore, Xanthomonadaceae were found to be more abundant in soil treated with DB; this group of bacteria might play an important role in the control of bacterial plant pathogens (Wu et al. 2014). On the class level, the dung beetles significantly increased the abundance of Gammaproteobacteria, Alphaproteobacteria, Bacilli, and Bacteroidia when compared with cow dung or control. These taxa have been studied for their potential as biocontrol agents against plant diseases, and as biofertilisers to improve plant growth (Parham et al. 2002). *Bacillus* is involved both in suppressing plant pathogens and promoting plant growth (Algam et al. 2010). Gammaproteobacteria were shown to play a major role in the decomposition of various organic substrates including glucose, phenol, caffeine and naphthalene in a field study (Padmanabhan et al. 2003). They were also found to importantly contribute to the decomposition of labile C compounds and increased soil respiration (Cleveland et al. 2007). It has been concluded that Gammaproteobacteria are particularly favoured by the increase in soil organic content compared to other bacterial taxa (Ai et al. 2008). Overall, our results show that the changes in the soil microbial composition and enhancement of soil microbial diversity critically depend on the burrowing activity of the

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dung beetle *C. molossus*, adding to a previous report on the similar effects of the dung beetle genera *Aphodius* and *Geotrupes* (Slade et al. 2016) and contributing to the body of evidence on the beneficial impact of dung beetles on the soil microbiome.

Additionally, although the growth of Chinese cabbage plants was enhanced in the CD treatment in our study, addition of the dung beetles to the cow dung further enhanced the parameters related to plant growth, suggesting that the addition of dung beetles was beneficial to overall plant growth. Beneficial outputs of *C. molossus* beetles on Chinese cabbage, as well as for bok choy as reported in our previous study (Kaleri et al. 2020) are likely due to the improvement in soil properties, as plant height in our current study directly correlated with an array of soil parameters related to the abundance of organic and inorganic nutrients and activity of soil enzymes – especially in the DB treatment. The mechanistic link between the tunnelling activity of dung beetles and its output on plant growth was demonstrated when Nervo et al. (2017) documented the dung beetle-driven incorporation of the stable ^{15}N isotope from the cow dung into the soil, and its subsequent mineralisation and uptake by growing plants.

Beside enhancing the plant growth parameters, the dung beetles positively affected the flavonoid, polyphenol and vitamin C content in the leaves of Chinese cabbage. Multiple factors can affect the total antioxidant capacity of plant tissues, with soil type and soil humus content playing an important role: higher humus compounds content in soil has been linked to stronger antioxidant content in plants (Rimmer 2006). It was reported that high nutrient content in soil correlated with increased vitamin C in blueberries, strawberries and maize (Asami et al. 2003). Furthermore, cow dung alone did not contribute to an increase in chlorophyll content in our study, but the addition of dung beetles led to a significant increase

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in chlorophyll as compared to both other treatments. The soil manure with dung material can have an important role to increase the chlorophyll content by supplying a fraction of Mg^{2+} ions (Wang et al. 2017). Similar results were reported in our previous study, wherein the presence of dung beetles increased the content of polyphenols, vitamin C and chlorophyll in bok choy (Kaleri et al. 2020). It can thus be argued that in addition to plant growth, the antioxidant and chlorophyll content, as well as the nutritive quality of plants can be enhanced by growing them in dung beetle-treated soil.

The addition of cow dung and dung beetles to the soil caused changes in the phytohormone composition of Chinese cabbage. The content of ZR, GA_3, and IAA was found higher in DB plants when compared with CD or control, while a reduction was noted in ABA content when compared with the CD or control plants. Different phytohormone composition of CD- and DB-treated plants compared to control plants suggests a significant difference in their physiological status as plants are able to readily change the phytohormone content in their tissues in response to environmental cues (Santner et al. 2009). The dung beetle-driven changes in soil nutrient content and availability thus reflected on the phytohormonal status of the plants in our study. Nutrient availability, which is increased by the presence of dung beetles and their effects on soil properties, represents an important environmental factor that alters the physiological responses of the plant through affecting its phytohormone levels. Cytokinins, and ZR in particular, act as molecular messengers for N availability. In maize, ZR is synthesised in roots and transported through the xylem to the plant shoot in response to NO_3^- availability in the soil (Takei et al. 2001). Another plant hormone that signals N availability is auxin (Walch-Liu et al. 2006). Thus, the higher content of ZR and IAA in dung-beetle treated plants is a direct consequence of increased N availability. The auxin IAA

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is also a common product of L-tryptophan metabolism by soil fungi and bacteria, including those that can stimulate plant growth (Arshad and Frankenberger 1991). ABA, on the other hand, acts as a signalling molecule in a variety of abiotic stresses (Tuteja 2007), consistently with the lower ABA content that is found in DB and CD treatments in this study. On the other hand, it has been suggested that some plant growth regulators can be synthesised by the microorganisms in the compost and uptaken by plants (Ravindran et al. 2016). Positive correlation has been found between the production of several plant growth regulators and subtilin in organic and bio-dynamic manures, that not only supplies valuable nutrients but also shows significant effects on crop growth and disease resistance (Tomati et al. 1988). We also showed in our previous study (Kaleri et al. 2020) that plant growth regulators gibberellins, which promote germination, growth and yield of plants, were synthesised more in dung beetle applications. Taken together, our results suggest that the physiological responses of the plants, mediated by endogenous levels of various phytohormones, can be affected by the presence of organisms such as dung beetles, thanks to the complex biological outputs that dung beetles exert on the soil environment even without directly interacting with the plant.

5 Conclusions

Our study provides evidence for the beneficial effects of the dung beetle *Catharsius molossus* on soil health and fertility. By facilitating the transfer of the cow dung into the soil, these tunnelling dung beetles maximise the nutritive inputs from the cow dung on an array of soil properties, including soil mineral and organic nutrient content, soil enzyme activities and bacterial diversity. Scientific reports on the effects of dung beetles on soil enzyme activities and bacterial diversity are scarce, and our results contribute to the body of evidence on their

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ecological functions. We show, using Chinese cabbage as a model system, that dung beetles also enhance the beneficial outputs of cow dung on plant growth and antioxidant content. Thus, the combined use of dung and beetles can be used as an effective, low-cost approach to enhance soil health and fertility, and improve growth characteristics and nutrient content of edible crops.

Acknowledgments

We would like to thank Dr. Yinan Yao and Yugui Liu for their help in experimental setup.

Funding

This work was supported by the National Natural Science Foundation of China (no. 30902005), Sichuan Science and Technology Program (no. 2017JY0163) and Longshan academic talent research supporting program of SWUST (no. 18LZX561) and by the Ministry of Education, Science and Technological Development of Republic of Serbia, Contract No. 451-03-68/2020-14/200007.

Compliance with Ethical Standards

Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Fig. 1 Effect of dung beetles and cow dung on soil microbial biomass N (MBN) (**a**), C (MBC) (**b**), and C:N ratio (**c**) under control (CO), cow dung (CD), and dung beetles (DB) treatment. The values are mean ± SD (n = 3). Means are significant at P-value (*, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$) as determined by the LSD test

Fig. 2 Effect of dung beetles and cow dung on soil enzyme activity: the cellulase (**a**), β-glucosidase (**b**), catalase (**c**), acid phosphatase (**d**) and urease (**e**) activity under control (CO), cow dung (CD), and dung beetles (DB) treatment. The values are mean ± SD (n = 3). Means are significant at P-value (*, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$) as determined by the LSD test

Fig. 3 The Pearson correlation coefficient analysis heat map showing correlation among soil and plant parameters: overall correlation among all parameters considering all data of all treatments (n = 12) (**a**) and correlation of plant height and various soil enzymes with other soil quality parameters under individual treatment groups (n = 3) (**b**). The darkest scale shows highest significantly positive correlation while the lightest scale shows highly negative correlation

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Fig. 5 Principal coordinate analysis (PCoA) of bacterial community composition in control (CO), dung beetle (DB) and cow dung (CD) treated soil. In addition to the scatter plot

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representation of the PCoA (**a**), boxplot representations of the distribution of principal component 1 (PCA1) (**b**) and principal component 2 (PCA2) (**c**) are shown in the figure. The values are mean ± SD (n = 3)

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Fig. 8 Effect of dung beetles and cow dung on endogenous levels of phytohormones: *trans*-zeatin riboside (ZR) (**a**), gibberellic acid (GA_3) (**b**), abscisic acid (ABA) (**c**), and indole-3-acetic acid (IAA) (**d**) in fresh leaf samples of Chinese cabbage grown in control (CO), cow dung (CD), and dung beetles (DB) treated soil. The values are mean ± SD (n = 3). Means are significant at P-value (*, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$) as determined by the LSD test

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Table 1 Soil properties and mineral content under dung beetle, cow dung treated and control (untreated soil). Each value represents mean ± SD (n = 3). Means are significantly different from control values at P-value (* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$) as determined by the LSD test. EC = electrical conductivity; Ca = calcium; Mg = magnesium; N = nitrogen; P = phosphorus; K = potassium

Treatments	Dung beetles	Cow dung	Control
Soil EC (μS cm^{-1})	325.90 ± 2.96***	329.37 ± 0.02***	425.17 ± 0.07
Soil pH	7.93 ± 0.11*	8.22 ± 0.12	8.12 ± 0.13
Total Ca (%)	1.30 ± 0.01***	1.16 ± 0.02**	1.02 ± 0.02
Total Mg (%)	5.45 ± 0.03** *	5.00 ± 0.06*	4.93 ± 0.01
Total N (%)	0.38 ± 0.05*	0.34 ± 0.05*	0.24 ± 0.03
Total P (%)	0.23 ± 0.01*	0.18 ± 0.01	0.17 ± 0.00
Total K (%)	2.41 ± 0.01**	2.17 ± 0.05	2.08 ± 0.11

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Table 2. Effects of dung beetles, cow dung and control soil on the germination and growth of Chinese cabbage plants. Each value represents the mean ± SD (n = 3). Means are significantly different from control values at p value (*$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$) as determined by the LSD test

Treatments	Dung beetles	Cow dung	Control
Germination (%)	88.0 ± 1.07**	84.1 ± 0.28**	53.7 ± 0.25
Plant height (cm)	10.0 ± 0.97***	8.18 ± 0.21**	6.20 ± 0.31
Leaf number	5.52 ± 0.41**	4.50 ± 0.16	4.22 ± 0.04
Leaf length (cm)	7.18 ± 0.20***	4.91 ± 0.16***	3.87 ± 0.09
Leaf width (cm)	4.51 ± 0.26***	3.25 ± 0.06*	2.90 ± 0.03
Root length (cm)	8.86 ± 0.25***	6.63 ± 0.11**	5.40 ± 0.20
Root fresh weight (g)	0.49 ± 0.06**	0.37 ± 0.01*	0.29 ± 0.02
Root dry weight (g)	0.08 ± 0.01***	0.05 ± 0.00*	0.02 ± 0.00

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Figure 1

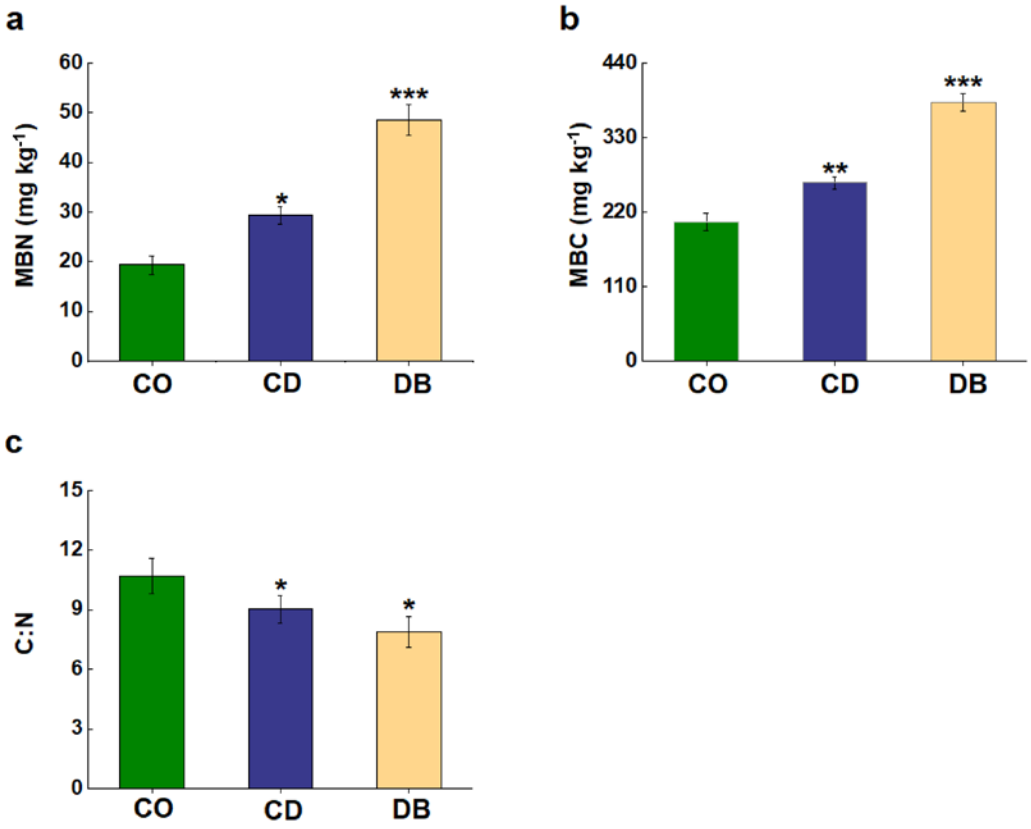

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Figure 2

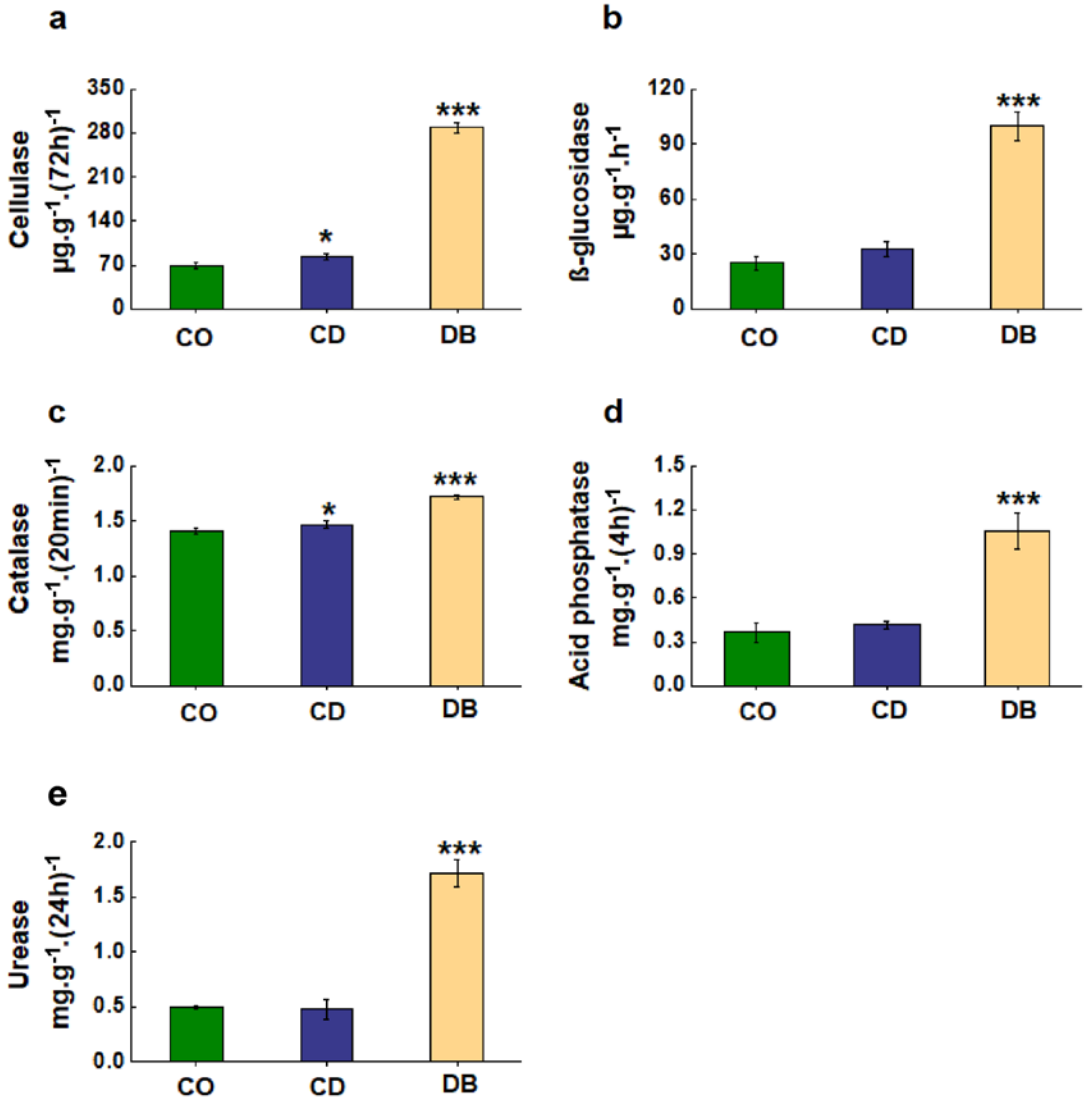

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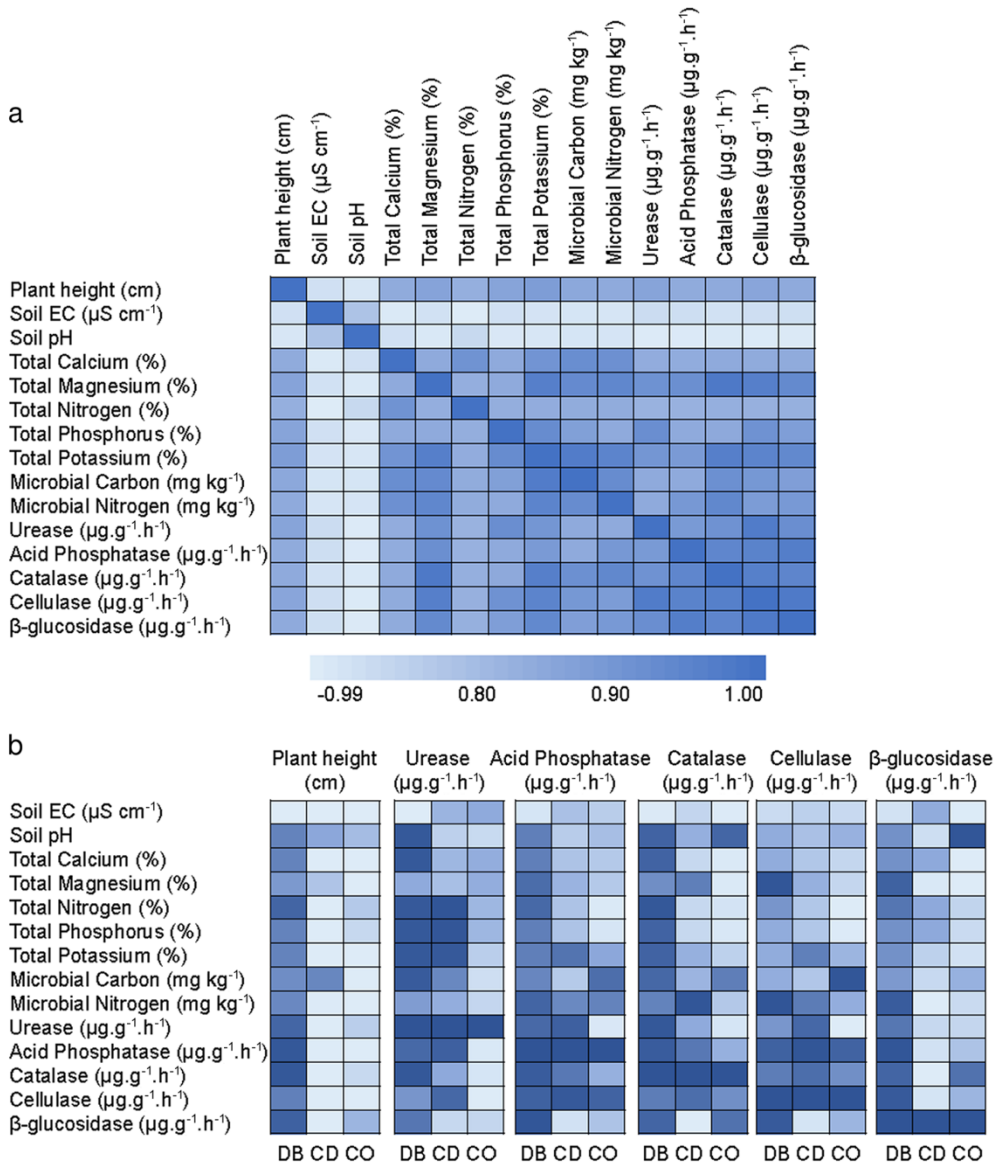
Figure 3



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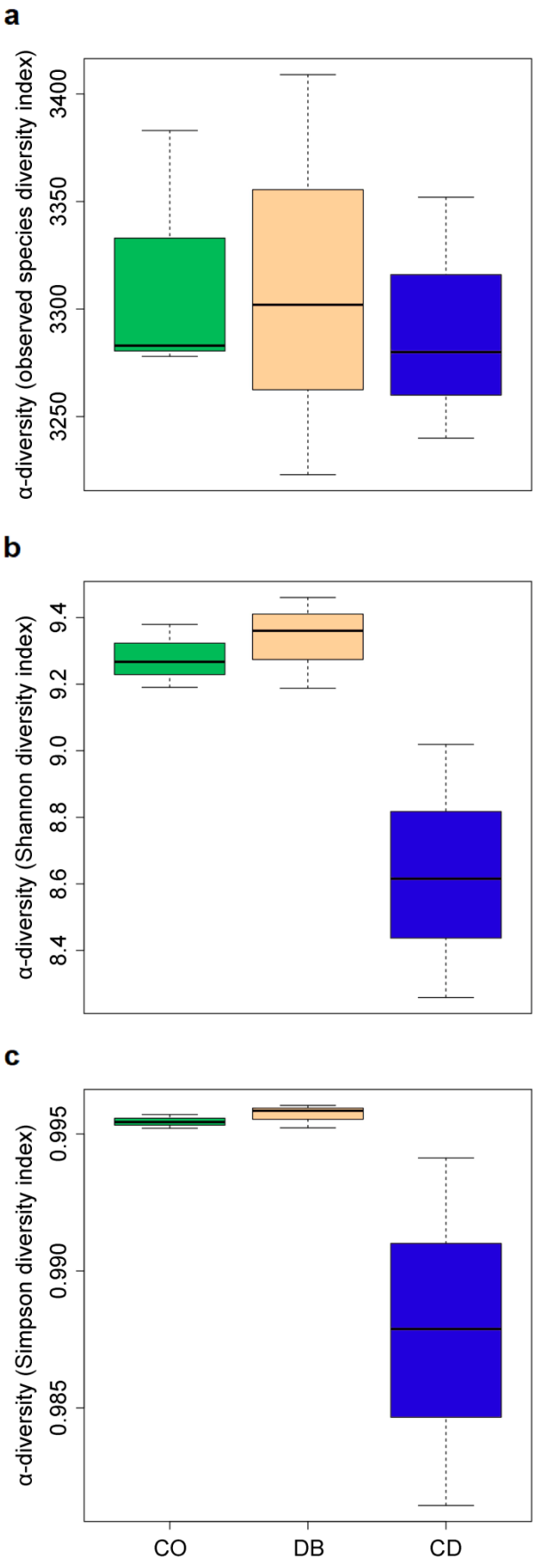

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Figure 5

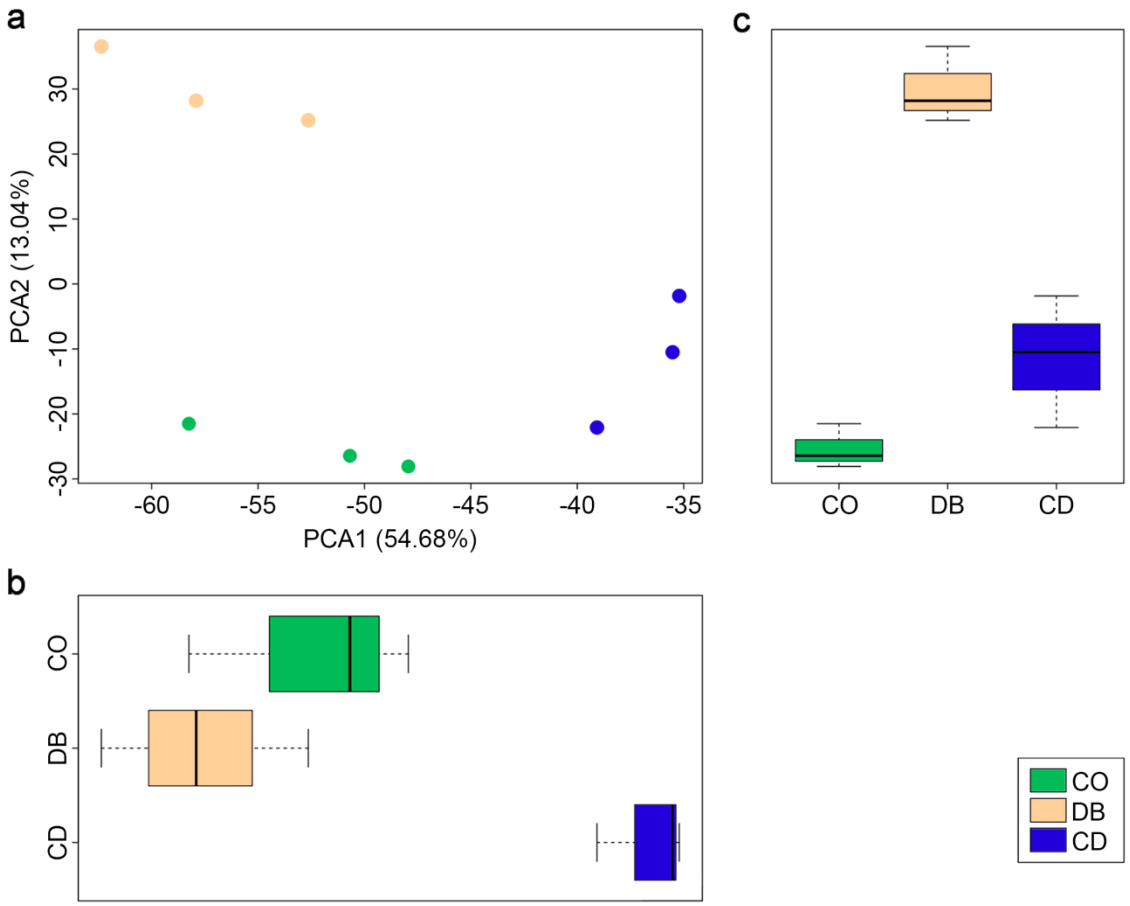

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Figure 6

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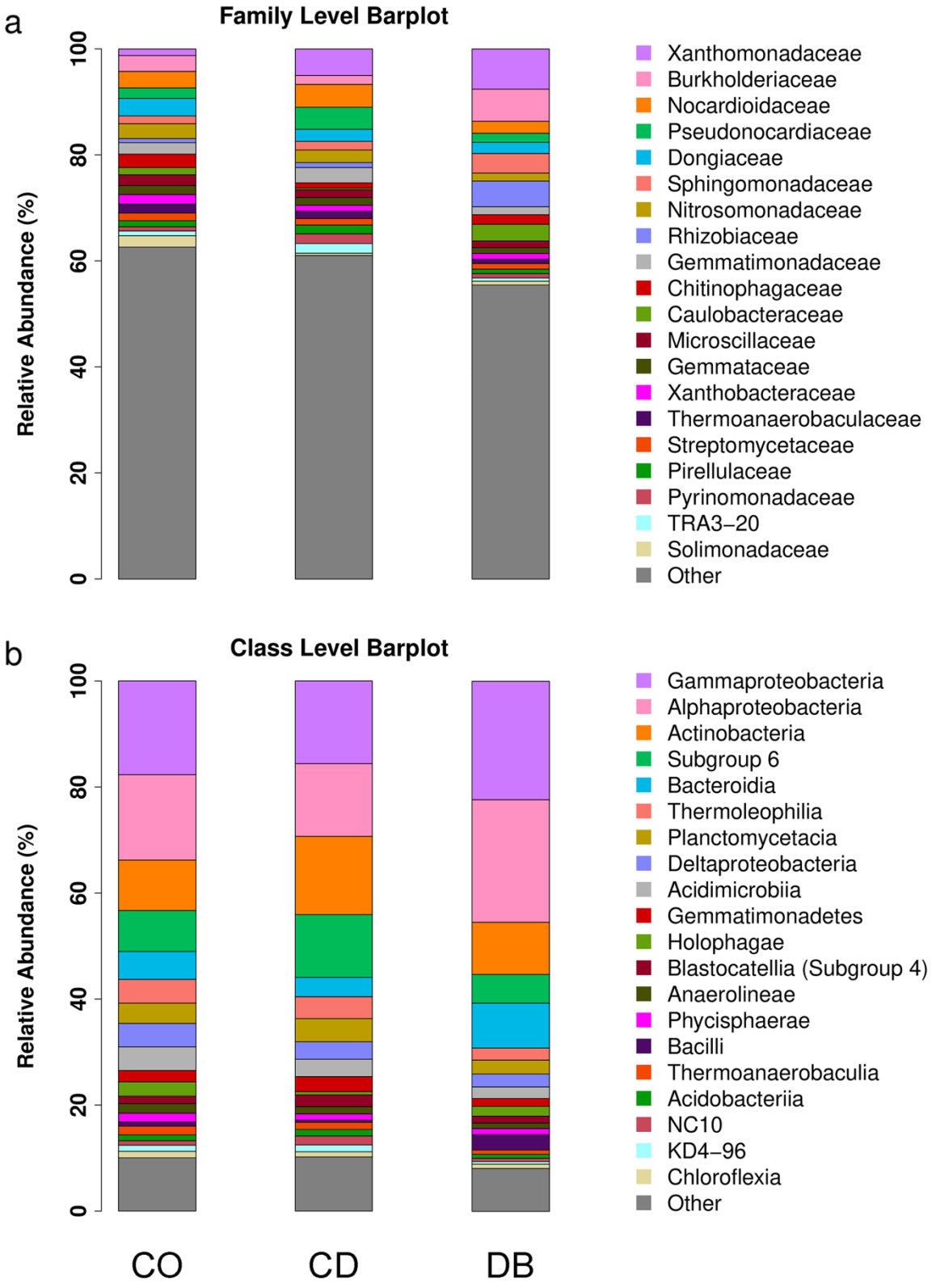

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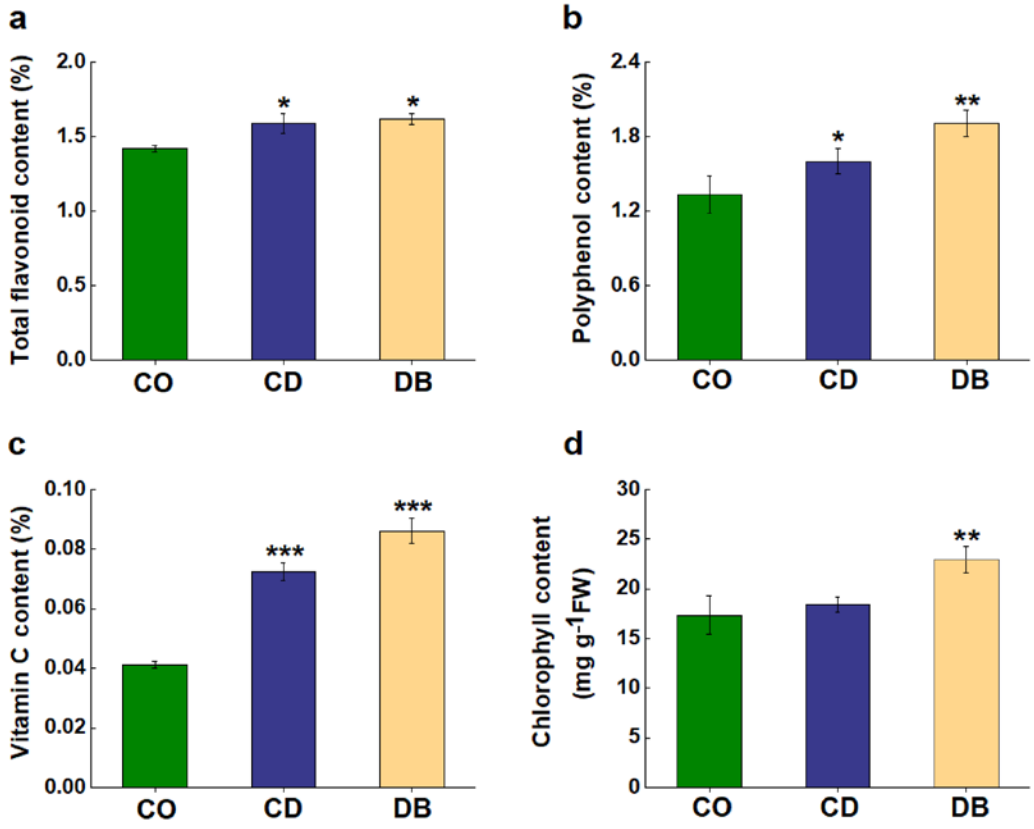
Figure 7



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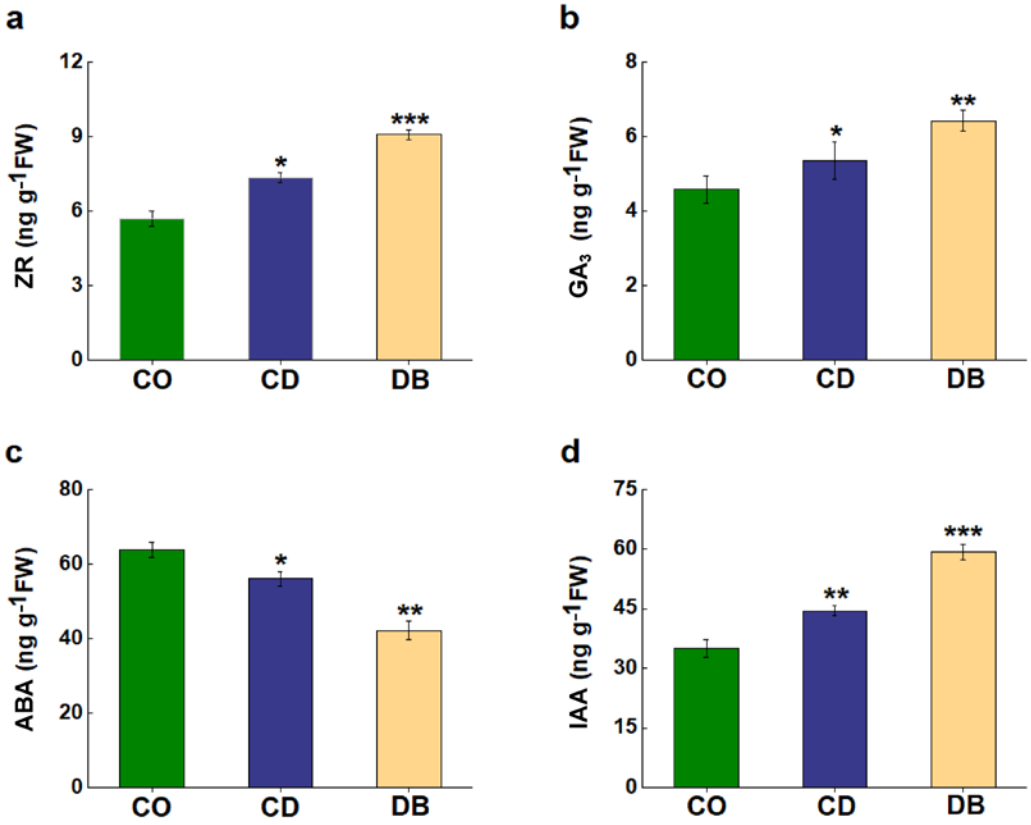
Figure 8