

# Endophyte mediated restoration of citrus microbiome and modulation of host defense genes against *Candidatus Liberibacter asiaticus*

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## Research Article

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# Abstract

## Background

Phloem limited non-culturable bacteria *Candidatus Liberibacter asiaticus* (*CLas*) affects the worldwide citrus production through causing citrus Huanglongbing (HLB). Despite the efficient colonization of citrus endophyte in the phloem as same niche as *CLas* pathogen, citrus microbiome manipulation and recruitment as well as citrus defense mechanisms in the presence of indigenous citrus endophyte against this pathogen are still unknown.

## Results

Endophyte-mediated microbiome manipulation may potentially play a significant role in restoration of disease suppressive endophytic microbiome in vascular pathogen affected diseased plants and positively influence the citrus defense. For this, citrus endophyte *Bacillus subtilis* L1-21 was introduced in *CLas*-infected citrus groves for one year and pathogen reduction from  $10^5$  to 10 copies/gram/leaves was recorded. Resident bacterial community composition in diseased host dramatically changed after introduction of *B. subtilis* L1-21 and positive enrichment of certain bacteria was recorded in diseased citrus host. These enrichments were predominantly driven by high and low relative abundance of *Bacillus* and *CLas* pathogen respectively, after one year of endophyte application. Moreover, endophyte application resulted in citrus defence gene induction against *CLas* pathogen and demonstrated key resistance genes (PR-1, PR-4, RPS5, RBOHD) in endophyte-pathogen interaction pathway in infected citrus. Upon introduction of *B. subtilis* L1-21 in the diseased citrus plants, we identified high level of up-regulated genes (> 2-fold) involved in defense pathway ( $p_{adj} < 0.05$ ) underpinning the fundamental defense mechanisms.

## Conclusion

Thorough evaluation of disease suppressive mechanism of endophyte against pathogen requires further exploration. However, introduction of *B. subtilis* L1-21 restructured citrus microbiome by regulating key bacterial communities which might help plant to control this pathogen. In addition, we highlight advanced insights regarding activation of multiple disease resistance and secondary metabolites encoding genes in endophyte treated HLB-infected citrus plants showing potential resistance against *CLas* pathogen. Conclusively, endophyte-mediated manipulation could play decisive role in restoration of microbiome to positively influence the citrus defense.

## Introduction

Citrus species are considered important for their great economical value all around the world due to their nutritional profile, contribution in foreign exchange, and industrialization [1]. Unfortunately, citriculture has been facing huge economical loses due to several diseases among which, Huanglongbing (HLB) also known as citrus greening is most devastating to citrus industry. The disease is associated with the

phloem limited α-proteobacteria *Candidatus Liberibacter asiaticus* (*CLas*), *Ca. Liberibacter africanus* (*CLaf*), and *Ca. Liberibacter americanus* (*CLam*) [2]. The *CLas* pathogen is vectored by Asian citrus psyllid *Diaphorina citri* and the pandemic has affected citrus hosts throughout citrus growing regions of the world [3]. The bacterium causes imbalances in host metabolism by consuming nutrients and creates a nutrient deficient environment which aggravates the disease symptoms [4]. The disease is further characterized by blotchy mottled and pale-yellow leaves, yellow shoots, corky veins, stunting, twig dieback and dramatic decrease in fibrous root mass [3].

The distribution of pathogen in diseased plant is highly patchy mostly being limited to leaf midribs and stems, which somehow makes the treatment difficult [5]. Current management practices include control of ACP through insecticide application, antibiotics treatment for *CLas* inhibition, plant defense inducers, combination of stock and scion, graft-based chemotherapy, and transgenic cultivars [6, 7]. Although, injudicious use of agrochemicals is threatening to environmental and human health which necessitates the eco-friendly disease control options. Recently, endophyte-mediated host microbiome manipulation has gained much attention being environmental friendly and effective disease control method against several plant pathogens [8, 9]. Endophytes are microbes who live in close association with host plant and regulate plant health, growth and safety [10]. Endophytes are considered to perform better due to internal colonization in plants and they exhibit versatile mechanisms to antagonize pathogens [11, 12].

*CLas* pathogen is known to disturb host microbiome composition which may directly link to disease progression. Endophytic microbiome was found significantly different in symptomatic, asymptomatic diseased citrus plants and healthy plants which highlights the role of endophytic microbiome in disease outcome. In this regard, manipulation of beneficial microbiome associated with diseased state may lead to ultimate cure [13]. Indigenous endophytic strains have proven to perform better as compared to alien strains isolated from other plant species due to their adaptation in host plant [14]. However, citrus defense response involved in the presence of introduced indigenous endophyte in the presence of *CLas* pathogen is completely unknown and proved to be crucial against pathogen attack. Large scale studies using transcriptomic analysis in citrus leaves during HLB infection investigated the host response [15, 16] and expression of these increased transcripts level was achieved through next-generation sequencing [17].

Here, vascular pathogen such as *CLas* is a major challenge for citrus industry that must be resist to save the citrus industry. Only a few studies evaluated the effect of long term in citrus infected fields with antibiotics and growing resistant varieties. However, introduction of native citrus endophytes to manipulate and recruit microbiome in endophyte-citrus-*CLas* pathogen interaction and to check citrus defense pathways in nature is scarce. To address these questions, initially, we investigated the long-term effect of endophyte *B. subtilis* L1-21 for more than one year in citrus infected field to understand the pathogen reduction. Further, we examined the effect of endophyte L1-21 on the residence microbiome enrichment that can influence the *CLas* pathogen in diseased citrus host. We provide multiple lines of evidence involved in citrus defence by determining the transcript abundance of the molecular mechanisms of pathogen resistance and citrus defense genes against *CLas* in citrus healthy and infected

host in the presence of indigenous citrus endophyte. This finding summarizes the latest advances in citrus-endophyte-CLas interaction in nature and highlight endophyte-mediated defense mechanism against vascular pathogen.

## Materials and Methods

### Bacterial strain and culture condition

The endophytic strain *B. subtilis* L1-21 whose genome has been sequenced was employed in the citrus field against CLas pathogen and in different greenhouse experiments to check the resident microbiome manipulation and transcript array in citrus cultivars. Endophyte L1-21 was stored in 50% glycerol (v/v) (Tansoole, Shanghai, China) at -80 °C, and stock culture was renewed every 4 months on Luria Bertani (LB) agar (Sigma Aldrich, USA) and cultivated for 24–48 hours to check stability of potential endophyte. Pure culture of L1-21 was grown until late log phase in LB broth for 24–48 hours at 37 °C with 150 rpm in a shaking incubator. The colony forming units (CFU/ml) of the endophyte was checked using different dilution in sterile distilled water and plated on LB agar before foliar spraying on the citrus trees.

### Citrus diseased fields and endophyte introduction

The experiment was conducted in the diseased citrus grove located at Gengma, Lincang City, Yunnan province, China (23.538 °N, 99.397 °E), with an average annual temperature of 24–26 °C and total annual rainfall of about 1300 mm. Five years old *Citrus reticulate* trees (120 trees) indicated 100% severe CLas disease incidence were used for endophyte application. Prior to endophyte *B. subtilis* application, a field survey was carried out to visually determine individual tree HLB severity and detected CLas pathogen load with qPCR analyses as confirmed through method elsewhere [18]. Foliar application of endophyte *B. subtilis* L1-21 ( $10^6$  cfu ml<sup>-1</sup>) included as a monthly foliar spray on 120 citrus trees for treatment until all leaves were wet, in the early morning or late evening to facilitate absorption. Citrus trees before endophyte application (zero times) were considered as negative control. Each month the leaves were randomly collected for DNA extraction and pathogen load were quantified as performed previously [14]. All the experiments were arranged as three replicates of three trees in a randomized complete block design and finally all the trees were merged into single pool at the end of experiment.

### DNA extraction and Illumina MiSeq sequencing

A 5-year-old citrus variety (*C. reticulate*) was grown in a greenhouse in a constant temperature of 30°C and light dark time was 12 h each for 2 months before endophyte application. The DNA of each citrus plants were extracted using CTAB method as performed previously and CLas pathogen was quantified accordingly following the optimized method in our laboratory [14]. The citrus trees with similar concentration of pathogen were sorted out. Endophyte *B. subtilis* L1-21 with concentration of  $10^6$  CFU/ml was sprayed on the upper and lower epidermis. The citrus leaves were collected before treatment of L1-21 (at 0 day; CK) and after treatment (at 3, 30 days, and one year) for Illumina Hiseq2500 high-throughput amplicon sequencing. Total genomic DNA was extracted from 1 g of leaf samples using the Plant DNA

Extraction Kit (Zymo Research Corp., Irvine, CA, United States), according to the manufacturer's instructions. Three biological replicates were used in each experiment and each replicate consist of three citrus plants. The V5-V7 variable region of 16S rRNA was amplified using primer pair 799F (5'-AACMGGATTAGATACCCKG-3') and 1193R (5'-ACGTCATCCCCACCTTCC-3') [19] and sequenced on an Illumina Hiseq2500 platform Gene Denovo Biotechnology Co., Ltd. (Guangzhou, China). Raw reads obtained from Illumina Hiseq2500 sequencer were initially quality controlled at 20% cut-off level using Trimmomatic (v.0.33) software [20] and UCHIME software was used for chimeras removal, dual-end splicing, and production of clean reads [21]. UPARSE pipeline was used to generate operational taxonomic units (OTUs) at 97% sequence similarity and blasted against SILVA database of bacteria using RDP for taxonomic annotation [22]. QIIME2 was used to compute the alpha diversity indices and beta diversity based on Bray-Curtis dissimilarity matrix and the results were visualized using boxplots and principal coordinate analysis (PCoA), respectively [23]. Permutational multivariate analysis of variance (PERMANOVA) was performed to check the overall differences among bacterial communities under different treatments (Tukey-HSD;  $p < 0.05$ ).

## Citrus tree growth conditions and treatments in the greenhouse

We looked for significant genes and pathways that were associated with elimination of *CLas* pathogen inside citrus in the presence of *B. subtilis* L1-21. Four groups of samples- Healthy (H) and diseased (D) (as control), healthy plants with endophyte treatment (LH), and diseased plants with endophyte treatment (LD) - were selected to check transcripts in each sample consisting of three biological replicates and three citrus trees for single application were used. A total of 4 treatments group were performed in the citrus plants to check the gene transcript level in each of the treatment citrus trees were categorized into different clusters: citrus samples without *CLas* pathogen before treatment (H1, H2, H3), with *CLas* pathogen (D1, D2, D3), non-pathogenic trees treated with endophytes (LH1, LH2, LH3), and pathogenic trees treated with endophytes (LD1, LD2, LD3). Citrus plants were grown under glasshouse conditions and diseased (D vs LD) and healthy trees (H vs LH) were selected based on qPCR analysis of pathogen copy density, as described previously [24]. Plants were grown in similar soil conditions in plastic pots (310 × 210 mm; height and width), and leaves were collected at 0 and 6 h after foliar spraying with the endophyte concentration of  $10^5$  CFU/ml. We randomly collected three replicates of six leaves from each citrus tree. In addition, same number of leaves were stored for each of the treatment at -80 °C for future gene validation.

## RNA extraction, library construction and sequencing

Frozen citrus leaves from each group were separately milled to a powder in a mortar with liquid nitrogen. Total RNA was extracted and mRNA was enriched by removing rRNA using a Ribo-ZeroTM Magnetic Kit (Epicentre). RNA fragmentation was performed in the presence of fragmentation buffer and cDNA was extracted by reverse transcription. Then, second-strand cDNA were synthesized by DNA polymerase I, RNase H, dNTP and a buffer, and the cDNA fragments were purified with a QiaQuick PCR extraction kit and end repaired, to which poly (A) was added and ligated to Illumina sequencing adapters. The ligation

products were size selected by agarose gel electrophoresis, amplified by PCR and sequenced using Illumina HiSeqTM2500 by Gene Denovo Biotechnology Co. (Guangzhou, China).

## Data processing assembly and annotation

High quality clean reads were obtained by removing reads containing adapters, removing low quality reads containing more than 50% of low quality (Q-value  $\leq 20$ ) bases, more than 10% of unknown nucleotides (N). Reference genome was downloaded from <http://citrus.hzau.edu.cn/orange/>. The reference genome was constructed for mapping reads to ribosome RNA (rRNA) using short reads alignment tool Bowtie2 [25]. The rRNA removed reads of each sample were then mapped to reference genome by TopHat2 [26] (version 2.0.3.12), respectively. After aligned with reference genome, the enriched unmapped reads were split into smaller segments which were then used to find potential splice sites. The reconstruction of transcripts was carried out with software Cufflinks [27], together with TopHat2. During the last step of assembly, all the reassembles fragments were aligned with reference genes and then similar fragments were removed. RSEM software was used to calculate number of reads mapped to each gene RSEM [28]. The gene expression level was normalized by using FPKM (Fragments Per Kilobase of transcript per Million mapped reads) method.

## Differentially expressed genes analysis and quantification of defense-related genes

The edgeR package (<http://www.r-project.org/>) was used to identify differentially expressed genes (DEGs) across three biological replicates per treatment. We identified genes with a fold change  $\geq 2$  and a false discovery rate (FDR)  $< 0.05$  as significant DEGs that were then subjected to enrichment analysis of GO functions and KEGG pathways [29]. All DEGs were mapped to GO terms in the Gene Ontology database (<http://www.geneontology.org/>), gene numbers were calculated for every term and significantly enriched GO terms in DEGs compared to the genome background were defined by hypergeometric distribution. The calculated p-value was FDR corrected, where FDR  $\leq 0.05$  was the threshold.

## Validation and comparison of genes using real time-qPCR

To validate RNA-seq results, the expression patterns of 10 randomly selected genes from various functions and regulation were analyzed by qPCR. *C. reticulate* plants were used for the qPCR validation. Three biological replicates were collected independently and immediately frozen in liquid nitrogen. Total RNA was extracted from citrus leaves using FastQuantity RT Kit (KR106) (Tiangen), and cDNA was obtained using a PrimeScript™ RT Reagent Kit with gDNA Eraser (Perfect Real Time). qPCR amplification was performed with SYBR Green Real-time PCR Master Mix (TaKaRa), and three replicates were amplified based on the  $2^{-\Delta\Delta CT}$  method [30]. The primers used in the qPCR amplification are listed in Table S1. The actin 11 and Ubiquitin-conjugating enzyme 28 gene was chosen as the constitutively expressed internal control for normalization; the other operations were the like those for the CLas pathogen detection procedure.

## Statistics

All the data were subjected and analyzed using GraphPad Prism 8.0.2 (San Diego, CA, USA) and TBtools software was used to generate the different heatmaps analyzed in the study. The edgeR package (<http://www.r-project.org/>) was used to make microbial cooccurrence network analysis. The illustrations were drawn using Adobe Illustrator CC2019 (Adobe Systems Inc., San Francisco, CA, USA). The Pathogen copies/g of diseased citrus leaf material were calculated using a standard curve value based on recombinant plasmid [31].

## Results

### Citrus plants infected with CLas pathogen recovered through *Bacillus subtilis* L1-21

In 2020-2021, we tested the efficacy of *B. subtilis* L1-21 against 120 citrus plants in a citrus field (100% infected) trial in Genma county of Yunnan Province, China. Symptomatology inspections showed that most of the citrus plants were diseased with different severity levels as shown in Fig. 1A. Disease severity were visually rated as healthy, symptomatic, and asymptomatic followed by quantification of CLas pathogen copies through quantitative real-time PCR by targeting the ribosomal protein L12 [rplL] as performed in our previous study [31]. Citrus plants with visible symptoms indicated higher pathogen load suggested CLas was the main source of infection leading to diverse symptoms in diseased citrus fields. We evaluated that the introduction of *B. subtilis* L1-21 once every month for a period of one year could provide significant exclusion of CLas pathogen from diseased plants (Fig. 1B). Within one month, endophyte-inoculated infected plant contributes to higher pathogen reduction. Initial pathogen load before the start of field experiment was higher with  $10^5$  pathogen copies. All the diseased citrus trees before the experiment were treated as control. Results revealed significant decrease of pathogen load to 10 copies per gram with diseased trees displayed more robust growth and plants survived for long time after introduction of endophyte L1-21 (Fig. 1C).

### Manipulation of citrus bacterial community composition towards pathogen resistance in the presence of potential *Bacillus subtilis* L1-21

Further, we explored the effect of introduced *B. subtilis* L1-21 on the citrus diseased plants for possible enrichment of potential groups of microbial communities that could influence the pathogen resistance. A total of 1,541,254 raw reads with an average of 128,438 reads per sample were obtained from 12 samples through sequencing of V5-V7 variable region of 16S rRNA on an Illumina Hiseq2500 platform. After quality control and chimeras removal a total of 1,198,019, high quality effective reads were obtained with an average of 99,835 reads per sample. The effective reads were then clustered into a total of 2,264 OTUs with an average of 189 OTU per sample (Table S2). First, we assessed the alpha diversity of bacterial communities of citrus bacterial communities before (0 d; CK) and after treatment (3 d, 30 d, and 1 year) of endophyte L1-21 introduction and no significant difference was observed among the treatments (Tukey-HSD;  $p > 0.05$ , Fig. 2A-B). A venn diagram further showed the variation between common and unique OTUs of native bacterial communities (Fig. 2C). Further, we assessed the changes in the structure of bacterial communities through PCoA based on Bray-Curtis dissimilarity matrix under

different treatment (0 d, 3 d, 30 d, and 1 year). Results of PCoA based on Bray-Curtis dissimilarity matrix explained a total of the 76.37% and 11.70% variation among bacterial communities (Fig. 2D), indicating that bacterial community composition significantly changed after introduction of *B. subtilis* L1-21 (PERMANOVA;  $p = 0.001$  and  $R^2 = 0.3804$ ).

The phyla Firmicutes, Proteobacteria, and Actinobacteria dominated the citrus bacterial communities and accounted for more than 95% of the citrus microbiome (Fig. 2E). The relative abundance of Proteobacteria and Actinobacteria was significantly higher at 0 d (before endophyte L1-21 treatment; CK) than in after 3 d, 30 d, and 1 year of endophyte L1-21 treatment (Tuckey-HSD,  $p < 0.05$ ; Fig. 2E). The relative abundance of Firmicutes was significantly increased with introduction of endophytic treatment (3 d, 30 d, and 1 year) compared with 0 d and relative abundance of Proteobacteria and Actinobacteria significantly decreased after L1-21 treatment (3 d, 30 d, and 1 year) compared with 0 d (Tuckey-HSD,  $p < 0.05$ ). At the genera level the patterns of relative abundance differences and taxonomic distribution for citrus bacterial communities became more obvious under different treatments (Fig. 2F). There were several genera such as *Candidatus*, *Pseudomonas*, *Tsukamurella*, and *Shewanella* were present in significantly high relative abundance and unique to CK compared to 3 d, 30 d, and 1 year (Tuckey-HSD,  $p < 0.05$ ). In contrast, the relative abundance of *Bacillus* was significantly increased and relative abundance of *Candidatus Liberibacter*, *Pseudomonas*, *Tsukamurella*, and *Shewanella* was significantly decreased after application of endophyte L1-21 at 3 d, 30 d, and 1 year compared with CK (0 d) (Tuckey-HSD,  $p < 0.05$ ). This suggested that bacterial community composition and structure of citrus plant significantly changed after application of *B. subtilis* L1-21. The application of endophyte to diseased host resulted in complex network after one year compared to untreated diseased plants. The co-occurrence network involving healthier microbe interaction with diverse *Bacillus* might have directly or indirectly reduced the CLas pathogen as reported in field experiments (Fig. 3A, B). Therefore, we reasoned that significant changes in microbial communities towards positive trends are due to some unknown positive interactions that are involved inside the diseased citrus plants in presence of *B. subtilis* L1-21. These interactions significantly increased the abundance of other beneficial *Bacillus* or unknown potential strains which reduced the disease incidence. Overall research results show that potential endophyte leads to restructuring of bacterial endophytic microbiome in diseased citrus groves and its application reduces the pathogen at genera level which are similar with results of qPCR (data not shown) that application of endophyte L1-21 significantly reduced the population of CLas in the citrus endosphere.

## Citrus transcriptomic response to *Bacillus subtilis* L1-21 inoculant introduction

RNA-seq results provided here provided an appropriate data set for further exploration of citrus transcriptome. A total of 38.1 million to 57.4 million reads generated from all the samples were directly mapped to the *C. sinensis* genome (<http://citrus.hzau.edu.cn/orange/>) with 82.08 % to 83.43 % were unique mapped reads matched annotated citrus genes, and the mapping ratio of these reads is 83.04-84.23% (Table S3). Notably, samples treated with endophytic bacteria L1-21 suggested obvious

molecular changes. Importantly, the correlation coefficient of all the samples were high indicating endophytes treatment showed marked molecular changes in each of the healthy and diseased trees, the molecular difference between health and diseased plants (Fig. S1). Hence, the genes in response to citrus defense response need to be characterized. Differences in Gene ontology gene enrichment were studied for three main categories, namely, the biological process, cellular component, and molecular function for H (healthy) versus D (Disease) (Fig. S2). The endophyte *B. subtilis* L1-21 was applied to both healthy and diseased citrus plants (Fig. S2A), and similar genes among both plants were sorted out (Fig. S2B). We found that the most significant biological process were the metabolic process and single organism process in which six and three genes were upregulated respectively. We choose the most significantly enriched 17 biological processes after treatment with endophyte L1-21 ( $p_{adj}<0.05$ ). Significantly enriched differentially expressed genes were found in the diseased (D vs LD) and healthy citrus trees (H vs LH) treated with endophytes (Fig. S2C, S2D).

## ***Bacillus subtilis* L1-21 manipulated disease responsive genes in infected citrus**

Despite the progress of colonization of citrus endophyte and *CLas* pathogen in the phloem as same niche [14], citrus defense mechanism in the presence of indigenous citrus endophyte to mitigate this pathogen is still unknown. Further, molecular mechanisms in citrus plants after introduction of endophytes to diseased trees has not been studied so far. Whole transcriptomic profile analysis showed that diseased (Endo+HLB affected) and healthy (Endo+HLB free) citrus plants subjected to endophyte L1-21. More expression matrix were revealed after 6 h compared to control group as depicted through principal component analysis (PCA) (Fig. 4A). Consistently, approx. 3000 DEGs were regulated in Endo+HLB free plants (1965 upregulated; 979 downregulated) while more than 2000 DEGs were found in Endo+ HLB affected plants (1442 up regulated; 715 downregulated) (Fig. 4B). Our untargeted analysis points out that endophyte L1-21 application in plant-pathogen interaction pathway in both diseased and healthy citrus plants induces significant genes with more pronounced defense response compared to control group (Fig. 4C), including important genes (FLS2, WRKY33, PR1, PR4, RPS5, and RBOHD) with significant expression statistics (Fig. 4D). Further, we selected the top 10 abundant KEGG pathways that were triggered following application of the endophyte (Fig. 5) on diseased trees. The major upregulated KEGG pathways included biosynthesis of secondary metabolites; plant-pathogen interaction; and phenylpropanoid biosynthesis ( $p_{adj}<0.05$ ) (Fig. 5A). In treatment of healthy citrus trees, major KEGG enrichment pathways comprised biosynthesis of secondary metabolites; metabolic pathways; biosynthesis of amino acids; and, phenylpropanoid biosynthesis ( $p_{adj}<0.05$ ) (Fig. 5B). Upregulated pathogen resistance genes (in response to *CLas*) following endophyte application are pathogenesis-related 4 (PR4: Ciclev10029328m.g, Ciclev10029327m.g, Ciclev10029528m.g, Ciclev10029536m.g), disease resistance protein (CC-NBS-LRR class) family (Ciclev10030667m.g, Ciclev10024849m.g, Ciclev10024854m.g), chitin elicitor receptor kinase 1 (LYSM RLK1) (Ciclev10017678m.g), and respiratory burst oxidase homologue D (RBOHD, Ciclev10027774m.g) (Fig. 6A: additional file Table S4) and downregulated genes involved in pathogen resistance were heat shock protein 70 (Ciclev10027981m.g)

and 17.6 kDa class II heat shock protein (Ciclev10009756m.g), which are responsible for protein folding. The control citrus plants in the absence of endophytes indicating clear differences were observed compared to diseased and healthy plants treated with *B. subtilis* L1-21.

## Secondary metabolites in citrus defense to CLas pathogen

Secondary metabolites play an important role in defense mechanism of citrus trees and other plants. Significant regulation was noted in the diseased citrus leaves after application with endophyte indicating the positive effect of these agents against CLas pathogen. Significant regulation of secondary metabolites genes such as terpenoids and polyketides were noted in the diseased citrus leaves after treatment with endophytes indicating the positive affect of these agents against CLas pathogen. Geranylgeranyl pyrophosphate synthase 1 (Ciclev10012067m.g), isopentenyl diphosphate isomerase 1 (Ciclev10012312m.g), hydroxymethylglutaryl-CoA synthase/HMG-CoA synthase/3-hydroxy-3-methylglutaryl coenzyme A synthase (Ciclev10020042m.g), and squalene synthase 1 (Ciclev10028537m.g) are the important genes upregulated in the diseased citrus trees after treatment with endophytes ( $p_{adj}<0.05$ ). Lipoxygenase 2 (Ciclev10014199m.g) involved in jasmonic acid mediated response in leaves, aldehyde dehydrogenase 3F1 (Ciclev10025492m.g), and GroES-like zinc-binding dehydrogenase family protein (Ciclev10020620m.g) are responsible for metabolism of lipids (Fig. 6B: additional file Table S5). The oxidative stress created by CLas pathogen inside citrus trees are detoxified through genes such as glutathione S-transferases (Ciclev10008944m.g, Ciclev10032702m.g, Ciclev10033001m.g, Ciclev10005808m.g, Ciclev10005812m.g), thus helping the citrus trees to show tolerance to CLas pathogen.

## CLas pathogen negatively affected the protein folding, chaperones, and heat shock proteins

The most important mechanism involved in the disease symptoms of HLB are the down regulation of heat shock proteins, and product of these genes protect the protein folding and function during pathogen attack. The correct function is maintained in the phloem and leaves in the presence of these genes. The most important mechanism involved in the disease symptoms of HLB are the down regulation of heat shock proteins, and product of these genes protect the protein folding and function during pathogen attack. Up regulation in the citrus trees were observed treated with endophyte such as Chaperone DnaJ-domain superfamily protein (Ciclev10002683m.g, Ciclev10016883m.g, Ciclev10009810m.g), and Chaperone protein htpG family protein (Ciclev10030743m.g). The fold change for all these genes were more than 1 and the FDR ratio was ( $p_{adj}<0.05$ ). Ubiquitin mediated protein degradation plays an important role in plant-pathogen interactions. There are 10 ubiquitin related genes regulated after endophyte treatment inside the citrus trees, 9 of them were up-regulated and 1 was down-regulated (Fig. 6C: additional file Table S6).

## Endophyte induced changes in photosynthesis and carbohydrate metabolism

The HLB affected citrus leaves results in downregulation of important genes involved in photosynthesis processes. In the present study, we also showed that when diseased citrus leaves were treated with indigenous endophyte, only one of the gene ferrodoxin 3 (Ciclev10029499m.g) related to photosynthesis was up-expressed while two genes, APE1 (Ciclev10012434m.g) and PSB28 (Ciclev10022322m.g) related to acclimation of photosynthesis to environment and photosystem II reaction center, respectively were down-expressed indicating the pathogen is present inside the diseased leaves. Significant genes responsible for ethylene (Ciclev10000608m.g, Ciclev10014617m.g, Ciclev10031204m.g) were up-regulated. *CLas* pathogen causes accumulation of starch inside the phloem and other photosynthetic cells, resulting in blockage of important nutrients inside leaves. We found that *B. subtilis* L1-21 treatment resulted in downregulation of beta glucosidase 46 (Ciclev10014887m.g) and beta glucosidase 11 (Ciclev10019719m.g), which are responsible for starch accumulation (Fig. 6D: additional file Table S7). It has been reported that *CLas* pathogen negatively affected the metabolism of carbohydrate inside citrus trees. Significant genes are aldehyde dehydrogenase 3F1 (Ciclev10025492m.g), Galactose mutarotase (Ciclev10012004m.g), GroES-like zinc-binding dehydrogenase (Ciclev10020620m.g), hydroxymethylglutaryl-CoA synthase / HMG-CoA synthase / 3-hydroxy-3-methylglutaryl coenzyme A synthase (Ciclev10020042m.g), Alpha amylase(Ciclev10007401m.g), arginosuccinate synthase (Ciclev10019860m.g), and phosphoglucose isomerase 1 (Ciclev10000603m.g) with log fold change (LFC) of 1.77, 1.69, 1.57, 1.47, 1.45, 1.14, and 1.01, respectively, with FDR (padj<0.05) (Additional file Table S7).

## Endophyte modulated the host cell wall genes

The genes involved in cell wall breakdown are mostly expressed in the citrus leaves affected with HLB, indicating the symptoms development are associated with these genes in HLB progression. The cellulose/transferases are all associated with the cell wall breakdown. Our study indicated that cellulose synthase/transferases (Ciclev10007586m.g, Ciclev10023570m.g, Ciclev10014586m.g) genes are down regulated after applying endophytes, which is the main genes in breakdown of cell wall (Additional file Table S8).

## Host transcription factors induced during endophyte introduction

A total of 23 important transcription factors were identified when citrus trees were treated with endophytes. Among them 18 TFs are upregulated and 5 are down-expressed. These transcription factors has an important role in plant defense response, biotic and abiotic stress, plant immunity, leaf senescence, stomatal movement, and jasmonate metabolism. Several families of transcription factors, such as WRKY (11, 28, 33, 40, 50, 55), MYB (1, 15, 116), and EIN3 (Ciclev10000608m.g) are associated with the plant immunity and defense up-expressed in the citrus trees after endophyte treatments (Fig. 6E: additional file Table S9). The WRKYs transcription factors are also involved in the tolerance to *CLas* pathogen in citrus. The other important TFs responsible for plant defense mechanism are respiratory burst oxidase homologue D (Ciclev10027774m.g) and leucine-rich repeat protein kinase family protein (Ciclev10019897m.g), expressed to a log fold change of 4.02 and 2.29, respectively (padj<0.05) (Table

S9). The expression profiles of 10 genes were compared with qPCR and RNA-seq data, and the results were consistent with corresponding log2fold values. Most of the genes exhibited the similar expression patterns using both methods. Similar results indicated that data are reproducible and reliable though the samples were collected from different batches (Fig. S3).

## Discussion

In recent years, the noncultivable but viable pathogenic bacteria results in devastating losses to crops including citrus. Our study demonstrated that native endophyte *B. subtilis* L1-21 not only serve as beneficial microbe to reduce the *CLas* pathogen in glasshouse and field experiments, additionally, it also modulated the citrus defense genes against pathogen causing citrus HLB. In citrus infected field, introduction of endophyte L1-21 inoculant for one year suggested higher reduction of *CLas* pathogen followed by enhanced citrus shoots. In this scenario, we speculated that the native endophytes emerged as a key player to mitigate the disease in large diseased citrus groves. The introduced endophyte on large scale in the citrus might exclude the pathogen directly or manipulate other keystone microbial communities to stop the pathogen in citrus. Despite the L1-21 have been shown to suppress various fungal pathogens (*Botrytis cinerea* and *Penicillium digitatum*) [32, 33] through volatiles production, endophyte L1-21 mechanism of direct inhibition against *CLas* are unknown. However, L1-21 strain can modulate significant metabolites (such as lysine and tyrosine) in citrus plants in the presence of *CLas* pathogen [18]. Endophyte colonization in citrus host reduce *CLas* pathogen or their colonization could be retarded due to substances in the form of antibiotics or lipopeptides, bacteriocin proteins produce via endophytes [34]. Core microbiomes present in citrus host may be regulated by these endophytes, creating possibility to stop colonization of pathogen, most probably through induced systemic resistance (ISR) [35, 36]. Further, systemic resistance in host against pathogen is generated through specialized bioactive compounds synthesized by endophytes [37].

Notably, the restoration of defeated microbial communities (citrus endophytes) appears to play a significant role in the natural environment to stop the pathogen. In our previous study, we suggested that long-term application (> 1 year) of indigenous endophyte *B. subtilis* L1-21 in diseased citrus groves help in reduction of pathogen with successful control effect (> 85%) with recovery of culturable endophytic bacteria [14]. The slow growing nature of the *CLas* pathogen in citrus plants also make it difficult to compete with the fast growing introduced endophyte. It is challenging to actively suppress or reduce the load of slow growing pathogen in citrus plants in the presence of introduced endophyte with diverse disease suppressive mechanisms. Besides, we found that the *in vivo* performance of endophyte *B. subtilis* L1-21 reduced the pathogen load in diseased citrus plants through manipulating of native bacterial communities. Since, the *CLas* pathogen negatively affected the native citrus microbial communities, and thus leading to perturbation in the healthy host [38]. Strong and potential association was noted in our study in the presence of introduced endophyte in the diseased host. The relative abundance of keystone bacterial genera *Bacillus* and *Pseudomonas* was the highest after 30 days and one year of endophyte application. Interestingly, the relative abundance of *Candidatus Liberibacter* was significantly reduced after initial 30 days followed by one year, which are strongly align with results, in

which we showed that more than 1 year field experiments successfully exclude the pathogen from diseased citrus groves. As reported previously, “microbiota hub” are important to reduce the incidence of plant pathogens [12] which provide protection against invading pathogens, promote plant health, directly antagonize pathogens, or indirectly activate plant defense [10].

Further, we employed the *B. subtilis* L1-21 in infected citrus plants that can induce significant genes and pathways that were associated with elimination of *CLas* pathogen. The physiological mechanism involved in the infected citrus trees is also important to study the host characteristics [39]. It has been suggested that endophyte application could inhibit pathogen indirectly through activation of plant defense. We found significant genes and pathways associated with elimination of *CLas* pathogen. Some pathways were effectively regulated in diseased host but not in healthy trees after endophytes treatment confirming different role of endophytes in host. The diseased citrus trees after application of endophytes resulted in up-regulation of important genes in citrus plants responsible for pathogen/disease resistance, chaperone family protein, and respiratory burst oxidase. The differentially expressed pathogen resistance genes involved in different pathways defend plants during biotic stress. Most important are dominant disease resistance (R) genes which encode for leucine-rich repeat (NB-LRR) proteins providing resistance to pathogens through avirulence (Avr) genes [40]. Activation of 3 disease resistance genes (CC-NBS-LRR) (RPS5) may have induced the defense response against pathogen. None of the RPS5 gene was activated in the citrus trees in the absence of endophyte. Resistance to *CLas* pathogen was generated in citrus through over expression of an *Arabidopsis* NPR1 [41] also supported our findings. Combination of these pathogen resistance genes can uplift the citrus plants against invading/reside pathogen more easily indicating the possible synergistic association of introduced endophyte with resident citrus microbiome. Nine molecular mechanisms were elucidated recently about the mechanisms involved in resistance genes against the pathogen attack [42].

HLB negatively affected the chaperone proteins present inside the citrus. Our transcriptomic analyses revealed several genes responsible for chaperone proteins that were upregulated suggesting degradation of misfolded proteins resulted from *CLas* infection as depicted previously [43]. One of the chaperone down-regulated gene indicated that *CLas* pathogen was already residing inside the citrus tree. Respiratory burst oxidase homologue D (RBOHD), and mitogen-activated protein kinase 1 (MPK1) are other important genes involved in defense response and plant immunity. Significant activation of these genes are reported in response to the *CLas* pathogen after reprogramming defense signaling pathways [44]. We identified one gene responsible for phloem protein (PP 2-A12) that was up-regulated in only one treatment (D vs LD), but not in the other treatment (H vs LH). Studies showed that PP2 is associated with sieve plates plugging in wound response, and involved in defense mechanisms against insects and pathogens [45]. Genes related to PP2 have an active role in defense against *CLas* invasion in the citrus [46]. Another speculation is that PP2 accumulation results in blockage of translocation stream contributes to development of HLB symptoms during *CLas* invasion [16, 46].

Physiological response of citrus to endophyte were studied through gene expression dynamics involving secondary metabolites. Diverse secondary metabolites related to metabolism of terpenoids and

polyketides, were differentially regulated. Secondary metabolites are known to be regulated in citrus plants in response to pathogen attack [47]. These metabolites are the critical components of plant defense during biotic and abiotic stresses [48]. Lipoxygenase 2 regulated in our study is involved in SAR response during hormonal crosstalk, as was previously observed for role in jasmonic acid mediated response in host. The upregulation of LOX-2 in the citrus indicated that defense response was induced because *C*<sub>Las</sub> pathogen [49], may be for its own benefit [50]. The resistance due to ethylene in plant defense is based on the plant-pathogen interaction [17, 51, 52]. We depicted that several ethylene biosynthesis genes were up-regulated in the diseased citrus leaves after endophytes treatment confirming the possible role of its plant defense against *C*<sub>Las</sub> attack. Other important defense pathway is phenylpropanoid pathway whose two genes coding for key enzyme, phenylalanine ammonia-lyase were up-regulated. Whereas *C*<sub>Las</sub> infection in Volkameriana and Navel orange down-regulated those genes [53].

The present results provide insight into the most important mechanism involved in disease symptoms of HLB with down regulation of heat shock proteins, as their genes product protect the protein functioning and folding during pathogen attack. HSP70 and HSP90 are the important proteins in PPI network that are negatively affected with *C*<sub>Las</sub> infection. Expression of heat shock proteins HSP90 through RPS2 up-regulation can help induce host immune responses, leading to strengthening of hypersensitive response (HR), thus resulting in tolerance to HLB. Previous study showed that transcription of HDP90 is regulated by *Bacillus* sp. [39] leading to HR response in the host plant [54]. Protein modification pathways are also changed significantly in the presence of *C*<sub>Las</sub> pathogen. Ubiquitins are important protein in these pathways which are downregulated in the leaves and roots of the diseased citrus hosts [17, 46]. Our study indicated that 9 of these proteins were upregulated in the diseased citrus trees after endophyte application.

Genes responsible for photosynthesis pathways were upregulated and downregulated in the presence of HLB and after endophytes treatment. Transcripts encoding different ferrodoxin 3 were regulated in the treated plants. Ferrodoxin proteins play significant role in metabolic reactions such as fatty acids, phytochrome, and chlorophyll biosynthesis, Nitrogen and Sulphur assimilation, and maintaining redox balance [55]. The downregulation of the photosynthetic genes in our study revealed that HLB pathogen was already present inside the citrus trees because HLB is known to negatively regulate the photosynthetic reactions [49, 56]. HLB also causes accumulation of starch inside the phloem and other photosynthetic cells, resulting in blockage of important nutrients inside leaves [57]. It has been reported that glucose-6-phosphate transporter (GTP2) is up-regulated in the *C*<sub>Las</sub> infected citrus, imports the substrate glucose-6-phosphate for starch biosynthesis [58]. Several genes were related to starch biosynthesis, and sucrose degradation were also found upregulated in the infected plants [56]. We found that endophytes in the diseased citrus trees reduced the expression of two beta glucosidase genes involved in starch synthesis inside the leaves during *C*<sub>Las</sub> infection.

It is fascinating that several TFs were upregulated in the citrus plants in the presence of endophytes indicating the possible plant defense response against the pathogens and activating plant innate

immunity. Several genes related to WRKYs, MYBs, and EIN3 were upregulated in the diseased citrus leaves after treatment with endophytes. WRKYs are activated in the plant during pathogen-triggered immunity (PTI) during activation of MAPK cascade. These cascades thus activate defense related genes via direct phosphorylation of WRKYs and ERFs, which regulate plant immunity [59, 60]. These TFs are involved mostly in environmental plant stress response [61, 62]. Hundreds of transcription factors have been reported in different crops [63], indicating the specific role of each in specific expression [61]. Three transcription factors belonging to MYB class were upregulated in our study, and are widely involved in the stress response and plant development as well as signal induction [64].

## Conclusions

Citriculture has huge economic significance, but currently under threat from many diseases. Vascular pathogen including *C*Las responsible for devastating losses to citrus industry worldwide requires sustainable solutions. The concluding figure depicting the overall research findings is present in Fig. 7. Importantly, the mechanistic understanding and manipulation of individual and community level features by introducing citrus endophytic microbes can offer maximum plant protection. Thus, these factors highlight the possibility for plant protection through complex interactions involved in microbiome mediated HLB-disease resistance. By cautiously coupling and integrating multi-omics techniques and new experimental designs will provide better understanding of the role of keystone microbiome members and their interactions mechanisms which shape the disease resistance of the host. Such knowledge can detangle multiple pathways of interactions and elucidate the specific function (e.g. disease suppression) of potential members of microbiome in citrus-pathogen-microbiome system. Our study highlights that indigenous citrus endophyte *B. subtilis* L1-21 could manipulate microbiome through recruiting the potential microbes in *C*Las-affected citrus host and regulate defense genes to mitigate the devastating disease HLB.

## Declarations

### Ethics approval and consent to participate

Ethical approval was not applicable since the present study involved the introduced endophyte host microbiome manipulation and recruitment as well as host defense.

### Consent for publication

Not applicable

### Availability of data and material

All data are available in the main text or the supplementary materials. The raw data for citrus microbial diversity produced in this study was deposited in the NCBI under Bioproject accession PRJNA891542 (<https://www.ncbi.nlm.nih.gov/bioproject/891542>). Raw sequencing data have been deposited to the

Sequence Read Archive (SRA) under BioProject PRJNA640485 (<https://www.ncbi.nlm.nih.gov/bioproject/PRJNA640485/>).

## Competing interests

The authors have no conflicting interests related to this manuscript.

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## Authors' contributions

Y.L., P.H., A.A. and S.M. conceived the research. Y.L., A.A., Y.H. and S.M. revised the manuscript. Y.L., Y.L., Y.W., P.H. and S.M. completed all the sampling and measurement work. Y.L., A.A., W.A. and S.M participated in designing and making all the illustrations used in the study, Y.L. and S.M. contributed to the data analysis and visualization section. Y.L., A.A. and S.M. drafted the initial version of the manuscript. All authors contributed to reviewing and finalizing the manuscript. The authors declare no competing interests. The author(s) read and approved the final manuscript.

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## Figures

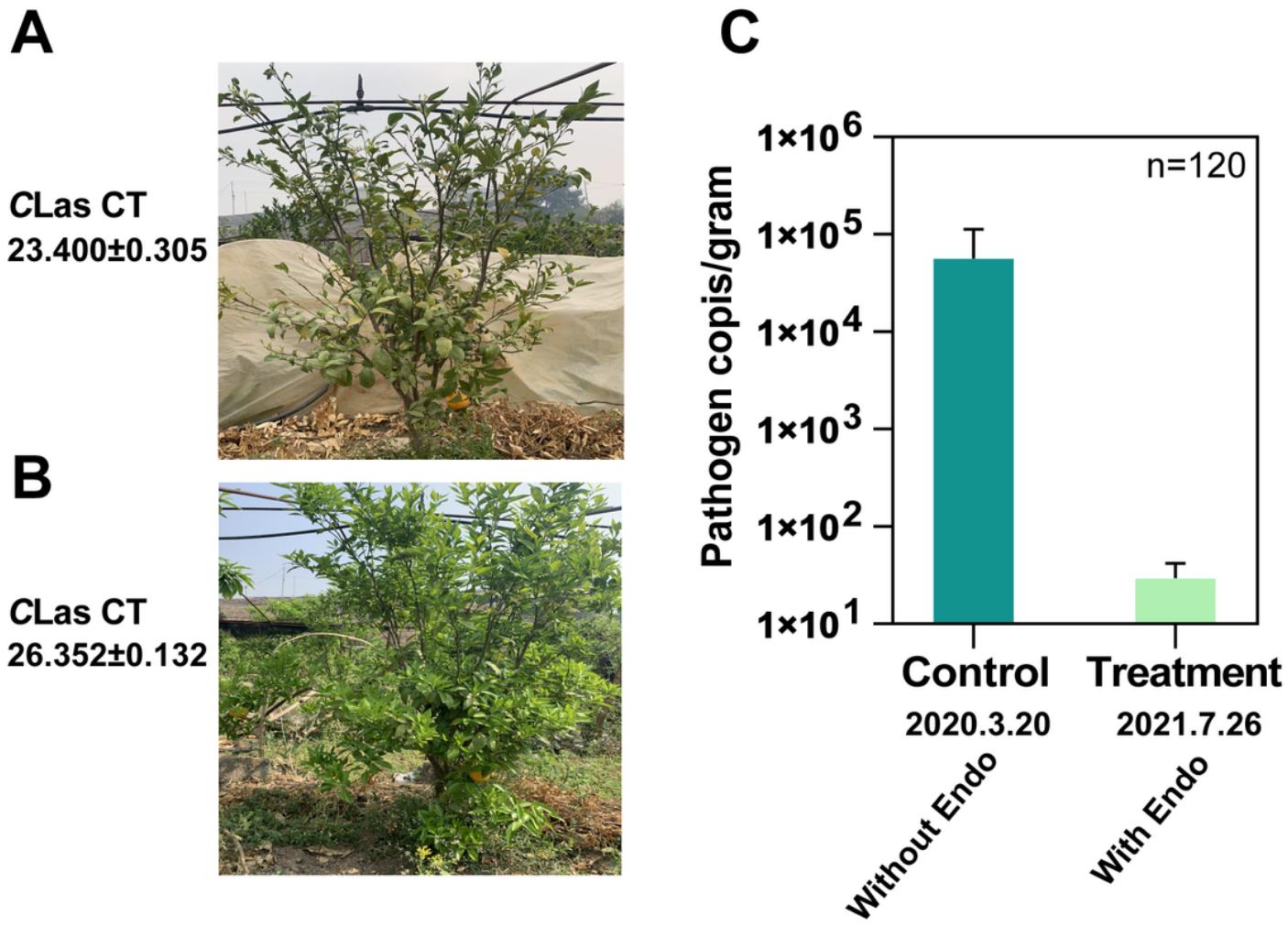


Figure 1

Huanglongbing affected diseased citrus groves before and after one year application of endophyte *Bacillus subtilis* L1-21 in Genma county, Yunnan province, China. The citrus groves were checked for CLas pathogen quantification before the start of experiment to make sure all citrus trees are affected with citrus HLB. **A** Diseased citrus plant tree before application. **B** Diseased plants recovered to healthy state after monthly endophyte application. **C** The pathogen was significantly reduced as mentioned with pathogen copies/gram of diseased citrus plant (total plants 120). The pathogen copies were calculated

based on the standard curve generated through recombinant plasmid of *CLas* pathogen gene as mentioned in methods.

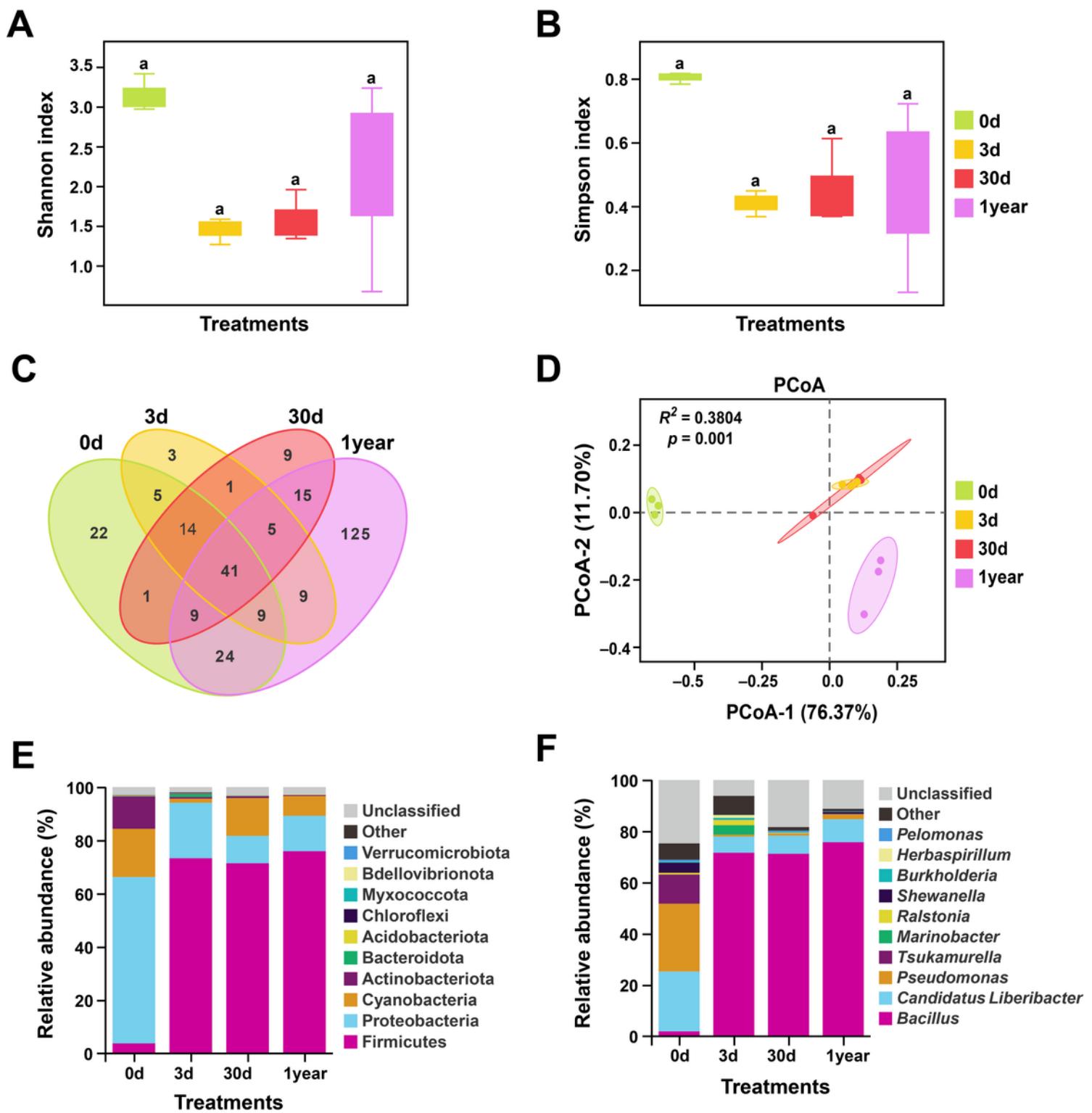


Figure 2

Effect of introduced *Bacillus subtilis* L1-21 on the manipulation of citrus bacterial microbiomes. **A-B** Alpha diversity indices of bacterial communities with bar plots displaying distribution of Shannon's and

Simpson indices of the citrus microbial communities during different time intervals. **C** Venn diagram showing OTUs variations among different time. **D** Principal coordinates analysis (PCoA) based on Bray-Curtis distances displaying separation between different time ( $R^2=0.3804$ ,  $P=0.001$ , PERMANOVA). Relative abundance of citrus community structure depicting **E** phyla and genus **F** restored in diseased citrus plants during different time intervals (3 d to 1 year) after application of *B. subtilis* L1-21.

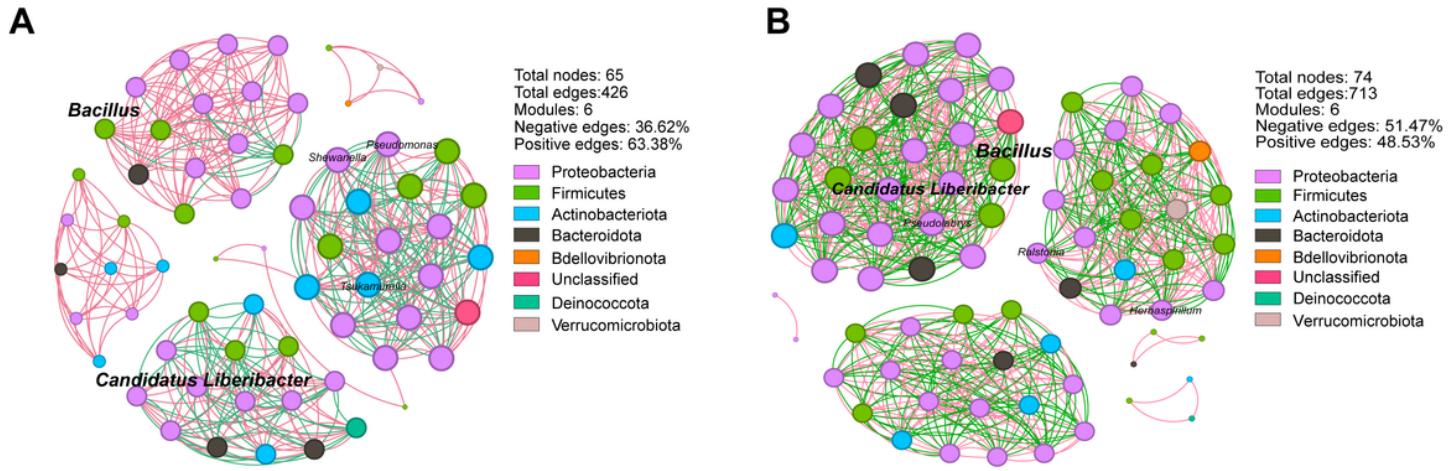
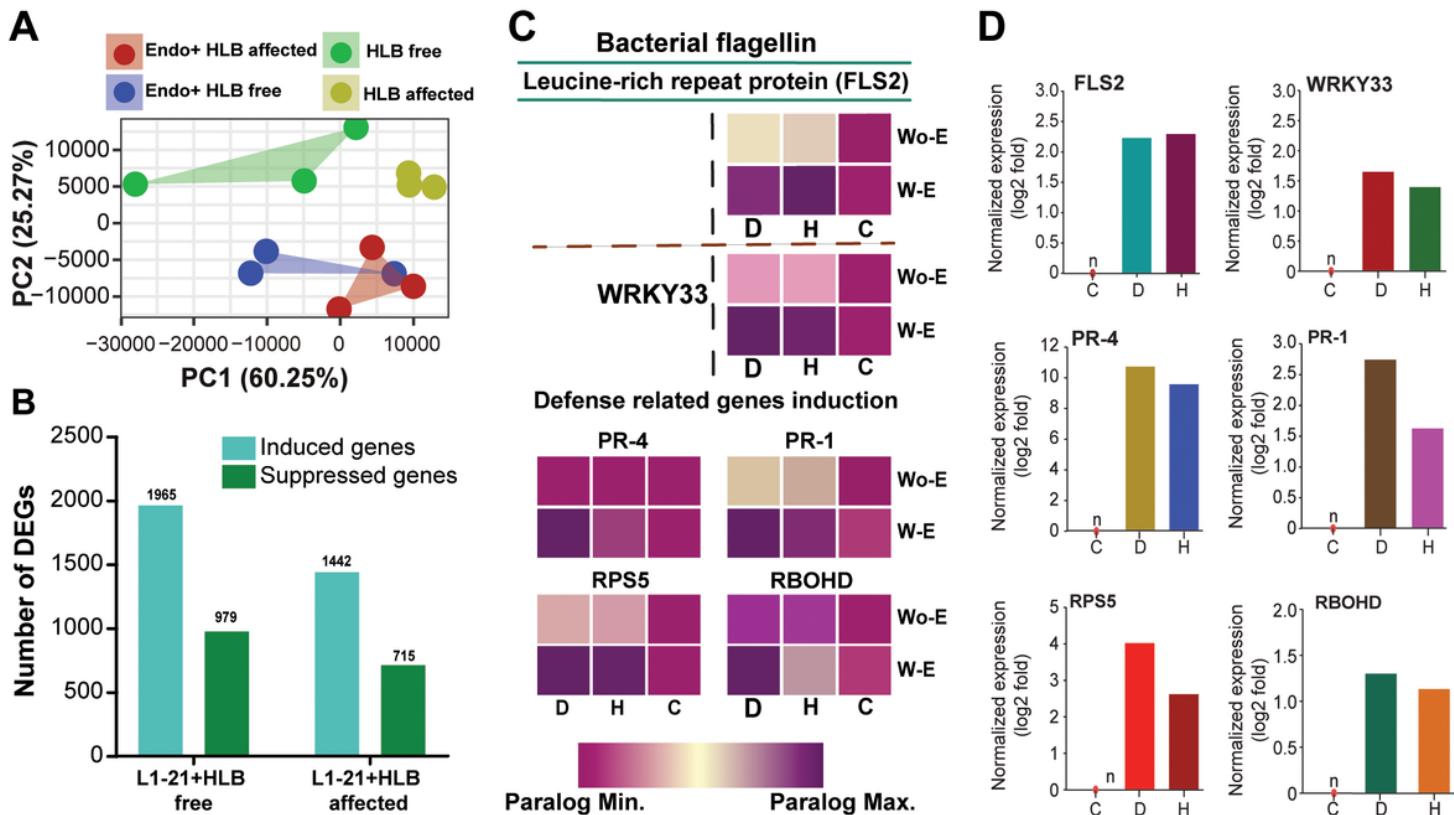


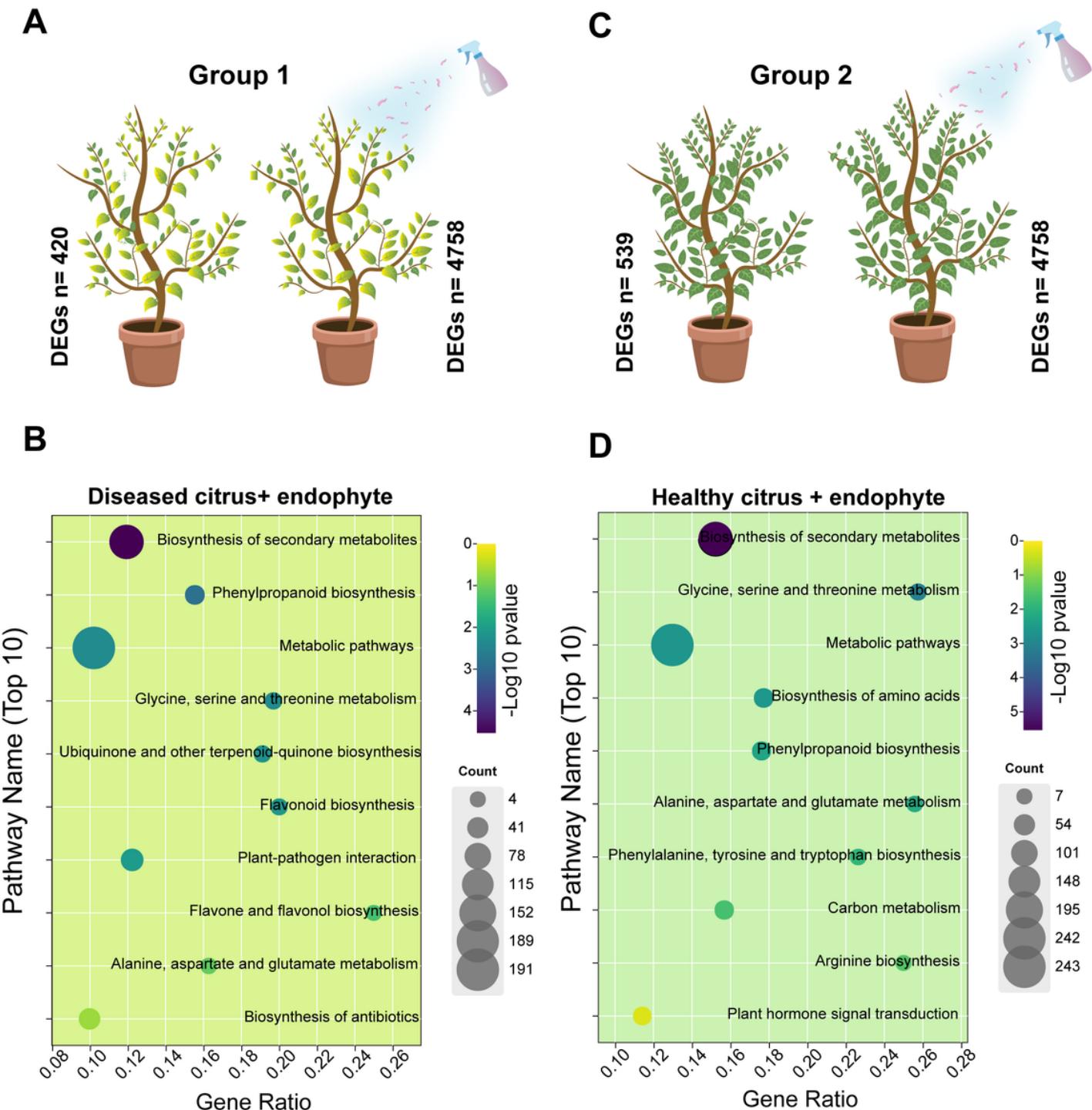
Figure 3

*Bacillus subtilis*L1-21 mediated manipulation of citrus bacterial microbiome. Co-occurrence network based on phyla and genus at **A** 0 time and **B** 1 year.



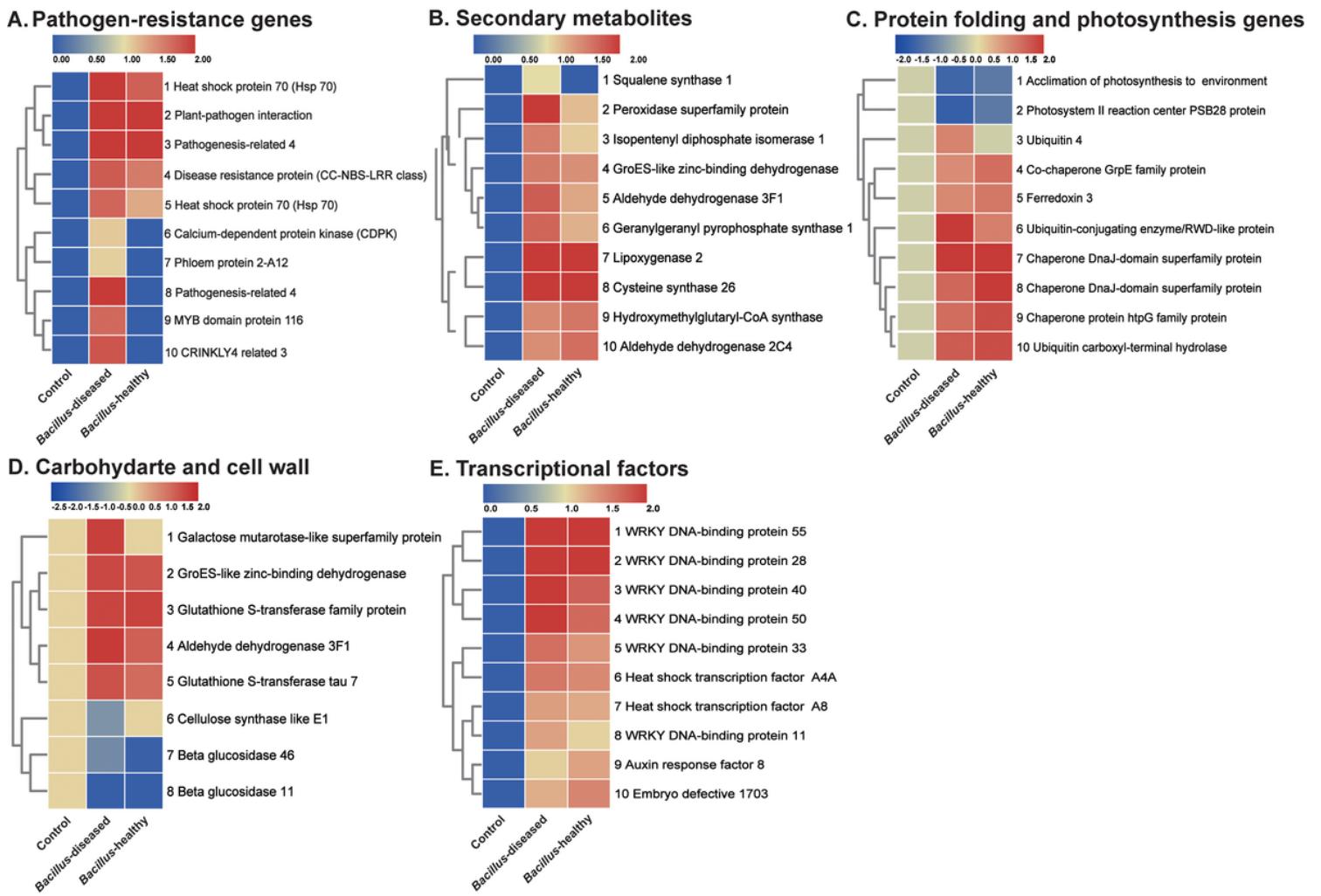
**Figure 4**

Untargeted transcriptomic analysis of diseased and healthy citrus host after 6 hours application of endophyte *Bacillus subtilis* L1-21 in the plant-pathogen interaction pathway. **A** PCA results of the endophyte (Endo) and HLB affected plants, endophyte and HLB free plants and control. **B** Summary of DEGs in HLB free and affected plants. **C** Expression of defense-related genes upon *B. subtilis* L1-21 application in plant-pathogen interaction pathway. **D** Normalized expression ( $\log_2$  fold change) of defense-related genes involved in endophyte L1-21 interaction in plant-pathogen pathway.



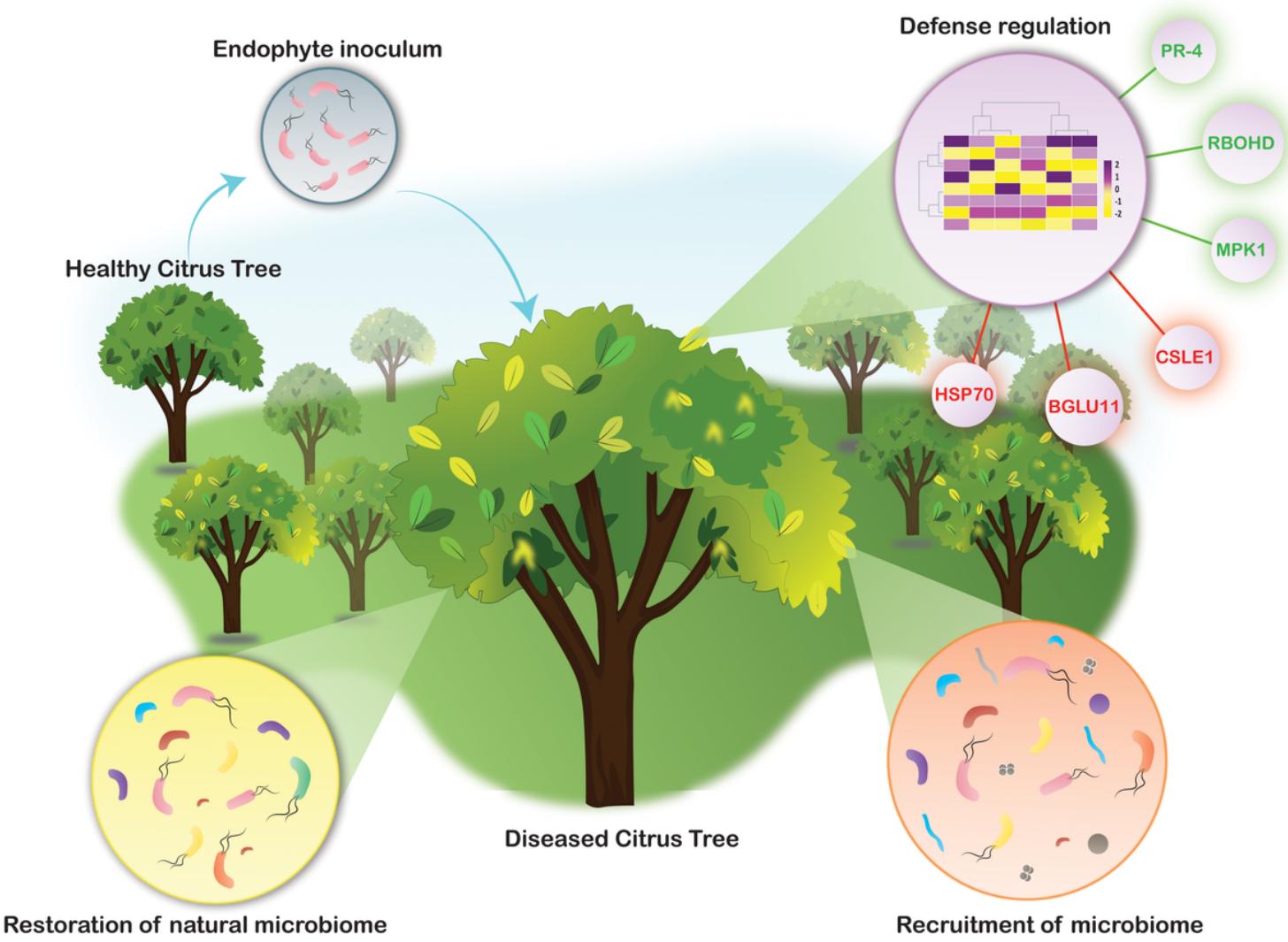
**Figure 5**

The top 10 KEGG enrichment pathway analysis of differentially expressed genes ( $p_{adj}<0.05$ ) in the presence of introduced *Bacillus subtilis*L1-21 endophytic strain. **A-B** In CLas pathogen affected citrus plants. **C-D** Healthy citrus plants treated with endophytes.



**Figure 6**

Expression profiles of upregulated and downregulated citrus transcripts after *Bacillus subtilis* L1-21 treatment. Citrus diseased and healthy plants were treated with endophyte L1-21 and samples were collected after 6 h. Citrus plants without endophyte treatment served as control. Heatmaps of the relative gene expression level (log2fold). **A** Pathogen-resistance genes. **B** Genes for secondary metabolites. **C** Protein folding and photosynthesis genes. **D** Carbohydrate metabolism and cell wall genes. **E** TF genes.



**Figure 7**

Concluding sketch illustrating the different events taking place in the diseased citrus plants by manipulation of individual and community level features by introducing citrus endophytic microbes, their microbial metabolites, and citrus defense genes.

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Supplementary20230420.docx](#)