Microbiome-wide association studies reveal correlations between the structure and metabolism of the rhizosphere microbiome and disease resistance in cassava

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Summary
Cassava is one of the most important staple food crops in tropical regions. To date, an understanding of the relationship between microbial communities and disease resistance in cassava has remained elusive. In order to explore the relationship among microbiome and phenotypes for further targeted design of microbial community, 16S rRNA and ITS of microbiome of ten cassava varieties were analysed, and a distinctive microbial community in the rhizosphere showed significant interdependence with disease resistance. Shotgun metagenome sequencing was performed to elucidate the structure of microbiomes of cassava rhizosphere. Comprehensive microbiome studies were performed to assess the correlation between the rhizosphere microbiome and disease resistance. Subsequently, the metagenome of rhizosphere microbiome was annotated to obtain taxonomic information at species level and identify metabolic pathways that were significantly associated with cassava disease resistance. Notably, cassava disease resistance was significantly associated with Lactococcus sp., which specifically produces nisin. To definitively explain the role of nisin and underlying mechanism, analysis of nisin biosynthesis-associated genes together with in vitro and in vivo experiments highlighted the effect of nisin on inhibiting the growth of Xanthomonas axonopodis pv. manihotis (Xam) and activating immune response in cassava. The new insights between cassava rhizosphere microbiome especially Lactococcus sp. and disease resistance provide valuable information into further control of cassava disease.

Introduction
The wild Manihot esculenta ssp. flabellifolia commonly known as cassava was domesticated in the Amazon basin and propagated by seeds (Olsen and Schaal, 1999; Pujol et al., 2005). In the past 500 years, cassava has been cultivated by stem cutting in Africa, Asia and South America (Olsen and Schaal, 1999; Pujol et al., 2005). Cassava is an important staple food crop, because it is a rich energy source that produces high yields and exhibits high tolerance to various environmental conditions, including drought, heat and nutrient-deficient soil (Ramu et al., 2017). At present, the starchy storage roots of cassava are a major staple food crop in tropical regions, feeding approximately 750 million people worldwide in Africa (55.5%), Asia (30.2%), Americas (14.3%) and Oceania (0.1%) (Food and Agriculture Organization of the United Nations, http://faostat.fao.org) (Bredeson et al., 2016; Wang et al., 2014).

Although traditional farmers maintain a high number of cassava varieties, cassava is susceptible to multiple diseases, such as cassava bacterial blight (CBB), cassava mosaic disease (CMD) and cassava brown streak disease (CBSD) (Bredeson et al., 2016). This may be due to the dependence on stem cutting for propagation and the limited commercial diversity of cassava varieties. Therefore, the selection, breeding and chemical control of disease-resistant cassava varieties has been extensively studied. In the evaluation of artificial disease resistance (Lu et al., 2013), most of cassava varieties were sensitive, and only very limited samples were resistant. Wang et al. (2014) presented the draft genome sequences of multiple varieties of cassava and identified 1584 and 1678 gene models specific to the wild and domesticated varieties, respectively. With the development of sequencing technology, Illumina resequencing data of cassava tolerant or susceptible varieties to CBSD have been analysed to characterize disease-related genes (Gomez et al., 2019; Shi et al., 2017). He et al. (2020) showed that the NUCLEAR FACTOR Y (NF-Y) transcription factor complex (MeNF-YA1/3, MeNF-YB11/16 and MeNF-YC11/12) plays an important role in the transcriptional activation of defence-related genes, contributing to the cassava plant defence response against CBB.

Since the colonization of land by ancestral plant lineages 450 million years ago (Hassani et al., 2018), plants and their associated microbes have interacted with each other, forming assemblages of species that are often referred to as ‘holobionts’. This is a functional entity of the multicellular host and its associated microbiota. The selective pressure acting on holobiont components has likely shaped plant-associated microbial communities and selected for host-
adapted microorganisms, with great effect on plant fitness (Berendsen et al., 2012; Hassani et al., 2018). Plants have diverse and abundant microbial communities that are referred to as the plant’s second genome. The microbiota plays important roles in the development, health and stress responses of the plant hosts (Berendsen et al., 2012; Spor et al., 2011; Subramanian et al., 2015). At present, the microbial diversity of soil, roots, leaves and aerial organs has been extensively investigated by high-throughput sequencing (Abdelfattah et al., 2017; Abdelfattah et al., 2016a; Abdelfattah et al., 2016b; Buee et al., 2009; Liu et al., 2018; Yuan et al., 2018). Microbial communities have been shown to be impacted by microhabitat (Jin et al., 2017), soil type (Bonito et al., 2014) and host genotype (Ofek-Lalzar et al., 2014; Zhang et al., 2019). In addition, plant rhizosphere microbiome has been reported to be closely associated with host phenotype (Jin et al., 2017). Plants and microbes interact to obtain nutrients for better growth and improved resistance, especially in root–microbes interaction (Edwards et al., 2015; Wu et al., 2020). On the one hand, host can also influence the overall composition of microbial communities. On the other hand, rhizosphere-associated microbes obtain essential nutrients from plants through host root exudates, and crucial nutrients can be converted to more usable forms by microbes before being assimilated by plants (Ryu et al., 2004). Furthermore, plants may adapt to the environment by regulating beneficial and harmful rhizosphere microbes. Rhizobacteria that colonize host roots can improve disease resistance and have important roles in plant health (Liu et al., 2019; Niu et al., 2011; Ryu et al., 2004), but the mechanisms underlying these phenomena remain elusive. Many plant-associated microbes are able to induce plant systemic resistance by generating secondary metabolites (Liu et al., 2019). For instance, Bacillus cereus AR156 is a plant growth-promoting rhizobacterium which induces resistance against a broad spectrum of pathogens (Niu et al., 2011).

At present, disease resistance is the main focus of cassava research (Gomez et al., 2019; He et al., 2020; Scusell et al., 2019; Shi et al., 2017), and Xanthomonas axonopodis pv. manihotis (Xam) is the primary pathogen that severely affects the production of cassava worldwide (He et al., 2020). The pathogen population structure, pathogen diagnosis, identification and expression of plant genes involved in cassava resistance have been carried out (Verdier et al., 2004). Several putative molecular pathogenicity effectors (Arrieta-Ortiz et al., 2013; Bart et al., 2012) and a number of quantitative trait loci (QTLs; Jorge et al., 2001; Verdier et al., 2004; Wydra et al., 2004) involved in the infection process of Xam were identified. Xam employs several effectors to promote bacterial growth and symptom formation during infection (Castiblanco et al., 2013; Cohn et al., 2014; Cohn et al., 2016; Kemp et al., 2004). Transmission of CBB caused by Xam in fields is typically by rain splash and infected cuttings. Disease incidence and severity are largely driven by environmental factors, primarily associated with periods of high rainfall (Banito et al., 2007; Dixon et al., 2002; McCallum et al., 2017). CBB resistance is likely polygenic and additive. Cassava putative resistance genes improve plant disease resistance against Xam (Fan et al., 2020; Wei et al., 2018a).

The prevention and cure of the effect of Xam in cassava is very limited. So far, cassava diseases are mainly controlled through the selection of disease-resistant varieties, rotation and spraying pesticides (McCallum et al., 2017). However, the overall disease resistance of cassava varieties is very low, and other methods may also bring the risk of pesticide residues (McCallum et al., 2017). In recent years, we have studied the mechanism of cassava resistance to CBB through reverse genetics and identified some disease resistance genes, including MebZIP3/5 (Li et al., 2017), MeHsf3 (Wei et al., 2018b), MeNF-Ys (He et al., 2020), etc. The objective of this study was to investigate the role of natural microbiota in protecting cassava from microbial pathogens, so as to provide novel insight into the development of probiotics to improve cassava disease resistance. Therefore, comprehensive microbiome studies were performed to assess the correlation between microbiome structure and disease resistance in cassava. The crucial microbial species related to disease resistance in cassava may be further used for the targeted design of microbial community in green and highly efficient agriculture.

Results

Compare analysis of the microbiota among leaves, stems and roots of cassava and the correlation between the host genotype and its microbiome

Here, three tissues (roots, leaves and stems) of ten representative cassava varieties (R01–R10) with contrasting disease resistance were chosen for analysis of the correlations between microbiome and disease resistance (Tables S1 and S2). The results of a 16S rRNA gene and ITS region amplicon analysis provided insight into the cassava microbiota at an OTU-based taxonomic level. After quality filtering, 4,570,658 bacterial reads and 5,425,715 fungal reads were generated across all samples, with an average of 46,168 bacterial reads (rhizosphere: 45,033 ± 8,106; stems: 45,523 ± 7,726; leaves: 47,947 ± 9,370) and 54,805 fungal reads (rhizosphere: 55,716 ± 14,282; stems: 54,386 ± 9,858; leaves: 54,312 ± 8,372) obtained in each sample. These reads were assigned to 106,097 bacterial and 22,339 fungal OTUs, respectively, according to 97% sequence similarity. Species abundance values indicated that a greater number of bacterial than fungal OTUs were obtained. The taxonomic assignment of OTUs resulted in the identification of 21 phyla and 547 genera (Figure 1a–h).

Amplicon sequencing-based microbial community structure and relative abundance analyses of the three cassava organs showed a high proportional abundance of bacterial and fungal phyla assigned to Proteobacteria and Ascomycota, accounting for more than 78 and 45% of the total number of detected sequences, respectively (Figure 1a–h). The average relative abundance of the arbuscular mycorrhizal fungi Glomeromycotina was 0.75%, 0.79% and 0.10% in cassava leaf, stem and rhizosphere, respectively (Figure 1d–f). At the genus level, the structure of core bacteria was similar (Figure 2a–c), while nearly half of their relative abundances were significantly different in rhizosphere, stems and leaves (Table S3). The structure and relative abundances of core fungi were significantly different in rhizosphere, stems and leaves (Figure 2a–c, Table S3). Members of the genus Aeromonas were the most abundant among the bacterial genera associated with cassava plants, accounting for 32%–39% of the detected sequences (Figure 2a–c). The dominant fungal genera found in rhizosphere were unidentified Ascomycota and Codinaeopsis with average relative abundance of 9.9% and 6.2%, respectively, whereas those of the core microbes of stems and leaves comprised unidentified Ascomycota, Fusarium and Cryptococcus with average relative abundance range from 7% to 13% (Figure 2a–c).

PERMANOVA test indicated the three cassava tissues significantly differ in their bacterial and fungal communities (Adonis...
<0.01). The principal coordinate analysis (PCoA) based on unweighted UniFrac metrics (Figure 3a,b) revealed the rhizosphere-associated bacterial and fungal OTUs to be clearly clustering according to habitat. There suggested that the rhizosphere was inhabited by a distinct microbial community compared with those present in the stems and leaves. The differences in the Shannon index values for the fungal diversity of the three organs were not significant (<0.05) (Tables S4 and S5). However, the Shannon index value for the bacterial diversity of the rhizosphere was significantly higher than that observed for the leaves (Figure S1). The ternary plot compares the abundances of the 48 bacterial and 37 fungal differential genera in three organs of the cassava samples, which were assigned to 13 bacterial and 10 fungal classes, respectively (Figure 3c,d, and Table S4). When comparing the different bacteria and fungi of the three organs, the ternary plot results indicated that most of the microbes had a tissue-specific distribution (spheres in the summit or along the edges of the triangles) instead of a generalist distribution (represented by the spheres in the middle of the triangles).

Subsequently, we explored the correlation between the cassava genotype and its microbiota distribution. All cassava varieties used in the present research were resequenced and annotated to obtain the coding sequence (CDS) abundance profile (Table S6). Most core microbes were regulated by multiple types of genes, including positive and negative effects. Microorganisms with a higher relative abundance in rhizosphere, stems and leaves of cassava, such as *Aeromonas*, *Enterobacter*, *Cryptococcus* and *Bullera* (Figure S2a,b), were more related to the host genotype.

**Interdependence between the phenotype and rhizosphere microbiome of cassava**

To determine whether the cassava phenotype is associated with its microbiome, we first conducted cluster analysis of the microbiomes of the cassava rhizosphere, stems and leaves and the measured their relatedness using Procrustes analysis. Principal component distribution plots (Figure 4a–c) show that the microbiomes of the cassava leaf and stem were not clearly differentiated through the cluster analysis. However, the rhizosphere microbiota showed obvious clustering among the different cassava varieties. It showed the samples R2 and R3 clustered in a cycle, and other 8 kinds of samples clustered in another cycle.

![Figure 1](image1.png)

**Figure 1** The composition of microbiota in the three organs of ten cassava varieties. 01 (SC7), 02 (SC124), 03 (ZM9710), 04 (KU50), 05 (SC5), 06 (SC11), 07 (SC6), 08 (Nanzhi199), 09 (SC9) and 10 (GR911) represents ten cassava varieties with the disease-resistant values of 2, 2, 4, 2, 3, 2, 1, 1, 1, 2, respectively. (a–f) The graph shows the number of microbial phyla with definite taxonomic status in 10 cassava varieties. The average relative abundance of phyla is in parentheses after their name. (a) Root-associated bacterial phyla. (b) Stem-associated bacterial phyla. (c) Leaf-associated bacteria phyla. (d) Root-associated fungal phyla. (e) Stem-associated fungal phyla. (f) Leaf-associated fungal phyla. (g) Number of microbial genera among the three organs of cassava. (h) Number of microbial genera among ten varieties of cassava.

![Figure 2](image2.png)

**Figure 2** The composition and correlation of core microbial genera in the rhizosphere (a), stems (b) and leaves (c) of cassava. The blue and yellow areas indicate bacterial and fungal genera, respectively (relative abundance >1%). The average relative abundance (%) is on the left side of the graph. The red and blue circles represent positive and negative correlations between microbial genera, ranging from −1 to 1 in the right.
Accordingly, the trend of cluster was consistent with the
phenotype of the cassava, especially with the index of disease
resistance to Xam. We subsequently performed Procrustes
analysis with respect to the phenotype of the cassava varieties
(Table S2) and microbiome of cassava. The Procrustes analysis
results revealed that the cassava rhizosphere microbiome and
phenotype content had similar clustering patterns, while those
observed for the microbiomes of the stems and leaves phenotype
were less similar. The fit of each Procrustes transformation over
the first four dimensions was reported as the $p$-value by 10 000
Monte Carlo Label Permutations ($p = 0.002, P = 0.55, P = 0.75$
in rhizosphere, stems and leaves, respectively). These results
supported the hypothesis that significant interdependence existed
between the phenotype and rhizosphere microbiome of cassava.
Therefore, further investigation was performed to assess this
relationship.

Co-occurrence of phenotype and rhizosphere
microorganisms

After identifying the correlation between the rhizosphere micro-
biome and the cassava phenotype, we further elucidated which
microorganisms were significantly related to the representative
phenotype through deep shotgun metagenomic sequencing and
redundancy analysis (RDA) (Figure 5a). An average of 10.82
gigabases (Gb) of high-quality paired-end reads were obtained for
each sample, totalling 316.25 Gb of high-quality data after deep
shotgun metagenomic sequencing and quality control. Taxonomic
information and the relative abundances of rhizosphere microbial
species were obtained by identifying shotgun reads using the
HUMAnN2 pipeline (Table S7). Heatmaps were generated to
represent the distribution of major rhizosphere microbial species in
each sample (Figure 5b), which exhibited the abundance of major
species in ten cassava varieties. At the species level, Enterobacter
cloacae, Aeromonas phage, Aeromonas unclassified and Aero-
monas caviae were identified as the primary microbial species
exhibiting high levels of abundance for all assayed cassava varieties
(Figure 5b), with average relative abundance of 26.5%, 28.2%,
13.0% and 7.6%, respectively. A partial RDA model was then
established to estimate the relationship between the rhizosphere
microbial species and phenotype of cassava (Figure 5a), the results
of which showed that Lactococcus sp., Pantoea dispersa and
Saccharomyces cerevisiae were closely associated with the disease
resistance of cassava. Enterobacter cloacae appeared to be associ-
ated with the amylose content of cassava, whereas Stenotropho-
monas maltophilia, Pseudomonas putida and Aeromonas caviae
were associated with starch content and viscosity. Despite the fact
that abundance of the Saccharomyces cerevisiae was associated with anti-Xam resistance in our study, Saccharomyces cerevisiae had a relatively low content in this study and had no competitive advantage over other bacteria. The extensive antibacterial action of Pantoea dispersa has been in-depth studied in tuber crops. Therefore, the relationship between Lactococcus sp. and disease resistance has been paid more attention.

Functional annotation of the microbiome with disease resistance of cassava

Based on preliminary observations that the rhizosphere microbiota was associated with the cassava phenotype, we further analysed the effect of microorganisms on the disease resistance or susceptibility of cassava. Then, we identified metabolic pathways that were significantly associated with cassava disease resistance based on the microbial metagenomic metabolic pathway map constructed using HUMAnN2 pipeline (Figure S3a–i). Among them, 15 metabolic pathways were positively correlated with disease resistance, whereas 16 pathways showed negative correlations. Although we are still unable to definitively explain the mechanism by which Lactococcus sp. were closely related to cassava disease resistance from metabolic pathway analysis, we focused on disease resistance associated with Lactococcus sp., which has the ability to produce nisin. Nisin is one of the best-studied lantibiotics. Therefore, six genes closely associated with nisin biosynthesis were obtained from the UniProt database, including nisC, nisB, nisR, nisT, nisK and nisP. The relative abundance of nisin biosynthesis-related genes in ten cassava varieties was calculated, as shown in Figure 6a. The results showed that nisin metabolism-related genes were present

![Diagram](image-url)
in each sample, although the abundance of these genes was significantly higher in the resistant cultivar samples than in those of the other cultivars. In addition, according to the taxonomic results from the metagenome of the microbiome, the abundance of Lactococcus sp. in the R03 cultivar was much higher than that observed in the other cultivars (Table S8), and the subsequent determination of the nisin content in cassava roots confirmed the concentration of nisin in relative resistant samples was significantly higher than the content of relative sensitive samples (Figure S4). These results indicated nisin is closely associated with the disease resistance of cassava.

Effect of nisin on Xam and plant disease resistance

To confirm our abovementioned hypothesis, we assessed the effect of nisin on inhibiting the growth of Xam, both in vitro and in vivo. Xam was cultured in LB liquid media containing different concentrations of nisin at 28°C, with the obtained OD600 values showing that nisin significantly inhibited the growth of the bacterial pathogen Xam (Figure 6b). The higher concentration of nisin used, the lower the OD600 value was observed, indicating the antibacterial activity of nisin towards Xam (Figure 6b). In addition, nisin solution at different concentrations was used to water the soils and roots of ten variety of cassava for 2 days. Generally, the nisin treatment significantly improved disease resistance against Xam in the variety of cassava cultivars, as evidenced by observations of decreased bacterial propagation and higher endogenous salicylic acid (SA) levels (Figure 6c,d). These results indicated that nisin confers improved disease resistance in cassava.

The cassava rhizosphere microbiome improved the host disease resistance

The objective of resequencing the genome of the ten representative varieties was to remove host DNA fragments of every variety to make the shotgun metagenomic sequencing reads more accurately. Since the host genome of every variety was resequenced, the relationship between host genome and 16S rRNA gene amplicon sequencing as well as shotgun sequencing data was also analysed. A co-occurrence network was established using host coding sequences (CDSs) associated with disease-resistant microorganisms and nisin metabolism-related genes to further assess how the rhizosphere microbe promotes cassava disease resistance. A significant positive correlation was observed between the presence of nisin-related genes in Lactococcus and disease resistance (Figure 7a,b). In addition, integrative network was also constructed to show the overall correlation between microbiome, cassava genotype, nisin metabolism-related genes and disease resistance (Figure S5). Therefore, we deduced that the mechanism of cassava disease resistance involves the cassava genome the rhizosphere-associated distribution of microbes.

Figure 6 In vitro and in vivo roles of nisin on Xam and plant disease resistance, respectively. (a) The relative abundance of nisin metabolism-related genes in ten cassava varieties. The abundance is expressed as Log Gene Relative Abundance. The colours of the columns depict 10 cassava varieties (R01-10) with the disease-resistant values of 2, 2, 4, 2, 3, 2, 1, 1, 2, respectively. There were significant differences in the relative abundance of nisin metabolism-related genes among 10 varieties by Kruskal–Wallis test (P < 0.05) and the R03 nisin metabolism-related genes was significantly higher than that of other varieties by ANOVA (P < 0.05). (b) The antibacterial activity of nisin towards Xam. Xam was cultured at 28°C in LB liquid medium containing different concentrations of nisin, with the OD600 value measured every hour. (c) The endogenous SA levels in cassava leaves after syringe infiltration of Xam. (d) Quantification of bacteria in cassava leaves after syringe infiltration of Xam. Solutions with different concentrations of nisin were used to water the soils and plant roots for 2 days. Thereafter, a Xam culture (10⁶ cfu/mL in 10 mM MgCl₂ and 0.05% silwet L-77) was syringe infiltrated into cassava leaves.

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microorganisms such as Lactococcus sp., Pantoea dispersa and Saccharomyces cerevisiae, were closely related to the plant disease resistance.

**Discussion**

The main purpose of the study was to reveal correlations between the structure and metabolism of the rhizosphere microbiome and disease resistance in cassava by using microbiome-wide association studies (MWAS). Therefore, ten representative varieties with the resistant values of 2, 2, 4, 2, 3, 2, 1, 1, 1, 2, respectively, were chosen for analysis. Here, a total of ninety 1.2mples (three tissues (rhizosphere, leaves and stems) of ten varieties with three biological replicates) and thirty samples (rhizosphere of ten varieties with three biological replicates) were used for amplicon sequencing and metagenomic sequencing, respectively. Although three biological repeats might be less robust statistical wise, we have to note that 180 samples of data (90 samples of 16S rRNA and 90 samples of ITS sequence) and shotgun metagenome data of 30 rhizosphere microbiome samples were used to reveal the correlation analysis. This analysis is just the beginning of correlation between the phenotype and cassava microbiome, and this is widely used as an effective method in preliminary correlation analysis (Kwak et al., 2018; Shi et al., 2019). Similarly, two tomato varieties with contrasting wilt resistance to the soil-borne pathogen Ralstonia solanacearum were used to analyse the rhizosphere microbiome of two tomato varieties (Kwak et al., 2018). Moreover, ten potato plants with different disease grades to potato common scab were selected to reveal the correlation between community composition and function of the geosaprobe soil microbiome (Shi et al., 2019). A significant interdependence was observed between the rhizosphere microbiome and the cassava phenotype from RDA results. The rhizosphere microbiome showed close correlation with disease resistance, starch content and viscosity. The correlation between the rhizoplane microbiota and frootil millet traits, such as the panicle weight of the primary stem, the grain weight per plant, the top second leaf width, the primary stem width and the panicle diameter of the primary stem has also been determined in the previous study (Jin et al., 2017). We hypothesized that rhizosphere microbes might be closely related to crop yield of block rhizomes.

As the fourth widely used crop after rice, sugarcane and corn, cassava is widely distributed in tropical and subtropical regions. However, cassava is susceptible to a variety of diseases, of which CBB caused by Xam is a major disease and poses production concerns, losses of between 12% and 100% (Tian et al., 2018; Verdier et al., 2004). Wydra et al. (2004) provided the first ecozonal account of cassava diseases and their association with agronomic and varietal characteristics. Among the 10 varieties assayed in this study, only R03 had high resistance to Xam, while R07, R08 and R09 showed sensitivity to Xam. However, an understanding of the structural differences in the cassava rhizosphere microbiome, which are caused by multiple factors, and their interactions with disease resistance remain elusive.

More and more studies have highlighted the impact of rhizosphere-associated microbial communities on plant health and development. Beneficial symbiotic relationships between host and rhizosphere microbiota as well as the detrimental effects of pathogens on plants have been recognized (Lareen et al., 2016; Mendes et al., 2011; Panke-Buissse et al., 2015). Rhizosphere-associated microorganisms can not only directly antagonize phytopathogenic microorganisms by secreting a series of secondary metabolites, such as antibiotics, or by spatial nutrient competition, they also indirectly inhibit the pathogenicity of pathogens by inducing plant resistance. Based on preliminary observations that the rhizosphere microbiota is related to cassava phenotype, we focused on the effect of microorganisms on the disease resistance or susceptibility of cassava. In this study, Lactococcus sp., Pantoea dispersa and Saccharomyces cerevisiae were closely shown to be closely associated with cassava disease resistance. Bacteria can have wide-ranging effects on the success of their host plants, influencing plant processes such as disease resistance, drought tolerance, life cycle phenotype and overall vigour (Bonito et al., 2014). Despite the fact that abundance of the Saccharomyces cerevisiae was associated with anti-Xam resistance in our study, Saccharomyces cerevisiae, a widely distributed industrial microbial fungus, had a relatively low content in this study and had no competitive advantage over other bacteria. An antagonistic function has been reported for the pathogen Pantoea dispersa, an indigenous endophytic bacterium of rice seeds that can produce auxins to inhibit the pathogen Fusarium oxysporum, allowing it to play an important role in the defence against pathogens (Verma et al., 2017). Moreover, the extensive antibacterial action of Pantoea dispersa has been in-depth studied in tuber crops. Pantoea dispersa have fungoidal and significantly inhibited Ceratocytis fimbriata growth on the leaves and tuberous roots of a susceptible sweet potato cultivar. It suggests that Pantoea dispersa strains could inhibit black rot in sweet potato plant (Jiang et al., 2019). As one of the best-studied lantibiotics, nisin is lanthionine-containing peptide antibiotic produced by Lactococcus sp. At present, nisin is considered to be a model system for lantibiotics. Nisin exerts a dual mode of bactericidal action (Mavaro et al., 2011). Nisin binds to bioactive lipid to inhibit bacterial cell-wall synthesis and permeabilize the cytoplasmic membrane of the target cell, leading to starvation and cell death. The nisin biosynthesis operon comprises genes that are involved in the modification (nisB and nisC), transport (nisT) and activation of nisin via processing (nisP), as well as exhibiting autoregulation via the two-component system nisRK (van Heel et al., 2016; Kuipers et al., 2004; Mavaro et al., 2011). Therefore, we evaluated all samples for the nisC, nisB, nisR, nisT, nisK and nisP genes, which are closely involved in nisin biosynthesis. Lactococcus sp. was shown to be closely associated with cassava disease resistance, mainly through the production of nisin. Further in vitro experiments confirmed the highly consistent nisin in the root surface of the relative disease-resistant varieties (R03, R05, R06 and R10) (Figure S4) and antibacterial activity of nisin towards Xam, and in vivo experiments showed that nisin treatment promoted endogenous SA levels and improved disease resistance against Xam. Nisin had a significant effect on inhibiting the growth of Xam and could promote endogenous levels of SA, one of the most important plant defence-related hormones in plant-pathogen interactions. Taken together, the exogenous nisin treatment conferred improved disease resistance against Xam. These results highlighted the crucial role of Lactococcus sp. and nisin producer in cassava disease resistance.

Plant-microbiome interaction plays more and more important roles in plant development and stress responses (Kandula et al., 2015; Kwak et al., 2018; Santhanam et al., 2015; Shi et al., 2019). Based on MWAS, biocontrol is considered as an ideal approach to control plant diseases and has been successful in many aspects of disease suppression (Kandula et al., 2015; Kwak et al., 2018; Panke-Buissse et al., 2015). A significant two-component system was closely shown to be closely associated with cassava disease resistance, mainly through the production of nisin. Further in vitro experiments confirmed the highly consistent nisin in the root surface of the relative disease-resistant varieties (R03, R05, R06 and R10) (Figure S4) and antibacterial activity of nisin towards Xam, and in vivo experiments showed that nisin treatment promoted endogenous SA levels and improved disease resistance against Xam. Nisin had a significant effect on inhibiting the growth of Xam and could promote endogenous levels of SA, one of the most important plant defence-related hormones in plant-pathogen interactions. Taken together, the exogenous nisin treatment conferred improved disease resistance against Xam. These results highlighted the crucial role of Lactococcus sp. and nisin producer in cassava disease resistance.
et al., 2018; Santhanam et al., 2015). For example, the dominant abundance of Flavobacterium in resistant tomato variety Hawaii 7996 is responsible for contrasting wilt resistance to the soil-borne pathogen Ralstonia solanacearum between resistant tomato variety Hawaii 7996 and sensitive tomato variety Moneymaker, highlighting the possible use of Flavobacterium in rhizosphere microbiome in controlling soil-borne pathogen Ralstonia solanacearum (Kwak et al., 2018). MWAS of ten potato plants with different disease grades to potato common scab revealed the possible use of geocaulosphere soil microbiome such
as Bacillus and Pseudomonas as well as some metabolic pathways in controlling Streptomyces scabies and Streptomyces acidiscabies (Shi et al., 2019). In addition, MWAS also identified the candidate crop productivity correlated beneficial bacteria in foxtail millet rhizoplane, including Bacillus, Falsibacillus and Paenibacillus (Jin et al., 2017). Besides MWAS, functional microbiota analysis also highlights the effect of some microbes in affecting the incidence of some diseases. Wang et al. (2019) revealed that Bacteriophage treatment could decrease the outbreak of Ralstonia solanacearum without affecting rhizosphere microbiota in tomato. Gu et al. (2020) found that the modulation of siderophore plays an important role in iron competition and biocontrol of soil-borne pathogen Ralstonia solanacearum. Here, the interaction between cassava disease resistance, nisin-related genes and Lactococcus sp. abundance was revealed by analysis of nisin biosynthesis-associated genes together with in vitro and in vivo experiments, highlighting the potential beneficial role in cassava disease resistance, at least partially. All these studies provided a series of potential beneficial microbes for further crop improvement of better performance and improved disease through modification of microbiota. To our knowledge, this is the first comprehensive microbiome studies in cassava, and the new insights of the interactions between the rhizosphere microbiome with cassava disease resistance provide valuable information for future biocontrol of CBB. There were also some limitations, and the follow-up experiments with more samples and larger sampling planning might identify more candidate beneficial microbes. In addition, the detailed verification of more potential microbes and the mechanism underlying the interaction between the microbiome and disease resistance need to be further elucidated.

In summary, the rhizosphere microbiome showed significant interdependence with the cassava phenotype. Notably, microbiome results revealed the association between cassava disease resistance and Lactococcus sp., which has the ability to produce nisin. The results of nisin biosynthesis-related gene analysis together with those of in vitro and in vivo experiments of all samples highlighted the crucial role of Lactococcus sp. in cassava disease resistance.

Methods

Experimental design and sample collection

In this study, plant samples of the same age in one field/soil type without proximity effects were chosen for analysis. In this study, ten cassava varieties were selected for analysis, including R01 (South China 7 (SC7)), R02 (SC124), R03 (ZM9710), R04 (KU50), R05 (SC5), R06 (SC11), R07 (SC6), R08 (NanZhi199), R09 (SC9) and R10 (GR911). These varieties were grown in the Danzhou Tropical Crop Station, Hainan University, Hainan Province, China (Table S1). Approximately four-month-old cassava plants grown under the same conditions including light, temperature, watering (once every week) and fertilization (once every month of compound fertilizer) were used for microbial collection and further microbiome analysis. Then, a QIAamp® DNA Mini kit (Qiagen, Hilden, Germany) was used for microbial sediment DNA extraction of the bacterial 16S rRNA gene V3-V4 region and the fungus ITS region.

Resequencing of cassava genome and coding sequencing annotation

For cassava resequencing, about 1 g of fresh cassava leaves of each accession were collected and immediately frozen in liquid nitrogen for further use. Generally, plant DNA was extracted using DNA extraction kit (Biomarker, Beijing, China) according to the manufacturer’s instructions. Then, the quantity and quality of the extracted DNA were further determined by NanoDrop (Thermo Scientific, Wilmington, DE, USA). At least 5 µg of genomic DNA for each accession was used to build paired-end-sequencing libraries with insert sizes of approximately 500 bp vendor-provided instructions (Illumina). An average coverage of 12.9x of the assembled genome with 125-bp paired-end reads was generated for each accession using an Illumina HiSeq 2500 platform. The CDS was annotated with Bowtie2, by mapping the sequencing reads to the cassava reference genome (NC_035161–NC_035178).

Microbial DNA extraction and high-throughput sequencing of the bacterial 16S rRNA gene V3-V4 region and the fungus ITS region

Roots were shaken to remove the loosely adhering soil. Then, roots, stems and leaves washed with PBS for 3 times, and the organs were re-dip in the PBS for 20 mins. The scrubbing solution was centrifuged for 10 min at 12 000 g for microbial sediment collection and further microbiome analysis. Then, a QIAamp® DNA Mini kit (Qiagen, Hilden, Germany) was used for metagenomic DNA extraction. The quality of the metagenomic DNA was assessed using spectrophotometry. We selected the bacterial V3-V4 region of the 16S ribosomal RNA (rRNA) gene as well as the fungus ITS region for high-throughput sequencing analysis (Dethlefsen and Relman, 2011). After PCR amplification, the amplicons were quantified, and all PCR products were then pooled to a final concentration of 100 nm. Next, samples were loaded onto an Illumina MiSeq high-throughput sequencing platform for sequencing (Shao et al., 2017). The QIIME (v1.9; Caporaso et al., 2010) platform was used for the bioinformatics analysis.
analysis. Before bioinformatic analysis, the primers and sequence barcodes were removed using Sickle software. Representative OTUs were selected and annotated using the Ribosomal Database Project (RDP) and Greengenes (version 13.8) to determine the phylogeny and relative abundance of the OTUs (Cole et al., 2007).

Deep shotgun metagenomic sequencing and quality control

Thirty microbial samples from roots (10 varieties × 3 biological repeats) were collected for deep shotgun metagenomic sequencing using an Illumina HiSeq 4000 instrument. Libraries were prepared with a fragment length of approximately 300 bp, and paired-end reads were generated using 150 bp in the forward and reverse directions. The reads were trimmed using FastQC (Brown et al., 2017) software and were subsequently aligned to the cassava genome to remove host DNA fragments. An average of 10.82 gigabases (Gb) of high-quality paired-end reads was obtained for each sample, totalling 316.25 Gb of high-quality data that were free of cassava DNA and adaptor contaminants.

Non-redundant gene catalogue construction and calculation of gene abundance

The shotgun reads were assembled into contigs and scaffolds using IDBA-UD (Peng et al., 2012), after which the contigs were used to predict the functional genes with MetaGeneMark (Zhu et al., 2010). Finally, a non-redundant gene catalogue was constructed using CD-HIT (Li and Godzik, 2006). The abundances of genes were determined by aligning the reads to the gene catalogue using Bowtie2 (Langmead et al., 2009). The genera with an average relative abundance exceeding 1% in the samples constituted the core microbial structures. Subsequently, for any sample N, we calculated the relative abundance as follows:

Step 1: Calculation of the copy number of each gene:

\[ b_i = \frac{x_i}{L_i} \]  

Step 2: Calculation of the relative abundance of gene i

\[ a_i = \frac{b_i}{\sum b_i} \]  

\[ a_i \] the relative abundance of gene i.

\[ b_i \] the copy number of gene i from sample N.

\[ L_i \] the length of gene i.

\[ x \] the number of mapped reads.

Functional annotation and metabolic pathway analysis

HUMAnN2 (the HMP Unified Metabolic Analysis Network 2, http://www.huttenhower.sph.harvard.edu/humann2), a pipeline used for the efficient and accurate profiling of gene abundance in microbial pathways from a community based on metagenomic sequencing data, was used for functional annotation and metabolic pathway analysis (Ai et al., 2019; Franzosa et al., 2018). In addition, the abundances of nisin metabolic-related genes, including nisP, nisK, nisT, nisR, nisB, and nisC, were calculated for the samples of each root using Bowtie2 (Langmead et al., 2009).

Analysis of the effect of nisin on Xam and plant disease resistance

For in vitro experiments, Xam was cultured at 28°C in LB liquid medium containing different concentrations of nisin, with the OD_{600} value measured every hour to analyse the antibacterial activity. For in vivo experiments, different varieties of cassava plants were cultivated in soil under a 16 h light/8 h dark cycle for 21 days, nisin solutions at different concentrations were used to water the soils and plant roots for 2 days. Thereafter, a Xam culture (10^8 cfu/mL) in 10 mM MgCl2 and 0.05% Silwet L-77 was infiltrated into cassava leaves with a syringe, after which plant disease resistance and endogenous SA levels were assayed as described by Wei et al. (2018). The concentration of nisin in the cassava root was determined according to the protocol of the nisin ELISA kit.

Statistical analysis

All statistical analyses were performed using R. The PCA was performed in R using the ggord and ggplot2 packages. Procrustes analysis was performed using the Procrustes function in the vegan R package. The differences in the abundances of genera and genes as well as phenotype indices were tested with the Wilcoxon rank-sum test and were considered significantly different at P < 0.01. The package ggplot2 was used to construct ternary and RDA plots (de Luna Souto et al., 2018), with the package ggrepdr used to construct the boxplot. The heatmap was constructed using the package pheatmap.

The networks were calculated using the Spearman’s rank correlation coefficient and visualized by networks in Cytoscape (Version 3.7). Specific methods are as follows: Based on above results, we have highlighted the Lactococcus sp., Pantoea dispersa and Saccharomyces cerevisiae potentially important role in disease resistance. Furthermore, cassava CDS with significant correlation with these three microorganisms were found by calculating the Spearman’s rank correlation coefficient (R > 0.4), and Lactococcus sp. was correlated with Nisin metabolism-related genes. Then, the Bundle pattern of Cytoscape software was applied to make the network.

Accession numbers

The sequence data reported in this manuscript have been deposited in the NCBI database (SRA accession: PRJNA565326).

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Conflicts of interest

The authors declare no competing financial interests.

Author contributions

HS and JZ conceived and designed the experiments. JZ, LZ, YW, WH, GL and HZ performed the experiments and analysed the data. JZ, LZ, YW, WH and HS wrote and revised the manuscript. All authors read and approved the final manuscript.

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**Supporting information**

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Figure S1** Differences in bacterial (left) and fungal (right) Shannon index diversity values for the three organs (leaves, roots, stems) of cassava.

**Figure S2** Correlation network of core microbes (A: bacteria and B: fungi) and CDSs of cassava.

**Figure S3** Comparative analysis of the root microbial metabolic pathways between R03 and other cassava species.

**Figure S4** The content of nisin in different cassava roots.

**Figure S5** Co-occurrence network between microbiome (blue), pathways between R03 and other cassava species.

**Table S1** Sample information and resequencing data of ten varieties of cassava.

**Table S2** Phenotype related indexes of ten varieties of cassava.
Table S3 The difference of core bacterial and fungal genera among samples.
Table S4 The bacteria and fungus relative abundance of each samples.
Table S5 The microbial taxonomic Shannon alpha diversity among samples.

Table S6 The relative abundance of coding sequence in cassava.
Table S7 The microbial metabolic pathway annotated by HUMAnN2 pipeline.
Table S8 The metagenomic species relative abundance annotated by MetaPlAn2.