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Ultrahigh-throughput screening of antagonistic bacteria against *Erwinia carotovora* Ecc15 based on droplet microfluidics

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ABSTRACT

Soft rot is a destructive disease that inflicts significant losses on agricultural production and the economy post-harvest. Biocontrol strategies based on antagonistic microorganisms have a broad application prospect to fight against plant pathogens. This study utilized fluorescence-activated droplet sorting (FADS) technology as an alternative to traditional plate culture methods to isolate microorganisms with antagonistic activity against the soft rot pathogen *Erwinia carotovora* Ecc15. Initially, the culture performance of the FADS platform was evaluated by analyzing bacterial diversity in droplet culture samples and agar plate culture samples, our data showed that droplet culture exhibited higher species richness and diversity than plate culture, and more than 95% of the operational taxonomic units (OTUs) in the droplet samples belonged to the rare biosphere. Additionally, we developed a green fluorescent protein (GFP)-Ecc15-based FADS screening system, which achieved an enrichment ratio of up to 148. Using this system, we successfully screened 32 antagonistic bacteria from rhizosphere soil sample of healthy konjac plants, and some may be novel microbial resources, including the genera *Lelliottia*, *Buttiauxella* and *Leclercia*. Notably, strain D-62 exhibited the strongest antibacterial ability against Ecc15, with an inhibition zone diameter of (20.86 ± 1.56) mm. *In vivo* experiments conducted on the corms of *Amorphophallus konjac* demonstrated that strain D-62 could effectively reduce the infection ability of Ecc15 to the corms, indicating that strain D-62 has the potential to be developed as a biocontrol agent. Our findings suggested that the FADS screening system showed a screening efficiency approximately 3×10^3 times higher than plate screening system, while significantly reducing costs of infrastructure, labor and consumables, it provides theoretical guidance for the screening of other plant pathogen biocontrol bacteria.

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1. Introduction

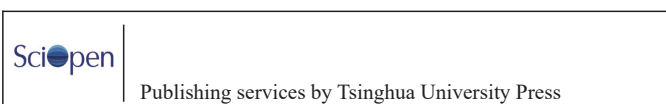
Amorphophallus konjac, a perennial herb, is valued for its corm rich in konjac glucomannan (KGM), with diverse applications in the medical, food, agriculture, and other industries^[1-2]. Additionally, its

growth in mountainous environments makes it a potential cash crop for local economic development^[2]. The identification of potential causal bacteria from rotten corms of *A. konjac* is crucial for preventing and controlling konjac diseases. Soft rot caused by soil-borne pathogens such as *Erwinia carotovora* subsp. *carotovora* (Ecc), *E. carotovora* subsp. *atroseptica* (Eca) and *E. chrysanthemi* (Ech) is a globally distributed bacterial phytopathogen and one of the most destructive diseases affecting vegetables and ornamental plants^[3-4]. Among these pathogens, Ecc has the largest number of hosts and is the most widely

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distributed, and it is considered the main pathogenic bacteria^[5]. Soft rot occurs in the succulent storage tissues and can cause significant yield loss in economically important crops^[6]. In addition, the disease can be found in the field, during transport, storage, and even during sale^[7]. The pathogen of soft rot primarily enters the plant tissue through the wound to produce pectin-decomposing enzymes, which causes the osmotic pressure disorder of plant cells and leads to cytoplasmic lysis, collapse, and death^[7-8]. Agrios et al.^[8] found that bacterial soft rot accounted for 15%–30% of harvested crop losses after harvest, making it a major contributor to total produce loss caused by bacterial diseases. Mansfield et al.^[9] listed the three pathogens as the top ten plant pathogenic bacteria based on scientific/economic importance in 2012. Therefore, there is an urgent need to address the hazards caused by soft rot pathogens. Although the use of chemical pesticides is still the primary means of effectively controlling plant diseases in crops worldwide, the residue of chemical pesticides would also cause bad effects on the health of human^[10-11]. Biological control strategies based on antagonistic microorganisms could represent a promising alternative to control soft rot disease. However, currently, the traditional microbial culture and separation technology typically has the characteristics of high labor intensity, high cost, low yield, and low efficiency^[12]. Thus, droplet microfluidic technology, which has emerged in the past decade, is characterized by its high throughput, highly parallelized and efficient cultivation of abilities has attracted attention, and can be used as a potential screening tool^[13].

Microfluidic fluorescence-activated droplet sorting (FADS) is currently a highly favored technology for conducting high-throughput screening of diverse biological libraries. The microfluidic chip serves as the main tool for droplet generation, which enables researchers to

manipulate minuscule liquid with precision, automation, and control. This capability facilitates multiple parallel experiments and minimizes the errors associated with manual operation^[14]. The essence of microfluidic droplet generation is to use the change of flow shear force or surface tension to split the discrete phase fluid in two immiscible fluids into droplets with a volume of nL or pL, or drive the movement of droplets^[15]. The droplets act as independent microbial reactor, which can not only prevent cross-contamination of reagents and interspecific competition in adjacent droplets, but also makes it possible to isolate, culture and identify microorganisms that have not been cultured and discovered so far^[16-18]. By generating monodisperse droplets surrounded by an immiscible oil phase, the FADS platform allows the encapsulation of detection reagents with single cells containing enzymes, antibodies, or small molecule targets in droplets, followed by quantitative detection and ultrahigh-throughput sorting in microfluidic devices. For instance, the FADS screening platform has successfully screened *Pseudomonas aeruginosa*^[19], *Actinobacteria*^[20], and *Bacillus* spp.^[21-22] with antimicrobial activity against the target strains. Therefore, establishing an ultrahigh-throughput screening system for antibiotic producing bacteria in a microbial reactor and the screening of multiple cell mixtures at the single-cell level is a promising method for finding new antibacterial target organisms. However, the FADS technology has rarely been reported in the field of plant disease biocontrol strains screening.

Based on the above facts, this study used *E. carotovora* Ecc15 as a pathogen indicator bacterium, and used a FADS platform to singularize, cultivate, and screen bacterial cells from complex environmental communities. The workflow featured 3 major phases (Fig. 1): a) Evaluating the cultivation performance of the FADS

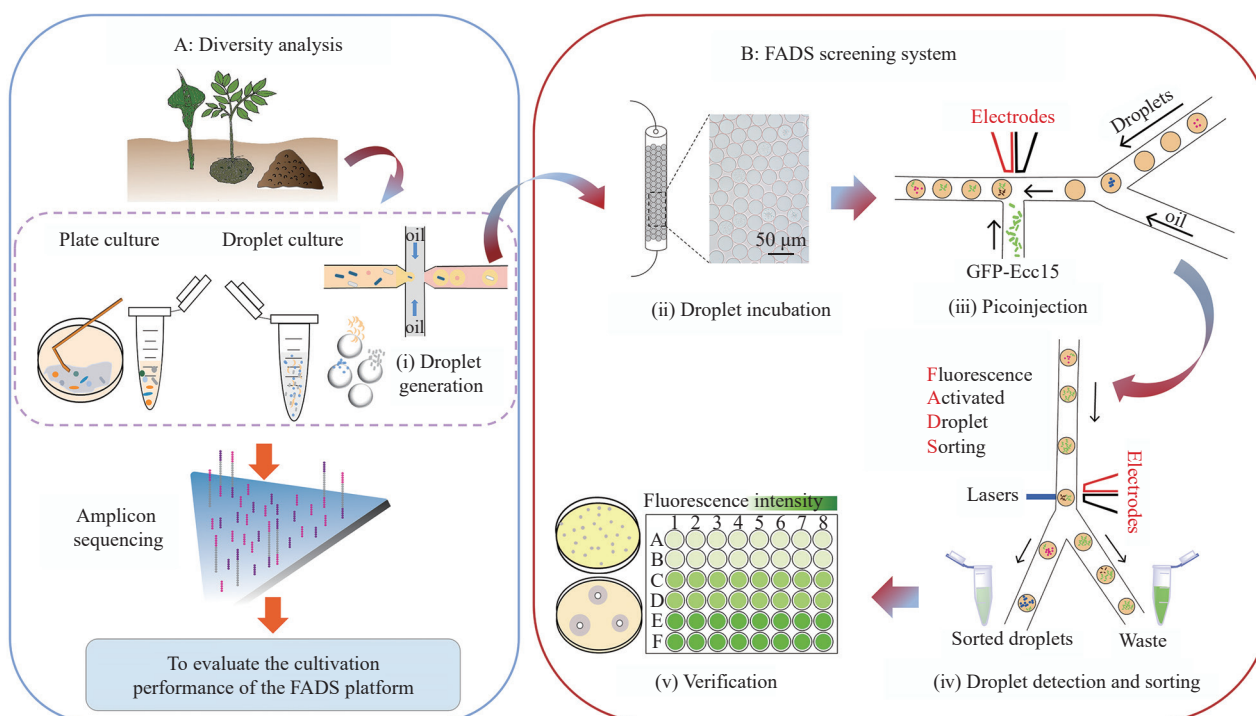


Fig. 1 Concept of microbial droplet cultivation and activity screening. (A) The workflow for comparing the bacterial diversity of droplet culture and agar plate culture of soil microorganisms. (B) The framework of the FADS screening system. (i) Single cells of the soil community were encapsulated in droplets. (ii) Droplets were incubated in Teflon tube. (iii) Droplets were encapsulated with the reporter strain GFP-Ecc15. (iv) Droplets fluorescence signal detection and sorting. (v) Antibacterial activity testing of isolated bacteria. The cell-free supernatant of isolated bacteria was tested for antibacterial activity by conventional well diffusion methods against *E. carotovora*.

platform by comparing the bacterial diversity of droplet cultivation and agar plate cultivation; b) Establishing an ultrahigh-throughput FADS screening system for antagonistic bacteria against *E. carotovora* Ecc15; c) Screening for antagonistic bacteria from soil sample with FADS systems. The throughput of FADS is a thousand times higher than traditional agar plate screening methods and has much lower infrastructure, labor, and consumables costs, and we can efficiently sort 3×10^5 cells within 3 h, and discover novel microbial resources from soil samples which are difficult to obtain through traditional plate screening methods, including the genera *Lelliottia*, *Buttiauxella*, and *Leclercia*. We subsequently evaluated the biocontrol assay of the antagonistic bacteria with the strongest antibacterial activity on the corns of *A. konjac*, our data showed that strain D-62 effectively reduced the infectivity of the pathogen Ecc15. The results showed that the FADS screening system of biocontrol bacteria provides theoretical guidance for the screening of other plant pathogen biocontrol bacteria.

2. Materials and methods

2.1 Soil sample collection and extraction

The soil was collected from the rhizosphere of healthy konjac plants in the konjac planting area of Bijie City, Guizhou Province, China (27°11'35" N, 105°19'3" E) in December 2021. After removing the surface soil, collected the soil from a depth of 10 cm and transported in a sealed self-sealing bag before returning it to the laboratory for further treatment.

Preparation of cold extracted soil extract (CESE): the soil was mixed with distilled water at a ratio of 1:1 (m/m), stirred at 300 r/min for 2 h at room temperature, and let settle for 2 h. The insoluble particles in the supernatant were removed by centrifugation at $5000 \times g$ for 30 min, and the supernatant was filtered twice with a bottle-top membrane filter with a pore size of 0.22 μm , then divided evenly and stored at -20°C .

The bacterial soil community was extracted following the method described by Mahler et al.^[21] with some adjustments. Initially, 20 g of freshly collected soil was spread evenly on a plate glass tray and dried at 37°C for 24 h. Subsequently, the dried soil was carefully screened using a 2 mm stainless steel sieve to remove any large particles such as stones or visible plant material, and 5 g of soil and 25 glass beads (2–6 mm diameter) were mixed in 45 mL of CESE and shaken with 220 r/min at 30°C for 2 h. The suspension was sonicated twice at 50 W for 1 min. Half of the resulting suspension was filtered through a 40 μm cell strainer, while the other half was allowed to settle at room temperature. After 1 h, the supernatant was decanted. Aliquots of both the filtered and settled cell suspensions were stored at -20°C . For use, a mixture consisting of a suspension rich in soil particles (settled) and a suspension containing less particles (filtered) was prepared using a ratio of 2:35 (V/V).

2.2 Strains and culture conditions

E. carotovora Ecc15 with green fluorescent protein (GFP) marker was constructed and presented by Yongxiang Liu (Guizhou Academy of Agricultural Sciences), hereinafter referred to as GFP-Ecc15; *E. carotovora* Ecc15, *Bacillus velezensis* and *Escherichia coli* were

isolated, identified and preserved by the Biotechnology Laboratory of Guizhou University. Polymerase chain reaction (PCR) primers used in this study were synthesized by GENEWIZ Biotech Co., Ltd. (China) (Table S1).

B. velezensis and *E. coli* were grown in Luria-Bertani (LB) broth at 37°C . Soil microbial community were grown in a medium containing 44% distilled water, 50% (V/V) CESE, and 6% (V/V) supernatant of soy mannitol medium at 30°C , as described by Mahler et al.^[21]. Reporter strain GFP-Ecc15 was grown in pre-cultures containing LB medium with 200 $\mu\text{g/mL}$ erythromycin at 30°C , 220 r/min for 18 h. Second preculture was inoculated to a defined start optical density at $\text{OD}_{600\text{nm}}$ of 0.1 and cultivated without selection markers until the logarithmic period. For generating the cell suspension, which was picoinjected into droplets, was re-suspended in $2.5\times$ terrific broth medium (TB, 30 g/L tryptone, 60 g/L yeast extract, 10 g/L glycerol, 5.78 g KH_2PO_4 , 31.32 g K_2HPO_4 , pH 7.2) + 1% glucose.

2.3 Droplet microfluidic culture and agar plate culture

Water-in-oil droplets were generated on a microfluidic chip of the droplet entrapping microfluid cell sorter (DREM cell; G3c, TMAXTREE, China), a device developed based on droplet microfluidic technology for single cell ultrahigh-throughput sorting. The graphics of the droplet generation chip, pico-injection chip, and sorting chip were created using AutoCAD and fabricated by soft lithography^[23–25]. The droplet generation chip features two inlets for the continuous oil phase and water phase, as well as one outlet for collecting the highly monodisperse emulsion. Two kinds of liquids confined in the microfluidic channel were gathered using the pressure pump on the chip, followed by droplet formation at the flow-focusing joint due to shear stress, and the droplet diameter was 30 μm (approximately 14 pL). To guide the generation of single-cell droplets on the microfluidic chip, we preliminary evaluated the bacterial concentration of the soil microbial community samples, which was 8×10^7 CFU/mL, and further selected the λ value of 0.1 to calculate the concentration of the microbial community suspension according to the Poisson distribution. At this concentration, we aimed to reduce the number of droplets containing multiple bacterial cells. Firstly, the soil community suspension was incubated with an equal volume of 1 $\mu\text{g/mL}$ DAPI staining solution (E607303, Sangon Biotech Shanghai, China) at 30°C for 30 min, centrifuged and resuspended, and diluted to approximately 7×10^6 CFU/mL with CESE medium to generate droplets. Then, the number of cells in the droplet was observed using an inverted fluorescence microscope to verify if the distribution of bacteria aligned with the theoretical value. To incubate the droplets, about 3 mL of the emulsion was encapsulated in a Teflon tube (approximately 305 m, $\phi_{i,d} = 0.45$ mm, $\phi_{o,d} = 0.75$ mm, Suzhou, China) to ensure the stability of the droplets during the incubation.

For cultivation on agar plates, the soil microbial community was diluted with 0.9% NaCl solution to $\text{OD}_{600\text{nm}}$ of 0.1 (approximately 7×10^6 CFU/mL), the soil diluent was further diluted 10 times with NaCl solution, and two concentrations of soil diluent (100 μL) were applied on agar plates, and 15 plates of each concentration were taken as a set. Droplet populations were generated with three replicates and compared to three plate sets. All samples containing soil communities were incubated at 30°C . After 7 days of incubation, all collected droplets were fused with an equal volume (1:1, V/V) 1H, 1H, 2H,

2H-perfluoro-1-octanol (PFO) to destroy the droplets and release the cells. The mixed sample was shaken and allowed to stand, then the upper water phase, including the cells, was transferred into the new EP tube. On the plate, the collected colonies were suspended in 2 mL of 0.9% NaCl solution. In the solution, colony cell library was formed, and DNA was extracted and sequenced.

2.4 Construction of microplate screening system of antagonistic bacteria against *E. carotovora*

GFP-Ecc15 was used as the reporter, strain *B. velezensis* with strong antibacterial activity against reporter as antagonistic bacteria, and *E. coli* with no inhibitory effect on the reporter strain as non-antagonistic bacteria. The microplate screening model was performed in 48-deep-well plates (Fig. S1). Firstly, about 1 mL LB medium was added into each well, *B. velezensis* and *E. coli* cultures were adjusted to OD_{600 nm} of 0.4, and 15 μ L bacterial dilution of both strains was inoculated in consecutive wells, LB medium was used as control. All wells incubated at 37 °C for 2–3 h and 800 r/min on the well plate shaker, the 2 model strains adapted to the new environment and began to grow. Then 15 μ L pre-cultured reporter strain GFP-Ecc15 bacterial solution (OD_{600 nm} was 0.4) was added to each well, after 24 h of incubation at 30 °C, the model antagonist strains produced antimicrobial metabolites, and inhibited the growth of reporter by antimicrobial substances or competition (nutrition, space). Finally, the fluorescence value of each well was measured using a microplate reader. We further evaluated whether the system can be used in droplet system by the fluorescence intensity of *B. velezensis* and *E. coli* co-cultured with reporter respectively.

2.5 Construction and validation of the FADS screening system

The conditions for constructing the FADS screening model were optimized as follows (Fig. S2): Strain GFP-Ecc15 was able to grow normally in droplets without affecting droplet stability, and the fluorescence signal in the droplets could be detected. In the FADS platform, the oil/water pressure for stable droplet formation was set at 320/360 mbar, as these generated 30- μ m droplets at a speed of 2 400 Hz/s, the concentrations of antagonistic and non-antagonistic cultures for droplet formation were OD_{600 nm} = 0.03 and OD_{600 nm} = 0.01, respectively, the droplets were incubated for 2–6 h, and the concentration of GFP-Ecc15 culture used for picoinjection was OD_{600 nm} = 0.4. The FADS screening system was constructed as shown in Fig. S3. The 6-step screening process involved: i) preparing the bacterial solution for droplet generation; ii) using the prepared bacterial solution as the water phase and fluorinated oil as the oil phase to generate water-in-oil droplets; iii) collecting and culturing the droplets at 37 °C; iv) reinjecting the cultured droplets into the picoinjection chip, encapsulated with the reporter strain GFP-Ecc15 at a speed of 5 nL/s, and culturing for 24 h; v) reinjecting the fusion droplets into the sorting chip to collect droplets with low fluorescence signals; vi) verifying the antibacterial activity of the isolated bacteria.

In our study, the high fluorescent droplets were discarded while the low fluorescent droplets were collected. Mahler et al.^[20] believed that the fluorescence intensity of the co-cultured droplets was less than 1/3 of the droplets containing only the reporter after 24 h, the droplet supernatant was considered to have an inhibitory effect. An equal volume of PFO was added to the droplet culture, and the vortex

was shaken to break the droplets and recover the bacterial cells in the medium. These bacterial cells were transferred to agar plates for culture, and single colonies were selected for streak purification.

Next, we will demonstrate the feasibility of the screening system through three repeated screening experiments. In General, the traditional antibacterial activity was evaluated using well diffusion assay. In the above screening model, only three strains were involved. The colony of the reporter (GFP-Ecc15) could be excluded by fluorescence plate observation, and the rest only needed to verify the ratio of *B. velezensis* and *E. coli* isolated. To determine the enrichment ratio of two kinds of strains accurately and quickly, we designed primers *ricT*-F and *ricT*-R based on the whole genome of *B. velezensis*, which can only amplify specific fragments of *B. velezensis* but not the *E. coli* (Table S1). Randomly selected 100 single colonies except for GFP-Ecc15 on the plate for PCR validation, evaluated the proportion of antagonistic bacteria after sorting. The model for enrichment rate was developed based on the Poisson distribution, and the enrichment rate (η_m) of antagonistic bacteria is calculated as the equation below^[26]:

$$\eta_m = \frac{1}{1 - e^{-\varepsilon_0 \lambda / (1 + \varepsilon_0)}}$$

Where ε_0 is the initial ratio of active to inactive cells and λ is the initial mean number of cells per droplet. The enrichment rate (η) is defined as the ratio of ε after sorting (ε_1) to ε before sorting (ε_0).

2.6 Screening for antagonistic bacteria from soil sample with FADS systems

To demonstrate the applicability of the screening model to complex environmental samples, we screened bacteria with bactericidal activity from soil microbial communities. Single cells were encapsulated in droplets (λ approximately equal to 0.1) and incubated at 30 °C for 3 days, usually with secondary metabolite production in droplets before GFP-Ecc15 cells were picoinjected into the droplets. After 24 h of cultivation, droplets were sorted in the sorting chip, and we enriched the droplets with low fluorescence intensity and distributed on agar plates. However, there may be a small amount of false negative droplets in the collected droplets. Therefore, it's necessary to further verify the antibacterial activity of the isolated bacteria.

We utilized the same medium components as the droplet system to isolate bacteria with antibacterial activity against strain Ecc15 from soil samples on agar plates. The soil suspension was diluted with 0.9% NaCl solution to OD_{600 nm} of 0.1, and 50 μ L was coated on CESE agar plate, and cultured at 30 °C for 3 days. The single colony on the plate was picked and streaked for purification, and the bacteria that inhibited Ecc15 strain were screened by the well diffusion method.

2.7 Antibacterial activity test

The test of antibacterial activity of isolated bacteria was performed according to the well diffusion method described by Hassan et al.^[27], with minor modifications. The isolated strains were cultured in LB medium at 30 °C for 48 h. The culture was centrifuged at 12 000 r/min for 15 min, and the supernatant was filtered using a 0.22 μ m filter membrane to obtain the cell-free supernatant. The tested pathogen Ecc15 was cultured to logarithmic phase under the same conditions as before, cell density was then adjusted to 1×10^6 CFU/mL with sterile water. bacterial solution with 100 μ L was added to aseptic and uncoagulated

LB solid medium and shaken well to make a plate containing pathogens. Three holes were equally punched on the medium containing bacteria with a diameter of 6 mm, and the small pieces of the medium were carefully removed to make a round hole, then 100 μ L cell-free supernatant was injected into the hole and cultured at 30 °C for 16–18 h, and measured the inhibition zone diameters to determine the antibacterial activity of each strain. *B. velezensis* was used as the positive control and the medium containing only pathogens as the negative control. It is generally believed that the inhibition zone diameter > 15 mm is highly sensitive, 10–15 mm is moderately sensitive, and < 10 mm is lowly sensitive^[28]. The antagonistic bacteria cultures obtained from single colonies were stored in 25% glycerol at –80 °C.

2.8 Identification of antagonistic bacteria

All antagonistic bacteria were revived from frozen stocks, streaked onto LB agar, and inoculated into LB broth. The cultures were incubated at 30 °C and 220 r/min. DNA was extracted from overnight broth cultures according to the kit instructions (Solarbio, Beijing, China). 16S rRNA was amplified by PCR amplification with the universal primers 27 F and 1492 R^[29], then directly sequenced.

2.9 Biocontrol assay of the *E. carotovora* Ecc15 by strain D-62

Biocontrol assay of the *E. carotovora* Ecc15 by strain D-62 was performed according to the method described by Azaiez et al.^[30] and Nian et al.^[31] with some modifications. Fresh, healthy and mature corms of *A. konjac* were used in the *in vivo* biocontrol experiments conducted in this study. The corms underwent a series of steps including washing with sterile distilled water, wiping their surfaces with 75% ethanol, rinsing with sterile distilled water to remove any residual ethanol, and finally being sliced into 4 equal parts. Artificial wounds were created at the center of the corms using sterile toothpicks, and the corms were then randomly divided into 4 groups. 1) Group E: the corms with the lesion inoculated with 10 μ L *E. carotovora* Ecc15 culture (1×10^7 cells/mL) and 20 μ L H₂O; 2) Group D: the corms with the lesion inoculated with 20 μ L D-62 culture (1×10^8 cells/mL) and 10 μ L H₂O; 3) Group D + E: the corms with the lesion inoculated 20 μ L D-62 culture (1×10^8 cells/mL) and 10 μ L *E. carotovora* Ecc15 culture (1×10^7 cells/mL); and 4) Group H: the corms with the lesion inoculated with the 30 μ L H₂O. All samples were transferred to sterile constant temperature chamber and incubated at room temperature at 70% humidity for 4 days. The changes of the corms were documented at 0, 24, 48, 72, and 96 h. The diameter of disease spot was measured using cross-crossing method^[31].

2.10 Statistical analysis

All experiments were performed in triplicate and expressed as mean $\pm$ standard deviation (SD). Microsoft Excel 2021 was used for data processing, all experiments were expressed as mean $\pm$ standard deviation (SD). One-way analysis of variance (ANOVA) was performed for data analysis using IBM Statistics SPSS version 22.0, the differences among groups were evaluated by performing Duncan's test, and *P*-values < 0.05 were considered statistically significant. Pairwise comparisons utilized unpaired Student's *t*-tests, ns *P* > 0.05, **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

3. Results

3.1 Evaluation of the culture performance of the droplet microfluidic platform

The concept of microbial droplet culture and agar plate culture was shown in Fig. 2A. To isolate and culture individual bacterial cells from soil microbial community, we used the DREM cell to generate droplets containing bacterial cells, and the number of cells follows Poisson distribution in each droplet (Fig. 2B). To ensure the presence of a single cell in each droplet, we encapsulated DAPI-stained bacterial cells at a concentration of 7×10^6 CFU/mL, the distribution of bacteria cells in droplets was visualized using fluorescence microscopy (Fig. 2C). Our results revealed that approximately 8.95% of the droplets contained only one cell, which was close to the theoretical value (9.05%), and the occurrence of droplets containing more than 2 cells was minimal, leading us to select the concentration for final droplet generation. Following culture, biomass of colonies was collected and metagenomic DNA was extracted for sequencing of 16S rDNA amplicons on the Illumina MiSeq platform, based on 97% sequence similarity, an operational taxonomic unit (OTU) table containing the distribution information of each OTU in all samples was generated and classified.

To evaluate the cultivation capability of our droplet microfluidic platform, we compared the bacterial diversity of the original soil samples (WS), the droplet pooled cultures (WD1-WD3) and the agar plate pooled cultures (WP1-WP3) for all samples. Firstly, the results of principal component analysis (PCA) showed that there was no overlap between the three samples, indicating that the bacterial community of three samples differed significantly, and they were relatively independent in the PCA (Fig. 2D). The rarefaction curves of each sample also further confirmed the advantage of droplet-based single cell culture (Fig. 2E), with droplet samples having higher species richness compared with agar culture samples. Besides the PCA and rarefaction curves used above, the advantages of droplet cultivation were further confirmed by the α -diversity indices (ACE, Chao1, Shannon, and PD_whole_tree). In addition, the result suggested that all α -diversity indices values were greater for droplet samples than plate samples, indicating that the droplet culture samples showed higher species diversity (Fig. S4). In conclusion, these results suggested that the droplet microfluidic platform is a favorable tool for cultivating microorganisms compared to traditional agar plates.

At different taxonomic levels, the differences in community structure among all samples were visible. Fig. 2F showed the bacterial composition at the phylum level, and 33 and 17 bacterial phyla were detected in the droplet sample and the plate sample, respectively. And 4 phyla including Spirochaetota, Deferrobacter, Entothionellaeota, and Thermoplasmata were only detected in droplet samples were not found in original soil samples or plate samples. Proteobacteria was the dominant phylum for all sample species in droplet culture and plate culture, while Actinobacteriota was the dominant phylum in the original soil samples. In addition, the relative abundance of Cyanobacteria was about 10 times higher in droplets ((6.06 $\pm$ 5.06)%) than on agar plates ((0.62 $\pm$ 0.54)%). At the genus level, it can be seen from Fig. 2G that *Azospirillum* was highly enriched in droplet samples, and the relative abundance reached (52.35 $\pm$ 6.79)%, while in the plate samples and original soil samples, *Azospirillum* bacteria were absent, and *Variovorax* was

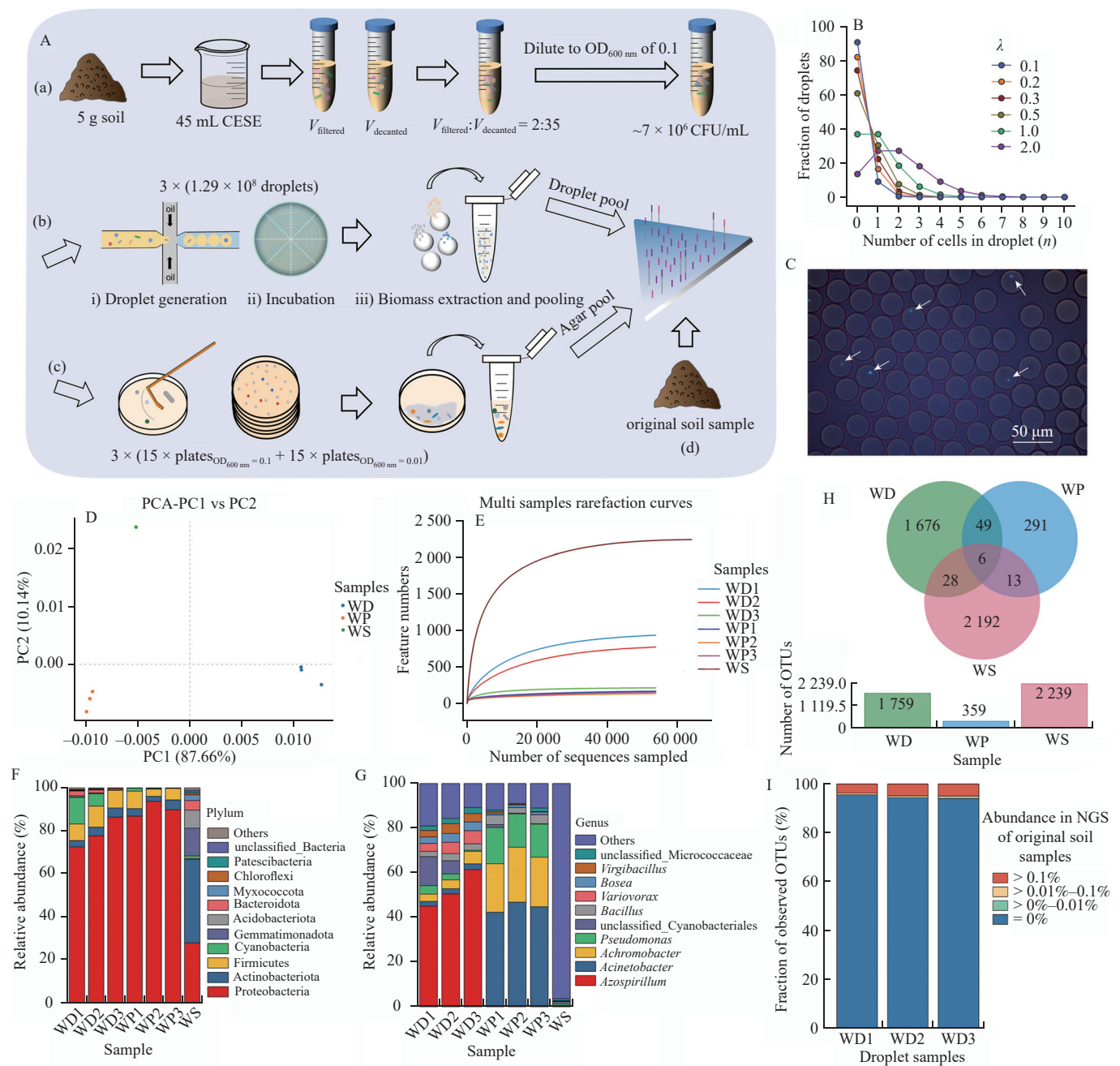


Fig. 2 Comparison of bacterial diversity between droplet culture and agar plate culture. (A) Concept of microbial cultivation in droplets and on agar plates. (a) The extraction of bacterial soil community. (b) General workflow of droplet culture. (i) Single cells of the soil community were encapsulated in droplets; (ii) Droplets were incubated in Teflon tube; (iii) Biomass extraction and pooling. (c) General workflow of agar plate culture. (d) The 16S rDNA gene amplicon sequencing of the original soil samples. (B) Poisson distribution of the original soil samples, droplet sample and plate sample. (C) The distribution of bacteria cells in the droplets was visualized by fluorescence microscopy. White arrowheads point toward individual bacterial cells. (D) PCA representing the differences among the original soil sample, droplet sample and plate sample. (E) Rarefaction curves of bacterial communities of three treatment samples. The relative abundance of the dominant bacterial taxa in all samples at phylum level (F) and genus level (G). (H) Venn diagram indicating the communal and unique OTUs of different sample types. (I) The bar chart constructed with all OTUs of the three droplet samples indicates the ability of the droplet culture to reveal the rare biosphere. > 0.1%, > 0.01%–0.1%, > 0%–0.01% and 0% represent the abundance of OTUs in original samples; 0% represents that these OTUs are detected in the droplet samples but absent in the original samples. NGS represents next-generation sequencing technology.

mainly distributed in the droplet samples. Additionally, *Acinetobacter* ((44.66 ± 1.85)%), *Achromobacter* ((22.81 ± 1.27)%) and *Pseudomonas* ((15.33 ± 0.62)%) were the predominant bacterial genera in the plate culture samples, accounting for 82.80% of all OTUs. The obtained data showed that the microbial composition obtained by different culture methods was significantly different.

The importance of the droplet microfluidic platform in the recovery of rare microbial was further confirmed by in-depth study of the OTU table. According to Hu et al.^[18], rare species referred to microbial with relative abundance less than 0.01%. As shown in the Venn diagram in Fig. 2H, a total of 4 255 OTUs were detected in all samples in this study. Although the original samples constituted the majority of OTUs detected

in this study, many OTUs did not present in the original samples, but they were detected in the droplet samples, these OTUs belonged to rare microbial species below the detection limit of current sequencing methods. In addition, agar plate culture also obtained some undetected bacterial genera from the original soil samples, but the frequency was much lower. The results depicted in Fig. 2I showed that over 95% of the OTUs in the droplet samples were part of the rare biosphere, with a relative abundance lower than 0.01% in the original soil samples. Our data suggested that droplet culture has great potential for revealing rare biospheres in complex microbial communities.

In conclusion, results from the present study again demonstrated that the droplet culture method is superior to conventional agar plate and has a higher ability to reveal rare biospheres. By combining both methods with high-throughput sequencing, researchers can gain a more comprehensive insight into microbial diversity in the environment. This integrated approach can also serve as a valuable tool for isolating new strains from extreme environments, particularly those that necessitate prolonged cultivation.

3.2 Construction of the GFP-Ecc15-based FADS platform for single-cell screening

In our study, using the model strains *B. velezensis* and *E. coli* as an example, and tested the fluorescence intensity of the two strains co-cultured with the reporter in microplate screening model. As shown in Fig. 3A, the fluorescence intensity of *B. velezensis* co-cultured with GFP-Ecc15 was close to the background fluorescence (LB medium), which indicated that antagonistic bacteria significantly inhibit the growth of the reporter strain. The fluorescence intensity of *E. coli* co-cultured with reporter strain exhibited showed significant differences compared to the background fluorescence ($P < 0.01$). Moreover, in the wells containing only GFP-Ecc15 cells,

due to the absence of other microbial interference, the GFP-Ecc15 strain grew well and the fluorescence value was the highest, which could simulate the state of empty droplets in the droplet system.

Based on these results, we established an ultrahigh-throughput FADS screening system. To demonstrate that the FADS system can be used to screen antagonistic bacteria, we performed the FADS experiment to enrich strain *B. velezensis* with antibacterial activity (low fluorescent droplets). As depicted in Fig. 3B, the fluorescence signal distribution of droplets was primarily concentrated in three ranges: the 2.5–4 V (droplets containing strains *B. velezensis* and GFP-Ecc15), 4–6.5 V (droplets containing strains *E. coli* and GFP-Ecc15), and > 7 V (droplets only containing strain GFP-Ecc15). Furthermore, the fluorescence signal below 2 V represented the background fluorescence. Fig. 3C showed that most of the droplets exhibited strong fluorescent signals, the growth of bacteria was visible in a few droplets in the bright-field, but the expression of the reporter strain in these droplets was not observed in the dark-field. It is believed that the *B. velezensis* significantly inhibited the growth of GFP-Ecc15 strain, so that the fluorescence signal of the reported strain in droplets was weak or even couldn't be detected. After recovering droplets below the threshold (direct current (DC) = 4 V), and cultured on an agar plate to form colonies. Colony PCR was performed on 100 randomly selected single colonies using *B. velezensis* and *E. coli* as positive and negative controls, respectively. The results of the agarose gel electrophoresis pattern revealed an increase in the proportion of *B. velezensis* and *E. coli* strains from approximately 1:1 before sorting to 96:4 after sorting (Fig. S5). Additionally, the average enrichment rate of antagonistic strain in the FADS screening system was 148 times (Fig. 3D). These findings demonstrated the effectiveness of the FADS screening system in isolating bacteria with inhibitory effects on the reporter strain, highlighting the feasibility of this screening approach.

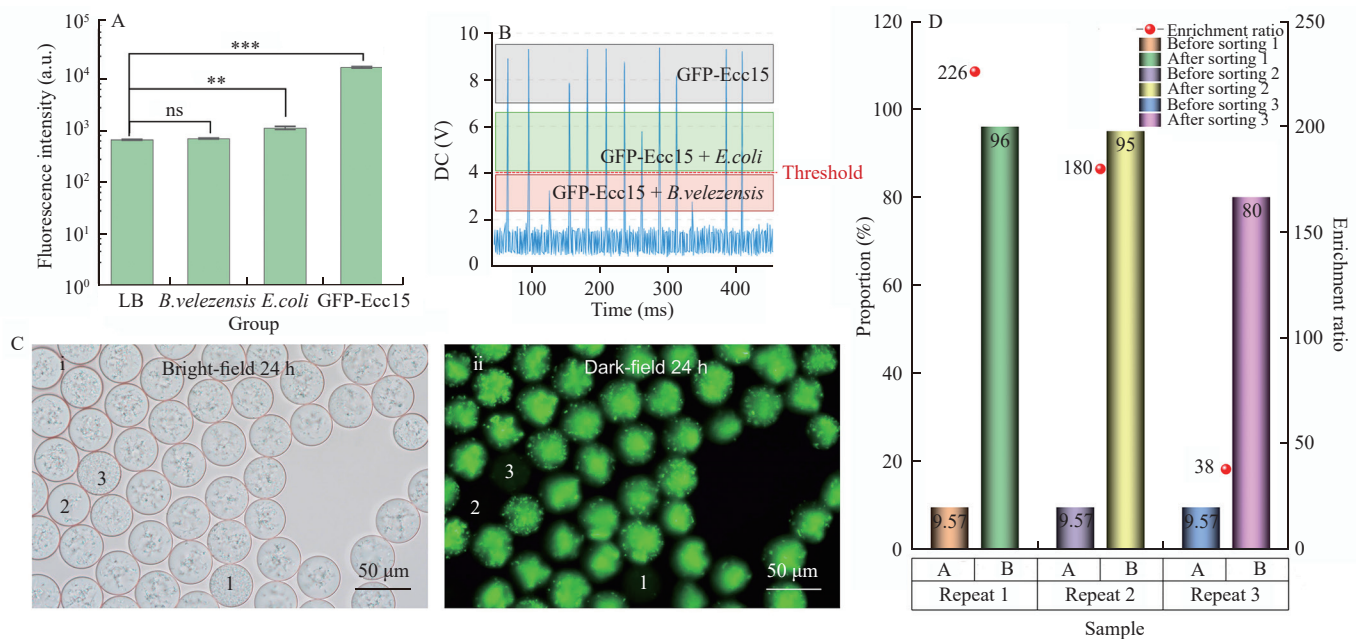


Fig. 3 FADS screening for *B. velezensis* and *E. coli*. (A) Fluorescence values of strains *B. velezensis* and *E. coli* co-cultured with GFP-Ecc15 for 24 h, respectively. ** $P < 0.01$, *** $P < 0.001$, ns $P > 0.05$. (B) Distribution of fluorescence intensity in droplets. (C) Bright-field (i) and dark-field (ii) of *B. velezensis* and *E. coli* co-cultured with GFP-Ecc15 for 24 h in droplets. (D) Enrichment rate of the FADS screening system. A and B represent the proportion of *B. velezensis* before sorting and after sorting, respectively.

3.3 Screening for antagonistic bacteria from soil sample with FADS systems

The study utilized the FADS platform to cultivate, isolate, and screen bacterial cells from complex environmental communities, and combined the cultivation of soil microorganisms with screening of bioactive strains to achieve the selective isolation of bacteria producers of antibacterial substances. Droplets with low fluorescence intensity were collected and distributed on agar plates. The plates were then incubated, and colonies were picked and purified. We tested the antibacterial ability of 102 strains against the pathogen indicator Ecc15 using the agar diffusion method, and the inhibition zone diameter was used to judge the antibacterial ability. Our results of Fig. 4A showed that the cell-free supernatant of 32 strains exhibited inhibitory effects on Ecc15, and D-62 strain had the largest inhibition zone diameter (20.86 ± 1.56 mm), indicating that it had the strongest antibacterial ability against Ecc15 and was most likely to produce antimicrobial compounds. We also obtained 13 strains that inhibited the growth of pathogenic bacteria by agar plate screening, among them, P-70 strain exhibited the strongest antibacterial ability against Ecc15 strain (Fig. 4B). However, it is worth noting that the antibacterial ability of all antagonistic strains was weaker compared with CK (*B. velezensis*) except strains A-19 and D-62 ($P < 0.05$). This could be attributed to the treatment of the *B. velezensis* with atmospheric and room temperature plasma (ARTP) mutagenesis, resulting in enhanced antibacterial activity. Overall, ARTP mutation technology provides valuable insights for the improvement of antagonistic strains in terms of their antibacterial capabilities.

The preliminary identification results of all antagonistic bacteria determined by the 16S rRNA gene as presented in Table S3. The 32 strains of antagonistic bacteria screened by FADS system were divided into 3 phyla, 6 families, and 9 genera, namely *Stenotrophomonas*, *Serratia*, *Lelliottia*, *Buttiauxella*, *Enterobacter*, *Leclercia*, *Pantoea*, *Rhodococcus*, and *Bacillus*, respectively, and Proteobacteria was highly enriched, while Actinobacteria and Firmicutes had only 1 genus each. The analysis of bacterial diversity in droplet culture and plate culture revealed that *Rhodococcus*

bacteria were only detected in droplet samples with a relative abundance of $(0.38 \pm 0.04)\%$. On the other hand, *Lelliottia*, *Buttiauxella*, and *Leclercia* were not detected in 3 different samples, so we hypothesized that the relative abundance of the three bacterial genera in the three samples was low and undetectable by high-throughput sequencing methods, and they were speculated to be rare biosphere microorganisms, with few literature reports on their antibacterial activities. Surprisingly, all antagonistic bacteria screened by agar plate system were identified as genus *Bacillus* spp., underlining the strong bias towards Firmicutes caused by intermediate cultivation on agar plates. These findings suggested that traditional agar plate system exhibited lower diversity of antagonistic bacteria compared with FADS screening, which further illustrating the potential of droplet culture to restore microbial diversity in complex environments.

3.4 Biocontrol assay of the *E. carotovora* Ecc15 by strain D-62

We further tested the biocontrol effect of the D-62 strain with the strongest antibacterial ability on Ecc15, results of Fig. 5 showed the biocontrol assay of strain D-62 on the corms of *A. konjac* against strain Ecc15. As shown in Fig. 5A, the lesions gradually expand with the extension of storage time, which can be found that the lesions of Group E samples were obvious on the 96th h. However, the lesions of Group H and Group D samples were changed slowly, there was no significant difference both two groups ($P > 0.05$), and there were no visible symptoms of rot were developed on the corms of *A. konjac* treated only with the strain D-62. In Fig. 5B, it was observed that the lesion diameter of Group E samples was higher than Group D + E at the 24th, 48th, 72nd and 96th h of storage. The difference between Group E and Group D + E indicated that D-62 had the potential to decrease the infection ability of *E. carotovora* Ecc15 to the corms, thereby delaying the decay of samples. In addition, our data of the lesion diameter of Group D and Group H revealed that strain D-62 did not exhibit pathogenicity to the corms of *A. konjac*, indicating D-62 possessed potential biocontrol performance.

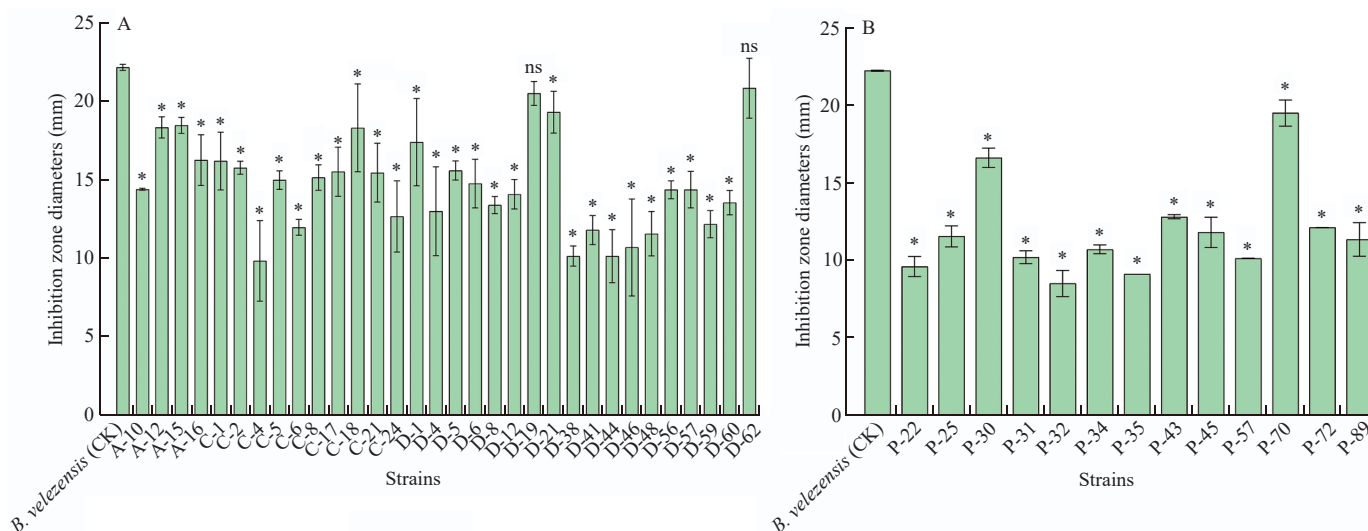


Fig. 4 Antibacterial activity of strains isolated from the FADS system (A) and agar plates against Ecc15 (B). Compared with CK group, * $P < 0.05$, ns $P > 0.05$.

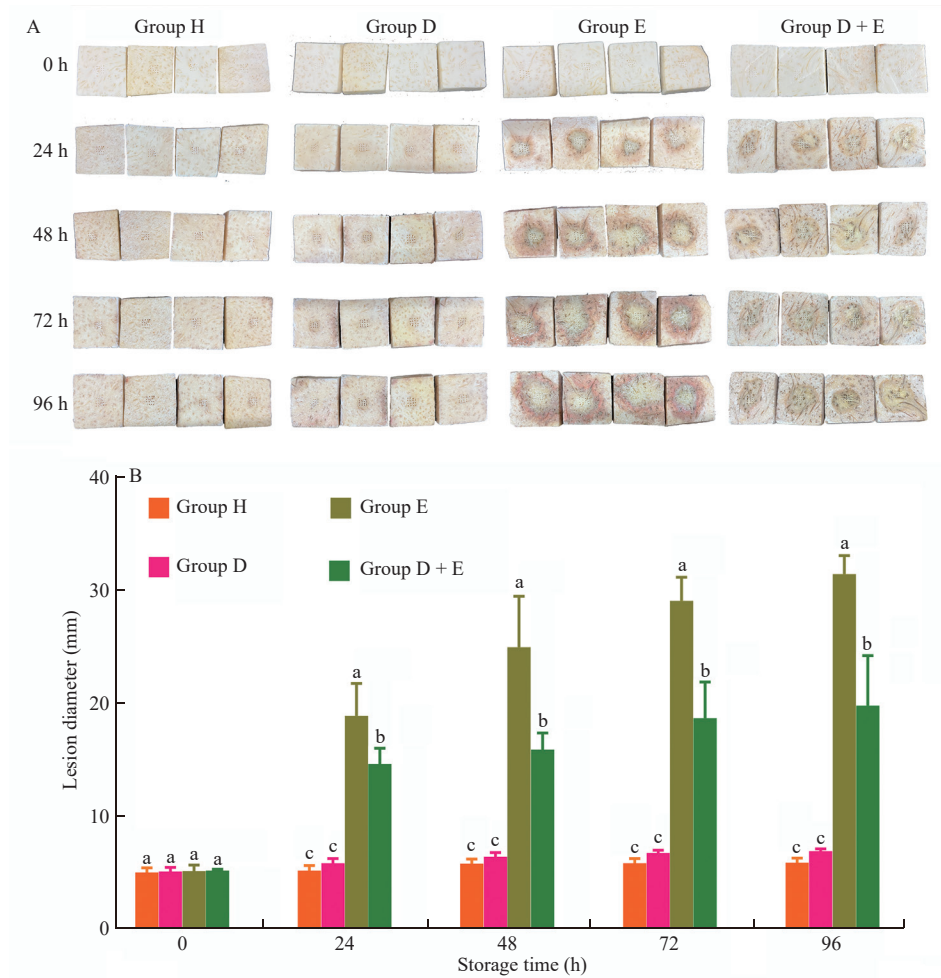


Fig. 5 Appearance of the corms of *A. konjac* (A) and lesion diameter of the wounds (B) during storage. Different lowercase letters indicate significant differences between the means of lesion diameter of the 4 groups samples at the same storage time in Fig. 5B ($P < 0.05$).

4. Discussion

The cultivation of microorganisms is critical for microbiological research, as it is a valuable source for discovering and producing various target substances with a wide range of industrial applications. However, studies have shown that only 1%–15% of bacterial diversity can be cultured under standard laboratory conditions^[32]. Microbial communities in different environments are typically composed of rich and rare biospheres, with the rare biosphere accounting for 75% of the total biomass and mostly having a relative abundance below 0.1%, these rich and rare microorganisms play an essential role in maintaining community structure and function^[33–34]. In the traditional method of cultivating and isolating microorganisms, the number of colonies per plate is usually limited to a few hundred due to competition for nutrients and space, fast-growing microorganisms can outcompete rare or slow-growing species, hindering their growth^[35]. However, advancements in microbial culture and separation technology, such as dilution to extinction culture^[36] and media of mimic natural conditions^[37], have allowed for the discovery of previously uncultured microorganisms and expanded the pool of rare microbial resources. Despite these advancements, challenges remain in fully understanding microbial diversity.

Droplet microfluidic provides a favorable tool for the cultivation and isolation of microorganisms due to its capabilities

of high-throughput screening and single-cell sorting. Compared with other culture and separation technologies, the droplet generated by the microfluidic chip can be regarded as an independent microbial reactor, and the isolation in droplets has many advantages, including preventing interspecific competition and eliminating deviations caused by differences in growth rates^[38], and it is more likely to restore species under clear cultivation conditions to expand their scale. In our study, we used the FADS platform to encapsulate the microorganisms from soil samples and incubated in approximately 14 pL droplets. High-throughput sequencing data revealed the presence of 33 bacterial phyla in our droplet culture samples, while deep-sea sediment culture samples showed 19 bacterial phyla using the microfluidic streak plate platform developed by Hu et al.^[18]. Furthermore, our FADS platform successfully cultured a higher number of bacterial genera (638) compared to the 235 genera detected in the droplet samples by Mahler et al.^[21]. It is worth noting that different droplet platforms may favor different microorganisms. Overall, our findings indicated that the FADS platform demonstrates superior culture performance compared to other droplet platforms, offering great potential for uncovering rare biospheres in complex microbial communities, which enables the recovery of microbial species with high diversity from environmental samples.

On the other hand, natural antimicrobial compounds derived from microorganisms are one of the effective strategies to protect the sustainable development of crops^[39]. To obtain target compounds of interest from natural microorganisms, different screening methods are used, and target strains are selected from mutant libraries. *In vitro* confrontation culture is the traditional plate-based screening mode, but it is usually time-consuming and inefficient^[40]. It is urgent to explore innovative technologies that can replace traditional isolation and screening methods. Currently, microtiter plate-based screening is the most commonly used screening method, although it has limitations in throughput, typically around 10^3 – 10^4 per round^[41]. FACS can perform screening of single cells with extremely high throughput (approximately 10^7 cells/h), which has become an attractive option^[17]. However, FACS is restricted to analyzing intracellular or membrane-bound products that produce fluorescence signals correlating with the target compounds, posing challenges for studying extracellular products or interactions between different strains^[42]. FADS, a combination of microfluidics and cell sorting, can overcome the limitations of FACS, its cost-effective cultivation and separation methods make it a promising alternative^[43]. In our study, we proposed an efficient FADS system that combined the fluorescent signal of a reporter strain with droplet microfluidics. As shown in Fig. 6, compared to the agar plate screening system, the FADS system can achieve the sorting of 3×10^5 cells within 3 h, the screening efficiency of the droplet microfluidic platform was 3×10^3 times higher than the plate system, with a reduction in medium consumption by 1.2×10^7 times and a significant decrease in experimental costs. The results illustrated that the FADS screening strategy is an effective methods to improve the screening efficiency of single cells with higher throughput and lower cost than traditional agar plate screening.

The FADS screening system was used to screen bacteria with inhibitory effects on *E. carotovora* Ecc15, resulting in the isolation of 32 antagonistic bacteria, and they were classified into 9 genera. Interestingly, certain bacterial genera, such as *Lelliottia*, *Buttiauxella* and *Leclercia* may be considered rare biosphere microorganisms due to the relative abundance of them in soil was less than 0.01%. Conversely, the 13 antagonistic bacteria isolated by the agar plate screening system were all identified as *Bacillus* spp., which were ubiquitous bacterial genera in nature. These findings further demonstrated the potential of the FADS platform for both restoring rare microorganisms and screening new antagonistic bacteria, showcasing its applicability across various samples from different environments. However, we realized that during the process of transferring the low-fluorescence droplets to agar plates for culture, it

was observed that some microorganisms present in the droplets may not thrive in solid medium, potentially leading to a loss of valuable antagonistic microbial resources. To address this issue, incorporating microwells with liquid media for strain recovery could be beneficial. However, isolating single droplets from FADS systems for microwell culture poses significant challenges. As a result, other upscaling strategies for liquid culture are currently being developed. Recently, microfluidic devices like the single-cell microliter-droplet screening system (MISS Cell; TMAXTREE, China) have been introduced. These devices enable the screening of target droplets based on fluorescence intensity or optical density values^[44], providing powerful tools for research in microbiology and other fields.

Earlier studies have shown that at least 13 genera of bacteria have biocontrol potential, with *Bacillus*, *Pseudomona*, and *Agrobacterium* genera, and the genus *Bacillus* and Fluorescent pseudomonads have antagonistic activity against soft rot bacterium Ecc *in vitro*^[45]. The majority of antagonistic bacteria we isolated belonged to the *Serratia* spp., which has long been considered as a potential biocontrol agent. Some strains of *Serratia* spp., such as *Serratia liquefaciens*, *S. plymuthica*, and *S. rubidaea*, have been displayed to possess antibacterial activity, and in some cases, even exhibited potent antifungal properties^[46–47]. *Stenotrophomonas* spp. strains A-10 and D-56 were identified as *S. maltophilia*, a bacterium that has been previously considered as a conditional pathogen in numerous studies^[48–49]. However, a recent study discovered that *S. maltophilia* was isolated from plants as an endophytic bacterium, the antibacterial activity assay revealed that the inhibition zones of *S. maltophilia* extract against *Staphylococcus caseolyticus* and *Acinetobacter baumannii* were (15.11 ± 0.11) and (11.30 ± 0.16) mm, respectively^[50]. *Bacillus* species are well known producers of bioactive substances with a broad spectrum of antimicrobial activity such as surfactin, fengycin and iturin exhibiting remarkable antifungal, antitumor and antiviral activities^[30]. Rahman et al.^[10] isolated a *Bacillus* sp. strain E-65, which significantly inhibited the growth of potato soft rot Ecc P-138, it was found that E-65 reduced the soft rot infection to 22-week storage potatoes of different varieties by 32.5%–62.5% *in vitro* potato storage experiments. Azaiez et al.^[30] isolated a strain *B. amyloliquefaciens* Ar10, which had the control effect on the potato pathogen *Pectobacter carotovorum*. Many researchers also exploited *Bacillus* spp. to control soft rot bacteria in various plants, such as pepper^[51], *A. konjac*^[52], cucumber^[53–54], Chinese cabbages^[55], tomato^[56] and so on. In the present study, antagonistic strain D-62 was identified as a member of the genus *Bacillus* by molecular biological methods, and displayed strong antagonistic actions against

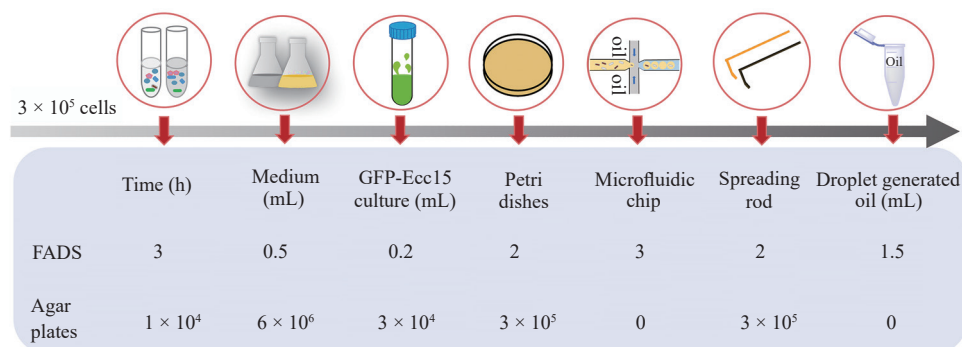


Fig. 6 Comparison of droplet microfluidic and plate screening system.

E. carotovora Ecc15 in storage experiment, and the ability of strain D-62 to inhibit the growth of Ecc15 makes it a potential biocontrol agent.

Bacteria are one of the important infection sources of plant diseases. The challenge is greater when dealing with bacterial diseases compared to fungal diseases because there have always been fewer bactericides than fungicides^[57]. Despite the high efficiency of biocontrol bacteria identified in laboratory settings, their control effects in the field can be unstable and heavily influenced by environmental factors, and the colonization capacity often decreases post-application, leading to a significant weakening of disease control effectiveness^[58]. As a result, genetic engineering to modify antagonistic bacteria and the exploration of new combinations of antagonistic bacteria have emerged as important strategies to enhance control efficacy^[59–60]. For instance, genetic engineering has been used to increase the production of antibacterial substances, such as phenazine-1-carboxylic acid^[61], 2-hydroxyphenazine^[62], and phenazine-1-carboxamide^[63]. The *in vitro* experiments results of Feng et al.^[64] indicated that some combination of two bacterial strains inhibited the growth of *Phytophthora infestans* mycelium better than a single bacterium, indicating that certain strains complemented each other and enhanced their inhibitory effect on pathogens. To our knowledge, we used FADS system to screen antagonistic microorganisms of plant pathogenic bacteria for the first time, which can quickly obtain target antagonists from complex environments. Our data demonstrated that FADS system is suitable for screening large collections of bacteria in search of potential antagonists against bacterial plant pathogens, as well as other types of bacterial pathogens. The discovery of novel microbial antagonists offers valuable strain resources for developing combinations of antagonists, which is of great significance for the control of plant pathogens.

5. Conclusion

In conclusion, we developed a FADS-based screening system for ultrahigh-throughput screening of antagonists against the *E. carotovora* Ecc15 strain. The system includes droplet generation, cultivation, reporter strain picoinjection, and droplet sorting based on fluorescence intensity. We applied the screening strategy to a complex microbial community and successfully obtained 32 antagonistic strains, they were classified as 3 phyla, 6 families and 9 genera. Compared with the plate screening system, the diversity of antagonistic bacteria enriched by the FADS system was more abundant, and some bacterial genera that were not isolated by the plate were observed, including *Lelliottia*, *Buttiauxella*, and *Leclercia*. Moreover, the FADS system can achieve the sorting of 3×10^5 cells within 3 h, the screening efficiency was increased by approximately 3 000, and the volume of medium and the consumption of other disposable consumables were also reduced by more than 1×10^5 , which significantly reducing experimental cost, these data confirmed the efficiency and robustness of the FADS method. The present study demonstrated the safety of strain D-62 for the corms of *A. konjac* and its effectiveness in controlling bacterial *E. carotovora* infection in postharvest *A. konjac* following their pretreatment with strain D-62. We believe that the screening system of antagonistic bacteria based on FADS can be widely used in the high-throughput screening of various environmental samples and mutant libraries, it has the potential to become a useful tool for biocontrol bacteria screening of other plant pathogens.

Conflict of interest

Chong Zhang is an editorial board member for *Food Science and Human Wellness* and was not involved in the editorial review on the decision to publish this article. The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary information

Supplementary data associated with this article can be found, in the online version, at <http://doi.org/10.26599/FSHW.2024.9250263>.

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