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Optimization, application effects and improved microecology of a composite microbial agent containing oyster shells

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Oysters are one of the largest marine shellfish species worldwide. However, oyster shells are treated as waste, accumulating on coastal shores and seriously polluting the ecosystem. In this study, a composite microbial agent was developed using calcined oyster shell powder, *Beauveria bassiana* spore powder, and ecological chitosan. To release the active ingredients, oyster shells were treated by calcination. The optimal application ratio of the agent was determined by detecting tomato growth indicators. Application of the optimized agent to vineyards increased 100-grain weight, carotenoids, and total amino acids by 10.55%, 8.71%, and 29.40%, respectively. Further detection of soil microbial population changes showed that the application of the agent increased the abundance and diversity of soil microbial populations, promoting the metabolism of bacterial amino acids, polysaccharides. These findings suggest that the agent not only enhanced plant growth and fruit quality, but also enriched the diversity and abundance of soil microbial communities.

Keywords Oyster shell, *Beauveria bassiana*, Composite microbial agent, Soil Microbiome

Oysters are one of the most productive marine shellfish in the world mainly found along the coasts of Europe, Asia, and the Americas[1]. Global oyster production reached 8.12 million tons in 2022[2]. However, the edible or valuable parts of oysters constitute only 10% of their total mass, with the majority discarded as waste and accumulating on coastlines[3]. The accumulation and decomposition of waste shells lead to bacterial proliferation, causing soil and water contamination and posing significant environmental challenges for coastal cities[4]. At present, oyster shells are mainly used in environmental protection and medical industry fields[5]. Li et al.[6] used coprecipitation to produce $MnFe_2O_4$-loaded oyster shells for phosphorus removal in wastewater. The bead layer and gill of oysters are rich in bioactive peptides and proteins; exhibit antioxidant, antimicrobial, antihypertensive, anticancer, antifatigue, and anticoagulant properties; and promotes osteoblast differentiation[7]. Currently, their high processing costs and low utilization rates have limited oyster shell use in pollution management[8]. Therefore, low-cost and more efficient handling methods are key to the sustainable development of oyster farming.

Renowned for its distinctive toxicity and human safety, the entomopathogenic fungus *Beauveria bassiana* has been extensively used for the biological control of pests[9]. A total of 58 types of *B. bassiana* insecticides are registered worldwide, representing 33.9% of all fungicides[10]. *B. bassiana* primarily infects and exterminates insects by penetrating their exoskeleton with conidia[11]. Moreover, it can parasitize more than 700 species of insects, mainly controlling Hemiptera and Lepidoptera pests[12]. Upon colonization of plants by *B. bassiana*, the likelihood of pest infestation is reduced, while the resistance of the parasitized plants to diseases is enhanced[13]. A study elucidating the interaction between tomatoes and *B. bassiana* revealed that the fungal colonization bolsters the plant's resilience against gray mold and reduces oxidative damage to plants[14]. By analyzing the tomato leaf proteome, Barra-Bucarei et al.[15] found that molecular pathways related to protein/amino acid turnover were upregulated, suggesting that the colonization of *B. bassiana* can promote plant growth. Furthermore, inoculation with *B. bassiana* augmented plant resistance to diverse abiotic stressors, such as salinity, high temperatures, and drought[16,17].

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The extensive use of pesticides and fertilizers leads to soil moisture loss, acidification, excessive heavy metal accumulation, and reduced fertility. Pesticide residues in fruits pose a threat to human health[18]. Recently, physical controls, organic fertilizers, and microbial agents have been commonly used as alternatives for pesticides and fertilizers[19]. Common microbial agents include *Bacillus*, *Ampelomyces quisqualis*, *Trichoderma* sp., and *Pseudomonas* sp[20]. *Bacillus* is often used to control agricultural pests, such as aphids and whiteflies, and is a food-safe genus that can be used in the production of industrial enzymes and food additives[21]. *Trichoderma* sp. is mainly used to control fungal and nematode diseases and some leaf and spike diseases in various plants, promotes plant growth, and increases plant resistance to microbial pathogens[22]. With more advancements in research on microbial agents, increasing choices are available to promote green agriculture. In addition, ecological chitin can be combined with other natural polymers, fertilizers and nutrient elements. On the one hand, it can provide the nutritional needs of plants, on the other hand, it also helps to improve the texture of the soil[23].

This study aimed to develop a composite microbial agent containing oyster shell powder (obtained by calcination), *B. bassiana* spore powder, and ecological chitin, and to evaluate its effects on plant growth and soil microbiota. The purpose is to provide theoretical basis and practical guidance for solving the problem of efficient utilization of waste oyster shells and promoting sustainable development of agriculture.

Materials and methods

Calcination of oyster shells

First, waste oyster shells were washed and dried, followed by calcination at temperatures of 500 °C (OS_{500}), 700 °C (OS_{700}), 900 °C (OS_{900}), and 1100 °C (OS_{1100}) in a muffle furnace (SXL-1200C, SIOMM, China) for 2 h. The calcined oyster shells were sieved through a 0.25-mm mesh and securely stored in sealed containers. The pH of the oyster shell powder (OSP) was determined using a pH meter (pHS-3C, INESA, China). Total nitrogen (TN) content was analyzed using the Kjeldahl method; total phosphorus (TP), $HClO_4$–H_2SO_4 method; and total potassium (TK), NaOH method and flame atomic emission spectrometry[24]. Scanning electron microscopy (SEM) (sigma500, ZEISS, Germany) was used for the structural and morphological examination of the powder post-sputter coating to capture images.

Construction and optimization of composite microbial agent

B. bassiana spore powder (BBSP) was purchased from Guangxi Kanglv Biotechnology Co., Ltd. The composite microbial agent (CMA) was prepared from mixing OSP, BBSP, and ecological chitosan, and the 'millennium' tomato was cultivated with different proportions of the three substances. Seedlings were nurtured in conventional trays until they reach the 4–5 true leaf stage before transplantation. Five pots were allocated for each treatment, with a fertilizer application rate of 1.0 g/500 g soil during planting. The ratios are M1:1:1:1, M2:1:2:1, M3:1:1:2, M4:2:1:1, M5:1:2:2, M6:2:1:2. And plant management was uniform for all treatment groups. Upon the ripening of tomato fruits, plant height, stem thickness, chlorophyll content (measured by spectrophotometry), and root vitality (TTC method)[25] were measured. Fruit quality at the same fruit node was evaluated, including soluble sugar (anthrone method) and titratable acidity (acid–base titration) contents[26].

Field trial design

The field trial was conducted at Taiyihu International Winery (Shandong Province, China; 36° 59′ 27″ N, 121° 21′ 54″ E). The test field has a flat topography with moderate fertility, characterized by Shandong brown soil (generally acidic to slightly acidic and no free calcium carbonate) and with an annual precipitation of 436.2 mm and an average temperature of 12.2 °C. Two-year-old chardonnay grape vines were planted at a spacing of 1.2 m between plants and 2 m between rows. Meticulous water and fertilizer management ensured a consistent growth pattern.

The experiment used the grape variety chardonnay as the experimental material, utilizing the root irrigation method. The experiment spanned from September 26, 2022 to September 6, 2023 as one cycle, with a treatment group (Treat) and a control group (CK). There were three replicates each, each of which covered an area of 0.667 hm^2. Every 4 months, grape plants received CMA treatment, with 3 kg of CMA irrigated into the grape root zone per 0.0667 hm^2. All other agricultural practices were the same between the grape plants in the treatment and control groups.

Determination of physicochemical properties and aroma components of grapes

During the grape ripening stage on September 6, 2023, 3 kg of grape berries were randomly harvested for each treatment, and measurement was replicated three times. After weighing 100 grapes, grape color difference was measured using a handheld colorimeter. The total amino acid (TAA) content was determined using an amino acid analyzer (L-8900, HITACHI, Japan). Total soluble solids (TSS), titratable acidity, chlorophyll, and carotenoid content in grapefruits were measured[27]. The sugar and acid contents of the grapes were determined by the Shandong Province Analysis and Testing Center using high-performance liquid chromatography (H-class, Waters, USA).

After juicing the grapes, 20 µL of 2-octanol and 2 g of NaCl internal standard were added. Dichloromethane was used for simultaneous distillation–extraction for 2 h. The extract was evaporated to 1 ml under rotation, filtered through a 0.25-mm membrane, placed into a vial for liquid injection. Aroma components were analyzed using a Shimadzu gas chromatography-mass spectrometry system (QP2020, Shimadzu, Japan).

Soil sampling and microbiological population studies

Root soil samples were collected using a five-point sampling method, situated approximately 10 cm away from the plant at a depth of 20 cm. Five collections were combined to form a composite sample for each group, which included the fertilization and control groups with three replications each. The collected samples were transported

to the laboratory and sieved through a 2-mm mesh. Subsequently, a portion of the samples underwent air-drying for physical and chemical property analysis, and another portion was stored in an ultra-low temperature freezer for microbial determination.

Total DNA was extracted using a kit (Nobelab, China). Primers B341F_B785R and ITS1-F_ITS2-R were used to amplify the bacterial 16S rDNA V4 region and fungal ITS1 region. High-throughput parallel sequencing was performed on the Illumina Novaseq 6000 platform in PE250 mode. Low-quality sequences (those containing N bases or less than 100 bp long) were filtered out using the fastq_mergepairs command of VSEARCH (version 1.9.6. https://github.com/torognes/vsearch). Paired sequences obtained from the dual-end sequencing were reverse-joined, and high-quality sequences with a homogeneity of ≥97% were clustered into an operational taxonomic unit (OTU) using the fastq_command. The classify.seqs command of the mothur software (https://mothur.org) was utilized to annotate OTUs based on species information with a similarity and confidence level exceeding 80%. Alpha diversity indices of samples were calculated using QIIME (version 1.9.1. https://qiime2.org), used the Community Richness Index (Chao1) and the Community Diversity Index (Shannon) and functional prediction analysis of bacterial communities was conducted using PICRUSt2 (https://picrust.github.io/picrust/), it is difficult to predict the KEGG pathway because of the lack of fungal genomic data. LEfSe (Linear discriminant analysis (LDA) Effect Size, http://huttenhower.sph.harvard.edu/galaxy/). comparisons between two or more groups, emphasizing statistical significance and biological correlation, enable the search for biological markers with statistical differences between groups.

Data analysis

One-way analysis of variance (ANOVA) and Duncan's multiple range test were performed to determine the statistical significance of mean differences using the SPSS version 20.0 software (https://www.ibm.com/products/spss-statistics). Statistical significance was set at $P < 0.05$.

Results and discussion

Effect of different calcination temperatures on the properties of oyster shells

The structure and properties of oyster shells changed after different calcination temperatures (Fig. 1). At OS_{500}, a smooth surface with a microporous structure was evident. When the temperature was increased, the OSP appeared coarse and black-gray with intensified surface cracks. At OS_{900}, the OSP surface was uniformly dispersed, having honeycomb-like porous structures with wrinkles, and its color was gray-white, advantageous for heavy metal adsorption and other applications. There was no significant change in the structure after the temperature increased to 1100 °C (Fig. 1a). This structural alteration is attributed to the conversion of $CaCO_3$ in oyster shell to CaO and $Ca(OH)_2$ as calcination temperature was increased. Increasing calcination temperatures reduced specific gravity post-calcination and increased weight loss, consistent with the findings of Tongwanichniyom et al[28] . The TN and TK contents of OS_{700} and OS_{900} were not significantly different; however, TP content was higher in OS_{900} than in OS_{700} (Fig. 1b).

Post-calcined OSP use was associated with increased soil pH, organic matter, TN, and NO_3^--N, exhibiting a positive correlation with temperature[4]. After the application of OS_{800}, Bi et al.[29] observed a decrease in exchangeable Cd in soil from 60 to 27%, and the Cd content in Chinese cabbage decreased from 2.80 to 0.048 mg/kg. Although higher calcination temperatures efficiently liberated oyster shell components, the presence of calcite and lime on the surface of OS_{700} facilitated a micro-precipitation mechanism for phosphate adsorption[30]. Using OSP and bone meal as soil amendments could stabilize heavy metals and increase soil nutrients, and calcined OSP exhibited pH regulation, water retention, and calcium enrichment properties[4]. The adsorption capacity of oyster shells is primarily attributed to chemical precipitation, electrostatic adsorption, and hydrogen bonding effects[31]. Furthermore, oyster shells and phosphoric acid have been used to develop triple superphosphates as a sustainable alternative to phosphate fertilizer[32].

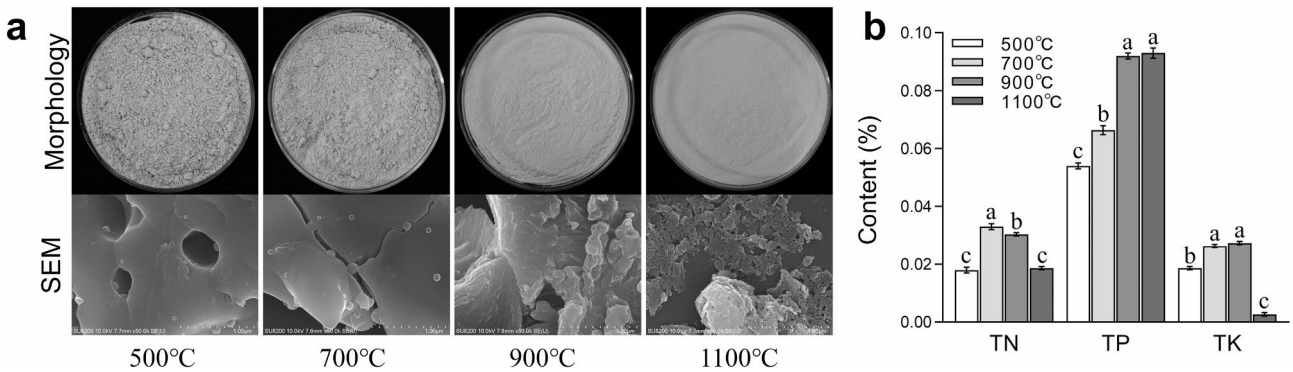

Fig. 1. (**a**) OS_{500}, OS_{700}, OS_{900}, OS_{1100} morphology and SEM images. (**b**) Effect of different calcination temperatures on the release of TN, TP and TK from oyster shells.

Group	Plant height (cm)	Stem thickness (mm)	Chlorophyll (mg/g)	Root activity (%)	Soluble sugar (%)	Titrable acid (%)
CK	71.23 ± 0.74e	8.64 ± 0.10b	1.374 ± 0.002 g	49.09 ± 0.18 g	2.78 ± 0.05f	0.33 ± 0.02a
M1 (1:1:1)	75.45 ± 0.51bc	9.18 ± 0.24a	1.544 ± 0.003d	53.37 ± 0.28b	3.15 ± 0.04b	0.28 ± 0.01c
M2 (1:2:1)	73.82 ± 0.28d	8.71 ± 0.08b	1.406 ± 0.003f	50.45 ± 0.09f	2.87 ± 0.03e	0.32 ± 0.02a
M3 (1:1:2)	76.84 ± 0.27a	9.26 ± 0.07a	1.533 ± 0.003e	52.89 ± 0.08c	3.01 ± 0.06 cd	0.31 ± 0.01b
M4 (2:1:1)	76.20 ± 0.49ab	9.21 ± 0.09a	1.625 ± 0.003b	51.36 ± 0.18e	2.94 ± 0.05d	0.28 ± 0.01c
M5 (1:2:2)	74.99 ± 0.67c	9.25 ± 0.08a	1.587 ± 0.003c	52.45 ± 0.19d	3.04 ± 0.03c	0.30 ± 0.03b
M6 (2:1:2)	76.47 ± 0.40a	9.23 ± 0.04a	1.634 ± 0.002a	55.21 ± 0.09a	3.26 ± 0.04a	0.26 ± 0.01d

Table 1. Effect of different ratios of CMA on physico-chemical properties of tomato. Different lowercase letters indicate significant differences between groups, $P < 0.05$.

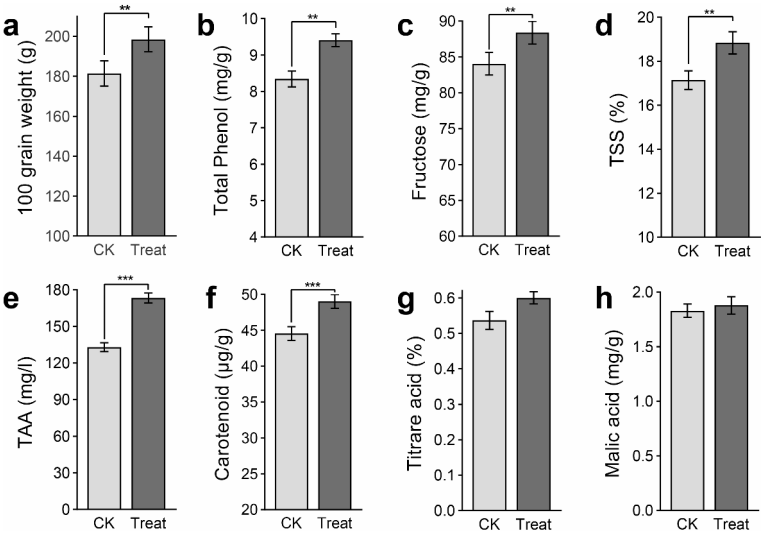

Fig. 2. Effect of application of CMA on grape 100-grain weight (**a**), total phenols (**b**), fructose (**c**), TSS (**d**), TAA (**e**), carotenoids (**f**), titratable acid (**g**) and malic acid (**h**). (**$P < 0.01$, ***$P < 0.001$).

Role of different CMA combinations on tomato growth

The application of various ratios of CMA on tomato plants affected plant height, stem thickness, chlorophyll content, root activity, soluble sugar levels, and titratable acid (Table 1). Plant height and stem thickness in all CMA treatments were significantly higher than those in the control group, without significant variance among the treatments. The use of different ratios of CMA associated with chlorophyll content, root activity, soluble sugar content, and titratable acid content. Notably, the M6 group outperformed the other treatments, showing increases of 18.95%, 12.48%, and 17.44% and a decrease of 21.21% in titratable acid compared to the control group. Consequently, the optimal ratio of OSP, BBSP, and ecological chitosan was 2:1:2 (subsequent experiments will be applied in this proportion). The increase in chlorophyll content plays an important role in starch accumulation and photosynthesis[33]. The improvement in tomato root vigor indicated enhanced soil adaptability, increased metabolism, and improved root cell membrane integrity[34].

The primary constituents of ecological chitosan are chitosan and poly-γ-glutamic acid (γ-PGA). Chitosan has a synergistic effect on the growth of *B. bassiana*[35]. γ-PGA is a biopolymer that can improve fertilizer efficiency and plant growth[36]. It has excellent water absorption property and is often used in the medical field as a bio-adhesive for hemostasis and healing[37]. Combined with OSP, chitosan and γ-PGA enhanced the water absorption and moisture retention abilities of plant roots. These components work synergistically to promote plant growth more efficiently.

Improvement in grape quality with CMA

The grapes were cultivated by root application with the optimal ratio of compound microbial agents (2:1:2), and the effects on grape growth and quality were further investigated. The 100-grain weight (Fig. 2a) and total phenol (Fig. 2b), fructose (Fig. 2c), and TSS (Fig. 2d) contents of grape berries were increased by 10.55%, 8.50%, 12.78%, and 5.72%, respectively. Carotenoids are essential as precursors for various volatile aroma compounds and play a significant role in the aromatic profile of grapevines[38]. The TAA content in the treatment group increased by 29.40% compared to the CK group (Fig. 2e); TAAs are crucial for grape quality and subsequent fermentation processes[39]. Inadequate amino acid levels during fermentation necessitate additional supplementation. The Treat group showed an 8.71% increase in carotenoid content compared to the CK group (Fig. 2f), thereby containing

more aroma compounds in grape wine. However, those of titratable acid and malic acid did not show any significant difference compared to those of the control (Fig. 2g,h).

Aroma is a pivotal indicator of grape and wine quality, the application of CMA affected the main aroma components of grapes (Fig. 3). Following the application of the CMA, the content of alcohol compounds increased by 11.89%, and that of norisoprene compounds increased by 40.47%. The low threshold of norisoprene compounds is the main aroma substance of chardonnay grapes, and the increase in their content determines the aroma of grapes and wines. Furthermore, the treatment group showed greater color differences in a* and b* values compared to the CK group (Table S1), indicating a richer reddish-yellow hue in grape color, reflective of a higher level of ripeness. The color difference in the grapes is related to anthocyanins, the accumulation of which contributes to the organoleptic properties of the wine[40].

Effect of CMA application on soil microorganisms

The alpha-diversity index of soil microbial communities is listed in Table S2 ($P < 0.05$). The Chao1 indices of bacteria were 5084.00 ± 55.20 and 5197.02 ± 89.74, and the Shannon indices were 6.58 ± 0.71 and 6.96 ± 0.68 in the CK and treatment groups, respectively. For fungi, the Chao1 indices were 666.45 ± 40.31 and 794.03 ± 28.65, and the corresponding Shannon indices of 3.19 ± 0.14 and 3.67 ± 0.72 were in the CK and treatment groups, respectively. The Chao1 and Shannon indices in both bacteria and fungi were higher in the treatment group than in the CK group, suggesting that the application of the composite fungicide increased the abundance and diversity of the microbial community in the vineyard soil.

The effect of the CMA application on the composition of the microbial community of the vineyard soil is shown in Fig. 4. The dominant phyla of the soil bacterial community are Proteobacteria, Actinobacteria, Acidobacteria, and Gemmatimonadetes (Fig. 4a). The CMA treatment increased the relative abundances of Gemmatimonadetes and Proteobacteria by 17.86% and 6.26%, respectively, but decreased that of Actinobacteria by 23.68%. There was no significant change in that of Acidobacteria.

Gemmatimonadetes play a crucial role as phosphate-solubilizing bacteria in cultivated soil[41]. Their relative abundance significantly increased with the application of the CMA, enhancing phosphorus utilization efficiency

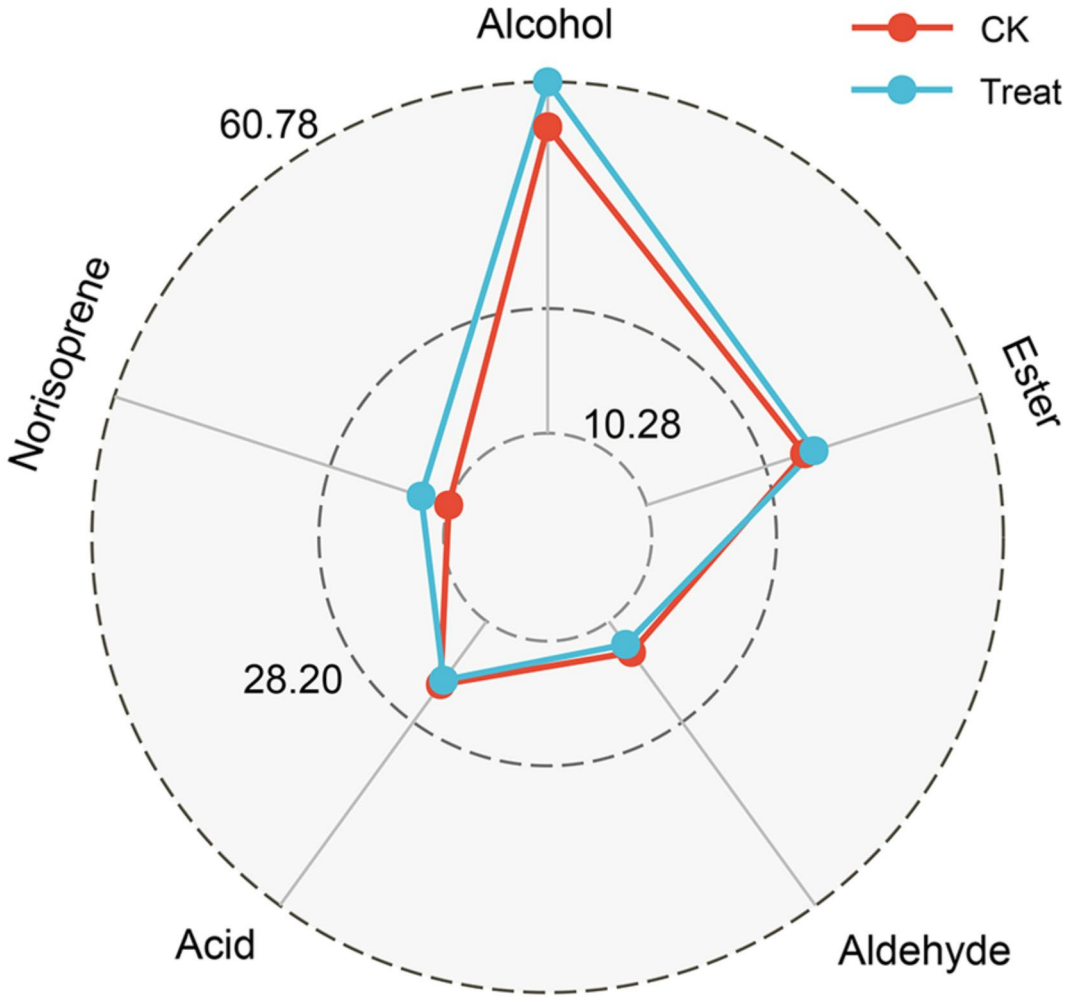

Fig. 3. Effect of application of CMA on grape aroma compound content. Asterisks indicate differences between the two groups.

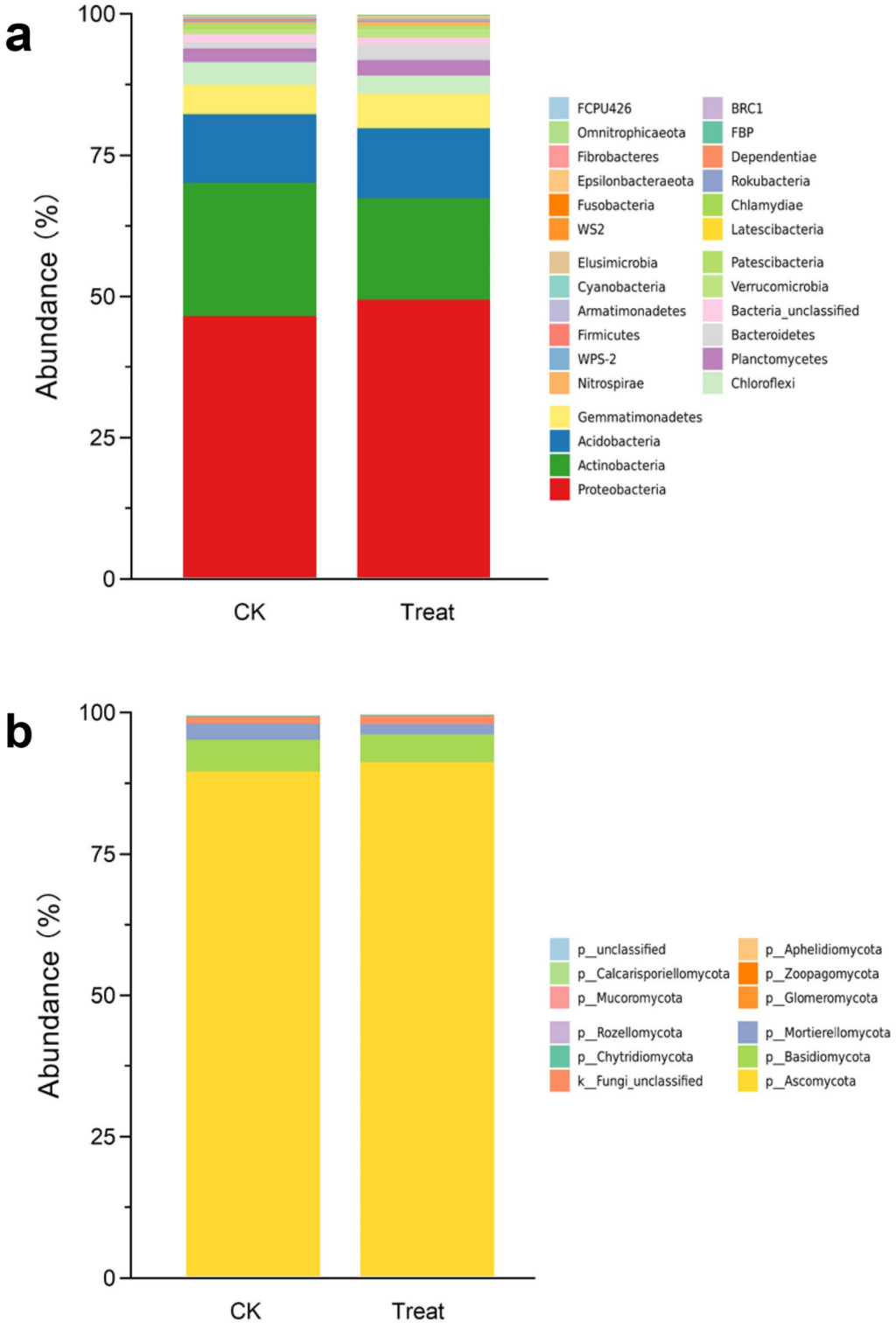

Fig. 4. Differences in the bacterial (**a**) and fungal (**b**) composition of grape-growing soils at the gate level after application of the composite fungicide.

in the soil and promoting grape growth. The abundance of Proteobacteria is related to pH, and its increased abundance could effectively slow down the biotic and abiotic stresses of plants[42]. Actinobacteria, common endophytic fungi in roots, produce numerous secondary metabolites; however, the precise reason for the decline in Actinobacteria abundance remains unclear.

In the grape cultivation soil, the dominant fungal phyla include Ascomycota, Basidiomycota, Mortierellomycota, and Fungi_unclassified (Fig. 4b). The relative abundance of Fungi_unclassified increased by

27.83%, whereas that of Mortierellomycota and Basidiomycota decreased by 38.85% and 10.85%, respectively. That of Ascomycota was not significantly changed. Oxalic acid secretion decreased with decreasing abundance of Mortierellomycota[43]. Basidiomycota is associated with nutrient transporters in plants[44]. Further investigation revealed that after the application of CMA, fungi showed a higher increase in abundance relative to that of bacteria. Adamczyk et al.[45] suggested that readily available substrates can enhance bacterial abundance, and fungal biomass influences fungal abundance growth, as fungi efficiently utilize substrates as biomass, with bacterial cell fragments serving as food for some saprotrophic fungi. This may explain the relative increase in fungal abundance compared to bacterial abundance after the application of CMA.

The LEfSe analysis method was used to analyze the microbial population (Fig. 5). In bacteria, at the phylum level, the CK had three biomarkers: Cyanobacteria, Firmicutes, and Planctomycetes. The treatment group had four biomarkers: Bacteroidetes, Chlamydiae, Latescibacteria, and Verrucomicrobia (Fig. 5a). In the treatment group, the CMA application elevated the abundance of Bacteroidetes, which is linked to organic compound

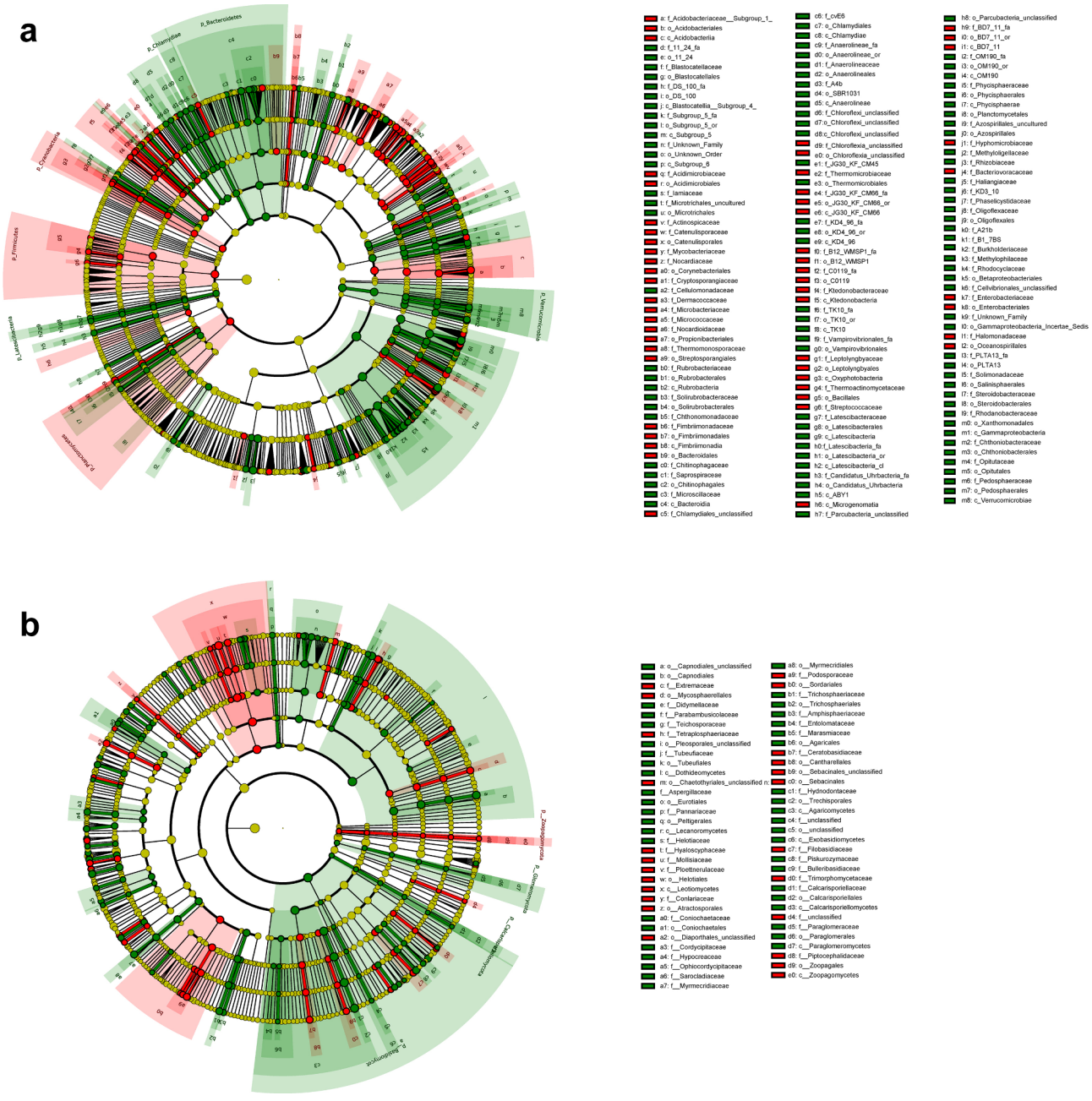

Fig. 5. LEfSe evolutionary map analysis of differences in abundance of bacterial (**a**, LDA > 2) and fungal (**b**, LDA > 2) communities in grape-growing soils treated with composite fungicides. Circles radiating from inside to outside represent taxonomic levels from phylum to genus, species without significant differences are coloured yellow, red nodes indicate microbial taxa that play an important role in the CK group, and green nodes indicate microbial taxa that play an important role in the Treat group.

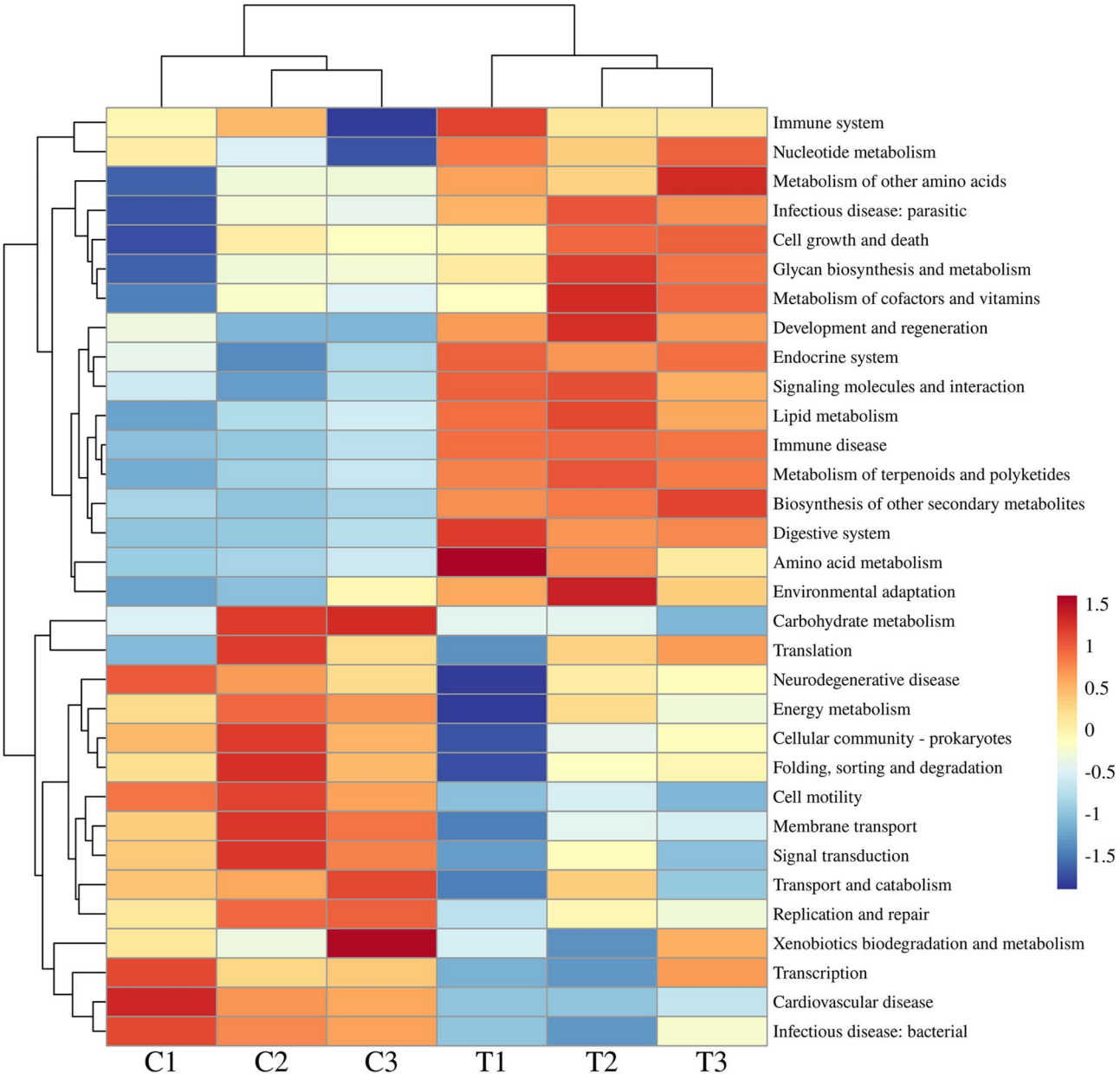

Fig. 6. Predicted functional abundance of KEGG_pathways_L2, a soil bacterium from grapevine cultivation.

transformation, nitrogen fixation, and denitrification[46]. Chlamydiae and Verrucomicrobia exhibited sensitivity to soil pH and moisture levels[47]. In fungi, at the phylum level, the CK group had one biomarker: Zoopagomycota. The treatment group had three biomarkers: Glomeromycota, Calcarisporiellomycota, and Basidiomycota (Fig. 5b). Glomeromycota plays a crucial role in establishing symbiotic relationships with grapevines, enhancing mineral nutrient provision to the grapes. Basidiomycota is important in algae and moss networks, offering resilience against diverse abiotic stresses[48]. The treatment group showed a significantly higher diversity of bacterial and fungal phyla than the CK group in terms of biomarker count.

The predicted functional abundance of soil bacterial communities from the KEGG database is illustrated in Fig. 6. The application of CMA enhanced the metabolic activities of bacterial amino acids, lipids, terpenoids, polysaccharides, glycans, and nucleotides and stimulated the biosynthesis of additional secondary metabolites, cellular processes such as growth and death, and environmental adaptation. KEGG pathway enrichment analysis is commonly used for annotating the functions of differentially expressed genes. In the soil of the treatment group, there was a notable increase in bacterial metabolism and secondary metabolites. Secondary metabolites play a vital role in grape development, aroma compound production, and other physiological functions. Therefore, the utilization of CMA not only has a beneficial effect on grape growth, but also has a certain effect on the accumulation of grape aroma components.

Conclusion

In this study, the CMA containing OSP, BBSP and ecological chitin was developed, and the calcination temperature of oyster shells was 900 ℃. The CMA was optimized by cultivating tomatoes in different proportions. The application of the CMA in the field trials significantly increased grapevine yield and quality and improved the diversity and abundance of inter-root soil microorganisms. These results showed that the CMA can significantly promote grape growth and improve soil microbiology. This study reports a valuable agent for the efficient use of waste oyster shells, contributing to the sustainable development of agriculture.

Data availability

Data is provided within the manuscript or supplementary information files.

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Declarations

Competing interests

The authors declare no competing interests.

Additional information

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