Huanglongbing (HLB), the most destructive citrus disease, is caused by three species of phloem-limited Candidatus Liberibacter. Chemical control is a critical short-term strategy against Candidatus Liberibacter asiaticus (Las). Currently, application of antibiotics in agricultural practices is limited due to public concerns regarding emergence of antibiotic-resistant bacteria and potential side effects in humans. The present study screened 39 antimicrobials (non-antibiotics) for effectiveness against Las using an optimized graft-based screening system. Results of principal component, hierarchical clustering and membership function analyses demonstrated that 39 antimicrobials were clustered into three groups: “effective” (Group I), “partly effective” (Group II), and “ineffective” (Group III). Despite different modes of action, 8 antimicrobials (aluminum hydroxide, D,L-buthionine sulfoximine, nicotine, surfactin from Bacillus subtilis, SilverDYNE, colloidal silver, EBI-601, and EBI-602), were all as highly effective at eliminating or suppressing Las, showing both the lowest Las infection rates and titers in treated scions and inoculated rootstock. The ineffective group, which included 21 antimicrobials, did not eliminate or suppress Las, resulting in plants with increased titers of Candidatus Liberibacter. The other 10 antimicrobials partly eliminated/suppressed Las in treated and graft-inoculated plants. These effective antimicrobials are potential candidates for HLB control either via rescuing infected citrus germplasms or restricted field application.

Citrus Huanglongbing (HLB) is an serious citrus disease and has caused enormous economic losses to citrus industry in the world1,2. Citrus HLB has been in China for at least 100 years3. In Florida, USA, since HLB was first discovered in August 2005, citrus acreage and production in Florida have declined from 750,000 acres and 170 million boxes to 520,000 acres and less than 80 million boxes in 2015–2016, respectively4. And the Florida citrus industry has lost over 50% of its citrus plants, and production is decreasing at an alarming rate5.

HLB is caused by three species of uncultured, phloem-restricted proteobacteria in the Candidatus Liberibacter genus, L. asiaticus (Las), L. americanus, and L. africanus1,6,7, and is transmitted by either Diaphorina citri or Trioza erytreae8. Effective strategies against Las bacterium in citrus production are still limited, and breeding resistant citrus varieties is considered to be the most efficient and sustainable strategy against HLB. Thus, traditional citrus breeding has often been limited, due to polyembryony, pollen-ovule sterility, sexual and graft incompatibilities, and extended juvenility9. To date, there are still no commercial genetically modified citrus varieties available due to lack of consumer acceptance of genetically modified organisms. Therefore, it will likely take many years to release an HLB-resistant citrus cultivar.

Chemical control is considered to be an effective short-term strategy for combating citrus HLB. In our previous studies, a graft-based chemical control method was developed and applied for screening novel effective
antibiotics against HLB\textsuperscript{10,11}. These antibiotics (ampicillin, carbenicillin, penicillin, cephalxin, rifampicin, and sulfadimethoxine) have been confirmed to be effective against Las bacterium\textsuperscript{11}. The results of oil-in-water and water-in-oil nanoemulsion delivery of the effective antibiotics into citrus phloem from bark and foliar, respectively, indicated that these nanoemulsions enhanced the therapeutic efficacy of the antibiotics against Las bacterium\textsuperscript{12,13}. In addition, several studies also demonstrated application of antibiotics and plant defense inducers by trunk-injection also suppress Las titer in HLB-affected citrus in the field\textsuperscript{14-16}.

Currently, application of antibiotics in agricultural practices has become limited due to public concerns regarding the emergence of antibiotic-resistant bacteria and potential side effects in humans. In recent, under the emergency Exemption provisions of Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA), Florida has declared an HLB crisis that allows use of the antibiotics including: streptomycin sulfate (Fire Wal 50WP AgroSource, Inc.), oxytetracycline hydrochloride (FireLine 17WP AgroSource, Inc.), and oxytetracycline calcium complex (Mycoshield, Nufarm America, Inc.) for controlling citrus HLB by foliar application in Florida. In the previous studies, streptomycin sulfate and oxytetracycline can suppress Las titer in greenhouse and field\textsuperscript{11,19}, thus, tetracycline and streptomycin were only bacteriostatic rather than bactericidal\textsuperscript{11,19}. It is necessary for continuous application of these two antibiotics to suppress the disease, thus, frequent applications are high cost and may result in the emergence of antibiotic-resistant bacteria. Although the antibiotics screened in our previous study have been shown to be effective against Las and less phytotoxic\textsuperscript{11}, their application to citrus crops in commercial groves has not yet been approved by the US Environment Protection Agency or other regulatory agencies. As with any new active ingredient, registration of these active ingredients would take many years. Considering the long approval period, potential health risks, and lack of evidence regarding their superiority to other chemicals currently used in plant agriculture, use of these antibiotics is not viable/practical for HLB. Nowadays, HLB is seriously threatening citrus industry in Florida and other regions of the world. Therefore, screening of non-antibiotic or other chemical compounds that have already been registered for fruit tree production and can reduce the emergence on antibiotic-resistant bacteria is urgently needed for the survival of the Florida citrus industry. In the present study, 39 antimicrobial (nonantibiotic) compounds (including natural product, antimicrobial metals, and commercial product), which can reduce risk of emergence of antibiotic resistant bacteria and potential side effects in humans, were evaluated for effectiveness against HLB and phytotoxicity via an optimized graft-based assay.

Materials and Methods

Antimicrobial compounds and working concentrations. Antimicrobial compounds and their concentrations used for screening were selected according to suggestions from the InnoCentive group who have cooperated with the Citrus Research and Development Foundation in Florida (USA). A call was solicited worldwide for suggestions of antimicrobial compounds that may combat Las bacterium infection. Based on the suggestions of range of the concentration received, the citrus scion (rough lemon, \textit{Citrus limonum}) was soaked into antimicrobial compounds solution at different concentration for 24 hours. Then, based on observation of phytotoxicity (such as leaf wilting) on citrus scion, the concentration of antimicrobial compounds would be determined for optimized graft-based assay. In this study, antibacterial activity of 39 antimicrobial compounds were evaluated by optimized graft-based assay. Important information pertaining to each compound is provided in Table 1.

Graft-based assay. Antibacterial activities of the compounds against Las and their phytotoxicity to citrus were evaluated by graft-based assay, according to our previous reports, with minor revisions\textsuperscript{10,11}. Briefly, HLB-infected budsticks were collected from symptomatic rough lemon trees (\textit{Citrus limonum}, “lemon #76”) at the US Department of Agriculture-Agricultural Research Service-US Horticultural Research Laboratory farm in Fort Pierce, FL (USA), and confirmed positive for Las by real-time quantitative polymerase chain reaction (\textit{qPCR})\textsuperscript{10,21}. The budsticks were soaked in one of the chemical treatments listed in Table 1 (30 scions/treatment/concentration and two scion grafted into each rootstock) overnight in a fume hood under ventilation and continuous fluorescent light. Each soaked budstick was cut into a 2-bud scion and grafted onto individual 2-year-old HLB-free grapefruit (\textit{Citrus paradisi} var. Duncan) rootstock seedlings. Then, grafts were covered with plastic tape for 21 days. To improve scion growth, new flush from the rootstocks was pruned immediately after grafting. Grafted plants were kept at 25 ± 2°C under shade in an insect-proof greenhouse.

Evaluation of chemical antibacterial activity and tree health. The antibacterial activities of chemicals tested against Las bacterium was determined by measuring the Las titer in both the grafted scion and rootstock via \textit{qPCR}, according to Zhang's protocol with minor modifications\textsuperscript{10,11}. Briefly, five leaves were collected from both scion (rough lemon) and rootstock (grapefruit) at 6 months after grafting. Each leaf was rinsed three times with sterile water. Midribs were separated from the leaf samples and cut into 1.0 to 2.0 mm pieces. DNA was extracted from 0.1 g (fresh weight) of leaf midrib tissue using a DNeasy Plant Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. \textit{qPCR} was performed with primers and probes (HLBas, HLBr and HLBP)\textsuperscript{21} for Ca. \textit{L. asiaticus} using an ABI PRISM 7500 sequence detection system (Applied Biosystems, Foster City, CA, USA) in a 20μl reaction volume consisting of the following reagents: 300 nM (each) target primer (HLBas and HLBr), 150 nM target probe (HLBP), and 1 × TaqMan \textit{qPCR} Mix (Applied Biosystems). The amplification protocol was as follows: 95°C for 20 s followed by 40 cycles at 95°C for 3 s and 60°C for 30 s. All reactions were performed in triplicate and each run contained one negative (DNA from healthy plant) and one positive (DNA from \textit{Ca. L. asiaticus}-infected plant) control. The positive control was same for all the runs, and was checked to make sure that the \textit{Ct} remained constant. Data were analyzed using the ABI 7500 Fast Real-Time PCR System with SDS software.

After grafting 6 months, the scion survival, scion grown rate, scion infected, Las transmission, and disease index were calculated according to our previous studies\textsuperscript{11,22}.
Data analysis. Data analysis was conducted similarly to our previous study, with minor revisions. Variance analysis was conducted to analyze the antibacterial activity and phytotoxicity of chemical compound. The data of antimicrobial compound treatments were analyzed by Duncan’s multiple range test at \( P < 0.05 \). In the further evaluation, the antibacterial and phytotoxicity of the chemical treatments were carried out by principal component and hierarchical cluster analyses using the SAS/STAT procedure in PRINCOMP and CLUSTER, respectively. The membership function for each index was calculated using the following equation: \( U(X_i) = (X_i - X_{\text{min}})/X_{\text{max}} - X_{\text{min}} \).

| Code | Chemical name | Company | Work conc. | Solvent | Type | Mode of action |
|------|---------------|---------|------------|---------|------|----------------|
| AL   | Aluminum hydroxide | Sigma Aldrich | 200 mg/l | water | metal | disruption of membrane structure |
| Amp  | Ampicillin | Fisher Scientific | 1000 mg/l | water | positive control | disruption of membrane structure |
| AZA  | Azadirachtin | Sigma Aldrich | 100 mg/l | ethanol | natural product | disruption of membrane structure |
| CARV | Carbocrol | Sigma Aldrich | 100 mg/l | water | natural product | disruption of membrane structure |
| MESO | Meso-erythritol | Fisher Scientific | 3000 mg/l | water | natural product | disruption of membrane structure |
| PCY  | P-cymene | Santa Cruz Biotechnology | 100 mg/l | water | natural product | disruption of membrane structure |
| PD  | Poly-D-lysine | Sigma Aldrich | 100 mg/l | water | natural product | disruption of membrane structure |
| PLA  | Poly-l-arginine | Sigma Aldrich | 100 mg/l | water | natural product | disruption of membrane structure |
| SFC  | Surfactin from bacillus subtilis | Sigma Aldrich | 10 mg/l | ethanol | natural product | disruption of membrane structure |
| THU  | Thujone | Sigma Aldrich | 100 mg/l | ethanol | natural product | disruption of membrane structure |
| NS   | Nanosilver | Attotstat | 5 mg/l | water | metal | disruption of membrane structure and energy metabolism |
| SC   | Silver colloidal | Fisher Scientific | 50 mg/l | water | metal | disruption of membrane structure and energy metabolism |
| SD   | SilverDyne | World health alliance,international inc. | 2 ml/l | water | metal | disruption of membrane structure and energy metabolism |
| SDN  | Silver,nanoparticle | Fisher Scientific | 50 mg/l | water | metal | disruption of membrane structure and energy metabolism |
| SN   | Silver nitrate | Fisher Scientific | 50 mg/l | water | metal | disruption of membrane structure and energy metabolism |
| ABC  | DL-2-aminobutyric acid | Sigma Aldrich | 100 mg/l | water | natural product | induction of pathogenesis protein |
| SAR  | Proprietary SAR Inducer 2018A | Bayer CropScience | 0.75 ml/l | water | commercial product | induction of pathogenesis protein |
| BSO  | DL-buthionine-sulfoximine | Sigma Aldrich | 100 mg/l | DMSO | natural product | interference of activated oxygen metabolism |
| BER  | Berberine chloride | Sigma Aldrich | 8 mg/l | ethanol | natural product | interference of energy metabolism |
| MET  | 2-methyl-4-isothiazolin-3-one | Sigma Aldrich | 4 ml/l | water | natural product | interference of energy metabolism |
| NSC  | Nicotine | Fisher Scientific | 100 mg/l | ethanol | natural product | interference of energy metabolism |
| EBI-601 | EBI-601 | Echelon Biosciences,Inc. | 200 mg/l | water | commercial product | interference of nucleic acid metabolism |
| EBI-602 | EBI-602 | Echelon Biosciences,Inc. | 200 mg/l | water | commercial product | interference of nucleic acid metabolism |
| 2AC  | 2-amino-5-chlorobenzoxazole | Acros Organics | 100 mg/l | DMSO | natural product | interference of nutrition metabolism |
| HYD  | Hydroxyurea crystalline | Fisher Scientific | 500 mg/l | water | natural product | interference of nutrition metabolism |
| INH  | Isonicotinic acid hydrazide | Sigma Aldrich | 100 mg/l | water | natural product | interference of nutrition metabolism |
| QUI  | Gossypol | Sigma Aldrich | 100 mg/l | ethanol | natural product | interference of nutrition metabolism |
| CRE  | M-cresol | Fisher Scientific | 4 ml/l | water | natural product | interference of other metabolism |
| FA   | Formic acid | Sigma Aldrich | 1 ml/L | water | natural product | interference of other metabolism |
| ZINEB | Zineb | Sigma Aldrich | 250 mg/l | DMSO | commercial product | interference of other metabolism |
| 80WG | 80WG | Bayer Crop Science | 500 mg/l | water | commercial product | interference with cell wall synthesis |
| BITC | Benzyol isothiocyanate | Sigma Aldrich | 50 mg/l | water | natural product | interference with cell wall synthesis |
| QUAD | Quadrix (cyproconzole) | Sigma Aldrich | 1200 mg/l | ethanol | natural product | interference with cell wall synthesis |
| SAP  | Saponin | Santa Cruz Biotechnology | 1000 mg/l | water | natural product | interference with cell wall synthesis |
| 20WP | 20WP | Stamer 20 WP in Japan | 2000mg/l | water | commercial product | unknown |
| EcoClean | EcoClean | EcoUSA | 50 ml/l | water | commercial product | unknown |
| FT33 | FT33-3 | OCION | 2000mg/l | water | commercial product | unknown |
| MA   | MA-3 | Magnalson | 1000 mg/l | DMSO | commercial product | unknown |
| Proud | Proud | BioHumaMetrics | 10 ml/l | water | commercial product | unknown |
| PT81 | PT81-3 | OCION | 1000 mg/l | water | commercial product | unknown |
| DMSO | DMSO | Fisher Scientific | 1 ml/l | water | negative control |
| CK-1 | Water | — | — | — | negative control |
| CK-2 | Ethanol | Fisher Scientific | 1 ml/l | water | negative control |

Table 1. Information of chemical compounds screened for control of citrus Huanglongbing.
The comprehensive evaluation value of efficacy of antimicrobial compound against HLB were calculated by following equation:

\[ D(X) = \sum_{j=1}^{n} U(X_j) \times W_j(j = 1, 2, 3 \ldots n). \]

Furthermore, seven variables of antibacterial activities and phytotoxicity (scion survival, scion growth, infection rates; Las transmission; Ct values in scions and rootstocks; and disease index) were accessed at each step of the stepwise discriminant analysis process. All the data analysis was run in SAS software package (SAS V.9.1, SAS institute, NC, USA).
Results

Survival and growth of scions treated with antimicrobial compounds. CRE treatment displayed significant phytotoxicity to scions. Only 18.75% of the scions survived and little new flush (12.50±0.0%) was produced with this treatment. However, more than 50% of the scions that were treated with the other remaining chemicals survived. Although scion growth rates with CRE, as well as AL, ABC, 20WP3, EcoClean3, SN, 80WG, SAR, EBI-601, EBI-602, and MA were all less than 20%, the scion survival rate with the latter antimicrobial compounds was 54.55–99.47%. In addition, several chemicals, including MET, BER, HYD, INH, SAP, THU, SD, and PT81, demonstrated higher scion survival and growth rates, as well as the positive control Amp (Table 2).

Effect of antimicrobial compounds against Las bacterium. Variance analysis indicated that the chemicals had significant effects on Las titer in scions ($P=0.0001$) and rootstocks ($P=0.0001$), as well as the percentage of infected scions ($P=0.0032$) and Las transmission ($P=0.0001$), in the fixed model. Plants grafted with Las-infected scions soaked in antimicrobial compounds Amp, EBI-601, and NIC showed a significant reduction in Las in both scions and rootstocks, resulting in a $C_T>36.0$ (Table 2). However, the scion infection rate (10–66.67%), Las transmission rate (13.64–23.24%), and DI (10.69–20.46) of EBI-601 and NIC were much higher than those of Amp (Table 2). Las-infected scions treated with 80WG, EBI-602, and SC displayed a marked reduction in Las ($C_T=36.08–37.19$), and the Las infection rate, Las transmission rate, and DI were 5.57–50%, 40–65.19%, and 17.77–25%, respectively. Las titer in rootstocks grated by Las-infected scions soaked in SD and AL were also remarkably reduced by 11–15% Las transmission and 13.49–16.67% DI (Table 2). Some antimicrobial compounds, including 2AC, MET, AZA, FA, INH, MESO, QUAD, SAP, THU, ZINEB, PT81, and FT33, were not effective in suppressing Las, leaving more than 70.0% of the rootstocks infected (Table 2) and 24.32 to 72.75% DI. None of the negative controls (0.1% DMSO, 0.1% CK-1 and CK-2) had a significant effect on Las titer in the inoculated rootstocks ($C_T=24.83–26.20$) or scions ($C_T=24.99–32.7$), and the DI of these solvents ranged from 56.25 to 80.21%.

Principal component, hierarchical cluster, membership function and stepwise discriminant analyses. Principal component analysis was used for the data obtained from the 39 tested compounds and 4 controls (CK) after standardization as described in the Methods. The first principal component accounted for 58.30% of the total variance in the data set, while the second principal component explained 19.18% (Table 3). The contribution of each variable, their relationships, and the resulting principal components are shown in (Table 3). The scion infection rate, Las transmission rate, and disease index contributed primarily to the first principal component, as did the percentage of $C_T$ values in scions and rootstocks, but with opposite values to $C_T$. Scion growth and survival contributed primarily to the second principal component, as did the scion infection and Las transmission rates, but with opposite values to the scion infection and Las transmission.

In the principal component, hierarchical cluster, and membership function analyses, antimicrobial compounds were classified by scion infection rate, Las transmission rate, $C_T$ values of scions and rootstocks, and DI without considering information regarding the antimicrobial compound class. The result indicated that 39 antimicrobials were divided into three groups: “effective” (Group I), “partly effective” (Group II), and “ineffective” (Group III). Group I was composed of 9 antimicrobial compounds (AL, SD, EBI-601, BSO, SFC, NIC, SC, EBI-602, and Amp) which displayed high antibacterial activity against Las, resulting in the lowest Las titers in scions and rootstocks of grafted plants (Tables 4 and 5). Group III consisted of 24 antimicrobial compounds and had the least antibacterial effect; this group had the highest scion infection rate, Las transmission rate, and Las titers in citrus grafted-scions (Tables 4 and 5). Group II compounds (BITC, CARV, QUI, PDL, PCY, PLA, SN, SDN, 80WG, and SAR) partially suppressed Las as compared to Groups I and III (Tables 4 and 5). In addition, the scions and rootstocks of plants grafted to scions treated with Group I compounds, did not display HLB-like symptoms (Fig. 1), while those grafted to negative control or Group III solvent-soaked Las-positive scions had typical HLB symptoms, such as yellow shoots, corky leaves in rootstocks, or blotchy mottled leaves on the scion.

The results from stepwise discriminant analysis indicated the scion infection rate, $C_T$ of the inoculated rootstock, and DI were selected for discriminant function based on Wilk's lambda and the F-value ($P=0.000010$ and $\chi^2=74.942$; Table 6). By using these three variables as predictors, 100% of the antimicrobial compounds were correctly classified into hierarchical cluster analysis groups from all seven variables. Also, 23 out of 39 compounds were correctly classified as having an overall a posteriori probability greater than 90.0%.

Discussion

Citrus HLB is a devastating disease of citrus worldwide. Chemical control is considered to be an effective short-term strategy against Las bacterium. Antibiotics have been used in several agricultural practices for decades, and their use has only begun to peter out due to public concerns about emergence of antibiotic-resistant bacteria and potential side effects on humans. Thus, non-antibiotic chemical compounds that can reduce or
eliminate the risk of creating antibiotic-resistant bacteria and have little to no negative effects on humans are needed to rescue the citrus industry. In the present study, 39 antimicrobial compounds (including natural product, antimicrobial metal, and commercial product) that are already approved and being applied to commercial agricultural products were evaluated for their efficacy against Las and phytotoxicity to citrus trees. Principal component, hierarchical cluster and membership function analyses clustered these compounds into three groups based on their anti-Las activity and citrus phytotoxicity. Group I compounds (AL, BSO, NIC, SC, SFC, SD, EBI-601, and EBI-602) were highly effective, along with the positive control (Amp), yielding the lowest Las titers in inoculated plants. Group II compounds (BITC, CARV, QUI, PDL, PCY, PLA, SN, SDN, 80WG, and SAR) were partly effective; and Group III chemicals (2AC, MET, AZA, BER, ABC, FA, HYD, INH, CRE, MESO, QUAD, SAP, EcoClean, MA, Proud, NS, CRE, 20WP, INH, HYD, 2AC, BER, THU, DMSO, ABC, ZINEB, AZA, CK-1, PT81, MET, Meso, FA, CK-2, QUAD, SAP) were ineffective.

| Chemical compounds | U (1) | U (2) | Integrated assessment value (D) | Rank | Group | Posteriori probability |
|--------------------|-------|-------|---------------------------------|------|-------|------------------------|
| Amp                | 0     | 1     | 0.192618                        | 1    | I     | 0.9995                 |
| NIC                | 0.066726681 | 0.63919921 | 0.083788765 | 2    | I     | 0.9657                 |
| BSO                | 0.160210512 | 0.671083 | 0.034825458 | 3    | I     | 0.8211                 |
| EBI-602            | 0.203226923 | 0.77348967 | 0.029194502 | 4    | I     | 0.8606                 |
| SD                 | 0.187632435 | 0.71332287 | 0.026797572 | 5    | I     | 0.9464                 |
| SFC                | 0.180956997 | 0.64393869 | 0.017367815 | 6    | I     | 0.8339                 |
| AL                 | 0.157842255 | 0.57289262 | 0.017308209 | 7    | I     | 0.7483                 |
| EBI-601            | 0.15057129 | 0.47288647 | 0.002331145 | 8    | I     | 0.8606                 |
| SC                 | 0.2419232035 | 0.70418244 | 0.006964583 | 9    | I     | 0.8293                 |
| CARV               | 0.259164878 | 0.55511956 | 0.045840531 | 10   | II    | 0.652                  |
| PLA                | 0.30675162 | 0.69019481 | 0.04782924 | 11   | II    | 0.8719                 |
| PCY                | 0.371844055 | 0.80732273 | 0.063680228 | 12   | II    | 0.8216                 |
| PDL                | 0.405332041 | 0.819992 | 0.08098059 | 13   | II    | 0.9792                 |
| 80WG               | 0.322969323 | 0.53854676 | 0.086624728 | 14   | II    | 0.8297                 |
| QUI                | 0.357814556 | 0.61367064 | 0.092712284 | 15   | II    | 0.9401                 |
| SN                 | 0.353535074 | 0.58855754 | 0.095026949 | 16   | II    | 0.9404                 |
| SAR                | 0.38598435 | 0.66211184 | 0.09986519 | 17   | II    | 0.8726                 |
| BITC               | 0.418049028 | 0.7250177 | 0.106980444 | 18   | II    | 0.7348                 |
| SDN                | 0.424181151 | 0.58663404 | 0.137040272 | 19   | II    | 0.8992                 |
| FT33               | 0.516570875 | 0.6923799 | 0.171131487 | 20   | II    | 0.5476                 |
| EcoClean           | 0.525288333 | 0.51672721 | 0.210198949 | 21   | III   | 0.7774                 |
| MA                 | 0.575431064 | 0.63207467 | 0.217442923 | 22   | III   | 0.5706                 |
| Proud              | 0.604251783 | 0.65615671 | 0.22979285 | 23   | III   | 0.8926                 |
| NS                 | 0.613156984 | 0.66195796 | 0.233924658 | 24   | III   | 0.9142                 |
| CRE                | 0.433003255 | 0.25523679 | 0.25523679 | 25   | III   | 0.9876                 |
| 20WP               | 0.595457378 | 0.46907026 | 0.260645144 | 26   | III   | 0.9735                 |
| INH                | 0.779211966 | 0.96680824 | 0.273087279 | 27   | III   | 0.9955                 |
| HYD                | 0.7120975 | 0.73020343 | 0.279100532 | 28   | III   | 0.996                  |
| 2AC                | 0.649761097 | 0.47436371 | 0.291635238 | 29   | III   | 0.9655                 |
| BER                | 0.789806888 | 0.82848614 | 0.305975805 | 30   | III   | 0.9989                 |
| THU                | 0.805168547 | 0.70316684 | 0.33918055 | 31   | III   | 0.9995                 |
| DMSO               | 0.803102278 | 0.6469855 | 0.348773206 | 32   | III   | 1                     |
| ABC                | 0.639927983 | 0.09995999 | 0.357959395 | 33   | III   | 0.9991                 |
| ZINEB              | 0.810816425 | 0.59682085 | 0.362982978 | 34   | III   | 0.9987                 |
| AZA                | 0.824305796 | 0.5641215 | 0.377232866 | 35   | III   | 1                     |
| CK-1               | 0.874537774 | 0.67931554 | 0.384654011 | 36   | III   | 0.9996                 |
| PT81               | 0.960182813 | 0.91776699 | 0.389208055 | 37   | III   | 1                     |
| MET                | 0.832967106 | 0.52569784 | 0.389376241 | 38   | III   | 1                     |
| Meso               | 0.884108083 | 0.65975749 | 0.394005188 | 39   | III   | 1                     |
| FA                 | 0.893962621 | 0.50323147 | 0.430040915 | 40   | III   | 1                     |
| CK-2               | 0.980486116 | 0.75240821 | 0.43302794 | 41   | III   | 0.9999                 |
| QUAD               | 0.90139187 | 0.50998677 | 0.43309917 | 42   | III   | 1                     |
| SAP                | 0.73985166 | 0.44684253 | 0.44684253 | 43   | III   | 1                     |

Table 4. Membership function of principal component, comprehensive evaluation, hierarchical cluster and stepwise discriminant analyses. SDA: stepwise discriminant analysis: the group is classified by SDA.
THU, ZINEB, 20WP3, Proud, EcoClean, NS, PT81, FT33, and MA), along with negative controls (DMSO, CK-1, and CK-2), were relatively ineffective and showed the highest Las titers.

With antibiotic-resistant bacteria posing a significant public health challenge, interest in understanding the antimicrobial properties associated with certain metals, such as silver and aluminum, is increasing. In ionic or nanoparticle forms, silver displays strong activity against microorganisms and has been used as a medicinal and antibacterial agent since the nineteenth century. Silver can influence a broad range of biological processes in microorganisms, such as cell membrane structure and function. The expression of proteins involved in ATP production is also inhibited by silver. In the present study, SC and SD were highly effective at suppressing Las bacterium and showed little phytotoxicity to citrus (Tables 2 and 5). SN and SDN (Silver, nanoparticle from Fisher Scientific) belonged to Group II and were partly effective against Las bacteria, while nanosilver from Allostat was not effective (Tables 2 and 5). Although the various antibacterial activities of silver compounds obtained from different companies likely result from their various chemical and physical characteristics, the mechanism(s) by which they exert their effect on Las bacterium is unknown. Both SC and SD are colloidal forms of silver. Generally, SC is a suspension of submicroscopic silver nanoparticles in water, with diameters ranging from 10 to 100 nm. Furthermore, SC reportedly has a broad effect against a wide spectrum of bacteria, including antibiotic-resistant forms. However, the safety of SC in humans and the environment is still a public concern. Bactericidal doses of silver ions range from 1000 to 10,000 mg/L in water; at higher doses, silver can be toxic to mammals and freshwater and marine organisms. Silver concentrations of less than 200 mg/L have no harmful effects on humans. The present study used SC and SD concentrations less than 100 mg/L (Table 1). Therefore, their use against HLB in citrus can be considered safe for humans. In the future, application of SC and SD on HLB-affected citrus trees in greenhouses and the field will be conducted, as well as a more intensive evaluation of their safety in humans and the environment.

Aluminum (AL) was also effective against Las bacterium and showed little phytotoxicity towards citrus. The Ct of inoculated rootstocks and infected scions treated with AL were 36.45 ± 0.57 and 33.87 ± 1.09, respectively (Table 2), and the DI was only 16.17. However, the scion growth rate of HLB-infected scions soaked in AL was less than 20% (Table 2). The antimicrobial activity of aluminum due to the release of metal ions has been addressed in a few previous studies. Positively charged aluminum ions attach to the surface of bacteria due to their negative surface charge at physiological pH. Therefore, aluminum plays an important role in bacterial toxicity. Although AL shows bactericidal activity against Las, its toxicity to citrus plants, humans, and the environment must be evaluated further.

NIC, 3-(1-methyl-2-pyrrolidinyl) pyridine, is a colorless to light-pale yellow, hygroscopic, yet oily liquid naturally present in the leaves of Nicotiana tabacum. It is considered to be a highly toxic chemical, which was belonged to the tobacco alkaloids. Several studies have demonstrated that NIC can suppress growth of microorganisms, including bacteria. In our study, NIC was found to effectively suppress Las titers in inoculated rootstocks and scions, with a DI of only 20.46 (Table 2). Previously, 45 °C thermotherapy combined with NIC applied to HLB-affected citrus by bark painting was shown to have a much higher therapeutic efficiency against Las bacterium than this combination treatment at 40 or 42 °C. The increase in therapeutic effect was attributed to an increased ability to uptake NIC through the bark at higher temperatures. Therefore, how different antimicrobials, especially NIC, are delivered into the citrus phloem will be investigated in future studies.

BSO has been shown to reduce glutathione levels and is being investigated as an adjunct to chemical control for the treatment of cancer. Glutathione has a broad range of biochemical functions. In particular, it is a major cellular antioxidant and determinant of redox state. Glutathione can prevent damage to plant caused by...
displayed effective antibacterial activity against Las bacterium and demonstrated that SFC from *Bacillus subtilis* showed low phytotoxicity in citrus plants (Table 2), likely resulting from its induction of systemic resistance. Therefore, tetracycline can suppress Las bacterium, it shows serious citrus phytotoxicity. Our research demonstrated plants can also interact with plant to reduce infections caused by *Pseudomonas syringae* on *Arabidopsis*.

**Table 5.** Chemical compound classification of antibacterial activity against Las bacterium. Different letter by group indicated that the significance at 0.05 level.

| Variable                      | Group I     | Group II    | Group III    |
|-------------------------------|-------------|-------------|--------------|
| Scion survival (%)            | 67.92 ± 16.22a | 72.85 ± 12.85a | 80.98 ± 20.92a |
| Scion grown rate (%)          | 25.55 ± 12.72a | 30.39 ± 13.31a | 40.62 ± 22.58a |
| Scion infected (%)            | 23.06 ± 21.01c | 41.27 ± 7.71b | 68.29 ± 16.65a |
| Las transmission (%)          | 21.7 ± 13.97c | 35.73 ± 14.28b | 77.19 ± 16.28a |
| Ct value in scion             | 36.36 ± 1.71a | 33.31 ± 1.57b | 28.9 ± 2.66c |
| Ct value in rootstock         | 36.32 ± 2.18a | 33.79 ± 1.5b   | 27.14 ± 2.65c |
| Disease index                 | 18.42 ± 9.25c | 33.16 ± 9.3b   | 49.24 ± 17.45a |

**Table 6.** Selected variable of antibacterial activity by stepwise discriminant analysis at *Chi* = 74.942 and P = 0.00010.

| Variable                      | Wilks Lambda | F value | Selected (N/Y) |
|-------------------------------|--------------|---------|----------------|
| Scion survival (%)            | 0.9975       | 0.0462  | N              |
| Scion grown rate (%)          | 0.9029       | 1.9887  | N              |
| Scion infected (%)            | 0.6325       | 11.0376 | Y              |
| Las transmission (%)          | 0.9665       | 0.6417  | N              |
| Ct value in scion             | 0.9687       | 0.5978  | N              |
| Ct value in rootstock         | 0.7334       | 6.9064  | Y              |
| Disease index                 | 0.8156       | 4.2948  | Y              |

by reactive oxygen species (ROS). Therefore, BSO is a glutathione-depleting agent that can enhance production of ROS that have potent antimicrobial activity against bacteria. Our results showed that Las titers were reduced in inoculated rootstocks and scions soaked in BSO, and BSO had phytotoxicity (Table 2). This may be attributed to production of reactive oxygen species effective against Las bacterium and related damage to citrus tree cells. Furthermore, this probable bactericidal mechanism of BSO is not likely to result in the emergence of antibiotic resistant bacteria. Therefore, BSO may have a great value in the rescue and maintenance of citrus crops.

SFC is an antimicrobial lipopeptide family member produced by *Bacillus subtilis* that displays antibacterial, antiviral, antitumor, and hemolytic action. Given its biological origin, SFC is generally considered to be of lower risk to humans and the environment than antibiotics. SFC’s ability to penetrate the cell membrane of all types of bacteria gives it very significant antibacterial activity. In previous studies, SFC from *Bacillus subtilis* was shown to reduce infections caused by *Pseudomonas syringae* on *Arabidopsis* plants. SFC can also interact with plant cells as a bacterial deterrent by stimulating induction of systemic immune resistance. Our current results demonstrated that SFC from *Bacillus subtilis* displayed effective antibacterial activity against Las bacterium and low phytotoxicity in citrus plants (Table 2), likely resulting from its induction of systemic resistance. Therefore, the eco-friendly antimicrobial SFC is a potential candidate for control of citrus HLB in the field.

EBI-601 and EBI-602 also belonged to Group I, being highly effective against Las bacterium (Tables 2 and 5). These chemicals are both degradation products of tetracycline. Previous studies have demonstrated that although tetracycline can suppress Las bacterium, it shows serious citrus phytotoxicity. Our research demonstrated that EBI-601 and EBI-602 could not only suppress Las titers in inoculated rootstocks and scions, but also had low phytotoxicity to citrus plants (Table 2). As degradation products of tetracycline, the physical and chemical characteristics of EBI-601 and EBI-602 may be different from that of tetracycline. Thus, the mechanism of their effect on Las and citrus plants is unknown.

Two other chemical compounds that were screened included the essential oils CARV and PCY. Essential oils are extracted from aromatic plants. Therefore, essential oil is one of most important natural compounds and have antioxidant, antibacterial, and antimicrobial properties. Currently, they have been largely utilized in food, cosmetics, and pharmaceuticals. Several essential oils, including CARV and PCY, displayed strong antibacterial activity. This has been attributed to their ability to permeabilize and depolarize cytoplasmic membranes. Several previous studies have demonstrated that essential oils also have insecticidal properties. However, the effect of essential oils, especially CARV and PCY, on the citrus psyllid, which is a vector for transmitting Las bacterium, is still unknown. Our current results indicated that CARV and PCY were partially effective against Las bacterium. Furthermore, CARV and PCY can be prepared as nanoemulsions, enhancing their delivery efficiency and antibacterial activities. Therefore, CARV and PCY are ideal candidates for combating HLB due to their eco-friendly, antibacterial and insecticidal properties, nanoemulsion characteristics, and ability to reduce Las titers.
Public concerns regarding emergence of antibiotic resistant bacteria due to the overuse of antibiotics on plants in the open environment and over large expanses of land has limited their applications in agricultural practices. In the present study, several effective and partially effective non-antibiotic antimicrobial compounds against Las bacterium were identified. These antimicrobials include metals and natural products that may reduce the risks associated with emergence of antibiotic resistance. However, the anti-Las activities of Groups I and/or II are still lower than that of Amp (positive control). These antimicrobials have anti-Las activity, low citrus phytotoxicity, and are generally considered safe for humans and the environment. It is possible that using a nano-delivery system or combining their application with thermotherapy would enhance the bactericidal activity of these compounds. The present research identified several highly and partially effective antimicrobials that may be effective for control of citrus HLB in the field by foliar spray or trunk injection. Future studies must assess potential risks these antimicrobials may pose to humans and the environment.

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**Author Contributions**

Conceived and designed the experiment: C.P., Y.D., and M.Z. Performed the experiments: C.Y. and Y.Z. Analyzed the data: C.Y., M.Z. Contributed reagents/materials/analysis tools: Y.Z., M.D., and Y.H. Wrote the paper: C.Y., M.Z., C.P. and Y.D.

**Additional Information**

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